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Heterogeneity of ILC2s

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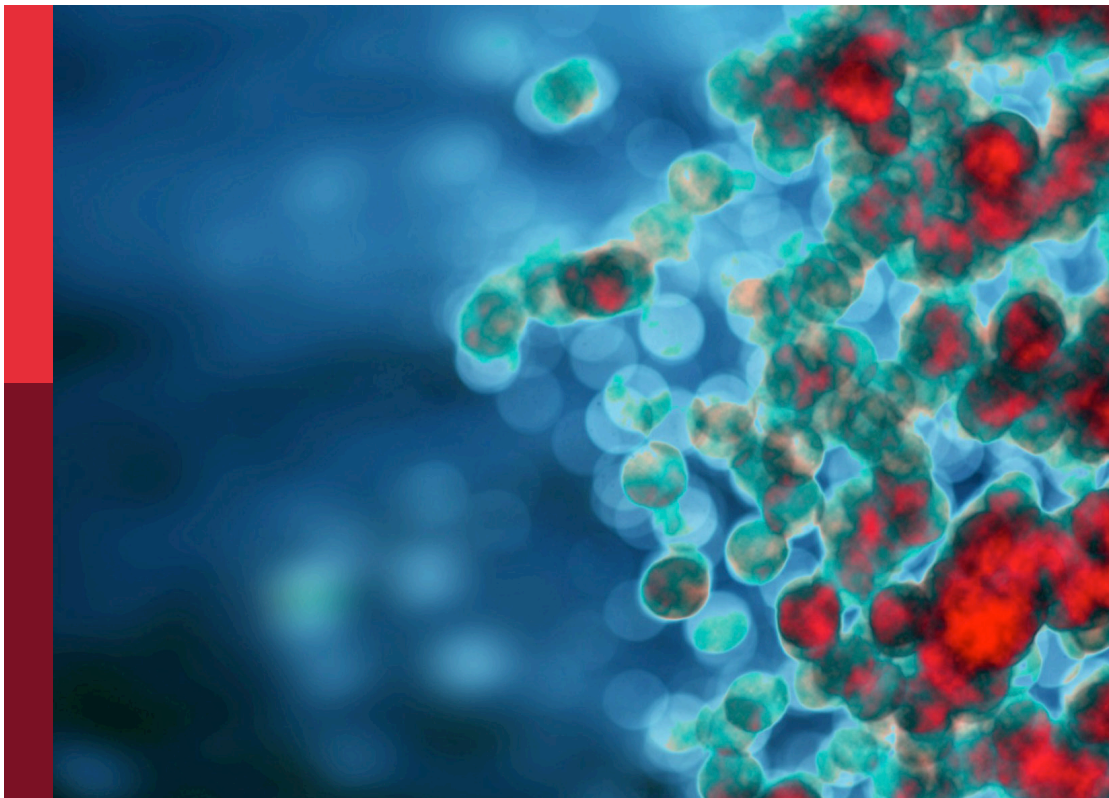

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Heterogeneity of ILC2s

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Editorial: Heterogeneity of ILC2s

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KEYWORDS

group 2 innate lymphoid cells, diversity, IL-33, tissue-specific, allergy

Editorial on the Research Topic
Heterogeneity of ILC2s

In 2010, novel subsets of lymphocytes were discovered in various tissues and were respectively named natural helper cells (1), nuocytes (2), and innate helper type 2 cells (3). Due to the similarities in transcription factors and cytokine production, these cells were subsequently consolidated into a unified group termed group 2 innate lymphoid cells (ILC2s) in 2013 (4). Further research has uncovered that ILC2s exhibit diverse functions and participate in allergic inflammation, parasite infections, tissue repair, and metabolic homeostasis (5). However, as our understanding of ILC2s deepens, their heterogeneity is once again attracting attention. For example, in 2015, a distinct subset of ILC2s known as inflammatory ILC2s was identified (6). In 2016, the first single-cell RNA-seq analysis of ILC2s demonstrated that intestinal ILC2s can be further categorized into multiple subgroups (7). During this period, it was also discovered that ILC2s possess the ability to modify their phenotype in response to the surrounding cytokine environment, which renders it uncertain whether there exist discrete subsets of ILC2s or a mixture of ILC2s at various stages of activation or development (8). Nevertheless, numerous recent studies have furnished evidence for the varied expression of tissue-specific markers, cytokine secretion profiles, and transcription factors in ILC2s (9). These findings imply that ILC2s exhibit distinct characteristics in different tissues and pathologies, potentially playing intricate roles *in vivo*.

To shed light on the complex heterogeneity of ILC2s, this Research Topic presents a compilation of original research articles and review articles under the title "Heterogeneity of ILC2s." Firstly, Kogame et al. discuss the latest advancements in understanding the differentiation and maturation process of ILC2s, which are closely associated with their heterogeneity. Secondly, ILC2s are tissue-resident cells with distinct characteristics in each tissue. Misawa et al. summarize the diversity of ILC2s in adipose tissue, Kobayashi et al. in skin, Sunaga et al. in the intestinal tract, and Asaoka et al. in the lung. These review articles provide readers with up-to-date knowledge on the diversity of ILC2s in each specific tissue. Furthermore, ILC2s exhibit functional heterogeneity. Ikutani and Nakae and Nakagome and Nagata summarize the roles of ILC2s in cancer, obesity, cardiovascular disease, and viral infections, respectively. Matsuyama et al. outline therapeutic strategies targeting ILC2s in asthma. These review articles highlight the functional diversity of ILC2s in the context of various disease processes. Lastly, Topczewska et al. using *Nmur1*$^{iCre\text{-}eGFP}$ mice generate ILC2-specific conditional knockout mice. Their work elegantly uncovers the role of IL-33 in ILC2s across multiple tissues.

This Research Topic not only presents the latest discoveries in basic research but also highlights the potential for future treatment strategies. Targeting the activation or suppression of specific subgroups of ILC2s holds promise as a novel approach in treating allergic and immune-related diseases. Advancing our understanding of the molecular mechanisms underlying ILC2 heterogeneity will be crucial for developing more precise control methods and identifying therapeutic targets, ultimately paving the way for personalized therapeutic strategies in the future.

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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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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REVIEW
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Heterogeneity of ILC2s in the Intestine; Homeostasis and Pathology

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Group 2 innate lymphoid cells (ILC2s) were identified in 2010 as a novel lymphocyte subset lacking antigen receptors, such as T-cell or B-cell receptors. ILC2s induce local immune responses characterized by producing type 2 cytokines and play essential roles for maintaining tissue homeostasis. ILC2s are distributed across various organs, including the intestine where immune cells are continuously exposed to external antigens. Followed by luminal antigen stimulation, intestinal epithelial cells produce alarmins, such as IL-25, IL-33, and thymic stromal lymphopoietin, and activate ILC2s to expand and produce cytokines. In the context of parasite infection, the tuft cell lining in the epithelium has been revealed as a dominant source of intestinal IL-25 and possesses the capability to regulate ILC2 homeostasis. Neuronal systems also regulate ILC2s through neuropeptides and neurotransmitters, and interact with ILC2s bidirectionally, a process termed “neuro-immune crosstalk”. Activated ILC2s produce type 2 cytokines, which contribute to epithelial barrier function, clearance of luminal antigens and tissue repair, while ILC2s are also involved in chronic inflammation and tissue fibrosis. Recent studies have shed light on the contribution of ILC2s to inflammatory bowel diseases, mainly comprising ulcerative colitis and Crohn’s disease, as defined by chronic immune activation and inflammation. Modern single-cell analysis techniques provide a tissue-specific picture of ILC2s and their roles in regulating homeostasis in each organ. Particularly, single-cell analysis helps our understanding of the uniqueness and commonness of ILC2s across tissues and opens the novel research area of ILC2 heterogeneity. ILC2s are classified into different phenotypes depending on tissue and phase of inflammation, mainly inflammatory and natural ILC2 cells. ILC2s can also switch phenotype to ILC1- or ILC3-like subsets. Hence, recent studies have revealed the heterogeneity and plasticity of ILC2, which indicate dynamicity of inflammation and the immune system. In this review, we describe the regulatory mechanisms, function, and pathological roles of ILC2s in the intestine.

Keywords: ILC2 - group 2 innate lymphoid cell, IBD - inflammatory bowel disease, Crohn’s disease, ulcerative colitis, mucosal immunology

INTRODUCTION

The intestine is one of the largest organs continually exposed to the external environment and it harbors an immune system to protect the host from pathobionts (1). Innate lymphoid cells (ILCs) are newly classified lymphocyte subsets that serve as a frontline defense, particularly in the mucosal tissues (2, 3). Unlike T- and B-cells, ILCs do not express adaptive antigen recognition receptors, and as such their expansion and activation are not driven in an antigen-specific manner, but rather by cytokine signals in the local microenvironment in each tissue. Although ILCs cannot induce antigen-specific reactions, they quickly respond to external antigen from the local microenvironment and rapidly produce various cytokines including interleukins (IL) and interferon (IFN) to maintain tissue homeostasis. ILCs are classified into three groups based on lineage-determining transcription factors and cytokine production, mirroring T helper cell subsets (2, 3). Group 2 ILCs (ILC2s) require transcription factors GATA3 (4, 5) and RORα (6, 7) for differentiation and produce signature "Type 2" cytokines, such as IL-4, IL-5, IL-9, and IL-13, as well as IL-6, IL-10, IL-17, and amphiregulin (AREG) (8–12). ILC2s were first reported as natural helper cells, nuocytes, and innate type 2 helper cells, and were detected in mesenteric adipose tissue, mesenteric lymph nodes, spleen, liver, lung, and small intestine (13–15). More recently, ILC2s have been found in various organs that are confronted with external antigen, such as the intestine, respiratory system, and skin, and also in those that are not continually challenged, such as liver, heart, muscle, and brain (16). Recent advancement of single cell omics and mass cytometry technologies have revealed that ILC2s possess tissue-specific phenotypes and contribute to the tissue-specific regulation of inflammation, allergic immunity, parasite infection, metabolism, and tissue repair (16–19).

In the intestine, epithelial cells respond to bacteria, parasites, and allergen within the intestinal lumen, and produce alarmins, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which subsequently activate ILC2s to proliferate and produce cytokines (16). Recent studies have reported abundant regulation of ILC2s beyond alarmins, including neuro-immune crosstalk, which is mediated by neurotransmitters and cytokines. Activation of tissue-resident ILC2s causes not only local inflammation but also subsequent tissue remodeling and organ fibrosis associated with intestinal chronic inflammatory conditions, such as inflammatory bowel disease (IBD), including ulcerative colitis (UC) or Crohn's disease (CD) (17). However, negative clinical trial results regarding targeting type 2 immune responses have encouraged us to explore the complexity of ILC2 and other type 2 immune cells, and their cytokine production. New technologies, including single-cell analysis, have been used to better decipher the functions and heterogeneity of ILC2s. Initially, ILC2s were thought to have roles in defending against parasitic infection and promoting allergic pathology (20), whereas studies of ILC2s in the context of IBD are developing. In this review, we focus on the roles of ILC2s in the intestine and discuss their regulation, neuroimmunology, fibrosis, and contribution to IBD.

REGULATION OF ILC2

ILC2s are localized in the lamina propria below the epithelial layer and are activated following epithelial damage by parasites and allergens in the mucosal tissues. This process is mediated by alarmins, such as IL-25, IL-33, and TSLP, which initially activate expansion and cytokine production in ILC2s when triggered by mucosal barrier damage (**Figure 1**). ILC2s responding to IL-33 produce the growth factor AREG, which binds to epithelium-expressed epidermal growth factor receptor (11). AREG has a critical role for epithelial cell proliferation and differentiation through the epidermal growth factor receptor pathway (21, 22). A recent study demonstrated that secretion of IL-33 was significantly accelerated in the colons of mice treated with dextran sulfate sodium (DSS) and injecting recombinant murine IL-33 improved epithelial damage, pro-inflammatory cytokine secretion, and loss of barrier function in DSS-induced colitic mice (23). Anti-colitic effect of IL-33 was observed in RAG2-/- or diphtheria toxin-treated DEREG mice where whole T cells or Tregs are depleted respectively (23). This suggests that ILC2 has significant roles in anti-colitic effect upon stimulation of IL-33 which is also known to enhance suppressive function of Foxp3+ regulatory T cells (Tregs) through its receptor ST2 (24) or stimulate CD103+ dendritic cells (DCs) to produce IL-2 and expand Tregs (25). In the small intestine, tuft cells, which exist in the epithelial layer of the intestinal tract and project microvilli into the lumen, produce IL-25 to sustain ILC2 homeostasis in the resting lamina propria (26). Together with high expression of IL-17RB (27), tuft cell-derived IL-25 activates ILC2s to produce IL-13, which affects epithelial crypt progenitors to promote differentiation of tuft and goblet cells, resulting in further activation of ILC2s in a positive feedback circuit of type 2 inflammation (26). This feed-forward pathway is constrained by CISH, a suppressor of cytokine signaling family member (28) and CISH-deficient ILC2s show excessive proliferation and cytokine production, resulting in increased tuft cells in the small intestinal (29). TSLP, which belongs to the IL-2 family with structural similarities to IL-7, is released from epithelial cells (30). TSLP incorporates the TSLP receptor (R) and the α-subunit of the IL-7R, and this ternary molecular complex activates multiple signaling pathways, such as the JAK1 and 2, STAT3 and 5, MAPK, PI3K, and NF-κB pathways (31–33). TSLP enhances the type 2 immune response, in particular the activation of ILC2s, resulting in increased type 2 cytokines IL-4, IL-5, and IL-13 (34–39). These effector cytokines are also regulated at the post-transcriptional level. Tristetraprolin, encoded by *Zfp36*, is an RNA-binding protein that destabilize mRNA. In the *Zfp36*-/- mice, ILC2 produces excessive *Il5* and *Il13* in the small intestine and other organs (40). Taken together, ILC2 in the intestine is regulated at the transcriptional and post-transcriptional level upon cytokine stimulation.

Neuroimmunology: ILC2 Neuro-Immune Crosstalk

The gastrointestinal tract is one of the most innervated organs, particularly by enteric neurons and extrinsic sympathetic and parasympathetic nerves, such as the vagus nerve (41). This

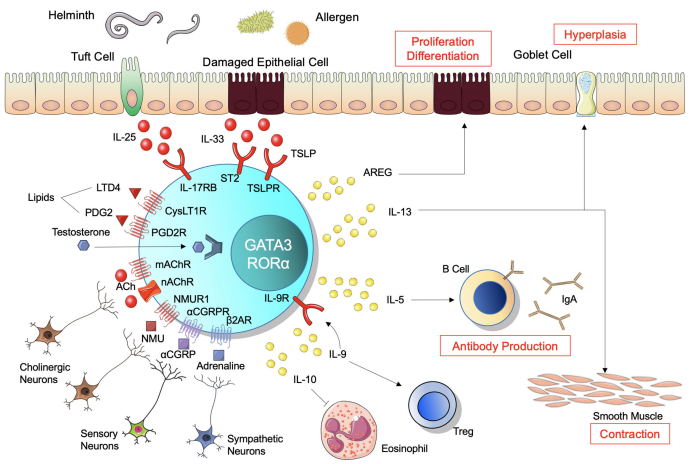

FIGURE 1 | Regulation and function of ILC2. Alarmins, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), activate innate lymphoid cells 2 (ILC2) to expand and produce cytokines. Neural systems regulate ILC2s bidirectionally through neuropeptides and neurotransmitters. ILC2s require transcription factors GATA3 and RORα, and subsequently produce various cytokines. IL-13 induces hyperplasia of goblet cells and contraction of smooth muscle in the intestine, involved in clearance of antigens in the lumen. IL-5 regulates B-cell antibody production and enhances IgA production. Amphiregulin (AREG) promotes epithelial cell proliferation and differentiation. IL-9 and IL-10 contribute to resolution of inflammation, while IL-9 also promotes regulatory T-cell activation and IL-10 decreases eosinophil recruitment. ILC2 expresses IL-9 receptor and thus receives IL-9 autocrine feedback. Regarding ligands and receptors, red indicates activation, blue indicates inhibition, and purple indicates both functions.

neuronal regulation shapes the levels of inflammation and homeostasis in the gut *via* controlling the epithelia, stroma, and immune cell compartments. The immune and neuronal systems interact bidirectionally, namely through neuropeptides and neurotransmitters that regulate immune cell functions, while inflammatory mediators from immune cells enhance neuronal activation. This "neuro-immune crosstalk" plays critical roles in tissue homeostasis (42–44). In addition to the intestinal macrophage (45), T-cell (46, 47), and ILC3 (48), ILC2s have been investigated for neuro-immune crosstalk from the early stages following identification (49, 50). ILC2s express receptors for neuropeptides and neurotransmitters, and are regulated through these receptors. Neuromedin U (NMU), a neuropeptide secreted from sensory cholinergic neurons, is detected in the intestine with high levels of expression and exerts biological activities through two G protein-coupled receptors: NMU receptor 1 (NMUR1) and 2 (NMUR2). NMUR1 is distributed in the peripheral tissues while NMUR2 is mainly observed in the central nervous system (51). Among the immune cells reported to express NMUR1 at a significant level, ILC2s predominantly express NMUR1 compared to other immune cell subsets, such as T cell, mast cell, and other groups of ILCs (52–56). NMU induces activation, proliferation, and type 2 cytokine secretion in ILC2s through NMUR1 (54–56). Although NMU regulation of ILC2s has been elucidated mainly in the field of allergic respiratory diseases, this relationship has also been revealed in the mouse gastrointestinal tract, indicating that NMU induces ILC2 activation, proliferation, and secretion of the type 2 cytokines IL-5, IL-9, and IL-13 (55). Calcitonin gene-related peptide (CGRP) is a later-identified neuropeptide that regulates ILC2s and is expressed and released by sensory neurons and ILC2s themselves (57–59). ILC2s express the receptor for α-CGRP in homeostatic and inflammatory conditions, and α-CGRP suppresses ILC2 proliferation by activating a cAMP response module, while promoting IL-5 expression (59). Single-cell RNA sequencing has revealed that expression of *Calca*, which encodes α-CGRP, is induced in intestinal killer-cell lectin like receptor G1 (KLRG1)-positive ILC2s in a food allergy model, but it is expressed in choline O-acetyltransferase $(ChAT)^+$ sensory neurons in the steady state (59). These paradoxical functions of CGRP in terms of pro- and anti-inflammatory influence on immune responses may represent key roles for maintenance of epithelial cell homeostasis by adjusting immune responses to neuronal signals. In particular, IL-5 enhanced by α-CGRP promotes repair of epithelial cell damage, while α-CGRP prevents excessive type 2 inflammation by suppression of ILC2 proliferation (59). Of note, $ChAT^+$ ILC2s are strongly induced by type 2 inflammatory conditions, such as helminth infection, *Alternaria* sensitization, and IL-25 and IL-33 treatment (38). In addition, ILC2s purified from the small intestine or cultured under IL-2, IL-7, and IL-33, express both muscarinic (*Chrm4* and *Chrm5*) and nicotinic (*Chrna2*, *Chrna5*, *Chrna9*, and *Chrna10*, *Chrnb1* and *Chrnb2*) acetylcholine (ACh) receptors. Therefore, ILC2s can respond to ACh to produce IL-5 and IL-13, and induce goblet cell hyperplasia, eosinophil accumulation, and helminth expulsion in the small intestine, which are partially abrogated in ILC-specific deletion of ChAT. Tuft cells also have the capacity to produce ACh and contribute to the regulation of ILC2s (26, 60). Studies suggest that the expression of CGRP and ChAT in ILC2s is similarly induced by type 2 inflammation and positive autocrine loops of ILC2-ACh or ILC2-CGRP potentially modify intestinal inflammation and

homeostasis, and also raise an interesting question to identify the distinct roles of neuropeptides like CGRP and ACh released by ILC2s and sensory neurons. Although ILC2s respond to ACh both in the intestine and lung, ILC2s in the lung express the α7-nicotinic ACh receptor in contrast to intestinal ILC2s, suggesting tissue specificity of ACh receptor usage in ILC2s (61). Similar to the CGRP, another vasodilative neuropeptide, vasoactive intestinal polypeptide (VIP) is also involved in the regulation of ILC2s. Intestinal ILC2s express the VIP receptor and produce IL-5 when incubated with IL-7 and VIP (62). Reciprocally, IL-5 from ILC2s directly activates nociceptors, such as TRPV1 and TRPA1, on afferent $Na_V1.8^+$ neurons and upregulates the release of VIP, which induces ILC2s and T-cells to release more IL-5 and form a positive feedback loop of type 2 inflammation (63).

ILC2s are regulated by not only cholinergic neurons but also adrenergic neurons. The β2-adrenergic receptor, a catecholamine receptor expressed on ILC2s, recognizes noradrenaline released from sympathetic neurons and downregulates ILC2 function and type 2 inflammatory responses (64). Hence, both parasympathetic neurons releasing ACh and sympathetic neurons releasing noradrenaline affect suppression of ILC2-derived type 2 inflammation.

ILC2 IN INFLAMMATORY BOWEL DISEASES

IBD, mainly comprising UC and CD, is chronic inflammatory disease of the gastrointestinal tract, although the etiology of IBD remains unclear. To date, more than 200 IBD-associated genes have been identified and impinge on the pathways associated with cytokine signaling, bacterial recognition, and barrier function (65–67). Accumulation of many studies reveals that abnormal immune responses against microorganisms of the gut flora initiates chronic intestinal inflammation in genetically susceptible individuals (68). Furthermore, dysregulation of both innate and adaptive immune pathways contributes to the pathogenesis of IBD (69) (**Figure 2**). Detailed elucidation of the innate immune system, including ILCs, is required to gain new insights into the immunologic mechanisms of intestinal inflammation.

Compared with ILC1s and ILC3s, the role of ILC2s in IBD patients is less well understood (19). This might be attributed to the very low frequency of ILC2s in the entire human gastrointestinal tract compared with the relatively high abundance of ILC1s in the upper gastrointestinal tract and ILC3s in the ileum and colon (70). At the time of IBD diagnosis, the frequency of ILC1s is increased in patients with CD, and the frequency of NKp44+ ILC3s in inflamed tissue is decreased in both CD and UC patients (71), consistent with the previous literature showing that NKp44+ ILC3s produce IL-22 and IL-22-producing ILC3s are decreased in IBD (72–76). In contrast, the frequency of ILC2s is increased in patients with UC at diagnosis (71), while both ILC1s and ILC2s are increased in patients with IBD established for at least 1 year (71). Although reports of increased ILC2 frequency are traditionally present in CD but not UC (77), a recent study suggests the involvement of ILC2s with mucosal inflammation in both CD and UC. Impressively, ILC2s show plasticity towards an ILC1 cytokine profile with IL-12 stimulation, and some ILC2s in the mucosa of CD patients acquire

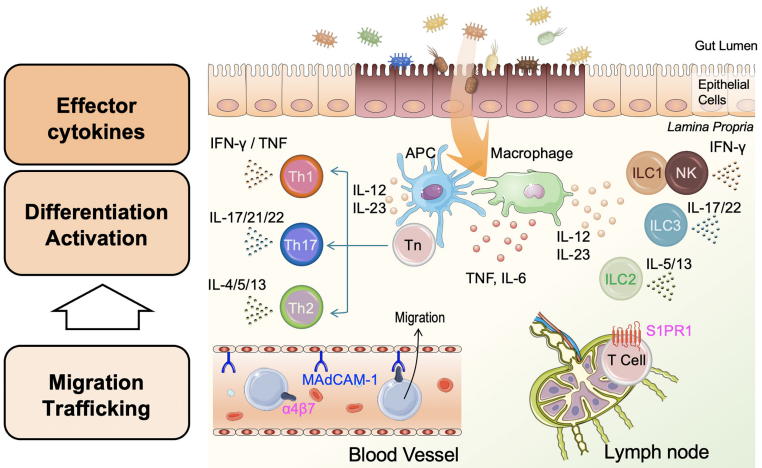

FIGURE 2 | Pathophysiology of inflammatory bowel disease (IBD). Damaged epithelial cells enhance antigen presenting cell (APC) and macrophage uptake of antigens such as bacteria in the gut lumen, leading to APC/macrophage activation. APCs and macrophages produce pro-inflammatory cytokines, including tumor necrosis factor (TNF), IL-6, IL-12, and IL-23. Activated APCs present processed antigens to naïve helper T-cells (Tn) and promote the differentiation of Tn to effector T-cells, helper T-1 (Th1), Th2, and Th17 cells. Th1 and Th2 release type 1 cytokines (interferon [IFN]-γ and TNF), and type 2 cytokines (IL-4, IL-5, and IL-13), respectively. Independent of these, Th17 cells release IL-17, IL-21, and IL-22. Innate lymphoid cells 1 (ILC1s) and natural killer (NK) cells, ILC2s, and ILC3s are activated in a non-antigen-specific manner in tissues and produce cytokines corresponding to adaptive Th cell phenotypes, Th1, Th2, and Th17, respectively. Inflammation in the lamina propria is involved in the migration and trafficking of lymphoid cells from blood vessels and lymph nodes. Circulating lymphoid cells bearing integrin-α4β7 bind to the mucosal vascular epithelium through mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) and migrate to the inflamed intestine. T-cells expressing shingosine-1-phosphase receptor 1 (S1PR1) in the lymph node are recruited to the site of inflammation by stimulation with S1P. Each cytokine or molecule has been targeted for IBD treatment.

capacity to produce IFN-γ in addition to IL-13, which could potentially contribute to intestinal inflammation (78). IL-12 is expressed and actively released in CD intestinal tissues (79), and is the therapeutic target of ustekinumab, which is used to treat CD and UC (80, 81). Notably, in IBD patients receiving vedolizumab, a monoclonal antibody that targets integrin α4-β7 and blocks gut-homing of activated immune cells, the frequencies of ILCs in peripheral blood remained unchanged, suggesting that distribution of ILCs is due to local proliferation or plasticity rather than recruitment of ILCs to the intestine (71). Therefore, the finding of increased frequency of ILC2s secreting IFN-γ may indicate that the plasticity of ILCs depends on the local mucosal microenvironment.

IL-33 expression is enhanced in the inflamed mucosa of IBD patients (24, 82) and experimental models of colitis (83), and has been previously shown to play both protective and detrimental roles in colitis, based on different models of colitis and analyses of cell types. Genetic ablation of ST2, a receptor of IL-33, resulted in amelioration of colitis induced by DSS or trinitrobenzene sulfonic acid (84). In addition, ILC2s expand and produce more Th2 cytokines during DSS-induced colitis, which is repressed in the steady state by E-cadherin on colonic epithelial cells and KLRG1 on ILC2s (85). Conversely, treatment with IL-33 or transfer of ILC2s improve intestinal mucosal damage through the AREG pathway in the DSS-induced colitis model (11). A recent study has reported that the intracellular pattern recognition receptor NOD2 drives early IL-33-dependent expansion of ILC2s during CD ileitis, based on CD patient samples and an established murine model of CD-like ileitis, the SAMP1/YitFc mouse strain (86, 87). In addition to alarmins, CC chemokine ligand 1 (CCL1) exerts unique roles on ILC2s in the intestine. ILC2s express high levels of the Th2-type chemokine receptor, C-C motif chemokine receptor 8 (CCR8), both in mouse intestine and human peripheral blood. In addition, the expression of CCR8 and its ligand CCL1 is upregulated in patients with UC and in the DSS-induced colitis model (88). In a helminth infection model, mice lacking CCR8 exhibit reduced type 2 cytokines IL-5, IL-13, and IL-9, and greater worm burden in the small intestine (89). This is not attributed to aberrant migration but to impaired proliferation and cytokine production in ILC2s in the lung and intestine, although CCL1/CCR8 signaling contributes to mediating monocyte and lymphocyte chemoattraction and is implicated in vascular regulatory T-cell recruitment and function (90). Of note, ILC2s are the major producers of CCL1, which forms a paracrine CCL1/CCR8 feed-forward loop during helminth clearance (89). Unlike parasite infection, the major source of CCL1 during DSS-induced colitis is macrophages rather than ILC2s, but CCL1/CCR8 signaling similarly protects hosts from both parasite infection and acute intestinal damage in a DSS colitis model (88). In addition, mice lacking CCR8 exhibit comparable numbers of ILC2 and tissue-repairing cytokines, IL-10 and AREG, but reduced numbers of intestinal IFN-γ-producing ILCs (88). However, these IFN-γ-producing ILCs may also have dual roles in colitis as discussed above. Further studies are needed to disentangle the complex results of previous reports regarding the roles of ILC2s in colitis and clinical trials targeting Th2 cytokines, and to further enhance our comprehension of the contribution of ILC2s to immune mechanisms in IBD.

ILC2 Contribution to Intestinal Fibrosis

Inflammation and impaired tissue repair induce accumulation of myofibroblasts, which produce extracellular matrix components, resulting in organ fibrosis (91, 92). In the intestine, fibrosis can lead to stenosis or perforation. Th2 cells produce type 2 cytokines, IL-4, IL-5, and IL-13, generating various pathological changes, such as infiltration of eosinophils, increased mucus production, and fibrosis (93). Recent studies have revealed that not only Th2 cells but also ILC2s producing type 2 cytokines in an antigen non-specific manner play an important role in immune-mediated fibrosis and modulation of tissue remodeling, causing dysfunction in various organs. Regarding the lung, expression of IL-25 and the ILC2 population increase in the lungs of idiopathic pulmonary fibrosis patients (94). Other alarmin cytokines, IL-33 and TSLP, are elevated in idiopathic pulmonary fibrosis, cystic fibrosis, and steroid-resistant asthma sufferers (95–98). These studies suggest that alarmin cytokines have critical roles in lung fibrosis and remodeling. In patients suffering from liver fibrosis of various etiologies, such as virus infection, alcoholic liver disease, non-alcoholic steatohepatitis, autoimmune hepatitis, and primary cholangitis (99), numbers of liver-resident ILC2s are activated and expanded followed by expression of IL-33 (100). A recent study has revealed the contribution of ILC2s in skin fibrosis within systemic sclerosis (101). Following activation by IL-33, ILC2s express the growth factor AREG and participate in epithelial barrier function and tissue repair in the intestine (11).

IL-13 produced by ILC2s is involved in expression of the tumor necrosis factor family cytokine TL1A, overexpression of which brings about intestinal fibrosis (102). Constitutive expression of TL1A in lymphoid and myeloid cells leads to spontaneous inflammation and fibrosis in the small intestine and colon (103, 104). TL1A is a ligand for death receptor 3 and enhances secretion of pro-inflammatory cytokines through multiple cell lineages (105). ILC2s highly express death receptor 3 and overexpression of TL1A activates ICL2 expansion, independent of IL-25 or IL-33 (106). Notably inhibition of TL1A function by either anti-TL1A neutralizing antibody or deletion of death receptor 3 reduces numbers of intestinal fibroblasts and myofibroblasts in murine DSS colitis, the model of human IBD (107). Deficiency of another tumor necrosis factor family cytokine, LIGHT, in mice exacerbates DSS colitis compared with controls and accumulates ILCs, suggesting LIGHT plays roles in regulating inflammation in the colon (108). Signaling through LIGHT receptor, lymphotoxin β receptor, in epithelial cells and dendritic cells protects against mucosal damage by inducing IL-22 from ILC3s (109). Although *Tnfsf14*, the gene encoding LIGHT, is highly expressed in not only ILC3s but also ILC2s (108, 110), the role of the LIGHT-lymphotoxin β receptor interaction in ILC2s has not yet been revealed and further research is needed.

Blocking IL-13 production from ILC2s by IL-25 neutralization enhances IL-22 production from ILC3s, which repair epithelial damage (111, 112). In murine models, IL-13 is associated with

chronic gut inflammation caused by trinitrobenzene sulfonic acid (113) and triggers transforming growth factor β 1-dependent fibrosis (114). Notably, IL-13 was identified as the key effector cytokine in UC by affecting epithelial apoptosis, tight junctions, and restitution velocity (115) and a promotor of collagen accumulation in CD by inhibiting fibroblast matrix metalloproteinase synthesis, resulting in fibrosis of intestine tissue (116). These studies indicate that blockade of IL-13 improves inflammation and subsequent fibrosis in IBD patients. However, clinical trials evaluating tralokinumab, an anti-IL-13 neutralizing antibody for moderate-to-severe UC (117), and anrukinzumab, an anti-IL-13 monoclonal antibody for mild-to-moderate UC (118), could not demonstrate statistically significant therapeutic effects compared with placebo controls. Although the effect of inhibiting IL-13 for IBD patients remains controversial, a recent study that found a high frequency of autoantibodies against integrin αvβ6 in UC patients suggests the possible contribution of type 2 immune responses in the pathogenesis in IBD (119).

THE ROLES OF ILC2 FOR INFECTION AND ALLERGY

As mentioned above, the exposure to pathogens such as parasites and allergens triggers ILC2 activation in mucosal tissue. Parasites and allergens contain catalytic enzymes that digest the mucosal barrier and provoke massive epithelial cell death, leading to release of IL-33, which rapidly activates ILC2s in the lung (120) and colon (121, 122). Since IL-33 rescues RAG2-/-, but not RAG2-/- γc-/-, mice from *Clostridioides difficile* (121) and amebic (122) infection, IL-33-ILC2 exerts host protection from these intestinal infections. In the nucleus of epithelial cells, endogenous IL-33 is highly expressed upon tissue inflammation (123). Additionally, lipid chemical mediators play critical roles in ILC2 activation (124, 125). ILC2s in the lung from wild-type, RAG2-/-, and STAT6-/- mice express cysteinyl leukotriene receptor 1 (CYSLTR1), and are induced to produce IL-4, IL-5, and IL-13 followed by stimulation of leukotriene D4 (124). Similar to the lung ILC2, small intestine ILC2 expresses CYSLTR1 and CYSLTR2, and produces IL-13 upon stimulation of leukotriene C4 and D4 (126). In small intestine, tuft cells become the essential source of cysteinyl leukotriene and activate ILC2s in cooperation with IL-25 following helminth infection (126). An *in vitro* study of ILC2s isolated from human skin showed that prostaglandin D2 induces ILC2 migration, production of type 2 cytokines and other pro-inflammatory cytokines, and upregulation of the expression of IL-33R and IL-25R (125). A subsequent study demonstrated that testosterone attenuates ILC2 function, and this result may explain the sex difference in prevalence of allergic disease (127). However, these ILC2 regulatory mechanisms have not yet been demonstrated in the intestinal tract.

Activated ILC2s exert inflammatory responses mainly *via* type 2 cytokines. IL-5, IL-6, and IL-13 are ILC2-derived cytokines that were identified when ILC2s were first discovered (13). IL-5 regulates B-cell antibody production and enhances IgA production from B-cells, while IL-5 and IL-13 are implicated in allergic inflammation and protection against helminth infection (13, 128). The recent study demonstrates that ILC2s predominate in the stomach, are induced by commensal bacteria, and protect against *H. pylori* infection through B cell activation and IgA production (129). IL-13 promotes intestinal smooth muscle contractility for exclusion of enteric nematode parasites and is required for expression of STAT6 (130). IL-13 derived from ILC2s induces hyperplasia of goblet cells, the columnar epithelial cell that lines gastrointestinal mucous membrane and contains abundant mucin, and participates in clearance of luminal antigens (131, 132). While activated ILC2s produce large amounts of IL-5 and IL-13, the level of IL-4 is generally low except in specific inflammatory conditions or disease models (14, 133, 134). IL-4 released from ILC2s promotes food allergy by blocking allergen-specific regulatory T-cells (135), and is required for type 2 helper T-cell (Th2) differentiation following helminth infection (136). Alternatively, ILC2s can also respond to IL-4 derived from eosinophils or basophils and accelerate proliferation and activation of ILC2s themselves. This feed-forward loop contributes to amplification of type 2 inflammation (137, 138). ILC2s also produce IL-9 following activation by IL-33, but not IL-25 (139). IL-9 derived from ILC2s promotes regulatory T-cell activation and effectively induces resolution of inflammation (140). Moreover, ILC2 simultaneously expresses IL-9R during helminth infection, suggesting an autocrine feedback of ILC2-derived IL-9 (139). Conversely, a molecularly distinct subset of ILC2s produce IL-10 following IL-2 activation and subsequently decrease eosinophil recruitment, suggesting downregulation of inflammation (12). Consequently, ILC2s interact with other immune cells through various cytokine crosstalk pathways and form amplification loops of type 2 immune responses with Th2 cells, eosinophils, and basophils.

HETEROGENEITY AND PLASTICITY OF ILC2

Recent studies have shown the heterogeneity of ILC2 subsets between tissues and implicated environmental factors in this variability. In the lung, the existence of two different ILC2 subsets —inflammatory ILC2 (iILC2) and natural ILC2 (nILC2)—have been identified, and these have different phenotypes, such as ST2 (a heterodimer of IL-33R), Thy1, KLRG1, and IL-17RB (10). iILC2 cells express more IL-25R and develop into nILC2-like cells, producing IL-5 and IL-13 after stimulation with IL-33 during worm infection. Moreover, iILC2 migrate from the intestinal lamina propria to other organs, including lung and liver, dependent on chemotaxis mediated by sphingosine 1-phosphate after injection of IL-25 or helminth infection (141). Although ILC2s are largely tissue-resident (142, 143), the ability of ILC2s to migrate suggests that ILC2s complement adaptive immunity by protecting both local and distant tissue against infection.

In the small intestine, IL-33 promotes the generation of iILC2s by induction of tryptophan hydroxylase 1, deletion of which results in increased susceptibility to helminth infection (144). However, in the colon, ILC2s express ST2. Following administration of IL-33, these cells proliferate and demonstrate

high expression of IL-5 and IL-13, with lower expression of IL-17 (145). Compared with other organs such as lung or skin, ILC2s in the small intestine express higher levels of IL-17RB, which forms the IL-25R together with IL-17RA (146, 147). Enriched IL-17RB in intestinal ILC2s suggests that IL-25 derived from tuft cells promotes efficient activation of ILC2s and defense against infection with helminths or other pathogens (26, 27, 148). Although TSLP is primarily expressed in skin keratinocytes, lung, and gut epithelial cells (31), the function of TSLP in the gut has not yet been clearly identified in contrast to its role in allergy and infection in the lung and skin (149).

The first cell population-specific RNA sequence study to characterize murine ILC subsets in the lamina propria of the small intestine identified the expression of genes associated with lipid metabolism, such as *Dgat2*, *Pparg*, and *Lpcat2*, and a gene associated with enteric neuron communication, *Bmp2* (147). In another single-cell sequencing study, graded expression of GATA3 characterized four different groups of intestinal ILC2s (150). ILC2s, which express high levels of marker genes, such as *Klrg1*, *Klf4*, *Ly6a*, and *Il2ra*, uniquely expressed high levels of *Il5*, *Csf2*, and *Areg* (150). As described above, single-cell RNA sequencing assists to determine the heterogeneity of ILC2s, particularly in the field of neuroimmunology. In studies of the lung, ILC2s in *Nippostrongylus brasiliensis*-infected mice are clustered into four subsets: resting nILC2s, *Il5*-high nILC2s, *Il13*-high nILC2s, and iILC2s (57). The expression of α-CGRP receptor is enriched within an *Il5*-high subpopulation of ILC2s and α-CGRP promotes IL-5 production only at early time point stimulations (57). Intestinal ILC2s express the components of the α-CGRP receptor at steady state, while α-CGRP suppresses the proliferation of ILC2s, but increases IL-5 levels during the early inflammatory phase (59). Furthermore, ILC2s of the small intestine express abundant NMUR1 gene, while adaptive immune cells, ILC1s, and ILC3s do not (55).

In vitro studies demonstrate the plasticity of human ILC2s, which switch phenotype between subsets such as ILC1s and ILC3s. IL-12 promotes the conversion of ILC2s into ILC1-like cells, characterized by expression of T-bet and production of IFN-γ (78, 138, 151). Conversely, IL-4 derived from eosinophils promotes ILC2 maintenance and proliferation by preventing IL-12-mediated ILC2 differentiation into the ILC1 phenotype (138). Furthermore, the ILC2 subpopulation that expresses c-Kit can convert into ILC3-like cells, producing IL-17 in response to IL-1β and IL-23 (152). Removal of the aryl hydrogen receptor, a transcription factor for ILC3, activates intestinal ILC2s, whereas increased aryl hydrogen receptor expression suppresses ILC2 function and enhances ILC3 function (153). As ILC3-to-ILC1 conversion has been reported (19, 154), ILC2s also demonstrate plasticity, resulting in ILC2 heterogeneity in the inflammatory gut.

CONCLUSION

ILC2s play important roles not only for protection against infection but also for promotion of chronic inflammation and tissue fibrosis. A variety of cytokines and cellular interactions with other immune cells and neuronal systems are involved in the homeostasis of ILC2s, suggesting complexity of ILC2 regulation. Recent studies revealed the potential of intestinal ILC2s, such as migration to other organs and plasticity of conversion to ILC1s or ILC3s. In particular, single-cell analysis can help our understanding of heterogeneity of ILC2s potentially attributed to pathological mechanisms and aid in discovery of therapeutic targets for chronic inflammation, including IBD. Understanding uniqueness and commonness of ILC2 between mice and humans, between the gut and other organs, and between health and disease may help answer important questions in gut biology: What role does ILC2 play in the contexts such as IBD, infectious disease, colorectal cancer, food allergy and intestinal fibrosis? What is the unique role of each ILC2 subset in the clinical settings? How are these ILC2 subsets dynamically regulated during the course of intestinal disease? How does each ILC2 subset interact with the other ILC2 subsets and other types of immune cells? What factors contribute to diversification of ILC2 subsets? Can these ILC2 subsets be targeted to develop effective therapeutic strategies for human intestinal diseases? Further research on ILC2s in different environments at different phases of intestinal inflammation will provide a clearer view on the roles of ILC2 during colitis, tissue regeneration, fibrosis, and cancer.

AUTHOR CONTRIBUTIONS

SS wrote the first draft of the manuscript and figures. JT wrote sections of the manuscript and drafted the figures. YM conceived, supervised, revised the text and figures. TK and TT supervised the study. All authors contributed to the article and approved the submitted version.

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REVIEW
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Heterogeneity of ILC2s in the Lungs

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Group 2 innate lymphoid cells (ILC2s) are GATA3-expressing type 2 cytokine-producing innate lymphocytes that are present in various organs throughout the body. Basically, ILC2s are tissue-resident cells associated with a variety of pathological conditions in each tissue. Differences in the tissue-specific properties of ILC2s are formed by the post-natal tissue environment; however, diversity exists among ILC2s within each localized tissue due to developmental timing and activation. Diversity between steady-state and activated ILC2s in mice and humans has been gradually clarified with the advancement of single-cell RNA-seq technology. Another layer of complexity is that ILC2s can acquire other ILC-like functions, depending on their tissue environment. Further, ILC2s with immunological memory and exhausted ILC2s are both present in tissues, and the nature of ILC2s varies with senescence. To clarify how ILC2s affect human diseases, research should be conducted with a comprehensive understanding of ILC2s, taking into consideration the diversity of ILC2s rather than a snapshot of a single section. In this review, we summarize the current understanding of the heterogeneity of ILC2s in the lungs and highlight a novel field of immunology.

Keywords: group 2 innate lymphoid cells, heterogeneity, development, plasticity, memory, aging

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INTRODUCTION

Group 2 innate lymphoid cells (ILC2s) express transcription factor GATA3 and produce type 2 cytokines upon stimulation with epithelial cell-derived cytokines, including IL-33 and IL-25. ILC2s, which were first characterized by three different research groups in 2010 (1–3), are distributed throughout the body, such as in the lungs, skin, intestine, liver, brain, bone marrow, and peripheral blood in mice and humans (1–13). In particular, ILC2s play a critical role in innate immunity-mediated type 2 airway inflammation (1–8, 10–12) and contribute to the repair of airway damage *via* amphiregulin production after influenza virus infection (14). Recent studies have shown that ILC2s are associated with lung fibrosis, chronic lung obstructive disease exacerbation, and lung cancer (15–23). Thus, ILC2s have various functions and are involved in the pathogenesis of several lung diseases.

ILC2s are primarily tissue-resident cells with different characteristics depending on the tissues in which they exist (24–27). In mice, ILC2s express higher levels of *Il1rl1*, which encodes the IL-33 receptor subunit ST2, in the lungs than in other tissues (27, 28). Conversely, the expression levels of *Il17rb*, which encodes an IL-25 receptor subunit, and *Il18r1*, which encodes an IL-18 receptor subunit, are lower in ILC2s in the lungs than in the small intestine and skin, respectively (27). Indeed, intranasal administration of IL-33 potently activates ILC2s in the lungs compared with IL-25 or IL-18 (27, 29, 30). Interestingly, when IL-25 is administered intraperitoneally (but not

intranasally), an intestine-derived unique ILC2 subset (called inflammatory ILC2s [iILC2s]), which highly expresses KLRG1 and IL-17RB, migrates to the lungs (31). Moreover, recent single-cell RNA-seq analyses in mice and humans have revealed the existence of diverse cell populations among lung ILC2s (27, 32–38). Although ILC2s were previously considered to be a single-cell population, these results confirm the presence of different subsets within ILC2s. Therefore, it is necessary to dissect individual cell populations to evaluate their molecular mechanisms and relationships with diseases.

In this review, we provide a current overview of the heterogeneity of ILC2s in the lungs, and summarize our understanding of the diversity of ILC2s and their contribution to immunity in the lungs.

DIFFERENCES IN ILC2s ACROSS TISSUES AND HETEROGENEITY OF LUNG ILC2s AT STEADY STATE

ILC2s are distributed in various organs of the body at a steady state. A recent study comparing the expression of various genes in murine ILC2s in various tissues, such as lung, skin, adipose, skin, intestine, and bone marrow, showed that the expression levels of *Gata3* and *Il7r* do not differ among ILC2s in each tissue; however, hundreds of genes are differentially expressed by ILC2s depending on the localized tissue (27). In particular, lung ILC2s display increased expression of *Il1rl1*, while intestinal ILC2s highly express *Il17rb* as well as *Ahr* and *Nmur1*, which encode aryl hydrocarbon receptor (AHR) and neuromedin U receptor 1 (NMUR1), respectively (27, 38, 39). Indeed, intranasal administration of IL-33 potently activates lung ILC2s compared with IL-25, while genetic ablation of *Ahr* or *Nmur1* modifies anti-helminth immunity *via* intestinal ILC2s (38, 39). In addition, most skin ILC2s that express lower levels of *Il1rl1* and *Il17rb* also show increased expression of *Il18r1*, which encodes an IL-18 receptor subunit (27). Interestingly, the tissue-specific features of ILC2s are independent of either the microbiome or epithelial cell-derived cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). However, neuropilin-1 (*Nrp1*) was recently identified as a candidate gene that determines the tissue specificity of lung ILC2s and enhances the expression of IL-33 receptor (40). NRP1 can be induced postnatally and its expression is maintained in the lung environment by TGF-β signaling, resulting in the establishment of tissue specificity by lung ILC2s (40). These findings suggest that the diversity of ILC2s depends on the tissue in which they reside, and that acquisition of tissue specificity may begin very early in development (**Figure 1**).

During the development of ILC2s in the lungs, ILC progenitors migrate to the lung before birth and differentiate into ILC2s depending on IL-7 signaling (41). These prenatal ILC2s persist after birth, and are detectable in adult mice, however, they account for a small proportion and decrease further with age. After birth, acute expansion and activation of ILC2s occur in the lungs between 2 and 3 weeks of age, and these

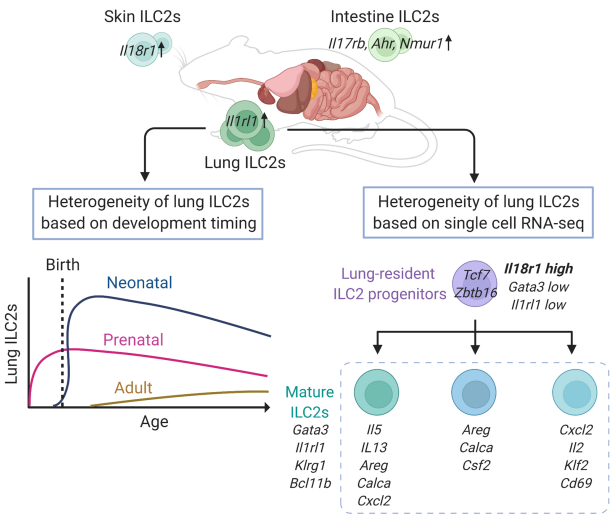

FIGURE 1 | Heterogeneity of lung group 2 innate lymphoid cells (ILC2s) in mice at steady state. Lung ILC2s have increased expression of *Il1rl1*, while intestinal ILC2s highly express *Il17rb*, *Ahr* and *Nmur1*, and *Il18r1* is enriched in skin ILC2s, respectively. Adult ILC2s in the lung have three developmental origins: prenatal, neonatal, and adult origin. The lung resident-ILC2 progenitor cells differentiate into at least three subtypes of mature ILC2s. This figure is created based on ref (27, 32, 41).

neonatal ILC2s have high expression of genes related to cytokine production, immunoregulation, and proliferation, such as *Il5*, *Il13*, *Nr4a1*, and *Mki67*, as well as *Cxcl2*, a neutrophil recruitment factor, which induces other immune cells into the lung and contributes to the development of the lung environment (41, 42). According to the fate-mapping approach, neonatal ILC2s are long-lived and tissue-resident and constitute the majority of lung ILC2s, even in adults (41). In adult mice, the proportion of neonatal ILC2s is gradually diluted by newly generated ILC2s, termed adult-derived ILC2s, and the adult lungs contain a diverse mixture of prenatal, neonatal, and adult-derived ILC2s. However, the turnover speed of ILC2s varies depending on the tissue and is slower in the lungs than in the skin, small intestine, and bone marrow. Therefore, the proportion of neonatal ILC2s is high in the lungs, and neonatal ILC2s play a major role in enhancing type 2 inflammation through local expansion in the lungs (41).

To assess the diversity of lung ILC2s at a steady state, a study performed single-cell RNA-seq analysis of lung ILC2s from adult mice, which revealed that lung ILC2s can be divided into several subgroups, including a group that highly expresses *Cxcl2*, *Il2*, *Klf2*, and *Cd69*; a group that expresses *Areg*, *Calca*, and *Csf2*; and a group that expresses *Il5*, *Il13*, *Cacla*, *Areg*, and *Cxcl2* (32). However, it is not clear whether these are groups of cells at different developmental stages or activation states. This study also identified a small number of ILC progenitors with low expression of *Gata3* and *Il1rl1*, and high expression of *Il18r1* in the lungs (32). These cells express ILC progenitor marker genes, such as *Tcf7* and *Zbtb16*, as well as genes that suggest a mixed lineage potential, such as *Rorc* and *Tbx21*. Although these

cells account for less than 2% of ILCs (Lin-IL7R+ cells), trajectory analysis suggests that these cells lose progenitor markers, such as *Cd7*, *Il18r1*, *Tcf7*, and *Zbtb16*, gradually increase the expression of *Gata3*, *Il1rl1*, *Klrg1*, and *Bcl11b*, and differentiate into mature ILC2s (**Figure 1**). Therefore, these data suggested that local ILC progenitors differentiated and matured towards ILC2s within the lung tissue (32).

While ILC2s are the dominant population of ILCs in murine lungs, percentage of ILC2s among lung-resident innate lymphocytes in human is around 30% (13, 35, 36, 43). Human ILC2s express *IL1RL1*, *IL17RB*, *KLRG1*, *GATA3*, and *PTGDR2*, which encode CRTH2, however, there is diversity in the expression of various genes depending on the localized tissues (33–36, 44). Specifically, human lung ILC2s show higher expression levels of *IL1RL1*, *IL17RB*, and *IL13* than tonsil and blood ILC2s (34–36). In addition, *SLAMF1*, *TNFRSF9*, *FFAR3*, and *PPARG* expression are upregulated in human lung ILC2s. SLAMF1 (CD150), encoded by *SLAMF1*, belongs to the signaling lymphocytic activation molecule (SLAM) family that modulates the activation of immune cells, including T cells (34, 45). *TNFRSF9* encodes the activation-induced surface receptor TNFRSF9, which was originally found in activated T cells (34, 46). *FFAR3* and *PPARG* are lipid metabolism-related genes that regulate the immune cells (47–49). Therefore, the upregulation of these four genes implies a tendency for cell activation. In contrast, human blood ILC2s show high expression levels of *PTGDR2*, *S1PR2*, and *CCR2*, which are migration markers (33, 34). While CRTH2 is one of the representative surface markers of human ILC2s, the expression of CRTH2 in blood ILC2s is downregulated after stimulation with a combination of IL-2, IL-25, IL-33, and TSLP, suggesting that CRTH2 expression is negatively correlated with ILC2 activation (34). Among lung ILC2s, approximately 35% of ILC2s lack CRTH2 expression (34). Based on these findings, human lung ILC2s may be relatively activated even in the steady state.

Although the diversity within human lung ILC2s has not been well studied due to their small number, a human study of single-cell RNA-sequence analysis using fetal samples following elective medical termination of pregnancy has been recently reported (33). In this study, fetal lung ILC2s were divided into five subgroups: Pre-ILC2s, CRTH2_ILC2s, PTGS2_ILC2s, CCR9_ILC2s, and KIT_ILC2s. Pre-ILC2s highly express *PRSS57* and *SPINK2*, which are associated with stem cell signaling and are suggested to be ILC2 progenitors. Pre_ILC2s were further divided into two subgroups according to the expression levels of *MKI67, PTGDR2*, and *BCL11B*, indicating that immature Pre_ILC2s differentiate into restricted ILC2 progenitors, thereby upregulating the expression of these three genes. CRTH2_ILC2s and PTGS2_ILC2s had high expression levels of *PTGDR2*, and can be considered as conventional ILC2s. In particular, CRTH2_ILC2s exhibited the highest expression levels of *IL1RL1* and *IL13*, and PTGS2_ILC2s expressed high levels of *PTGS2*, which encodes COX2. CCR9_ILC2s and KIT_ILC2s are unconventional ILC2s because of low expression levels of *PTDGR2*. CCR9_ILC2s are a rare population of lung ILC2s that express T cell marker genes, including *CD1E*, *CD2*, *CD3G*, *CD4*, and *CD8A*; however, their biological roles are unknown. KIT_ILC2s express *CCR6* and *LTB* and show plasticity, converting into IL-17^{+}ILC3-like cells, suggesting that KIT-ILC2s may be involved in ILC2 plasticity. Thus, it has been reported that there is diversity in the lung ILC2s of human fetuses; however, the relationship to disease and diversity in adults has not yet been determined.

HETEROGENEITY OF ACTIVATED ILC2s IN THE LUNGS

Various stimuli, such as allergens and viruses, induce the release of epithelial cell-derived cytokines, including IL-25 and IL-33. These cytokines enhance the phosphorylation of GATA3 *via* the NF-κB and MAPK signaling pathways in ILC2s (50), which induces cell proliferation and production of type 2 cytokines, such as IL-5 and IL-13. Although murine lung ILC2s express CD25, CD90.2, CD127, KLRG1, Sca-1, and ST2, the expression levels of these surface markers vary depending on the mouse strain and sex. Furthermore, they vary largely with the type of stimulation; intranasal administration of IL-33, house dust mite, or *Alternaria alternata* extract induced the different expression levels of surface markers, including CD25 and KLRG1, depending on the stimuli (51). In addition, a recent study evaluated the diversity of activated lung ILC2s in mice treated with IL-33 or IL-25 using single-cell RNA-seq analysis and flow cytometry (38). Intranasal administration of IL-25 or IL-33 increased the expression of *Il5*, *Il13*, *Klrg1*, *Arg1*, and *Areg* genes, as well as *Gp49* and *Batf*, and some subsets of ILC2s also increased MHC class 2 and CTLA4. Interestingly, *Nmur1* was highly expressed in lung ILC2s at the steady state and after IL-25 stimulation; however, it was downregulated by IL-33. Furthermore, the expression of semaphorin 4a was reduced by IL-33 stimulation. These results suggest that activated ILC2s can alter the expression of various genes and surface markers, depending on the stimulus.

Nippostrongylus brasiliensis (*N. brasiliensis*) causes migratory helminth infection in mice. Within hours of entry through the skin, *N. brasiliensis* stage 3 larvae migrate *via* the blood stream to the lung and alveolar space, causing local tissue damage and hemorrhage on days 1–2 post infection. Maturing larvae are then transported up the airways and swallowed to take up residence in the small intestine on days 4–7 post infection to allow adult reproduction before eventual expulsion. It has been reported that *N. brasiliensis* infection or an intraperitoneal injection of IL-25 induces a different subtype of ILC2s in the lungs and mesenteric lymph nodes, termed inflammatory ILC2s (iILC2s) (31). iILC2s are undetectable in the lungs at the steady state and have high expression of KLRG1 and IL-17RB; however, they have low expression of ST2 and Sca-1. Notably, iILC2s are circulating cells that arise from gut ILC2s residing in the intestinal lamina propria, and migrate to diverse tissues based on sphingosine 1-phosphate (S1P)-mediated chemotaxis (52). iILC2s contribute to the regulation of anti-helminth immunity by producing type-2 cytokines similar to lung ILC2s, that are called natural ILC2s

(nILC2s), to distinguish them from iILC2s. Furthermore, iILC2s express not only GATA3, but also intermediate levels of RORγt, suggesting that iILC2s carry out both nILC2-like and ILC3-like functions. Indeed, iILC2s produce IL-17 after an oral *Candida albicans* infection and contribute to antifungal immunity (31). Recently, IL-33 was reported to promote the generation of iILC2s *via* induction of tryptophan hydroxylase 1 (Tph1). Ablation of *Tph1* resulted in the impairment of iILC2 responses *via* modified expression of ICOS and increased susceptibility to *N. brasiliensis* infection (53).

Two studies investigated the heterogeneity of lung ILC2s during an *N. brasiliensis* infection using single-cell RNA-seq analysis (32, 54). In the first study, lung ILC2s were divided into four groups based on their gene clusters: resting nILC2s, *Il5*^{high}nILC2s, *Il13*^{high}nILC2s, and iILC2s (54). *Il5*^{high}nILC2 populations increased early after the *N. brasiliensis* infection (day 2), however, this proportion gradually decreased. On day 5, iILC2 populations transiently increased and became the major component of lung ILC2s. iILC2s, however, were undetectable on day 9, and *Il13*^{high}nILC2 populations increased thereafter (days 9 and 14) (**Figure 2**). Similarly, another study showed that the populations of ILC2s expressing *Calca*, *Csf2*, and *Cxcl2* increased on day 4 of the *N. brasiliensis* infection, whereas the populations of blood-derived circulating iILC2s expressing *Klrg1* increased on days 7 and 10 (32). On day 15, various subtypes of ILC2s were induced. Interestingly, fate mapping revealed that ILC2s on days 4 and 15 were differentiated from ILC progenitors in the lung rather than being replenished from the bone marrow. Thus, the diversity of ILC2s changes dynamically over time during *N. brasiliensis* infections.

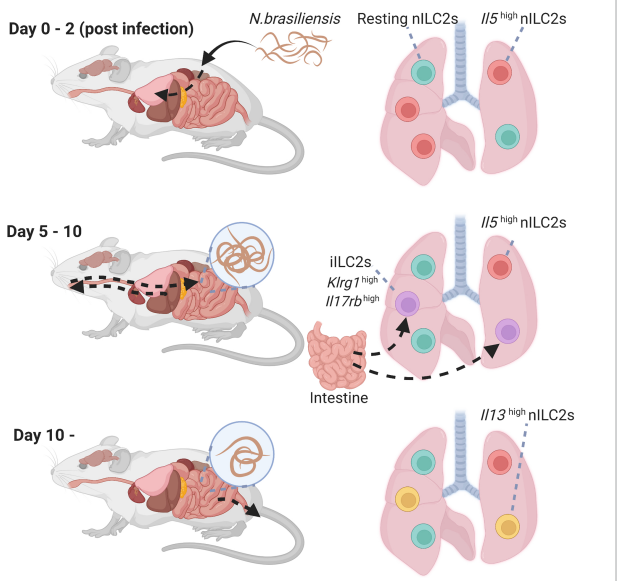

FIGURE 2 | Heterogeneity of lung group 2 innate lymphoid cells (ILC2s) during *N.brasiliensis* infection. Time course of activation of lung ILC2s in the lungs following a helminth infection. High levels of *Il5*^{high} natural ILC2s that appear shortly after the infection. Then, iILC2 populations transiently increased, and *Il13*^{high} natural ILC2 populations increased thereafter. iILC2, inflammatory type 2 innate lymphoid cell; nILC2, natural type 2 innate lymphoid cell; *N. brasiliensis*, *Nippostrongylus brasiliensis*.

Although there are no studies on the diversity of activated lung ILC2s in humans, a recent study reported that most resting ILC2s in humans express CD45RA; however, there is a large population of ILC2s expressing CD45RO in inflammatory mucosal tissues, including nasal polyps (55). The transcriptomic features of these cells were similar to those of mouse iILC2s, and peripheral blood-derived resting ILC2s expressed CD45RO in response to the stimulation. Thus, CD45RO may be a useful activation marker for human ILC2s.

As described previously, ILC2s produce type 2 cytokines in a GATA3-dependent manner upon stimulation with IL-33 or IL-25; however, their properties are known to be greatly altered by the surrounding cytokine environment. ILCs are divided into three groups according to their transcription factors and functions: ILC1s, ILC2s, and ILC3s; however, ILCs have plasticity, and can acquire the properties of other ILCs.

First, upon exposure to viruses, bacteria, or noxious agents, such as cigarette smoke, murine lung ILC2s convert to an ILC1-like phenotype with decreased expression of GATA3 and IL-33 receptor ST2, increased expression of T-bet, and the ability to produce IFN-γ (18). This conversion to an ILC1-like phenotype is mediated by stimulation with IL-1β, IL-12, or IL-18 (18, 56). Human ILC2s also acquire an ILC1-like phenotype *via* IL-1β, IL-12, and IL-18 (57, 58). Indeed, ILC1 populations are reported to increase in patients with chronic obstructive pulmonary disease (COPD), suggesting that ILC2s may be converted to ILC1s owing to the influence of cigarette smoke (18).

Second, a combination of IL-33 with leukotriene C4 or D4 can induce IL-17-producing ILC2s termed ILC2$_{17}$s in mice. Unlike Th17 cells or ILC3s, ILC2$_{17}$s are independent of RORγt expression (59). ILC2$_{17}$s produce IL-17, IL-5, and IL-13 by an intranasal administration of IL-33 or papain; however, Ahr-deficient ILC2s produce limited amounts of IL-17 since Ahr is essential for the induction of ILC2$_{17}$s. In humans, IL-2, IL-1β, IL-23, and TGF-β stimulation induces peripheral blood-derived ILC2s to convert to C-Kit^{+} NKp44^{-}ILC3-like cells that have the ability to produce IL-17 (60).

Recently, IL-10-producing ILC2s termed ILC2$_{10}$s were found in the lungs of mice after an intranasal administration of IL-33 or papain (61). In IL-33-treated mice, ILC2$_{10}$s represent a major proportion of IL-10-producing cells, and ILC2$_{10}$s can suppress activated ILC2s directly *via* IL-10. However, ILC2$_{10}$s also have the ability to produce type 2 cytokines upon stimulation by IL-33 and TSLP. Mechanistically, retinoic acid (RA) and IL-2 induce ILC2$_{10}$s, whereas TGF-β inhibits IL-10 production (62). RA also induces IL-10-producing ILC2s in human ILC2s, suggesting that RA plays an important role in ILC2$_{10}$s generation (61).

Together, ILC2s change their phenotype dramatically depending on the surrounding environment. These phenotypical changes may constitute a part of heterogeneity of ILC2s both in the steady state and activated state. However, while effector function of each phenotypical plasticity *in vitro* was reported, distinct role *in vivo* remained unclear. Therefore, further survey is awaited.

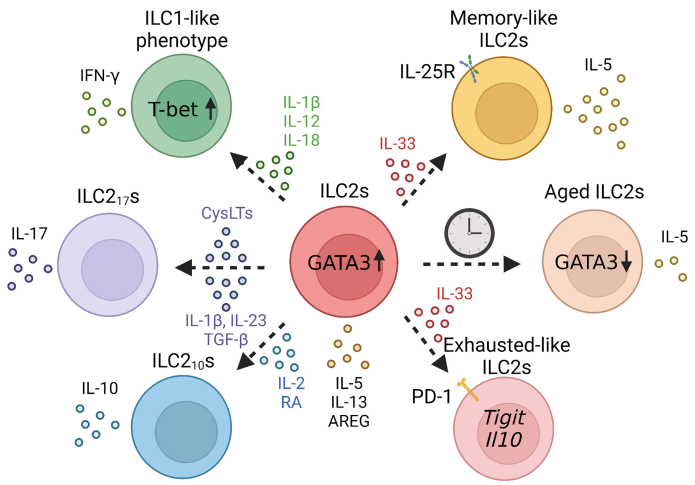

FIGURE 3 | Overview of plasticity and trained lung group 2 innate lymphoid cells (ILC2) in the lung.

HETEROGENEITY OF LUNG ILC2s POST INFLAMMATION

ILC2s are diverse even after they are activated once. Recent studies have shown that lung ILC2s can acquire immunological memory. In mice, some activated lung ILC2s mediated by an intranasal administration of papain or IL-33 have the ability to induce strong ILC2-mediated inflammation against secondary challenge. These cells exhibit immunological memory properties and are termed "memory-like ILC2s" (63). Memory-like ILC2s do not exhibit antigen specificity and respond strongly to a second stimulus regardless of the allergen type.

Memory-like ILC2s have some genetical similarities with activated ILC2s but they are resting cells unlike activated ILC2s. Memory-like ILC2s have higher expression levels of *Il1r2, Il5, Tnfsf18, Bcl2a1b, Bcl2a1d, Ler3*, *Syne1*, and *Il17rb* compared to those of naïve ILC2s, suggesting that memory-like ILC2s are activated. Since the expression of *Il17rb* is enhanced in memory-like ILC2s, they produce type 2 cytokines upon IL-25 stimulation, whereas naïve ILC2s do not respond to IL-25 stimulation alone (63).

Although *S1pr1, Il6st, Cd2, Cd7*, and *Sell* expression are lower in both activated ILC2s and memory-like ILC2s than naïve ILC2s, the expression of cell cycle-related genes including *Mki67* and *Ccnb2*, and the chemokine genes including *Ccl17, Ccl24, Cxcl3*, and *Ccl6* are lower in memory-like ILC2s compared to that in activated ILC2s, suggesting that memory-like ILC2s do not proliferate or produce chemokines as much as activated ILC2.

On the contrary, repetitive stimulation induces hyporesponsive phenotypes of ILC2s, which are termed "exhausted-like ILC2s" (64). This subset of ILC2s expresses high levels of *Il10* and *Tigit*, which are considered exhaustion markers. In addition, they express higher levels of PD-1, GITR, and KLRG1 than those in naïve ILC2s. In contrast to ILC2$_{10}$s, exhausted-like ILC2s are incapable of producing type 2 cytokines. A previous report showed that exhausted-like ILC2s could be collected from mice intranasally instilled with 100 μg papain every three days (64). On day 7 after the administration of the three papain doses, exhausted-like ILC2s emerged in bronchoalveolar lavage (BAL) fluid only. In addition, papain administration every three days for a month induced the generation of exhausted-like ILC2s both in the BAL fluid and lung. Thus, the intensity or duration of stimulation changes the fate of lung ILC2s from the acquisition of immunological memory to the loss of functional exhaustion.

HETEROGENEITY OF LUNG ILC2s IN AGING

Senescence is characterized by the progressive loss of physiological function in individuals with age (65, 66). DNA damage throughout life induces "cellular senescence", resulting in a poor proliferative capacity and irreversible cell cycle arrest, and results in a proinflammatory senescence-associated secretory phenotype (SASP) that leads individuals to low-grade, chronic inflammatory state termed as "inflamm-aging (67–71)". In mice, there is a marked increase in the expression of ILC2 progenitors in the bone marrow of aged (19–24 months old) mice compared with that in young (2–3 months old) mice; however, this increase was not observed in their progenitor cells, common helper-innate lymphoid progenitors (CHILPs) (72). Notch signaling could be involved in this increase in ILC2 progenitor expression with age; this pathway is specific to aged mice and is not involved in ILC2 progenitor differentiation in young mice. While the expression levels of ST2 are lower in aged ILC2 progenitors, young and aged ILC2 progenitors have the same expression level of Ki67, indicating that ILC2 progenitors of aged mice preserve the reproductive activity (72). In accordance with the increase in ILC2 progenitor expression in the bone marrow, ILC2

progenitor populations in the peripheral blood and ILC2 populations in the peripheral blood and small intestine also increase with aging. However, since bone marrow ILC2 progenitors are rarely transferred to the lung and lung ILC2s are highly dependent on local expansion of lung-resident ILC2 progenitors, lung ILC2 populations are reduced with aging.

Functionally, aged lung ILC2s show decreased expression of *Gata3*, *Il5*, and *Areg*, and reduced proliferative capacity and cellular function. Indeed, aging mice are susceptible to viral infections; however, transplantation of young mouse-derived ILC2s promotes recovery from viral infections (72). Furthermore, the expression of *Ehhadh* and *Cyp2e1*, which are involved in peroxisome proliferator-activated receptors (PPAR) pathway and cytochrome P450 (CYP) activity, respectively, is downregulated in aged ILC2s, and these genes enhance IL-5 production and Areg expression independent of GATA3 involvement (72). In addition, the levels of IL-12 and IL-18, that are able to suppress Cyp2e1 expression, were increased in the lungs of aged mice, suggesting that both intrinsic and extrinsic mechanisms cause cellular senescence in lung ILC2s (72).

The effects of aging on ILC2s in humans are poorly understood. In contrast to mice, peripheral blood ILC2 populations decrease with age in humans (73). Lung ILC2 populations in humans may also be negatively correlated with aging, similar to murine lung ILC2s (35). However, studies on human lung ILC2s are not sufficiently large to allow the estimation of this trend.

DISCUSSION

Since the discovery of ILC2s about a decade ago, a large number of studies on ILC2s have been carried out, and these studies have greatly contributed to our understanding of the immunology and unraveling of the underlying pathophysiology of various human diseases. Various molecules and regulators of ILC2s have been reported, and the general features of ILC2s are widely understood. However, ILC2s have received increasing attention in the last few years because they comprise a heterogeneous cell population rather than homogeneous.

Basically, ILC2s are tissue-resident cells, and the expression levels of transcription factors and receptors of ILC2s vary among tissues. Murine lungs consist of ILC2 progenitor cells and mature ILC2s at different developmental time points in the steady state, and tissue specificity of ILC2s develops postnatal due to the lung tissue environment. Lung mature ILC2s are not only activated by IL-33 due to the high expression of IL-33 receptors, but also by other stimuli, including IL-25 and allergens. However, besides "activation," changes in the expression of surface antigens and transcription factors vary depending on the stimulus that "activate" ILC2s, and in particular, migratory helminth infection or an intraperitoneal administration of IL-25 transiently induces intestinal-derived iILC2s in the lungs.

Recently, it has been reported that ILC2s have plasticity, and their properties are variable, such as the production of IFN-γ, IL- 10, and IL-17, depending on the surrounding environment, which also contributes to the diversity of ILC2s. While the plasticity is important and a well-known concept of ILC2s, its immunological role is unclarified. Moreover, boundaries between the concept of plasticity and heterogeneity of ILCs are unclear. Furthermore, after activation, ILC2s develop training immunity; however, they sometimes cause cell exhaustion. These diversities change with the aging time scale, and cellular senescence is induced by intracellular and extracellular factors in the lungs (**Figure 3**).

Despite the efforts of many studies to elucidate the diversity of lung ILC2s, several questions remain unclear: Does the diversity of lung ILC2s play a distinct physiological role *in vivo*? If so, what would happen if lung ILC2s are homogenous and lack plasticity? Does the transcriptional heterogeneity of ILC2s, especially at steady state have a unique biological role or does it merely reflect differences in mRNA expression? Additionally, does each ILC2 subtype affect lung immunity and have clinical significance in humans?

Although human studies are also insufficient, the studies summarized in this review contribute to our understanding of disease conditions in humans. The acquisition of immunological memory may contribute to allergen-specific asthma exacerbation and non-specific allergic inflammation. Exhaustion and aging of ILC2s may limit the type 2 inflammation response, especially in geriatric patients, because these changes attenuate the effector function of ILC2s. Therefore, a greater understanding of ILC2 subsets may provide new insights into the pathophysiology of lung diseases, including asthma. In addition, targeting specific ILC2 subsets may provide a new therapeutic strategy for lung diseases.

To date, most studies have only taken a snapshot of ILC2 diversity. Therefore, diversity among ILC2s should be considered in future investigations. It is expected that future research exploring the heterogeneity of ILC2s will shed light on a variety of life science mysteries.

AUTHOR CONTRIBUTIONS

MA and HK screened articles related to this topic and wrote the manuscript. KF reviewed the manuscript critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Tissue-Specific Diversity of Group 2 Innate Lymphoid Cells in the Skin

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Since the discovery of group 2 innate lymphoid cells (ILC2s), their developmental pathways, mechanisms of activation and regulation, and immunological roles in the steady state and in disease have been reported in various organs. ILC2s, which produce large amounts of IL-5 and IL-13 in response to tissue-derived factors and are essential in inducing and promoting allergic inflammation, have also been found to play multifaceted roles in maintaining tissue homeostasis. While T cells respond to foreign antigens, the activation of ILC2s is regulated by various tissue-derived factors, including cytokines, lipids, hormones, and neurotransmitters, and ILC2s show different phenotypes depending on the tissue in which they are present. In this review, we discuss tissue-specific characteristics of ILC2s in the skin. ILC2s, as defined in the lungs, intestinal tract, and adipose tissue, cannot be directly applied to cutaneous ILC biology, because skin ILC2s exhibit different aspects in the expression patterns of cell surface markers, the response to tissue-derived cytokines and the functions in both steady-state and inflammation. The skin contains ILCs with features of both ILC2s and ILC3s, and the plasticity between ILCs complicates their characters. Furthermore, the epidermis, dermis, and subcutaneous tissues contain ILCs with different characteristics; their localization has expanded our understanding of ILC function. Single-cell RNA-seq technology has further elucidated the role of ILCs in human skin and disease pathogenesis. Overall, this review discusses the phenotypical and functional heterogeneity of skin ILCs reported in recent years and highlights future directions within the field of ILC biology.

Keywords: skin, ILC2, diversity, allergy, dermatology

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INTRODUCTION

The skin is the largest barrier organ in the body and harbors a variety of innate and adaptive immune cells with diverse functions. Langerhans cells, γδT cells, and memory T cells are tissue resident immune cells and have been reported to communicate with keratinocytes and other stromal cells, playing crucial roles in both inflammation and tissue maintenance in the skin. Innate lymphoid cells (ILCs), a previously unappreciated family of innate immune cells, have provoked a

paradigm shift in our understanding regarding the roles of tissue-resident innate immune cells in the homeostatic maintenance of tissue physiology and in the induction, regulation, and resolution of inflammation (1).

Group 2 ILCs (ILC2s) were first reported as cells that produce type 2 cytokines, such as IL-5 and IL-13, in response to IL-33 and IL-25 in the adipose tissue and small intestine during helminth infection (2, 3). Subsequently, ILC2s were found to be present in various organs, including the lungs, tonsils, liver and muscle where they play an essential role in the development of allergic inflammation, tissue repair, and regulation of metabolism (4). ILC2s are also found in the skin (5–7). Skin, lungs and intestines are barrier organs, continuously exposed to different external stresses (mechanical and chemical) and invading microorganisms; thus, they have developed specific response mechanisms against various stimuli. The highly sophisticated tissue specificity of ILC2s is not surprising.

Important trademarks for defining ILC2s include the expression of the master transcription factor GATA3 and the ability to produce the type 2 cytokines, such as IL-5 and IL-13. However, the detection of ILC2-specific cell surface markers is used in a more versatile method in flow cytometry analysis. Lung ILC2s have been expansively researched over the past decade. They can be clearly separated from other ILCs by the expression of IL-33R (ST2). The lineage$^-$ Thy1.2$^+$ ST2$^+$ population has been commonly used for the stable detection of typical ILC2s in lungs by flow cytometry. In contrast, the definition of skin ILC2s varies from study to study, which has resulted in confusion. Early studies on skin ILC2s used ST2 as a cell surface marker for their detection (5). However, the number of ILC2s detected using ST2 as a marker was very low in the lineage$^-$ Thy1.2$^+$ population, and ILC2 research in the skin has been greatly hampered by the incomplete characterization of ILC2s. A recent comprehensive analysis of skin ILC subsets revealed that only a small fraction of ILC2s in the skin expresses ST2, and the true nature of skin ILC2s is being gradually revealed (8, 9).

TRUE CHARACTERIZATION OF SKIN ILC2

Advances in unsupervised transcriptome analysis have facilitated the detailed dissection of unknown immune cells. In particular, single-cell RNA-seq analysis has proven to be a powerful tool to comprehensively understand the characteristics and heterogeneity of immune cells in which cell surface markers have not been well defined. Single cell RNA-seq analysis of ILCs in different tissues, including the skin, lungs, and gut of mice expressing reporter alleles for IL-5 (*Red5*), revealed clear segregation of ILC2s by tissue, suggesting unique, tissue-specific characteristics of ILC2s (8). While *Gata3*, *Il7r*, and *Crlf2* (which encodes the TSLP receptor subunit TSLPR) are broadly expressed in ILC2s across tissues, expression of *Icos*, *Ccr6*, and *Itgae* is highly enriched in skin ILC2s. In contrast to high expression of *Il1rl1*, which encodes IL-33R, in lung and adipose tissue ILC2s, skin ILC2s show only minimal expression of *Il1rl1*. In addition, the expression of *Il17rb*, which encodes IL-25R, is abundant in gut ILC2s. These data demonstrate the tissue-organizing transcriptome identities of ILC2 subsets and differential expression of cell surface markers of ILC2s in each organ and also suggest the variable cytokine dependency of ILC2 activation.

Consistent with the analysis of IL-5-producing ILC2s, analysis of IL-13 reporter mice (*Il13*-DsRed) revealed that IL-13-producing skin ILC2s expressed ICOS and CD44, whereas expression of ST2, CD25, and KLRG1 was low (10). Half of IL-13-producing cells among skin immune cells are ICOS$^+$ ILC2s, and the steady state production of IL-13 is necessary for the development of CD11b^{low} type 2 dendritic cells and for the induction of allergic Th2 responses. The results of two studies showed that IL-5 and IL-13-producing skin ILC2s express ICOS, but not ST2. It should be noted, however, that ICOS is also expressed in other subsets of skin ILCs (9, 10).

Cytokine expression of ILC2s has been analyzed using various methods including *ex-vivo* stimulation (PMA+Ionomycin Ior specific cytokines), reporter mice and RNAseq of sorted cells; each method has advantages and disadvantages. Whereas *ex-vivo* stimulation forces cells to produce cytokines, steady state production of cytokines can be evaluated in reporter mice. L-13 expression was detected in both *ex-vivo* stimulation (8, 9) and reporter mice (*Il13*-DR and *Smart13*) (8, 10), suggesting production of IL-13 from ILC2s in steady state and activation state.

The fact that ILC2s express various cytokine receptors in different tissues is important when considering the development of therapies based on monoclonal antibodies for the treatment of allergies. While the use of anti-IL-4/IL-13 receptor antibodies has shown dramatic response in the treatment of atopic dermatitis (11), the failure of an anti-IL-33 treatment has suggested that IL-33-ILC2 axis may not be central in the pathogenesis of the disease. However, given that the majority of ILC2s in the skin do not express the IL-33 receptor, this result could have been expected. The efficacy of an anti-IL-33 therapy for the treatment of asthma was adequate but slightly weaker than that of anti-IL-4/IL-13 receptor antibodies, suggesting that consideration of the expression of cytokine receptors by ILC2s is essential for effective treatment (12).

ICOS expression has also been observed in migrating skin ILC2s (13). Tracking the migration of ILC2s in IL33tg-Kikume Green-Red mice revealed the migration of ILC2s from the skin to lymph nodes in the model of IL-33-induced dermatitis. This indicated that ICOS was expressed in both resident and migrating ILC2s, whereas CD103 expression was limited to skin-resident ILC2s. ICOS is a costimulatory molecule belonging to the CD28 superfamily and the importance of ICOS: ICOS-ligand interactions for the survival and cytokine production of ILC2s have been described in the lungs (14). However, the role of ICOS in skin ILC2s requires further investigation.

LAYER-SPECIFIC COMPARTMENTALIZATION OF SKIN ILC SUBSETS

The skin exerts its barrier function through a multilayered structure comprised of three distinct anatomical compartments: the

epidermis, dermis, and subcutis (or subcutaneous tissue). Each of these layers have distinct physiological functions and harbor resident immune cells. The epidermis, the outermost layer of the skin and the first line of physical and immunological defense, contains a unique subset of T cells and dendritic cells; $V\gamma5^+$ γδT cells (called dendritic epidermal T cells (DETCs) based on their morphological appearance) and Langerhans cells, respectively. The dermis primarily consists of dense connective tissues and contains an abundance of immune cells including αβ and γδ T cells, macrophages, dendritic cells, and mast cells. The subcutis is rich in adipose tissue and contains immune cells that interact with adipocytes. The layer-specific distribution of immune cells indicates that the immunological microenvironment differs in each layer, and it is therefore not surprising that ILC2s, which highly depend on tissue-derived factors, adapt their cellular phenotypes to the location in which they reside.

Analysis of ILCs in each skin layer revealed compartment-specific characteristics of ILCs (9). The ILC2 subsets in dermis and subcutis display different profiles. ILC2s that express ST2, CD25, Sca-1, and KLRG1, which are expressed on typical ILC2s in lungs, are enriched in the deepest layer of the skin, the subcutis. Considering that ILC2s contribute to adipose tissue metabolism (15, 16), the enriched distribution of ILC2s in subcutaneous tissue, which display a high adipose content, can be expected. Although the dermis is generally a major target for analysis in dermatology and subcutaneous tissue is removed from skin when the back skin of mice is analyzed, it can be an important reservoir for skin ILC2s. $ICOS^+$ $CCR6^+$ ILCs, on the other hand, are located in both the epidermis and dermis and exhibit ILC2 characters. Epidermal ILCs also display ILC3-like signature gene expression, such as *Rorc*, *Lta*, and *Tcf7*, in both bulk and single-cell RNA-seq analysis. ILC3s are abundant in mucosal tissues, the gut, and oral mucosa, which are constantly exposed to both resident and infectious microbes. IL-22-producing ILC3s are essential for the innate immune responses to fungi and extracellular bacteria as they promote the production of antimicrobial peptides from epithelial cells (17–20). The skin is also inhabited by microbiota and this balance needs to be properly maintained. Epidermal ILCs produce TNF and lymphotoxins and regulate the steady-state balance of the skin surface microbiota by inhibiting antimicrobial lipid production from the sebaceous glands (9). Although epidermal ILCs are ILC3-like cells as mentioned above, they express ILC2-signature genes such as *Il13* and *Il2*. Furthermore, while both epidermal and dermal ILCs express ICOS and CCR6, analysis of IL-5 and IL-13 reporter mice has revealed preferential expression of ICOS and CCR6 in skin ILC2s (8, 10). Therefore, it is probably difficult to divide cell types by the expression of cell surface markers, and the anatomical location may endow ILCs with unique site-specific functions. Despite of accumulating knowledge of ILC2s in murine skin, little is known about localization and cell surface markers of ILC2s in human skin. Immunohistochemical analysis of distribution of ILCs in human skin revealed ILC populations, mainly ILC1s and ILC3s in the upper dermis of healthy skin, and increased numbers of ILC2s in atopic skin (21). While increase of ILC2s in skin of patients with atopic dermatitis has been reported in other studies (7, 22, 23), their localization has not been extensively analyzed.

VARIETY OF ILC2 ACTIVATING CYTOKINES

The skin ILC2 landscape which has been unveiled by single cell transcriptome analysis not only facilitates flow cytometry detection of ILC2s by cell surface molecules, but also provides information regarding the cytokines that can activate ILC2s. In contrast to adaptive immune T lymphocytes, which require TCR-MHC class II interactions for their activation, ILC activation is directly triggered by tissue-derived factors that mount antigen-independent immune responses. Consistent with the marked expression of IL-33R in lung ILC2s, IL-33 is a key ILC2-activating cytokine in allergen-induced asthmatic airway inflammation (24, 25). As ST2 expression is limited to a subset of ILC2s located in subcutaneous adipose tissue (9), other cytokines may be involved in epidermal and dermal ILC2 activation.

The mechanism of ILC2 activation has been investigated in an atopic dermatitis model in which calcipotriol (MC903), a vitamin D3 analog, was applied to mouse skin. The results indicated that T lymphocytes were not necessary in MC903-induced dermatitis, but inflammation was markedly improved when ILC2s were removed by the administration of antibodies against CD25 or CD90.2 (5). Furthermore, deletion of RORα, which is necessary for the ILC2 development, significantly improved MC903-induced dermatitis (7), indicating that ILC2s are essential for MC903-induced atopic inflammation.

In a study using the same model, Kim et al. found that TSLP receptor-deficient ($Tslpr^{-/-}$) mice showed a markedly reduced response of ILC2s and improvement in MC903-induced skin inflammation, whereas disruption of IL-33 ($Il33^{-/-}$) or IL-25R ($Il17rb^{-/-}$) did not affect the ILC2 responses (5). In contrast, Salimi et al. reported that deficiency of IL-25R ($Il17rb^{-/-}$) or IL-33R ($Il1rl1^{-/-}$) significantly reduced both dermatitis and ILC2 activation (7). Mice overexpressing the IL-33 gene (*Il33*) in keratinocytes (hK14mIL33tg) were also shown to develop atopic-like dermatitis with a significant increase in ILC2s. In this study, ILC2 depletion by *RORα*-deficient bone marrow transplantation markedly improved dermatitis, indicating that keratinocyte-derived IL-33 causes atopic inflammation through ILC2 activation (26). Furthermore, a mouse model of antigen-driven allergic skin inflammation showed different aspects of ILC2 response. Dermatitis induced by epicutaneous sensitization with ovalbumin was significantly reduced by keratinocyte-specific IL-25 depletion (*K14*-Cre $Il25^{flox/flox}$). A study using IL-13 reporter mice showed that ILC2s serve as a major source of IL-13 and that *Rora*-Cre $Il17rb^{flox/flox}$ mice, which selectively lack IL-25R expression in ILC2s, demonstrate significantly decreased OVA-induced inflammation, suggesting that IL-25 acts directly on skin ILC2s to promote IL-13 production (27).

Unbiased transcriptomic analysis also revealed that skin ILC2s preferentially express the gene encoding the IL-18

receptor 1 (*Il18r1*) and that supplementation with TSLP and IL-18 induces a large amount of IL-13 production from skin ILC2s *in vitro*. In addition, IL-13 production in MC903-induced dermatitis was significantly reduced in IL-18-deficient mice (8). Consistent with this report, mice overexpressing murine *Il18* in keratinocytes under the control of the human keratin 14 promoter (KIL-18Tg) developed atopic dermatitis-like inflammation and type 2 inflammation independent of IgE/IgG1, suggesting that IL-18 induces type 2 inflammation through activation of ILC2s (28). On the other hand, in the lungs, Il18r1$^+$ Tcf7$^+$ ILCs exhibit capabilities of progenitors that can differentiate into ST2$^+$ ILC2s and ST2$^-$ non-ILC2s (29). It is not yet understood whether IL-18R$^+$ skin ILC2s also have progenitor-like properties, and how tissue-specific effects of IL-18 in skin and lung ILC2s are established.

Skin ILC2s exhibit different cytokine reactivity depending on the dermatitis models. Further, mouse genetic background may influence the results; TSLP for ILC2 activation in MC903-inflammatin was evaluated in the C57BL/6 strain (5), whereas the IL-25 and IL-33 were analyzed in the BALB/c strain (7). It is also unclear whether ILC2s that are responsive to IL-33, IL-25, TSLP, and IL-18 belong to different subsets. Therefore, it may not be feasible to identify a single cytokine that activates skin ILC2s. ILC2s can be flexibly activated in response to changes in the surrounding environment and various external factors that invade the skin. It would thus be interesting to determine the conditions under which TSLP, IL-33, IL-25, and IL-18 are produced by epithelial and other stromal cells, leading to the activation of ILC2s, and whether ILC2 stimulated by different cytokines contribute to different aspects of innate immunity. Furthermore, it should be noted that the receptors for these cytokines are expressed in various immune cells including T cells, basophils, and macrophages. For example, TSLP elicits IL-4 production of basophils, which subsequently promotes ILC2 responses (22). Therefore, it may not be sufficient to understand only direct action of cytokines on ILC2s; instead, it is important to reveal a network of cytokines in different subsets of immune cells.

Nevertheless, because these cytokines serve as therapeutic targets for the treatment of atopic dermatitis and the monoclonal antibodies to these cytokines have been studied in clinical trials (30), it is important to investigate their differential roles and redundancies in ILC2 activation. In translational research, we need better understanding of the receptor expression profiles on ILC2s in human skin.

MIXED ILC2–ILC3 PROFILES IN THE SKIN

Effector conversion among ILC subtypes during inflammation also contributes to the diversity of ILC2 composition, allowing flexible immune functions in the skin. ILCs are highly plastic and can differentiate from one subset into another. For example, human fetal intestine ILC3s can differentiate into ILC1s under the influence of IL-12 (31). IL-12 also induces the conversion of IL-1β activated human peripheral blood ILC2s to ILC1s (32, 33). Human tonsil ILC1s can also differentiate into ILC3s in the presence of IL-2, IL-23, and IL-1β (34). Immunological flexibility among ILC subsets may enhance tissue resilience but may simultaneously complicate disease pathology.

To characterize the potential transitions of skin ILC subsets, a study has been performed, which combines longitudinal scRNA-seq, scATAC-seq, *in vitro* experiments, and *in vivo* fate mapping in mouse models of psoriasis by injection of IL-23 or treatment with imiquimod (35). Since psoriasis is an IL-17-mediated inflammatory skin disease, increased ILC3s have been found in blood and skin of the patients (36, 37). In this study, IL-23 was found to trigger the conversion of tissue-resident skin ILCs, including quiescent-like cells and ILC2s, toward ILC3-like cells which are characterized by the co-production of IL-13 and IL-22 or IL-13 and IL-17A. This suggests effector transitions of ILCs and adaptation of mixed ILC2-ILC3 states. IL-23 is known to play a pivotal role in the expansion and survival of Th17, which has been considered a central effector in the pathogenesis of psoriasis (38). The conversion of ILC2s to IL-17-producing ILC3-like cells by IL-23 could be another important factor in this disease. Moreover, these findings underlined the limitations of the current standard experimental approaches that simply divide ILCs into three groups based on transcription factors and cytokine production and treat ILCs as discrete types. Tissues may have more partially committed cells that contribute to the flexibility of ILC responses.

Parasitic helminth infection or systemic IL-25 administration can induce a subset of migratory ILC2s that preferentially express the IL-25 receptor but not ST2. These cells that co-express GATA3 and RORγt, and have the capacity to produce IL-17A, have been referred to as inflammatory ILC2s (iILC2s) (39). In the skin, a subset of ILCs, which express both GATA3 and RORγt and produce IL-5, IL-13, and IL-17, has also been described in the inflammatory condition. Analysis of mice that exhibited hair loss revealed that microbial dysbiosis triggered ILC2-mediated inflammatory destruction of hair follicles. iILC2s circulated in an S1PR1-dependent manner and infiltrated the skin *via* the CCL20-CCR6 axis (40). Although the impact of iILC2s has been demonstrated in anti-helminth immunity (41), the contribution of iILC2s, particularly the potential plasticity in skin inflammation, is yet to be investigated. Co-production of type 2 and 3 cytokines in iILC2s would be of great interest to better understand mixed type of immune responses in inflammatory diseases.

A subset of skin ILCs that express CCR6 and RORγt and expand under inflammatory conditions have also been reported upon chronic ultraviolet exposure. These UV induced-ILC3s produce IL-22 and are associated with UV-induced keratinocyte carcinogenesis (42). Although ICOS is highly expressed in skin-resident ILC2s, UV-induced RORγt$^+$ ILC3s also expressed ICOS. It is unclear whether ICOS$^+$ ILC2s can be converted into ICOS$^+$ ILC3s and whether they share the characteristics of iILC2s. It would thus be interesting to study the fate of ILC2s under different inflammatory conditions and the factors that drive conversion of ILC phenotypes.

Mixed ILC2–ILC3 profiles are more evident among human skin ILCs. Isolated human dermal CD127$^+$ CRTH2$^+$ ILC2s

produce IL-17 after exposure to the hyphae of *Candida albicans* present in cell suspensions cultured from dermal samples (43). In the human peripheral blood, c-Kit$^+$ CCR6$^+$ ILC2s express RORγt at the baseline and produce IL-17 after exposure to IL-1β and IL-23, which is associated with differentiation of ILC3s. Furthermore, IL-17-producing ILC3s found in lesions of psoriasis, a type 3 immune-mediated inflammatory skin disease, have been found to switch back to ILC2s. Phenotypic switching of ILC2s to ILC3-like cells and of ILC3s to ILC2s may explain the highly plastic nature of human ILC2s.

Single-cell RNAseq analysis of human skin in atopic dermatitis further revealed the heterogeneous composition of human skin ILC2s (23). The results indicated that a discrete ILC2 cluster expressing *GATA3* and *RORA* was segregated from the NK, ILC1, and ILC3 clusters. ILC2s co-expressing ILC3 markers *RORC* and *AHR* were found in healthy human skin and these co-expressing cells were increased in atopic skin. Consistent with the expression of transcription factors, atopic ILC2s expressed type 2 cytokines (i.e. *IL5* and *IL13*) as well as type 3 cytokines (i.e. *IL22* and *IL26*). Th2/Th17/Th22 cytokine elevation has been observed in a subset of patients with atopic dermatitis, and the IL-23-IL-17 axis and IL-22 have been targeted by monoclonal antibodies for the treatment of atopic dermatitis (44, 45). The presence of ILC2s co-producing type 2 and 3 cytokines in atopic skin might therefore explain mixed cytokine profiles in patients and indicates that targeting type 2 cytokines alone might not be sufficient for the effective treatment. The mechanisms of coexisting ILC2 and ILC3 features in a single cell type, and the upstream signals that trigger and sustain the mixed status, need to be determined.

CONCLUSION

Over the past decade, our knowledge of ILC2s in the lungs and intestinal tract has been considerably expanded. In contrast, our understanding of the biology of ILC2s in the skin remains limited. ILCs have been commonly shown to demonstrate highly tissue-specific cellular characteristics that allow them to adapt to their surrounding environment. ILC2s play an important role in enhancing immune fitness of tissues locally by producing appropriate cytokines and modulating other immune and non-immune cells. This mini review discusses the highly heterogeneous features of skin ILC2s (summarized in **Figure 1** and **Table 1**). The heterogeneity and plasticity of skin ILC2s enable tissue-specific adaptation and might be related to the complex pathogenesis of inflammatory skin diseases, such as atopic dermatitis and psoriasis. Therefore, future research should not only use the experimental approaches that define ILCs as discrete subsets by cell surface markers, but rather require global and comprehensive analyses considering that ILCs are more diverse than previously estimated. Flow cytometry and microscopic analysis have accelerated the classification of immune cells over the past years. However, the limited numbers of parameters and our prior knowledge of cell-type definition have always posed as limiting factors in the study of the immune system complexity. Technological advancements of single cell analysis in

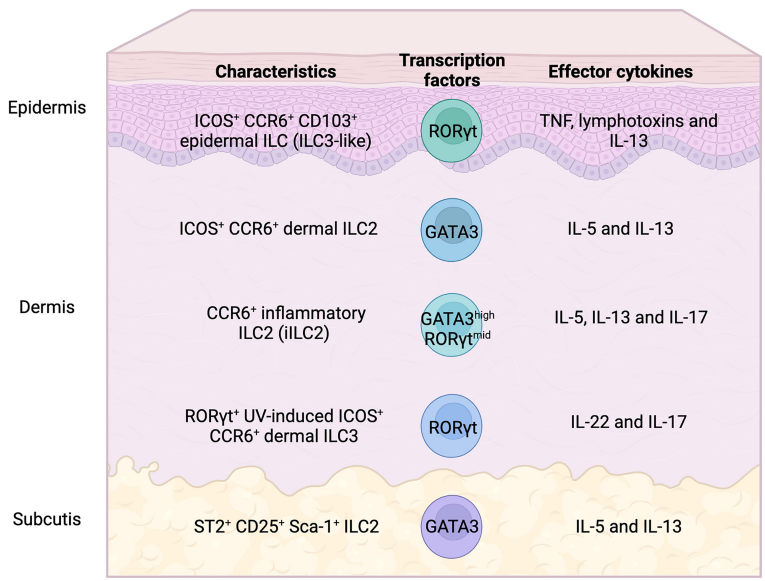

FIGURE 1 | Heterogeneity of skin ILC subsets in mice. Skin ILC2s, which produce IL-5 and IL-13, express ICOS and CCR6, but not ST2 (IL-33 receptor), and play an important role in atopic and allergic inflammation. ST2$^+$ ILC2s are only found in subcutaneous adipose tissues. Epidermal ILCs also express ICOS and CCR6, display the characteristics of both ILC2 and ILC3, and regulate microbiome balance. Inflammatory ILC2s that express both GATA3 and RORγt produce IL-5, IL-13, and IL-17, which infiltrate the tissues under inflammatory conditions. IL-22- and IL-17-producing RORγt+ ILC3s, which expand in UV-induced inflammation, also express ICOS and CCR6.

TABLE 1 | Heterogeneity of skin ILC2.

Cell identity			
Cell types	**Detection**	**Characteristics**	**References**
Mouse			
ILC2	Flow cytometry	Detected by expression of ST2 and CD25	(5, 46)
		Detected by expression of IL-7R, ICOS and c-Kit	(7)
IL-13 producing ILC2	Flow cytometry in IL-13 reporter mice		(6)
IL-5 producing ILC2	Single cell and bulk RNAseq in IL-5 reporter mice	High expression of *Gata3*, *Il7r*, *Crlf2*, *Icos*, *Ccr6*, *Itgae*, *Il18r1*, low expression of *Il1rl1*	(8)
IL-13 producing ILC2	Flow cytometry in IL-13 reporter mice	High expression of ICOS, low expression of ST2	(10)
Epidermal ILC	Single cell and bulk RNAseq and flow cytometry	High expression of CCR6, CD103, *Rorc*, *Lta*, *Il13* (ILC3-like)	(9)
Dermal ILC		High expression of ICOS, *Gata3*	
$ST2^+$ ILC2 in subcutis		High expression of ST2, CD25, Sca-1, KLRG1, *Gata3*, *Il5*	
Inflammatory ILC2	Single cell RNAseq and flow cytometry	Expression of GATA3, RORγt, CCR6, production of IL-5, IL-13 and IL-17A	(40)
Human			
ILC2	Flow cytometry	Detected by expression of ST2 and CD25	(5, 22)
		Detected by expression of IL-7Ra and CRTH2	(7)
ILC2	Immunohistochemistry	Expression of GATA3, IL-7Ra and CRTH2	(21)
ILC2	Single cell RNAseq	Expression of *GATA3* and *RORA*	(23)
Mixed ILC2 and ILC3		Coexpression of *GATA3*, *RORC*, *AHR*, *IL5*, *IL13*, *IL22*, *IL26* (increase in atopic skin)	
Cytokine response			
Activating cytokines	**Mice**	**Models**	**References**
TSLP	*Tslp*$^{-/-}$ (C57BL/6)	MC903 atopic-like dermatitis	(5)
IL-33	*Il1rl1*$^{-/-}$ (BALB/c)	MC903 atopic-like dermatitis	(7)
	Il1rl1$^{-/-}$	Wound healing	(46)
	hK14mIL33tg (C57BL/6)	IL-33 overexpression in keratinocytes	(26)
IL-25	*Il17rb*$^{-/-}$ (BALB/c)	MC903 atopic-like dermatitis	(7)
	K14-Cre *Il25*$^{flox/flox}$ (C57BL/6)	OVA allergic skin inflammation	(27)
	Rora-Cre *Il17rb*$^{flox/flox}$ (BALB/c)		
IL-18	*Il18*$^{-/-}$ (C57BL/6)	MC903 atopic-like dermatitis	(8)
	KIL-18Tg (C57BL/6)	IL-18 overexpression in keratinocytes	(28)

transcriptome, epigenome, and proteome enable identification of cellular heterogeneity in far greater detail than conventional methods. Although the single cell analysis still has opportunities for improvement such as costs, technical accessibility, and depth of analysis, the unbiased character of single cell analysis may break through the bottleneck of ILC research.

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ILC2s and Adipose Tissue Homeostasis: Progress to Date and the Road Ahead

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Group 2 innate lymphoid cells (ILC2s) were initially identified as a new type of lymphocytes that produce vigorous amounts of type 2 cytokines in adipose tissue. Subsequent studies revealed that ILC2s are present not only in adipose tissue but also in various other tissues such as lung and skin. ILC2s are generally recognized as tissue-resident immune cells that regulate tissue homeostasis. ILC2s express receptors for various humoral factors and thus can change their functions or distribution depending on the environment and circumstances. In this review, we will outline our recent understanding of ILC2 biology and discuss future directions for ILC2 research, particularly in adipose tissue and metabolic homeostasis.

Keywords: innate lymphoid cells, ILC2, adipose tissue, metabolism, cytokines, intestine

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INTRODUCTION

Innate lymphoid cells (ILCs) are predominantly tissue-resident lymphocytes that regulate tissue homeostasis at steady-state. Unlike T and B lymphocytes, ILCs do not express Rag1/2-dependent antigen receptors, but instead express receptors for a variety of humoral factors, including cytokines, metabolites, and neuropeptides. ILCs are classified into three major groups based on their function and developmental pathways; group 1 (natural killer (NK) cells and ILC1s), group 2 (ILC2s), and group 3 (lymphoid tissue inducer (LTi) cells and ILC3s) (1, 2). NK cells circulate in the blood and require the transcription factor eomesodermin (Eomes) for their differentiation, which is important for the expression of cytotoxic molecules such as perforin and granzymes. ILC1s are abundant in the liver and skin, and their differentiation is regulated by the T-box transcription factor (T-bet) but independent of Eomes. Both NK cells and ILC1s are capable of producing interferon (IFN)γ (3). The transcription factor GATA binding protein 3 (GATA3) governs the differentiation of ILC2s. Initially identified in visceral adipose tissue, ILC2s have been found to produce robust amounts of type 2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 (4–6). Subsequent studies have revealed that ILC2s are also present in various other tissues such as lung, skin and gut. Epithelial cell-derived IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) activate ILC2s, with IL-33 being the major activator (7–9). Although ILC2s are normally tissue resident cells, they can migrate from one tissue to another in response to exogenous IL-25 and helminth infection (10). LTi cells are known to produce lymphotoxin (LT), which plays a critical role in the development of lymphoid tissues during fetal stage. ILC3s are mainly found in the adult intestine, and the transcription factor RAR-

related orphan receptor γt (RORγt) is critical for their development. ILC3s play an important role in maintaining an optimal intestinal environment by producing the cytokines IL-17A and IL-22 (11).

Adipose tissue is one of the major energy storage sites, consisting of lipid-rich cells called adipocytes as well as stromal vascular fraction comprised of preadipocytes, fibroblasts, vascular endothelial cells, and various immune cells (12). Among immune cells, macrophages are the most abundant (typically 5-10% of stromal cells) and their number increases with obesity (13, 14). Consistently, in obese patients, approximately 40% of stromal cells are macrophages (14). These macrophages ultimately produce large amounts of pro-inflammatory cytokines, inducing chronic inflammation of adipose tissue, leading to insulin resistance, glucose intolerance, and ultimately type 2 diabetes. Furthermore, obesity is thought to increase the risk of severe disease caused by COVID-19 after SARS-CoV-2 infection (15). Therefore, it is an urgent need to establish effective strategies to prevent obesity-related comorbidities. ILC2s were initially discovered in adipose tissue and have been found to mediate type 2 immunity (usually considered as an "anti-inflammatory immune response"). Since then, researchers have begun to explore the key role of ILC2s in the regulation of adipose tissue homeostasis. In this review, we will highlight the recent findings on the interaction between ILC2s and adipose tissue. Furthermore, we aim to discuss the potential of ILC2s as therapeutic targets for metabolic disorders.

DEVELOPMENT OF ADIPOSE TISSUE-RESIDENT ILC2s

Like other ILCs, peripheral ILC2s emerge during development from common lymphoid progenitors (CLPs) in the bone marrow and fetal liver in a manner dependent on a transcriptional repressor, Id2 (1). Like other lymphocytes such as T cells and B cells, IL-7 and Notch signaling play an important role in differentiation from CLPs. Interestingly, relatively high concentrations of IL-7 and intermediate Notch signaling preferentially induce lineage commitment from CLPs to ILC progenitor cells (16). IL-33 negatively regulates expression of CXC chemokine receptor type 4 on ILC progenitor cells and promotes their exit from the bone marrow (17). Subsequently, ILC progenitor cells migrate to peripheral organs and differentiate into mature ILC2s in a STAT5-dependent manner. At the same time, especially in adipose tissue, platelet-derived growth factor receptor α $(PDGFR\alpha)^+$ and glycoprotein 38 $(gp38)^+$ mesenchymal cells are thought to further promote ILC2 differentiation, presumably by supplying IL-33. There are also mechanisms that regulate the survival and proliferation of terminally differentiated ILC2s in adipose tissue (18). $PDGFR\alpha^+$ multipotent stromal cells in adipose tissue activate ILC2s by producing IL-33. In addition, $PDGFR\alpha^+$ multipotent stromal cells directly interact with ILC2s *via* ICAM-1/LFA-1 axis to promote ILC2 proliferation. ILC2s-derived IL-4 and IL-13 stimulate $PDGFR\alpha^+$ multipotent stromal cells to produce IL-33 and recruit eosinophils by stimulating eotaxin production. ILC2s also produce IL-5, which further activates eosinophils and maintains a type-2 immune environment in adipose tissue. Collectively, the interaction between ILC2s and $PDGFR\alpha^+$ multipotent stromal cells plays a pivotal role in the maintenance of adipose tissue homeostasis.

FUNCTION OF ADIPOSE TISSUE-RESIDENT ILC2s

As described earlier, ILC2s produce IL-5 and IL-13 in response to IL-33 and recruit eosinophils to adipose tissue (19, 20). Furthermore, ILC2/eosinophil-derived IL-4 and IL-13 induce the differentiation of an anti-inflammatory M2 macrophages and maintain a type 2 immune environment in adipose tissue. On the other hand, numbers of pro-inflammatory M1 macrophages are increased in adipose tissue during obesity. These macrophages produce pro-inflammatory cytokines such as IFNγ, tumor necrosis factor (TNF)α, IL-6, or IL-1β, which significantly dampen the proliferation and function of ILC2s (21). In both mice and humans, the number of adipose tissue-resident ILC2s is markedly reduced in response to obesity (20, 22), and reduced ILC2 function subsequently leads to a decrease in eosinophils and M2 macrophages in adipose tissue, promoting pathological adipogenesis and insulin resistance after high-fat diet (HFD) feeding. On the other hand, adoptive transfer of activated ILC2s into obese mice suppresses HFD-induced weight gain and glucose intolerance (19). ILC2s together with regulatory T cells (Tregs) as well as eosinophils and M2 macrophages, are known to suppress adipose tissue inflammation. Adipose tissue-resident Tregs function as a unique cell population that highly expresses transcription factors GATA3 and peroxisome proliferator-activated receptor γ (PPARγ). IL-33 directly stimulates Treg proliferation, as adipose tissue Tregs highly express the IL-33 receptor ST2 (23, 24). In addition, ILC2s promote Tregs proliferation in adipose tissue through ICOSL and OX40L signaling in response to IL-33 (25).

Adipose tissue is generally categorized into white adipose tissue (WAT), which is found in the abdominal cavity and subcutaneously, and brown adipose tissue (BAT), which is found mainly in the interscapular region in mice and supraclavicular region, neck, para-aorta, paravertebral and suprarenal regions in humans (26, 27). While WAT stores excess energy as triglycerides, BAT produces heat in a way that does not cause shivering in cold conditions, and plays a pivotal role in maintaining body temperature (28). In addition to brown adipocytes, beige adipocytes, a distinct type of cell that regulates thermogenesis *in vivo*, have also been identified (29). Brown and beige adipocytes express uncoupling protein 1 (UCP1), which allows them to decouple mitochondrial respiration from ATP synthesis and dissipate energy as heat. Although brown adipocytes are most abundant in newborns and decrease with age, beige adipocytes can be temporally induced to differentiate within WAT in response to certain stimuli such as cold exposure. This phenomenon is referred to as "WAT browning". Therefore,

beige adipocytes are considered as an attractive target for ameliorating obesity and its related diseases. Two independent groups have reported that ILC2s are involved in WAT browning (22, 30). Artis and colleagues reported that IL-33-activated ILC2s produce methionine-enkephalin peptide (Met-Enk), which activates UCP1 in white adipocytes and promotes beige adipocyte differentiation under cold conditions. On the other hand, Chawla and colleagues showed a slightly different mechanism. Under thermoneutral conditions, ILC2s in WAT recruit eosinophils in adipose tissue in response to IL-33 and produce type 2 cytokines such as IL-4 and IL-13. These cytokines directly activate proliferation of adipocyte precursors expressing IL-4Rα and induce their differentiation into the beige adipocyte lineage. However, the physiological source of IL-33 was not clarified in any of the reports. Since then, studies have demonstrated that adipose-derived stromal cells including mesenchymal cells, mesenchymal stem cells, podoplanin$^+$ fibroblasts, or CD31$^+$ endothelial cells produce IL-33 at steady state (16, 24, 31–35). A recent study has also implied that eosinophils could be another source of IL-33 in adipose tissues (36). On the other hand, as mice age, mesothelial cells become major producer of IL-33. Therefore, the source of IL-33 might partially vary with age.

ILC2s in adipose tissue significantly decrease with age. Recently, Dixit and colleagues reported that the lethality of the aged mice subjected to cold conditions is considerably higher than that of young mice (37). This may be because ILC2-mediated WAT browning and subsequent heat production are not well induced in aged mice, and thus they are unable to maintain core body temperature. Taken together, these findings suggest that ILC2s play a critical role in maintaining adipose tissue homeostasis.

REGULATION OF ADIPOSE TISSUES BY ILC2s AND SIGNALS FROM OTHER ORGANS

Our group has previously demonstrated that ILC2s, which are also present in the small intestine, promote obesity in HFD-fed mice (38). Obesity was induced in HFD-fed *Rag2*$^{-/-}$ mice (lacking T cells, B cells, and NKT cells but with ILCs or NK cells), but not in *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ mice (lacking all lymphocytes). This result suggests that ILCs are involved in the induction of obesity. As mentioned above, adipose tissue ILC2s (WAT-ILC2s) suppress obesity-induced adipose tissue inflammation. Consistently, we found that adoptive transfer of WAT-ILC2s into *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ mice did not increase the number of M1 macrophages in adipose tissues after HFD-feeding. On the other hand, adoptive transfer of small intestinal ILC2s (SI-ILC2) into *Il2rg*$^{-/-}$*Rag2*$^{-/-}$ mice promoted HFD-induced obesity and subsequent adipose tissue inflammation. Unexpectedly, IL-33 and IL-25 were not involved in SI-ILC2s-dependent obesity induction. In addition to ILC2s, ILC3s (the major ILC population in the intestine) also seem to be partially involved in the process of diet-induced obesity. SI-ILC2s produce higher level of IL-2 than WAT-ILC2s. Interestingly, *Rag2*$^{-/-}$ mice lacking the β chain of the IL-2 receptor were less sensitive to HFD than *Rag2*$^{-/-}$ mice, implying that SI-ILC2-derived IL-2 plays an important role in the induction of obesity. Further analysis is required to elucidate the detailed mechanism of obesity promoted by SI-ILC2. Since the microbiota is one of the most important factors affecting lipid metabolism *in vivo*, it will be interesting to investigate the interaction between SI-ILC2s and the microbiota and its potential involvement in the regulation of obesity.

Recently, much attention has been paid to the relationship between ILC2s and the nervous system. The sympathetic nervous system innervating adipose tissue produces catecholamines, which increase the expression of UCP1 in white adipocytes and promote their differentiation into beige adipocytes (39, 40). ILC2s highly express receptors for a variety of neurotransmitters including catecholamines and neuron-ILC2 axis plays a pivotal role in homeostatic control of adipose tissue (41–46). The sympathetic nervous system promotes the production of IL-33 and activates ILC2s in adipose tissue under cold conditions. Conversely, surgical removal or drug-mediated ablation of sympathetic nerves greatly reduces the number of ILC2s and eosinophils in adipose tissue (47). Chronic obesity induces adipose tissue inflammation and severely damages neuronal function (48), resulting in the loss of ILC2s and consequent disruption of adipose tissue homeostasis. Intestinal ILC2s express the β_2-adrenergic receptors (β_2-AR) and co-localize with adrenergic neurons. Treatment of mice with β_2-AR agonist impairs the activation of ILC2s in the intestine (44). In contrast, β_2-AR signaling does not directly affect the ILC2 function in adipose tissues. Rather, sympathetic nerves stimulate adipose mesenchymal cells *via* adrenergic receptors to accelerate the release of glial-derived neurotrophic factor (GDNF). As a result, GDNF stimulates RET-expressing ILC2s in adipose tissue, promotes the production of IL-5, IL-13, and Met-Enk, and further regulate lipid metabolism (49). ILC2-intrinsic ablation of RET promoted HFD-induced obesity, insulin resistance, and glucose intolerance. These signals from sympathetic nerves are projected from the brain, including the paraventricular nucleus of the hypothalamus, suggesting that the "brain-fat circuit" regulates ILC2 function and adipose tissue homeostasis. In the lung and intestine, ILC2s express the receptor for neuropeptide NMU (41, 45, 46). Thus, NMU can directly activate the ILC2s-mediated type 2 immune response in these tissues. Studies in rats showed that NMU is involved in the regulation of UCP1 expression in adipose tissue, suggesting that the ILC2-NMU axis may also play an important role in adipose tissue (50). Since ILC2s are often located in close proximity to neuropeptide-producing nerves, it is possible that ILC2s produce factors that are beneficial for nerves and also induce proper lipid metabolism. Collectively, inter-organ communication critically influences lipid metabolism, and ILC2s play an important role in this process.

DISCUSSION

Here, we have outlined our current knowledge and perspectives on the function of ILC2s in adipose tissue (**Figure 1**). The regulatory mechanisms and functions of adipose tissue differ

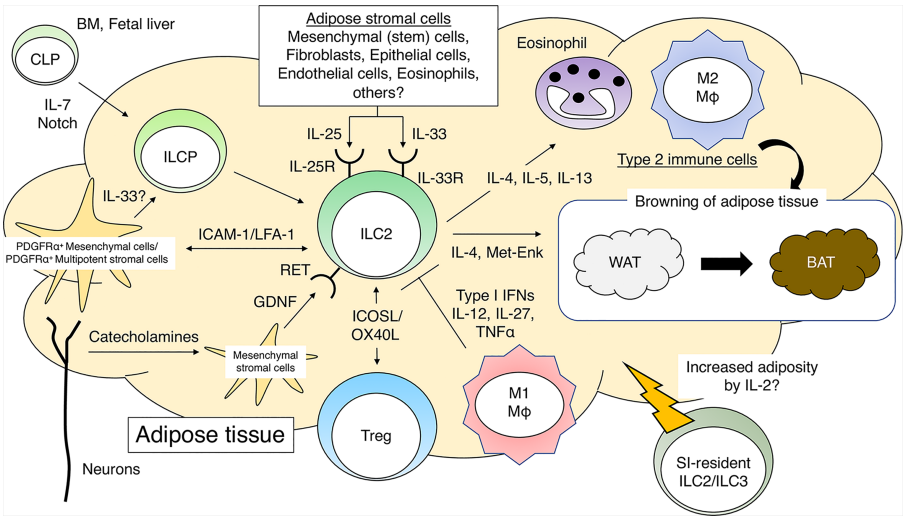

FIGURE 1 | Group 2 innate lymphoid cells (ILC2s) and adipose tissue homeostasis. IL-7 and Notch signaling promote differentiation of common lymphoid progenitors (CLPs) into ILC progenitor cells (ILCPs). PDGFRα$^+$gp38$^+$ mesenchymal cells further promote the differentiation of ILCP into mature ILC2s in adipose tissues. The direct interaction between ILC2s and PDGFRα$^+$ multipotent stromal cells is critical for the maintenance of adipose tissue homeostasis. ILC2s, when activated by cytokines such as IL-25 or IL-33, recruit eosinophils and M2 macrophages to sustain a type 2 immune environment in adipose tissues. ILC2s promote WAT browning by producing type 2 cytokines and Met-Enk. ILC2s promote Treg proliferation in adipose tissues in response to IL-33. Neuron-derived catecholamines activate adipose mesenchymal stromal cells to produce GDNF, which stimulates ILC2s and induces a type 2 immune response. During obesity, M1 macrophages produce pro-inflammatory cytokines and impair the function of ILC2s in adipose tissues. PDGFRα, platelet-derived growth factor receptor α; Met-Enk, Met-enkephalin; GDNF, glial-derived neurotrophic factor.

depending on the site. This is also true for ILC2s. ILC2-mediated adipose tissue browning is observed in subcutaneous WAT, whereas the neuron-ILC2 axis is observed in visceral WAT. Therefore, it should be emphasized that the effects of ILC2s in visceral WAT do not necessarily apply to subcutaneous WAT, and *vice versa*. Nevertheless, there is no doubt that ILC2s play an important role in adipose tissue homeostasis, and therefore, enhancing their homeostatic role may help prevent the development of metabolic abnormalities. Fine-tuning the interactions between ILC2s and adipose tissue components, including both immune and non-immune cells, is also important for maintaining metabolic homeostasis. Identifying the source(s) of cytokines that activate ILC2s, such as IL-33, seems to be one attractive approach.

Obesity reduces the number and function of ILC2s in adipose tissue, and their type 2 immune environment is compromised. In this situation, recruiting ILC2s to adipose tissue would be another attractive approach to prevent the development of adipose tissue inflammation and subsequent metabolic disorders. ILC2s migrate from the intestine to the lung in response to IL-25 and helminth infections, but this phenomenon has not been observed in adipose tissue. Further studies are necessary to fully understand the process by which ILC2s move between tissues to develop methods to recruit ILC2s to adipose tissue. In addition, it should be noted that the overactivation of ILC2s associated with the type 2 immune responses may sometimes be detrimental, as they induce allergic inflammation and intestinal ILC2s promote obesity. ILC2s can change their functions according to the environment and circumstances (51). Therefore, it is essential to study the heterogeneity of ILC2s in different types of adipose tissue in order to find appropriate ways to regulate ILC2-mediated metabolic abnormalities.

AUTHOR CONTRIBUTIONS

TM and SK conceived the contents and wrote the manuscript. MW discussed with TM to provide suggestions. All authors contributed to the article and approved the submitted version.

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Innate Immune Responses by Respiratory Viruses, Including Rhinovirus, During Asthma Exacerbation

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Viral infection, especially with rhinovirus (RV), is a major cause of asthma exacerbation. The production of anti-viral cytokines such as interferon (IFN)-β and IFN-α from epithelial cells or dendritic cells is lower in patients with asthma or those with high IgE, which can contribute to viral-induced exacerbated disease in these patients. As for virus-related factors, RV species C (RV-C) induces more exacerbated disease than other RVs, including RV-B. Neutrophils activated by viral infection can induce eosinophilic airway inflammation through different mechanisms. Furthermore, virus-induced or virus-related proteins can directly activate eosinophils. For example, CXCL10, which is upregulated during viral infection, activates eosinophils *in vitro*. The role of innate immune responses, especially type-2 innate lymphoid cells (ILC2) and epithelial cell-related cytokines including IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), in the development of viral-induced airway inflammation has recently been established. For example, RV infection induces the expression of IL-33 or IL-25, or increases the ratio of ILC2 in the asthmatic airway, which is correlated with the severity of exacerbation. A mouse model has further demonstrated that virus-induced mucous metaplasia and ILC2 expansion are suppressed by antagonizing or deleting IL-33, IL-25, or TSLP. For treatment, IFNs including IFN-β suppress not only viral replication but also ILC2 activation *in vitro*. Agonists of toll-like receptor (TLR) 3 or 7 can induce IFNs, which can then suppress viral replication and ILC2 activation. Therefore, if delivered in the airway, IFNs or TLR agonists could become innovative treatments for virus-induced asthma exacerbation.

Keywords: bronchial asthma, eosinophils, epithelial cell-related cytokines, innate lymphoid cells, rhinovirus

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INTRODUCTION

Viral infection is extensively involved in the exacerbation of asthma (1, 2). Viral infection is identified in 50–80% of patients with asthma exacerbation, and rhinovirus (RV) is detected in 50–80% of patients in whom the causative virus has been identified (3). Bronchial asthma is a chronic disease characterized by airway hyperresponsiveness (AHR), a variable degree of airway obstruction, and eosinophilic airway inflammation (4, 5). Although various types of cells are

involved in this process, both neutrophil and eosinophil inflammation may contribute to the development of viral-induced asthma exacerbation.

Innate immune responses contribute to the pathogenesis of eosinophilic airway inflammation. Type 2 innate lymphoid cells (ILC2) activated by epithelial cell-related cytokines such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) (6, 7) can induce IL-5 and IL-13 and thus eosinophilic inflammation. This indicates that innate immune responses including ILC2 may play important roles in virus-induced airway inflammation and asthma exacerbation.

In the present review, the role of viral infection and innate immune responses, including the role of ILC2, in asthma exacerbation is discussed.

ROLE OF VIRAL INFECTION IN THE DEVELOPMENT OF ASTHMA EXACERBATION

Viral infection, especially RV infection, plays an important role in the pathogenesis of asthma exacerbation. RVs have tremendous diversity, and there are about 100 classical serotypes that are classified into RV species A (RV-A) and RV-B (1, 2). With the development of molecular biological technologies such as PCR, more than 60 new RVs have been discovered (almost all are RV-C) (1, 2). RV viral capsid includes VP1, VP2, VP3 and VP4 proteins, and VP1 and VP3 are important for attachement to cell surface receptors. The receptor for the major group of RV-A and for all of RV-B is intercellular adhesion molecule (ICAM) 1, while that for the minor group of RV-A is low-density lipoprotein receptor, and that for RV-C is cadherin-related family member 3 (CDHR3) (8, 9) (**Table 1**). RV is taken up by receptor-mediated endocytosis and replicates in airway epithelial cells. Generally, the components of the viruses are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), melanoma differentiation-associated protein (MDA) 5, and retinoic acid-inducible gene (RIG) 1-like receptors. RV is a positive-sense single-strand (ss) RNA virus, however, it could be double-strand (ds) RNA during replication process. Therefore, dsRNA is recognized by TLR3 and ssRNA is recognized by TLR7/8 in endosomes of epithelial cells, which activate myeloid differentiation primary response 88 (MYD88) or TIR-domein-containing-adaptor-inducing interferon-β (TRIF) signaling pathways (10). Further viral RNA is recognized by MDA-5 or RIG-1 in the cytosol, however MDA-5 is much involved in the process of RV (11). Recognition of viruses by PRRs induces the translocation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and interferon regulatory factors (IRF) to the nucleus (10), which release various proinflammatory cytokines and chemokines such as IL-6, IL-8, CCL5, granulocyte macrophage colony-stimulating factor (GM-CSF), and interferons (IFNs) including IFN-λ (10, 12–14) (**Table 1**). However, RV infection inhibits antiviral responses. For example, it activates transforming growth factor (TGF) β, which can increase viral replication (15, 16).

Respiratory syncytial virus (RSV) is known to cause bronchiolitis with wheezing in infants. In adults, it can cause community-acquired pneumonia, and mortality rates comparable to influenza have been reported in elderly and high-risk patients (17). RSV is also known to be involved in the exacerbation of asthma. RSV is more frequently detected than RV in patients with wheezing less than 3 years of age. Although there is little data for adults, it has been reported that RSV is involved in 7% of asthma hospitalizations (18). RSV is negative-sense ssRNA virus and includes 10 proteins including 3 surface proteins such as fusion (F) protein, attachment (G) protein and small hydrophobic (SH) protein (**Table 1**). G protein is responsible for viral attachment and, glycosaminoglycans, CX3CR1, and annexin II are proposed for cell receptors of RSV G protein (19, 20) (**Table 1**). F protein is critical for cell fusion, resulting in viral entry and infection. A cellular receptor for RSV F protein is nucleolin (21) and TLR4, EGFR, and ICAM-1 are reported to be co-receptor (19, 20)

TABLE 1 | Characteristics of virus, important viral protein, entry molecules, recognition molecules and downstream cytokines/chemokines of RV, RSV and SAR-CoV-2.

Virus	Type	Important viral protein for infection	Entry molecules (Receptor)	Recognition molecules (PRRs)	Downstream cytokines/chemokines
RV	Positive-sense ssRNA	VP1 VP3	1) Major RV-A and all RV-B ICAM-1 2) Minor RV-A Low-density lipoprotein receptor 3) RV-C CDHR3	TLR3 (dsRNA) TLR7 (ssRNA) MDA-5 RIG-1	1) Proinflammatory cytokines/chemokines IL-1β, IL-6, IL-8, IL-12, TNF-α, CCL5, CXCL9, CXCL10, GM-CSF, IL-33, TSLP, IL-25 2) IFNs IFN-α, IFN-β, IFN-λ
RSV	Negative-sense ssRNA	G protein F protein SH protein	1) G protein Glycosaminoglycans CX3CR1 Annexin II 2) F protein Nucleolin, Co-receptor EGFR, TLR4 and ICAM-1	TLR3 (dsRNA) TLR7 (ssRNA) TLR4 (F protein) RIG-1 MDA-5	
SARS-CoV-2	Positive-sense ssRNA	S1 S2	ACE2 TMPRSS2 (for proteolytic cleavage)	TLR3 (dsRNA) TLR7 (ssRNA) MDA-5 RIG-1	

(**Table 1**). Fulin as a protease play a role in the intracellular cleavage of F protein, which is essential step for acquirement of RSV infectivity. RSV replicates in airway epithelial cells, and TLR3 and TLR7/8 in the endosome of epithelial cells play roles in the recognition of dsRNA and ssRNA in a similar way of RV. RSV RNA is also recognized by RIG-1 or MDA-5 in the cytosol. Further, F protein is recognized by TLR4 (22) (**Table 1**) expressed in the cell. Proinflammatory cytokine/chemokines and IFNs are then induced.

Clinically, it is not always symptomatic even if virus is detected, As a result of this, it is important to clarify the factors that determine the severity of viral infection. Candidates are (1) host-related factors, (2) virus-related factors, and (3) gene-related factors. As for host-related factors, several reports suggest that asthmatic patients are more susceptible to virus including RV, and their symptoms are easily exacerbated by RV infections (23, 24). As a potential mechanism, antiviral cytokines such as IFNs are produced at lower levels in asthmatic patients compared to non-asthmatic patients (25, 26), which is discussed later.

Virus-related factors have recently been highlighted. Several reports have noted differences in virulence between RV species; specifically, RV-C cause more serious pathogenic diseases than other RVs, including RV-B (13, 27–29). Furthermore, the reason why RV is much involved in the pathogenesis of asthma exacerbation has not been fully clarified. RSV infection is related to the induction of Th2-stimulated immune responses (30). CX3CR1, a receptor for RSV G protein, and its ligand CX3CL1 exacerbates allergic immune responses (31). However, if compared to the case of RV infection, IL-5 concentration in the serum during RSV infection is not increased (32), suggesting that the degree of type-2 bias may be lower than that in RV infection. There is a possibility that stronger viral responses, probably mediated by IFNs, suppress Th2-mediated immune responses and weaker viral responses by RV upregulates Th2 immune responses.

As for gene-related factors, recent studies suggest that interactions between genes and viral infection may play a role in the pathogenesis of asthma exacerbation. For example, a coding single nucleotide polymorphism (SNP) in CDHR3 (rs6967330; $C_{529}Y$) is associated with severe exacerbation in childhood asthma (33). Since then, CDHR3 has been found to be a receptor for RV-C (9). Moreover, this SNP enhances the protein expression of CDHR3 on the cell surface (9, 33), which increases the binding of RV-C and its replication (9). This SNP also increases RV-C illnesses *in vivo* (34), which suggests that asthma is easily exacerbated in patients with CDHR3-Y_{529} variants by increased susceptibility to RV-C.

Since 2020, the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that cause Coronavirus disease 2019 (COVID-19) has spread globally and created pandemic. SARS-CoV-2 is a positive-sense ssRNA virus and enters host cells by the surface S protein comprising S1 and S2 (35). The receptor for SARS-CoV-2 is angiotensin-converting enzyme 2 (ACE2) (**Table 1**). TMPRSS2 play a role in the intracellular proteolytic cleavage which induces a conformational change in S protein and allows for cellular entry *via* endocytosis. SARS-CoV-2 replicates in airway epithelial cells, and TLR3 and TLR7/8 in the endosome and MDA-5 or RIG-1 in the cytosol play roles in the recognition of viral components in a similar way. Severe cases of COVID-19 are assumed to be due to the defect or delay of IFN responses which unlease excessive expression of proinflammatory cytokines/chemokines, called cytokine storm syndrome (35). Furthermore, other mechanism such as increase in ACE2 signaling, observed in the case of SARS-CoV (36), may contribute to the immune response to SARS-CoV-2. ACE2 is not the only receptor mediating SAR-CoV-2 cell entry. For example, neuropilin-1 and DPP4 is reported to be potential receptor for SAR-CoV-2 (35). Asthma patients are reported to be less likely to suffer from COVID-19 or its severe disease (37). However, whether asthma is associated with severity of COVID19 is still controversial (37, 38). Zhu et al. reported that although the risk of severe COVID-19 is not elevated in patients with allergic asthma, it is significantly increased in those with non-allergic asthma.

In recent years, there have also been reports that mixed infections of viruses and bacteria (e.g., *Moraxella catarrhalis*) are involved in the exacerbation of asthma (39).

VIRAL INFECTION IN AIRWAY EPITHELIAL CELLS

1) General Roles/Responses of Airway Epithelial Cells in Viral Infection

Virus infects airway epithelial cells. Generally, virus is taken up by receptor-mediated endocytosis and replicates in epithelial cells as described above. The components of the viruses are recognized by PRRs including TLRs, MDA-5, and RIG-1. dsRNA is recognized by TLR3, and ssRNA is recognized by TLR7/8 in endosomes, and viral RNA is recognized by RIG-1 or MDA-5 in the cytosol. RSV F protein is recognized by TLR4 in the cell surface (22). Recognition of viruses by PRRs induces the translocation of IRF and NF-κB to the nucleus and the transcription of proinflammatory and antiviral genes such as IFNs

2) Status of Airway Epithelial Cells in Asthma Patients

When an asthmatic patient is infected with RV, the symptoms of the upper respiratory tract are similar to those of non-asthmatic subjects (23, 24). However, the symptoms of the lower respiratory tract such as coughing are exacerbated in asthmatic patients (23, 24). In addition, RV is frequently detected in the lower airways of asthmatic patients even during the stable period (40). As a potential mechanism, antiviral cytokines such as IFN-β and IFN-λ are produced at lower levels in asthmatic patients than in non-asthmatic patients (25, 26) (**Figure 2**), and thus viral replication is higher in asthmatic patients. However, the hypothesized lower production of antiviral cytokines from airway epithelial cells and higher viral replication in asthmatics is still controversial, as several reports suggest that these factors are almost the same as in non-asthmatics (41–43). In contrast, the expression of PPRs such as TLR3, MDA-5 or RIG-1 in

epithelial cells of asthma is similar to that of healthy volunteer (44).

As for the receptor of virus, the expression of ICAM-1 is upregulates in asthma (45) and its expression further increases after RV infection (46), which can induce the eosinophilic airway inflammation as described below. In contrast, expression of ACE2 is decreased by IL-4 or IL-13 (47) and its expression is decreased by allergen challenge (48), suggesting that the expression of ACE2 is lower in asthmatic patient than in healthy indivisuals.

INVOLVEMENT OF DENDRITIC CELLS DURING VIRAL INFECTION

1) General Roles/Responses of Dendritic Cells (DCs) in Viral Infection

DCs play important roles in innate immune responses during viral infection (10). DCs are professional antigen presenting cells, and present antigens such as viral components to naïve T cells. DCs are classified into myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs are involved in the initiation of the cytotoxic T-cell response and the activation of T helper cells. mDCs differentiate naïve T cells into effector T cells. Th1 cells contribute to the anti-viral responses, and Th2 cells contribute to allergic inflammation. mDCs express TLR4, and stimulation by TLR4 upregulates DC functions. RSV F protein can activate mDC functions through TLR4.

In contrast, pDCs contribute to the induction of tolerance and the maintenance of homeostasis in the lungs. pDCs produce large amounts of IFN such as IFN-α and IFN-λ through TLR7/8 for anti-viral immune responses (**Figure 1**) and induce regulatory T cells. Important role of pDC in the development of severe COVID-19 is proposed. SAR-CoV-2 can avoid or delay the stimulation of type I IFN-related responses *in vivo*. pDC tune down their capacity for IFN production, which can favor prolonged viral replication, termed "pDC exhaustion"

2) Status of DCs in Asthma Patients

mDCs of airway in asthmatic patients are increased and activated as compared with those in healthy individuals (49), which contribute to the exacerbated allergic immune responses in asthma. There is a possibility that it is also involved in the exacerbated viral-related immune responses.

IFN production from pDCs decreases in patients with high IgE levels or in those with asthma (50, 51) (**Figure 2**). For example, the production of the anti-viral cytokine IFN-α from influenza-stimulated pDCs is inversely correlated with the concentration of serum IgE (50). IgE cross-linking of peripheral blood mononuclear cells (PBMCs) suppresses the production of RV-stimulated anti-viral cytokines IFN-α and IFN-λ in asthmatic patients (51).

3) Discussion About the Status of DCs in Asthma Patients

Reflecting the reduced IFN production from pDCs, asthma is easily exacerbated by RV infection in patients with high IgE levels. Experimental infection with RV increases AHR and fractional exhaled nitric oxide (FeNO) in patients with high serum IgE (52). Asthma exacerbation is more likely to occur in patients with high concentrations of specific IgE during RV infection (53). Furthermore, anti-IgE Ab treatment reduces pDC surface receptor (FcεRIα) expression and restores RV-induced IFN-α production from pDC (54). Anti-IgE Ab decreases the duration of RV infection, peak RV shedding and the frequency of RV illnesses (55), Moreover, anti-IgE Ab reduces acute severity of RV-induced asthma exacerbation (56). Therefore, IgE plays an important role in the IFN production from pDC and RV-induced asthma exacerbation

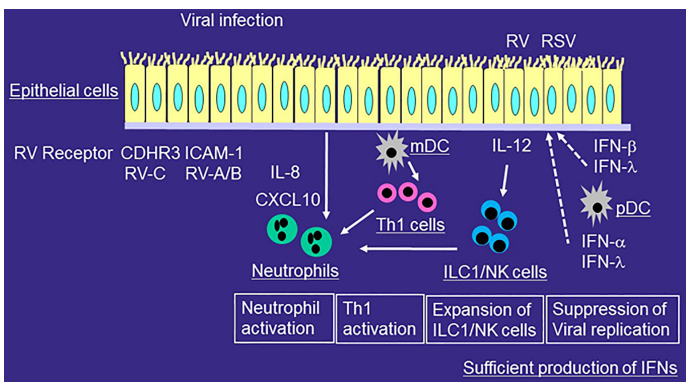

FIGURE 1 | Role of innate immune responses in the development of airway inflammation of healthy individuals during viral infection. RV or RSV infects airway epithelial cells and is taken up by receptor-mediated endocytosis. After recognition of virus components by PRRs, epithelial cells release proinflammatory cytokines and chemokines and antiviral cytokines including IFNs. mDCs present viral antigens to naïve T cells for differentiation, whereas pDCs produce IFN-α and IFN-λ. The production of IFN from airway epithelial cells or pDCs is sufficient for viral immunity. RV or RSV infection induces more IL-12 expression in airway epithelial cells, and more ILC1 and NK cells in the airways. Viral infection releases IL-8 from epithelial cells and thus induces neutrophilic airway inflammation. Airway neutrophils disappears relatively quickly and eosinophilic airway inflammation is not usually induced.

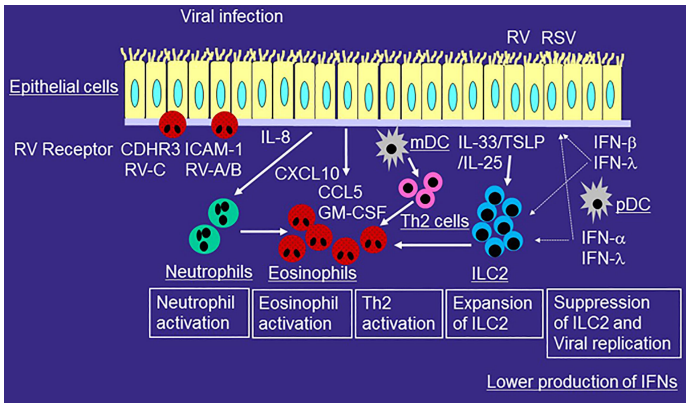

FIGURE 2 | Role of innate immune responses in the development of type 2-mediated airway inflammation of asthmatic patients during viral-induced asthma exacerbation. RV or RSV infects airway epithelial cells and is taken up by receptor-mediated endocytosis. After recognition of virus components by PRRs, epithelial cells release proinflammatory cytokines and chemokines including IL-6, IL-8, CCL5, GM-CSF, and CXCL10 and antiviral cytokines such as IFN-β and IFN-λ. mDCs present viral antigens to naïve T cells for differentiation, whereas pDCs produce IFN-α and IFN-λ. The production of IFN from airway epithelial cells or pDCs is lower in patients with asthma or with high IgE as compared with that of healthy individuals. RV or RSV infection induces more IL-33, IL-25, or TSLP expression in airway epithelial cells. Virus-induced IL-33, IL-25, or TSLP increase ILC2s in asthmatic airways and thus induce eosinophilic airway inflammation. IL-33 enhances RV-induced airway inflammation and suppresses IFN-β or IFN-λ expression and anti-viral immunity. Viral infection releases IL-8 from epithelial cells and thus induces neutrophilic airway inflammation. Activated neutrophils can accumulate eosinophils in the airway even without chemoattractants for eosinophils. Viral infection releases a variety of mediators including CCL5, GM-CSF, and CXCL10, which can directly activate eosinophils. Receptors of RV such as ICAM-1 and CDHR3 in airway epithelial cells also activate eosinophils.

INTERACTIONS OF VIRAL INFECTION AND NEUTROPHILS OR EOSINOPHILS IN THE DEVELOPMENT OF ASTHMA EXACERBATION

1) General Roles/Responses of Neutrophils or Eosinophils in Viral Infection

Viral infection induces neutrophilic airway inflammation mainly due to the release of cytokine/chemokines from epithelial cell. Neutrophils play roles in enhancing viral-induced inflammation through releasing granules, producing cytokines, and inducing the recruitment of other immune cells in the airways (**Figure 1**). IL-8 contributes to the accumulation of neutrophils in the site of inflammation, and IL-8 expression is upregulated in the airways of patients with viral infection (57, 58). However, in healthy individuals, airway neutrophils disappears relatively quickly (59). Eosinophilic airway inflammation is not usually induced (59).

2) Status of Neutrophils or Eosinophils in Asthma Patients

Both neutrophil and eosinophil inflammation may be involved in the pathogenesis of severe asthma. Viral infection induces neutrophilic airway inflammation as described above, which contributes to the exacerbations that frequently occur in severe asthma. The European Network For Understanding Mechanisms Of Severe Asthma (ENFUMOSA) reported that higher sputum neutrophil counts and mediators derived from eosinophils are observed in patients with severe asthma (60). In fact, IL-8 expression is upregulated in the airways of patients with severe asthma (61, 62).

The number of eosinophils also increases in the asthmatic respiratory tract during or after viral infection. Experimental infection with RV increases the accumulation of eosinophils in the airways after allergen challenge in patients with allergic rhinitis (63). Viral infection increases the eosinophil number in airway epithelium (59) and the levels of eosinophil cationic proteins in sputum (64) in patients with allergic asthma. This shows that eosinophils are actually activated and recruited in the asthmatic respiratory tract during or after viral infection (**Figure 2**).

3) Discussion About the Status of Neutrophils or Eosinophils in Asthma Patients

Recent studies have suggested that activated neutrophils can induce eosinophilic airway inflammation. For example, neutrophil extracellular traps (NETs) play a role in the development of eosinophilic airway inflammation of viral-induced asthma exacerbation. RV infection causes the release of dsDNA with the formation of NETs in a mouse model (65). In addition, in humans, the release of host dsDNA after RV infection is correlated with the exacerbation of type 2 allergic inflammation (65). Furthermore, neutrophil proteases, including elastase, directly activate eosinophil functions such as the production of superoxide anions and cytokines, and eosinophil cationic protein release (66, 67). Moreover, we reported that IL-8 or LPS-stimulated neutrophils induce the trans-basement membrane migration of eosinophils *in vitro* even without eosinophil chemoattractants (68, 69). Therefore, activated neutrophils can accumulate eosinophils in the asthmatic airways during viral-induced asthma exacerbation (70) (**Figure 2**).

To accumulate in asthmatic airways, circulating eosinophils need to adhere to vascular endothelial cells, migrate over cells, and be activated locally by inflammation (71). In this process, adhesion molecules such as vascular cell adhesion molecule (VCAM) 1 or ICAM-1, chemokines such as CCR3 ligands including CCL11 (eotaxin-1), and cytokines such as IL-5 play important roles. RV infection induces CCL5 (13, 14), a CCR3 ligand, and GM-CSF (12), an eosinophil growth factor/cytokine, which contribute to the migration and activation of eosinophils in the airway during viral infection.

Chemokines other than CCR3 ligands also play roles in the pathogenesis of RV-induced asthma exacerbation. For example, RV infection induces CXCL10, a CXCR3 ligand, in airway epithelial cells *in vitro* and *in vivo* (13, 72). Specifically, serum CXCL10 levels increase in virus-induced asthma and correlate with the severity of the disease, including airflow limitations (72). CXCL10 directly upregulates eosinophil functions *via* CXCR3 expressed in eosinophils (73).

As describe above, ICAM-1 is an adhesion molecule (74); however, it is also the cell receptor of RV-A and RV-B (8). ICAM-1 expression in epithelial cells increases after RV infection (46), and ICAM-1 directly upregulates eosinophil functions (75, 76). CDHR3 is a receptor for RV-C (9), and its SNPs are related to severe exacerbations (33). We recently reported that CDHR3 activates eosinophil functions (77). These findings suggest that ICAM-1- and CDHR3-mediated adhesion of eosinophils to epithelial cells may activate eosinophils during RV-induced asthma exacerbation (**Figure 2**). In addition, transfection of CDHR3-Y_{529} into HeLa cells increases eosinophil adhesion and superoxide anion production compared to CDHR3-C_{529} or negative controls, suggesting a possible role for CDHR3-induced eosinophil activation in the development of asthma exacerbation, especially with the CDHR3 variant.

Eosinophils are known to have an antiviral effect (78, 79). For example, eosinophils directly suppress the replication of parainfluenza virus through NO production (79). In addition, eosinophils directly suppress RSV replication through eosinophil-derived neurotoxin release (78). However whether the antiviral effect of eosinophils is actually exerted in the asthmatic airways is still unknown.

On the other hands, eosinophilic inflammation can also be a risk factor for RV-induced asthma exacerbations through the suppression of IFN production (80–84). Both high FeNO and sputum eosinophilia increase the risk of subsequent virus-induced asthma exacerbation (82). There are no reports that eosinophils directly suppress RV replication. Mathur et al. reported that eosinophils from allergic rhinitis can suppress the RV-induced IFN-λ1 expression from epithelial cells (BEAS-2B cells) *in vitro* and thus increase RV replication, probably through TGF- β (80). Recently, Dill-McFarland et al. reported that eosinophils or eosinophil supernatants inhibites RV-induced IFNα secretion from pDC of healthy volunteers *in vitro* (84). Furthermore, anti-IL-5 treatment increases (or restores) RV-induced IFNα secretion from pDC of asthmatic donor *ex vivo* as compared with that without anti-IL-5 treatment (84). Moreover, patients with eosinophilic inflammation demonstrate reduced TLR7 and IFN-λ expression in bronchial epithelial cells (81). Given these, IFN production from epithelial cells or pDC can be inhibited by eosinophils, and reducing eosinophil counts may be an important strategy for controlling RV-induced asthma exacerbations.

As for COVID-19, pre-existing eosinophilia is protective from COVID-19-associated admission in asthmatics, and development of eosinophilia during hospitalization is associated with decreased mortality (85), suggesting the protective role of eosinophils in SARS-CoV-2. However, the direct suppressive effect of eosinophils on SARS-CoV-2 replication has not been demonstrated until now. On the other hands, IFN-α or IFN-γ induces the expression of ACE2 in airway epithelial cells (86) and expression of IFNs is lower in patients with high eosinophil counts (87), suggesting that eosinophil-mediated suppression of IFN production in epithelial cells may be associated with suppression of ACE2 expression and thus COVID-19.

ROLE OF ILCS IN THE PATHOGENESIS OF VIRAL-INDUCED ASTHMA EXACERBATION

1) General Roles/Responses of ILCs in Viral Infection

ILCs play important roles in the viral-induced innate immune responses. ILCs do not express T cell receptor and thus cannot response specific antigens unlike T cells. ILCs are functionally classified based on expression patterns of transcription factor and cytokines; ILC1, which express the transcription factor T-bet and produce IFN-γ, ILC2, which expresses the transcription factor GATA3 and produce IL-5 and IL-13, and ILC3, which expresses the transcription factor RORγt and produce IL-17A or IL-22. In healthy individuals, ILC1 and NK cells, another effector lymphocytes of the innate immune system, are involved in the immune responses of viral infection as well as bacterial infection through the production of IFN-γ (**Figure 1**).

2) Status of ILCs in Asthma Patients

Innate immune responses are also involved in the pathogenesis of eosinophilic airway inflammation; this process includes ILC2 as well as epithelial cell-related cytokines such as IL-33, TSLP, and IL-25 (6, 7). The ILC2 activated by IL-33, TSLP, and IL-25 can produce IL-5 and IL-13 and thus induce eosinophilic inflammation. Several reports suggest that ILC2 is increased in the blood or airways of patients with asthma (88, 89) and highly increased in severe asthma as compared with mild asthma (90, 91). For example, ILC2 is increased in the blood and sputum of patients with severe asthma (91). However, in blood, the finding of increased amounts or frequency of ILC2 in asthma is controversial (89, 92), as the frequency of blood ILC2 has been found not to differ between well-controlled asthma and uncontrolled asthma (89). Rather, ILC2 in severe asthma is more activated than in mild asthma (89, 91). For example, IL-5^+ ILC2 in peripheral blood and sputum of severe asthma

patients is increased as compared to those with mild asthma or control patients (91). Furthermore, IL-13$^+$ ILC2 is increased in the peripheral blood of patients with uncontrolled asthma (89). As for the mechanism of ILC2 induction, allergen exposure increases ILC2 in the airways and decreases them in blood (93, 94), suggesting the accumulation of ILC2 from circulation into airways in response to allergens. Recently, the role of TSLP in the pathogenesis of severe asthma has been highlighted. TSLP contributes to the pathogenesis of corticosteroid-resistant airway inflammation by Bcl-xL expression *via* ILC2s (95, 96). From the above, it can be seen that ILC2 contributes to the development of airway inflammation in severe asthma.

Recent studies have demonstrated that innate immune responses including ILC2 play important roles in virus-induced asthma exacerbation. For example, Jackson et al. reported that RV-16 inoculation induces not only IL-4, IL-5, and IL-13 but also IL-33 in the asthmatic airway *in vivo*, and these are related to the severity of exacerbation; furthermore, IL-33 induction correlates with viral load and the induction of IL-5 and IL-13 (97). Dhariwal et al. examined the ratio of pulmonary ILC2 and ILC1 in asthma after RV-16 inoculation as compared with that of non-asthmatic subjects (98). They found that the ratio of ILC2 to ILC1 in bronchoalveolar lavage cells of asthmatics at baseline and after RV-16 inoculation is higher than that of non-asthmatic subjects, and it correlates with the severity of exacerbation and the induction of type 2 cytokines in nasal fluid (98). These findings suggest that ILC2 contributes to the development of type 2-mediated airway inflammation in viral-induced asthma exacerbation (**Figure 2**).

3) Discussion About the Status of ILCs in Asthma Patients

Epithelial cell-related cytokines such as IL-33, IL-25, and TSLP are induced by viral infection. RV produces IL-33 from airway epithelial cells or from bronchial smooth muscle cells *in vitro* (97, 99, 100), and supernatants of RV-infected bronchial epithelial cells induce type 2 cytokines from human T cells and ILC2 (97). RV induces IL-25 from airway epithelial cells of asthmatic patients *in vitro* (101). RSV induces TSLP in airway epithelial cells *in vitro via* activation of the innate signaling pathway (102). These findings suggest that viruses directly induce or produce epithelial cell-related cytokines, which can contribute to the induction of ILC2 and eosinophilic inflammation. However, the production of epithelial cell-related cytokines may depend on the type of virus, type of cells, the presence or absence of asthma, time of infection, and experimental conditions. Given the above, the actual role of epithelial cell-related cytokines in the pathogenesis of viral-induced asthma exacerbation needs to be further clarified. For example, the production of IL-33 by RV is much lower than that of other cytokines/chemokines such as IL-6 or IL-8 (100). We infected sinus or bronchial epithelial cells that were differentiated at the air-liquid interface with RVs, including RV-C, and measured the concentrations of IL-33, TSLP, and IL-25 in the basal medium; however, these factors were not induced (data not shown), in contrast to CCL5, CXCL10, CXCL11, IL-6, and IL-8 (13).

Mouse models are important for investigating host immunity during virus infection. However, mouse models of RV-infection have not always reflected human infection, because major groups of RVs such as RV-16 do not bind to mouse ICAM-1 and thus do not infect mice (103). As a result, minor groups of RVs such as RV-1B are often used in mouse models of RV infection. Using RV1B and RV-infected immature mice, Han et al. examined the roles of IL-33 and TSLP in RV-induced airway inflammation and ILC2 expansion; RV1B infection increased the expression of IL-33 and TSLP in the airway (104). RV1B-induced mucous metaplasia, expansion of ILC2, and AHR were suppressed by treatment with anti-IL-33 Ab or deletion of the TSLP gene (104). Beale et al. reported that RV1B infection increases pulmonary IL-25 expression, which is associated with increased type 2 cytokine production and increased viral load (101). Blockade of the IL-25 receptor reduces many RV-induced exacerbation-specific responses, including type 2 cytokine expression (101). Furthermore, Hong et al. reported that RV1B infection induces lung IL-13 and IL-25, and IL-13-producing ILC2 in neonatal mice, while an anti-IL-25 Ab suppresses ILC2 expansion, mucus hypersecretion, and AHR (105). As for RSV, Stier et al. reported that RSV infection upregulates IL-13-producing ILC2 with IL-13 expression in the lung (106). They also found that anti-TSLP Ab treatment or TSLP receptor deletion suppresses IL-13-producing ILC2 (106). These findings indicate important roles for IL-33, IL-25, and TSLP in the development of RV or RSV-induced ILC2-mediated airway inflammation in mice.

Recently, Rajput et al. developed a mouse model of RV-C infection; using immunofluorescence, they verified the colocalization of RV-C15 and CDHR3 in mouse ciliated airway epithelial cells (107). They reported that RV-C15-infected mice demonstrate greater eosinophilic airway inflammation; expression of IL-5, IL-13, IL-25, IL-33, and TSLP; and expansion of ILC2 compared to RV-A1B-infected mice (107). It was also found that RV-C-infected *Rora*$^{fl/fl}$ *Il7r*^{cre} mice deficient in ILC2 do not develop eosinophilic inflammation or the expression of IL-13 mRNA (107), suggesting that RV-C infection induces ILC2-mediated type 2 airway inflammation in mice. Different patterns of RV-induced airway inflammation among RV species are of great interest and should also be examined in humans.

Recent studies suggest that IL-33 exacerbates RV-induced airway inflammation and reduces anti-viral immunity. IL-33 increases the RV-16-induced inflammatory activity of human lung vascular endothelium and viral replication *in vitro* (108). IL-33 increases RV-induced type 2 cytokine production from PBMCs of asthmatics, but not of non-asthmatics (109). Werder et al. reported that an anti-IL-33 Ab decreases airway inflammation of cockroach-sensitized and challenged RV-infected mice (110). It also decreases RV replication and increases IFN-λ expression in mouse lungs *in vivo* and in human airway epithelial cells *in vitro* (110). In addition, Ravanetti et al. reported that IL-33 increases asthmatic airway inflammation and AHR in house dust mite-sensitized and challenged influenza-infected mice (111). They also show that an anti-ST2 Ab, which antagonizes IL-33, increases the expression of IFN-β in epithelial cells and DCs (111). As such, especially in RV infection, IL-33 plays roles in the augmentation of viral-induced asthma exacerbation.

POSSIBLE TREATMENT STRATEGIES FOR SUPPRESSING VIRAL INFECTION AND ILC2 ACTIVATION

Type I IFNs including IFN-α and IFN-β, type II IFNs including IFN-γ, and type III IFNs including IFN-λ have anti-viral capacity *in vitro* (112–115). IFN-β and IFN-γ also suppress the activation of ILC2 *in vitro* (116). This means IFN-β or IFN-γ can suppress not only viral replication but also ILC2 activation *in vitro*, which could be a novel strategy for treating viral-induced asthma exacerbation.

Low-dose IFN-α treatment improves lung function and allows for decreased corticosteroid dose in severe asthma (117). In a mouse model of allergic airway inflammation, IFN-γ attenuates RV1B-induced IL-13 expression and mucous metaplasia in immature mice, with a reduction in the expansion of ILC2s and the expressions of IL-5, IL-13, IL-17RB, ST2, and GATA-3 mRNAs in ILC2s (118). IFN-γ treatment also suppresses the allergen-induced overall immune response in a mouse model (119).

As systemic administration of IFN-α, IFN-β, or IFN-γ increases the risk of developing auto-immune diseases such as systemic lupus erythematosus (120) and liver dysfunction, the administration of IFN by inhalation has been developed. For example, Djukanović et al. examined the effect of inhaled IFN-β on cold-induced asthma exacerbation. Although IFN-β had no clinical benefit in treating asthma, it improved Asthma Control Questionnaire-6 responses in severe asthma patients in an exploratory analysis (121). Inhaled IFN-β tend to suppress RV load in sputum, whereas it boosted innate immunity as assessed by blood and sputum anti-viral biomarkers such as OAS1 and Mx1 (121). The effect of inhaled IFN-β on COVID19 has also been investigated (122), and available data from a phase II study demonstrate that inhaled IFN-β can accelerate recovery from the disease.

Another novel approach to suppress viral infection and ILC2 expansion is TLR agonists. For example, TLR7 agonists induce IFNs (123), which have anti-viral properties *in vitro*. TLR7 activation increases IFN-λ receptor mRNA expression in PBMCs (124). Furthermore, a TLR7 agonist has been reported to inhibit ILC2-dependent airway inflammation through interstitial macrophages producing IL-27 in a mouse model (125). Another potential approach is TLR3 agonism. TLR3 agonists induce IFN-β, which antagonizes STAT5-activating cytokines and suppresses ILC2 responses in lungs in a mouse model (126). These findings suggests that TLR agonism could be an important strategy for the suppression of viral-induced ILC2 activation, and thus viral-induced asthma exacerbation.

Anti-TSLP Ab treatment reduces asthma exacerbation in severe asthmatics (127) and clinical study demonstrates that anti-TSLP Ab decreases IL-5 and IL-13 concentrations in serum and eosinophilic inflammation in the airway (128). Although anti-TSLP Ab is assumed to suppress viral-induced ILC2-medicated eosinophilic inflammation as demonstrated in mouse model (106), actual effect of anti-TSLP Ab on viral-induced innate responses and ILC2 activation in patients with asthma should have been examined.

CONCLUSION

ILC2 plays important roles in the development of type 2-mediated airway inflammation in viral-induced asthma exacerbation. IFNs including IFN-β or TLR agonists can suppress not only viral replication but also ILC2 activation, which could become an innovative strategy for the treatment of virus-induced asthma exacerbation.

AUTHOR CONTRIBUTIONS

KN wrote the manuscript. MN edited the manuscript. All authors read and approved the final manuscript.

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Heterogeneity of Group 2 Innate Lymphoid Cells Defines Their Pleiotropic Roles in Cancer, Obesity, and Cardiovascular Diseases

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Group 2 innate lymphoid cells (ILC2s) are typically known for their ability to respond rapidly to parasitic infections and play a pivotal role in the development of certain allergic disorders. ILC2s produce cytokines such as Interleukin (IL)-5 and IL-13 similar to the type 2 T helper (Th2) cells. Recent findings have highlighted that ILC2s, together with IL-33 and eosinophils, participate in a considerably broad range of physiological roles such as anti-tumor immunity, metabolic regulation, and vascular disorders. Therefore, the focus of the ILC2 study has been extended from conventional Th2 responses to these unexplored areas of research. However, disease outcomes accompanied by ILC2 activities are paradoxical mostly in tumor immunity requiring further investigations. Although various environmental factors that direct the development, activation, and localization of ILC2s have been studied, IL-33/ILC2/eosinophil axis is presumably central in a multitude of inflammatory conditions and has guided the research in ILC2 biology. With a particular focus on this axis, we discuss ILC2s across different diseases.

Keywords: group 2 innate lymphoid cell, interleukin-5, interleukin-33, eosinophil, anti-tumor immunity, obesity, cardiovascular disease

INTRODUCTION

Recent expansion in our understanding of innate lymphoid cells (ILCs) began with several epoch-making reports in 2010 (1–4). The ILCs were originally indicated as interleukin (IL)-25 responsive non-B/non-T lymphocytes (5). ILCs are classified into five distinct cell populations based on their characteristics, including the profile of cytokines produced and the key transcription factors involved in their major immunological functions. These are the natural killer (NK) cells, group 1

ILC (ILC1), ILC2, ILC3, and lymphoid tissue inducer (LTi) cells (6). This classification should be observed with caution because ILCs possess a unique plastic ability to adapt to the surrounding milieu and can undergo transdifferentiation into another group of ILCs (7–9). ILC2s are tissue-resident cells (10), preferentially inhabiting the mucosal organs such as lung and intestine, and display tissue-specific transcriptional features influenced by the surrounding environment (11). The mucosal surface is the first line of defense against infectious pathogens; hence, ILC2s inherently display a prompt response through the secretion of IL-5 and IL-13. Unlike T cells, ILC2s lack antigen-specific receptors and instead express receptors for epithelial-derived cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, thereby ensuring signal recognition from exogenous agents. ILC2s not only participate in acute responses but also in the subsequent antigen-specific adaptive immune responses in cooperation with type 2 T helper (Th2) cells (12, 13). In addition to Th2 cytokines, ILC2s in the lung were found to produce IL-10 (14–16) and IL-10-producing ILCs, so-called ILCreg, have been reported in the intestine (17). Interestingly, like other antigen-presenting cells, ILC2s communicate through major histocompatibility complex class II molecules to activate acquired immune response (18). Thus, ILC2s provide a link between innate and acquired immunity (19).

ILC2s are involved in various immunological disorders and host defense (20). Asthma is a chronic airway inflammatory disease and one of the best-characterized allergic disorders associated with ILC2s (21, 22). ILC2s serve to establish predominant Th2 inflammation synergistically and/or competitively by interacting with other ILC subsets and immune cells (23). ILC2s in respiratory diseases are also evident in humans (24). To eliminate invading parasites, ILC2s mediate Th2 immune response in collaboration with adaptive Th2 cells (25, 26). In anti-viral immunity, although ILC2s exacerbate airway hyperreactivity through IL-13 production (27), they contribute to tissue repair by producing a wound-healing protein, amphiregulin (28). In most cases, IL-33 is considered a central cytokine for such ILC2-mediated immune responses.

Although the functions of IL-33 in allergies are well known (29, 30), the focus has currently shifted to its role in cancer (31–33) and cardiovascular diseases (34–36). IL-33 is one of the most effective cytokines for regulating ILC2s. In a steady state, IL-33 resides in the nucleus and is released by necrotic cells within damaged tissue (29, 37). When a tissue is injured/infected by pathogens, IL-33 acts by alarming the immune cells in the vicinity to mediate immune responses, and is thus called an "alarmin" or damage-associated molecular pattern. The IL-33 receptor comprises ST2 (IL-1 receptor-like 1) and IL-1 receptor accessory protein (38–40) which is expressed on various immune cells (41, 42). The binding of IL-33 to ST2 on the cell surface ensures Th2 responses, whereas soluble ST2 (sST2) in circulation inhibits excess IL-33-mediated responses and protects against disease development (29). In an allergic inflammation, platelets act as reservoirs and suppliers of IL-33 (43) and are capable of boosting ILC2 activities through direct interaction (44). In the lung tissue, platelets are supplied by the resident megakaryocytes (45) and may participate in regulation of ILC2 function.

IL-5 is a homodimeric cytokine and its engagement with its receptor, comprising an IL-5Rα and a common β-chain, plays critical roles in eosinophil biology starting from the early phase of its ontogeny in bone marrow (46, 47). Eosinophils store mediators such as major basic protein (MBP) in granules and are involved in both health and disease (48, 49). Genetic blockade of IL-5 signaling results in severe defects in eosinophil regulation (50, 51), and therefore treatments with anti-IL-5 or anti-IL-5Rα monoclonal antibodies (mAb) have been promising in patients with severe eosinophilic asthma (52–54).

In this review, we will discuss recent findings describing ILC2s in different types of disorders, such as cancer, obesity, and cardiovascular diseases. These findings suggest that roles of ILC2s are pleiotropic and diverse, largely depending on the surrounding environment. An ILC2-targeted therapeutic approach effective for one disease might be deleterious for another. This highlights the requirement for a detailed investigation and verification of the association and mechanisms of ILC2s.

Contradictory Roles of ILC2s in Tumor Immunity

Recent findings have shed light on both anti- and pro-tumor activities of ILC2s (55–60). The anti-tumorigenic activity of ILC2s appears to be largely dependent on the requirement of eosinophils at the site of malignancy. Histological evidence for the involvement of eosinophils in human cancers exists (61–63), however, the findings are controversial (64, 65). The number of infiltrated eosinophils in colonic or colorectal carcinomas significantly correlates with improved prognosis (63, 66–70). Conversely, in cervical cancer (71), nasopharyngeal carcinoma (72), and lymph node metastasis or lymphatic invasion (73), eosinophils were associated with unfavorable prognoses. In addition to the direct cytotoxic activity of the granules containing MBP on tumor cells (74, 75), eosinophils in tumor microenvironment (TME), when activated by interferon (IFN)-γ and tumor necrosis factor (TNF)-α efficiently promote mobilization of $CD8^+$ cytotoxic T cells from circulation (76). Eosinophils, however, display functional and phenotypical heterogeneity and their influence seems to rely on tumor types, TME, and cancer stages (64).

Involvement of IL-5-producing ILC2s in antitumorigenic activities was reported using an IL-5 reporter mouse (77), wherein lung ILC2s were required to retain sufficient number of eosinophils against tumor metastasis, and a blockade of IL-5 signaling resulted in an increased B16F10 metastasis. This is supported by the findings from a study that included three groups of mice deficient in C-C motif chemokine ligand 11 (CCL11), both CCL11 and IL-5, and eosinophils, respectively; all the three groups of mice exhibited increased tumor growth in chemically-induced fibrosarcoma (78). Antitumorigenic ILC2s are primed by their environment modulated by IL-33 (31–33). Mice inoculated with IL-33-expressing tumor cell lines, including EL4, CT26, and B16F10, resulted in a substantial

expansion of intertumoral ILC2, which inhibited tumor growth and induced apoptosis of tumor cells through the production of CXC chemokine receptor 2 ligands (79). IL-33-expressing A9, a murine lung tumor cell line, was also reported to augment the antimetastatic functions of ILC2s (80). Although ILC2s were not investigated in mice administered with IL-33, tumor growth and metastasis were inhibited *via* eosinophil recruitment (81). Mice deficient in ILC2s failed to control the incidence of experimentally induced colorectal cancers, whereas ILC2 infiltration correlated with better overall survival of patients with colorectal cancers (82). TME induces the expression of programmed cell death-1 (PD-1) on $CD8^+$ T cells as well as ILC2s, which results in the inhibition of cytokine production from these cells. Importantly, a blockade of PD-1 on the surface of ILC2s leads to revival of their antitumorigenic properties (83, 84). Interestingly, both serum IL-5 and IFN-γ levels are useful in predicting the efficacy of anti-PD1 mAb treatment in patients with non-small-cell lung and gastric cancer (85).

Accumulating evidence has also suggested pro-tumorigenic roles of ILC2s. In contrast to the previous study (77), IL-5 was reported to facilitate tumor metastasis (86). Additionally, IL-5 was suggested to enhance the migration of bladder cancer cells (87), and esophageal squamous cell carcinoma (88) in humans. Furthermore, IL-5 enhanced metastasis of breast cancer cells in obese mice (89). Consistent with these reports, ILC2s facilitated tumor metastasis in IL-33-treated animals by limiting cytotoxic activity of NK cells (90). Moreover, IL-13 derived from ILC2s promoted differentiation of myeloid-derived suppressor cells and were pro-tumorigenic in acute promyelocytic leukemia (91), bladder cancer recurrence (92), and metastasis of breast cancer (93).

Roles of ILC2s, eosinophils and IL-33 in tumor immunity show contrasting results, which poses a difficulty in understanding the distinct roles of these players in deciding the fate of tumor cells. However, the possibility of environmental cues as a key determinant for ILC2s to be antitumorigenic or pro-tumorigenic can be envisaged. For instance, lactic acid from tumor cells is pro-tumorigenic (94) whereas higher levels of IL-33 in TME are shown to induce antitumorigenic activities of ILC2s (81). This suggests that an assessment of the regulation of ILC2s by TME is essential for therapeutic intervention.

Anti-Inflammatory and Thermogenic Roles of ILC2 in Obesity

Obesity is a highly prevalent condition worldwide in which excess fat accumulates in the body. It is often associated with type 2 diabetes, high blood pressure, hyperlipidemia, and cardiovascular diseases (95). Apart from the roles of ILC2s in typical Th2 immune responses, they also contribute to homeostatic and metabolic regulation in adipose tissues (96, 97). Adipose tissues are categorized into white, brown, and beige. In comparison to white, beige and brown adipose tissues display higher and the highest thermogenic activity, respectively, and are thus specialized in generating heat. Initially, eosinophils were demonstrated to be the major IL-4-producing cells in white adipose tissue involved in inducing anti-inflammatory M2 macrophages (98) which prevents weight gain. Furthermore, ILC2s in adipose tissues were the major sources of IL-5 and IL-13 and recruited eosinophils to produce an anti-obese environment (99). Conversely, ILC2s in the small intestine were reported to induce obesity through the production of IL-2 (100), indicating the importance of the interplay between distal organs.

ILC2s also directly regulate adipocytes and participate in thermogenesis (101, 102). Adipose ILC2s promote beiging, conversion from white to beige, through production of methionine-enkephalin peptide, which can directly affect the adipocytes and upregulate the expression of uncoupling protein 1 (101), which was brought about by IL-33 (103). In response to cold stimuli, ILC2s are responsible for proliferation of platelet-derived growth factor receptors (PDGFR)-α^+ adipocyte progenitors and subsequent differentiation to beige adipocytes (102). IL-13 from ILC2s and/or IL-4 from eosinophils have been shown to stimulate PDGFRα^+ progenitors through their surface IL-4R.

Cell-cell interaction is important for the activation of adipose ILC2s. Both glucocorticoid-induced TNF receptor (104) and death receptor 3 (105) belong to the TNFR superfamily and are expressed on adipose ILC2s. Post ligand binding, ILC2s accelerate the production of IL-5 and IL-13 and improve glucose tolerance and insulin sensitivity, demonstrating their potential to be used in type 2 diabetes therapy. In contrast, IL-33 in the presence of TNF-α in obese conditions upregulates PD-1 expression on adipose ILC2s and limits their production of IL-5 and IL-13 (106). Recently, regulation of ILC2s by sympathetic nerves *via* adipose mesenchymal stromal cells was observed (107). Elucidation of the precise regulatory mechanism and knowledge on the specific activators of adipose ILC2s will aid in therapy for obesity or type 2 diabetes.

Reparative Roles of ILC2s in Cardiac Dysfunction

ILC2s are involved in healing cardiac tissue with cooperation from various types of immune cells to recover and regenerate cardiac tissue damage caused by myocardial infarction (MI) (108). ST2 is expressed on cardiomyocytes, and levels of sST2 in serum from animals and humans were elevated after MI (109). Therefore, IL-33 being the only known ligand of ST2 (38), its role in cardiovascular and vascular diseases (34–36) was investigated. In contrast to the known pro-inflammatory functions of IL-33, IL-33/ST2 signaling protected animals from experimentally induced cardiac failure by antagonizing angiotensin II-induced cardiomyocyte hypertrophy (110). Furthermore, IL-33 also dictates healing processes indirectly *via* ILC2s.

Under physiological conditions, ILCs reside in heart and display a progenitor-like phenotype (111). These heart resident ILCs are evident in biopsy samples from animals and humans with ischemic cardiomyopathy and myocarditis and are fated to differentiate to ILC2s in response to cardiac failure (111). ILC2s in pericardial adipose tissue (PcAT) proliferate in an IL-33 dependent manner in response to MI, and animals deficient in ILC2 exhibited incomplete recovery from heart dysfunction and a worsened mortality rate post-MI (112). Although the precise mechanism of ILC2s is unknown, the recruitment of eosinophils

by IL-5 is considered in the recovery of cardiac function. This is supported by the observation that animals deficient in eosinophils failed to ameliorate cardiac functions after MI and that IL-4 from eosinophils was essential for recovery (113). However, the infiltration of eosinophils into heart needs to be regulated in order to avoid eosinophilia which induces inflammatory dilated cardiomyopathy (114).

Interestingly, low-dose IL-2 (aldesleukin) administration in patients with acute coronary syndrome exhibited transient activation of blood ILC2s, with a concomitant increase in serum IL-5 and eosinophil counts, demonstrating recovery of cardiac function (112). Further research on ILC2s in cardiac diseases will provide beneficial insights into developing unprecedented therapies.

Protective Roles of ILC2s in Atherosclerosis

Atherosclerosis is an arterial disease characterized by the deposition of plaques on inner walls; and lipid modifications in plaques result in the generation of non-self-antigens, causing chronic inflammation. Atherosclerosis is the primary cause of most cardiovascular diseases. Administration of cytokines related to ILC2 activation were effective in reducing atherosclerosis in animals (115). TSLP (116), IL-25 (117), and IL-33 showed protective effects, and the effectiveness of IL-33 was largely dependent on IL-5 (118). ILC2s that were experimentally expanded with IL-2/IL-2R complexes protected from the development of atherosclerosis, although, the contribution of IL-5/eosinophils was limited (119). In contrast, ILC1 and NK cells were shown to play etiologic roles in disease development (120). This correlated well with a significantly high ILC1/ILC2 ratio in patients with acute cerebral infarction, commonly caused by rupture of atherosclerotic plaques (121). By selectively depleting ILC2s in an animal model of atherosclerosis, regional ILC2s that were in proximity to atherosclerotic lesions, sufficiently reduced atherosclerosis, possibly through phenotypic alteration of macrophages to anti-inflammatory M2 macrophages (122). Furthermore, transfer of ILC2s into mice that developed atherosclerosis led to an increase in B1 cell-derived atheroprotective IgM antibodies with reduction in plaque deposition (123). Collectively, ILC2s appear to be protective against atherosclerosis.

Etiologic Roles of ILC2s in Pathogenesis of Pulmonary Arteries

In contrast to the protective roles of ILC2s in cardiac failure and atherosclerosis, chronic inflammation in lungs possibly drives ILC2s to act in mediating disorders of blood vessels, including pulmonary arterial hypertension (PAH). PAH is a progressive vascular disease characterized by a severe obstruction such as hypertrophy of small pulmonary arteries with high pulmonary arterial pressure, thereby resulting in right ventricular failure. It is categorized as one of the five groups of clinical classification for pulmonary hypertension (PH) (124). PAH is an intractable rare disease and its development is multifactorial. Although the investigation of causative factors of PAH is ongoing, chronic inflammation may have a plausible role in the underlying mechanism (125). Evidence suggests chronic allergic conditions in mice, concomitant with eosinophilia, lead to the induction of vessel remodeling with remarkable collagen deposition and enhanced proliferation of α-smooth muscle cells (126, 127). Subsequently, Th2 cytokines (128) or IL-5 and eosinophils (129) were reported to be necessary for the initiation of arterial remodeling. In humans, parasitic infection, in which Th2 cytokines such as IL-4 and IL-13 are predominant, is believed to be the most common cause of PAH (130, 131), with Th2 cytokines inducing arterial hypertrophy and other arterial modifications (131, 132).

Pulmonary arterial hypertrophy can also be experimentally induced by prolonged administration of IL-33 in mice (133). Histological examination revealed that perivascular ILC2s and eosinophils were evident around hypertrophied arteries, and this hypertrophy was ameliorated with anti-IL-5Rα mAb that depleted eosinophils (134). The proximity of ILC2s to blood vessels in lungs, as visualized in collagen-rich (135) adventitial niches (136), may facilitate their vascular regulation through eosinophil recruitment. In this region, ILC2s are maintained by IL-33-expressing stromal cells (136) which possibly regulate ILC2s in case of arterial hypertrophy. Thus, elucidation of the precise regulatory mechanism will help to understand the initial phase of disease development.

Because of the lack of histological evidence in humans on initial phase of arterial hypertrophy, animal models of PAH are essential to reveal causative factors. Despite reports of advanced arterial hypertrophy in animal studies, severe PH or right ventricular hypertrophy is not evident (101, 102, 105). The establishment of animal models that are more relevant to human PAH will not only help us to understand the underlying mechanism but is also imperative in developing a therapeutic strategy.

DISCUSSION/CONCLUSION

Recent advances in ILC2 research have revealed their pleiotropic roles in various diseases (**Figure 1**). Due to heterogeneity in the function of ILC2s in various disease conditions, their clinical application faces many obstacles. A treatment that targets ILC2s in one disease may be detrimental to another. For example, therapy for obesity by activating ILC2s with low doses of IL-2 may result in excess amounts of IL-5 from the ILC2s and facilitate tumor metastasis (89). These may present a similar effect in related diseases such as atherosclerosis (119) and MI (112). Thus, understanding the precise action of ILC2s in a particular disease and the extent of its effect on other diseases is indispensable. Delicate procedures for regulating ILC2s are required in addition to careful analyses of experimental and clinical observations, which will ultimately lead to efficient therapeutic regimes.

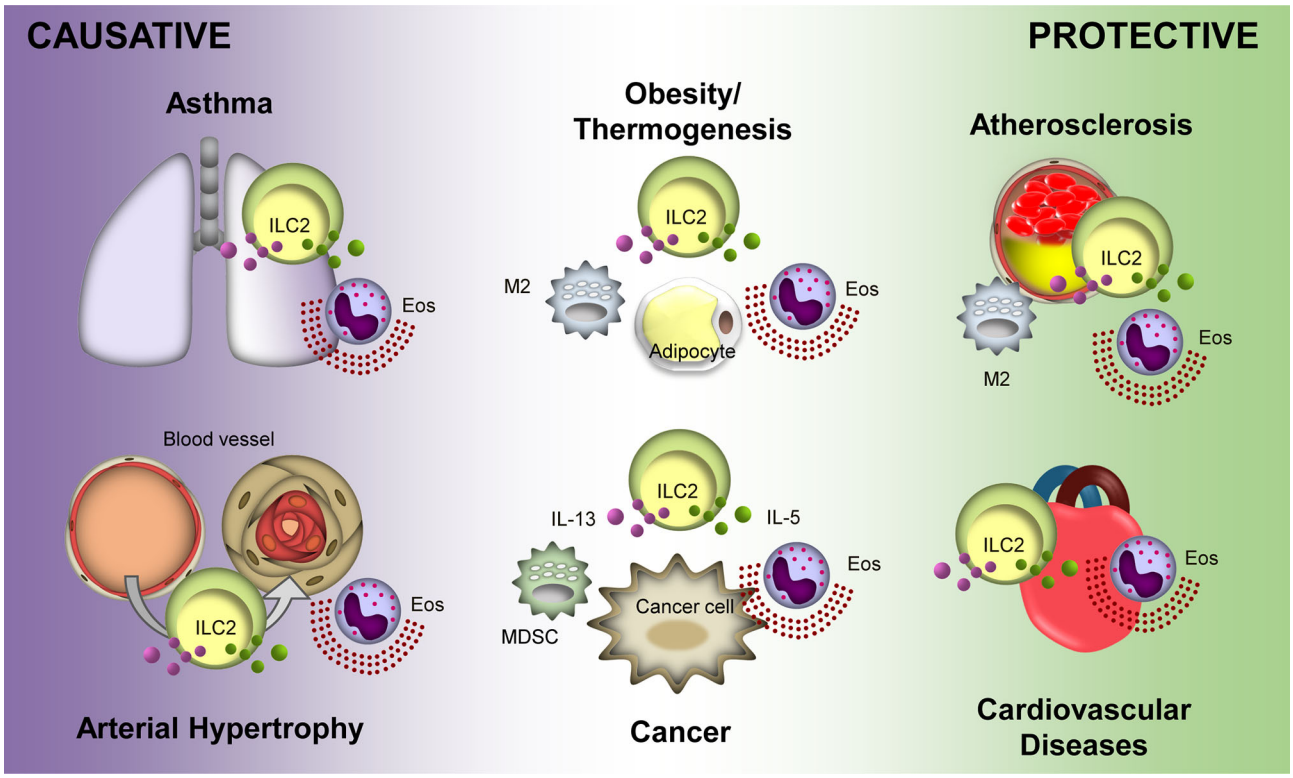

FIGURE 1 | Protective and causative roles of Group 2 Innate Lymphoid Cells (ILC2s) in different diseases. ILC2s display a protective role in atherosclerosis and cardiovascular diseases. Overall, ILC2s is protective in obesity and thermogenesis; however, distal ILC2s may cause obesity. Conversely, ILC2s potentiate asthma and arterial hypertrophy. Their roles in cancer are varied depending on the tumor microenvironment and type of cancer. Purple and green particles depicted in the figure are interluekin-13 (IL-13) and IL-5, respectively. Eos, eosinophil; M2, M2 macrophage; MDSC, myeloid-derived supressor cell.

AUTHOR CONTRIBUTIONS

MI designed and wrote the manuscript. SN reviewed and revised the manuscript prior to submission. All authors have read and approved the final version of the manuscript.

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The Therapeutic Potential for Targeting Group 2 Innate Lymphoid Cells in Asthma

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T helper type 2 cells (Th2 cells) and group 2 innate lymphoid cells (ILC2s) play an important role in the pathophysiology of asthma, including airway eosinophilic inflammation. ILC2s are activated by epithelial-derived cytokines [interleukin-25 (IL-25), IL-33, and thymic stromal lymphopoietin (TSLP)] from airway epithelial cells, leading to the release of high amounts of type 2 cytokines, such as IL-5 and IL-13. ILC2s induce airway inflammation in an antigen-independent manner, and ILC2s are considered to be involved in the pathogenesis of asthma exacerbation. Furthermore, ILC2 activation might also confer steroid resistance. Many recent studies in humans and mice are increasingly demonstrating that the function of ILC2s is regulated not just by epithelial-derived cytokines but by a variety of cytokines and mediators derived from innate immune cells. Furthermore, the biologics targeting these cytokines and/or their receptors have been shown to reduce asthma exacerbations and improve lung function and quality of life in asthmatics. This article reviews the current treatment landscape for type 2 airway inflammation in asthma and discusses the therapeutic potential for targeting ILC2s.

Keywords: group 2 innate lymphoid cells (ILC2s), airway inflammation, innate immune network, biologics, asthma

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INTRODUCTION

Asthma is a heterogeneous disease characterized by chronic airway inflammation, reversible airway obstruction, and airway hyperresponsiveness (AHR) (1). The prevalence of asthma is increasing in many parts of the world that have adopted aspects of the western lifestyle, and the disease poses a substantial global health and economic burden (2). Respiratory allergic diseases, including asthma, are associated with type 2 cytokines, such as interleukin-4 (IL-4), IL-5, and IL-13, which induce airway eosinophilia, mucus hyperproduction, AHR, and immunoglobulin (Ig)E class switching. There are allergic asthma phenotypes triggered by exposure to allergens and associated with allergic sensitization and adaptive immunity. There are also non-allergic asthma phenotypes triggered by exposure to environmental factors, such as air pollution, including ozone, cigarette smoke, and diesel particles; viral infection, and stress, which are associated with innate immunity independent of T helper type 2 (Th2) cells (3). Non-allergic asthma is induced by IgE-independent allergic reaction and the involvement of group 2 innate lymphoid cells (ILC2s). These different pathways

and phenotypes often coexist and act in synergy in patients, and distinct pathogenic mechanisms probably underlie each of these pathways and phenotypes (3).

In 2010, ILC2s were discovered in gut-associated mucosal tissues (4–6). ILC2s do not express hematopoietic lineage markers (Lin) and respond to epithelial cell-derived cytokines, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). Subsequently, activated ILC2s produce high amounts of IL-5 and IL-13 (7). Since ILC2s have been recognized for their ability to drive type 2 immune responses in experimental animal models in the absence of T cells, ILC2s are a T cell-independent source of type 2 cytokines, considered to play an important role in the pathogenesis of allergic diseases including asthma (7, 8). Furthermore, many studies in both humans and mice focused on ILC2s, investigating their main features such as development from a precursor, stimuli for their activation or inhibition, their plasticity, and their classification in different subsets (9).

Pharmacotherapy for asthma is based on inhaled corticosteroid (ICS), with add-on treatments if needed, such as long-acting β_2 agonist (LABA), leukotriene receptor antagonist (LTRA), and long-acting muscarinic antagonist (LAMA). We previously reported that LAMA suppressed innate-type-induced airway inflammation (10). This mechanism may contribute to the suppressive effect of asthma exacerbations. Approximately 5% of asthmatics have severe asthma that requires treatment with high-dose ICS plus a second controller and/or systemic corticosteroids to prevent asthma from becoming uncontrolled or that remains uncontrolled despite these therapies (11). Recently, patients with uncontrolled severe asthma have been considered candidates for biologics (12). In this review, we highlight the current treatment landscape for type 2 airway inflammation in asthma. Moreover, we discuss the therapeutic potential for targeting ILC2s (13).

THE ROLE OF ILC2S IN THE PATHOPHYSIOLOGY OF ASTHMA

ILC2 and Asthma Pathophysiology

In murine models, protease allergens, such as papain and house dust mites (HDM), damage the airway epithelial cell barrier *via* their proteolytic activity and release epithelial-derived cytokines, including IL-25, IL-33, and TSLP. Then, these cytokines induce ILC2 activation, leading to the production of high amounts of IL-5 and IL-13 derived from ILC2s. While T cells require priming by antigen-presenting cells upon first contact with an antigen, ILC2 activation is induced by epithelial-derived cytokines in an antigen-independent manner (14). Further, in a recent study, IL-33 and TSLP are shown to be produced from adventitial stromal cells other than airway epithelial cells. These cytokines derived from adventitial stromal cells are shown to accumulate ILC2 in regions around bronchioles and large blood vessels, where formed niches (15). ILC2s can control some of the features of asthma, such as AHR, goblet cell hyperplasia, and eosinophilia (8, 16, 17). Several groups have studied the role of human ILC2s in the pathophysiology of asthma. The number of ILC2s and the ability of cytokine production from ILC2s in peripheral blood were increased in asthmatics (18–21). Allergen exposure also increased the frequency of IL-5^+, IL-13^+, and prostaglandin D_2 (PGD_2) receptor chemoattractant receptor-homologous molecule expressed on Th2 cells-positive (CRTH2^+) ILC2s in the sputum of asthmatics (22). Furthermore, the frequency of ILC2s in bronchoalveolar lavage fluid (BALF) also was increased in asthmatics (23). The frequency of ILC2s positively correlated with sputum and peripheral blood eosinophils and fractional exhaled nitric oxide (FeNO) levels and negatively correlated with predicted FEV_1% (19, 24). Moreover, the numbers of total and type 2 cytokine-producing ILC2s in the blood and sputum of patients with severe asthma were significantly higher than those of mild asthmatics (20). In regard to epithelial-derived cytokines, the levels of IL-33 and TSLP were significantly increased in BALF from asthmatics. Furthermore, IL-33 levels in BALF were shown to positively correlate with the severity of asthma (23, 25). These findings suggest that ILC2s play an essential role in the pathophysiology of asthma.

ILC2 and Virus-Induced Asthma Exacerbation

ILC2s are also considered to be involved in the pathogenesis of asthma exacerbation. Viral respiratory infections are the most common cause of asthma exacerbations in both children and adults (26). Respiratory syncytial (RS) virus infection has been shown to result in not only neutrophilic inflammation but also eosinophilic inflammation (26, 27). RS virus enhanced type 2 cytokine production and pulmonary eosinophilic infiltration in an ovalbumin-induced murine asthma model (28). In a RS virus-inoculated murine model, RS virus also induced IL-13-producing ILC2 proliferation and activation in a TSLP-dependent manner (29). Furthermore, influenza A virus infection induced AHR, independently of Th2 cells and adaptive immunity. Influenza virus infection-induced AHR required the IL-33-IL-13 axis and ILC2s (30). In studies with rhinovirus (RV) inoculation, asthmatics increased the levels of nasal IL-33 and type 2 cytokines. Furthermore, human primary bronchial epithelial cells released IL-33, and ILC2s in response to the supernatants of RV-infected bronchial epithelial cell produced type 2 cytokines (31). In another study of patients with moderate asthma inoculated with RV, ILC2 numbers in BALF were also increased during viral infection, and ILC2-mediated inflammation in asthmatics was associated with the severity of asthma (32). Thus, these results support the potential role of ILC2s in the pathogenesis of virus-induced asthma exacerbations.

ILC2 and Steroid Resistance

TSLP is considered to promote the steroid resistance of murine and human ILC2s. In a murine model, the IL-33/ST2 (IL-33 receptor) pathways have been shown to be sensitive to steroids, and the induction of the TSLP/STAT5 pathway has been related to steroid resistance in IL-33-stimulated ILC2s (33). In human ILC2s, TSLP induced the steroid resistance in a MEK- and STAT5-dependent manner, and elevated levels of TSLP in BALF of asthmatics correlated with steroid resistance (25). Further, dexamethasone attenuated IL-5 production by the stimulation with tumor necrosis factor-like protein 1A (TL1A) or TSLP, but not by the combination of TL1A and TSLP in

human ILC2s. Thus, the interaction between TL1A/death receptor 3 (DR3) pathway and TSLP may be critical for steroid resistance in human ILC2s (34). A recent study also indicated that ILC2s expressing $CD45RO^+$ rather than $CD45RA^+$ were associated with steroid resistance. The frequencies of $CD45RO^+$ inflammatory ILC2s (iILC2s) in blood were increased in asthmatics and positively correlated with disease severity (24). These reports suggest that ILC2s might affect steroid resistance.

THE THERAPEUTIC POTENTIAL OF TARGETING THE INTERACTION BETWEEN ILC2S AND IMMUNE CELLS IN ASTHMA

Interaction Between ILC2 and Other Immune Cells

ILC2s function as initiators of adaptive immunity or as responders to signals produced by immune cells and structured cells (35). Many studies have demonstrated that a network exists between ILC2s and immune cells or non-immune cells, and that their interaction also plays an important role in orchestrating type 2 immune responses by cell-cell contacts or by communication *via* soluble factors, such as cytokines, lipid mediators, and hormones. In regard to the association between ILC2s and T cells, IL-2 derived from T cells can promote ILC2 proliferation and IL-13 production (36). ILC2s express MHC class II, and ILC2s by themselves might be identified as antigen-presenting cells and might modulate naive T cell activation (36, 37). Furthermore, ILC2s interact with other innate effector cells other than T cells to initiate type 2 inflammation. ILC2-derived IL-13 not only triggered mucus secretion and AHR directly but also promoted dendritic cell (DC) migration to the draining lymph nodes, leading prime naive T cells to differentiate into Th2 cells (38). Additionally, ILC2-derived IL-13 is shown to induce the production of CCL17 derived from DCs, leading to the attraction of $CCR4^+$ memory Th2 cells (39). Although DCs and macrophages have been known to produce TL1A to regulate the adaptive immune response by co-stimulating T cells through DR3 (40), recent studies have demonstrated that TL1A was able to directly stimulate ILC2s derived from human blood, murine mesenteric lymph nodes, and lungs to produce type 2 cytokines independent of IL-25 or IL-33 (41, 42). ILC2s also interact with granulocytes, such as basophils and mast cells, to regulate type 2 immune responses. Although eosinophils are activated by IL-5 derived from ILC2s, it has been reported that eosinophils produce IL-4 by the stimulation of IL-33 (43). Recent studies have demonstrated that basophils interacted with other immune cells and non-hematopoietic cells through cell-to-cell contact or cytokines and proteases, affecting the regulation of immune and allergic responses (44). In regard to the association with ILC2, basophils have been shown to trigger ILC2 proliferation and activation *via* basophil-producing IL-4 (45, 46). In the study of murine lungs, basophil-producing IL-4 had an important role in ILC2-derived type 2 cytokine production, subsequently leading to protease-induced airway eosinophilic inflammation (45). In regard to the interaction between ILC2s and mast cells, mast cell-derived IL-2 enhances the expansion of regulatory T cell (Treg) numbers by IL-33 stimulation, thereby suppressing ILC2-dependent allergic inflammation in a murine model. In this study, mast cells suppress protease-induced airway eosinophilic inflammation through a mast cell-IL-2-Treg-IL-10-ILC2 axis (47).

Therapeutic Potential of Targeting Cells Interacting With ILC2 in Asthma

In asthma, recent studies have indicated the therapeutic potential for targeting the crosstalk between ILC2s and other immune cells. Our group revealed that tiotropium, LAMA, attenuated ILC2-dependent airway eosinophilic inflammation by suppressing IL-4 production from basophils and subsequently regulating ILC2 activation in a murine model (10). Tiotropium is mainly attributed to muscarinic M3 receptor (M3R) inhibition. However, ILC2s hardly expressed M3R. *In vitro* study, tiotropium did not affect IL-33-induced IL-5 and IL-13 production from ILC2s, suggesting that tiotropium regulated ILC2s indirectly. In contract, basophils exhibited higher expression of M3R as compared with that of other immune cells. Tiotropium suppressed IL-33-induced IL-4 production from basophils. Moreover, in co-culture of basophils and ILC2s, the production of IL-5 and IL-13 was decreased by tiotropium. Furthermore, M3R was shown to be expressed on human basophils. Tiotropium also reduced the production of human basophil-derived IL-4 (10). Because the addition of tiotropium to ICS and LABA has been reported to reduce the frequency of asthma exacerbation in patients with severe asthma (48), the inhibitory effects of LAMA on ILC2-mediated airway inflammation may contribute to reducing the risk of asthma exacerbation (10). Toll-like receptors (TLRs) recognize components of viruses and bacteria to trigger an innate immune response. R848, a TLR7 agonist, stimulated IL-33-induced interstitial macrophages (IMs) to attenuate ILC2-mediated airway eosinophilic inflammation through IM-derived IL-27 (49), which suppressed the proliferation and type 2 cytokine production of murine and human ILC2s (50, 51). Other TLR7 agonists, AZD8848 and GSK2245035, have been evaluated as therapeutic agents in clinical trials for asthma. However, the effect of AZD8848 on the fall in FEV_1 of late asthmatic response after allergen challenge was not sustained for 4 weeks, and GSK2245035 also did not show clinical improvement (52, 53). ILC2-mediated airway inflammation has been associated with allergen-independent stimuli such as viral infection. Thus, TLR7 agonists, as well as LAMA, are expected to have an effect on regulating asthma exacerbation, especially those associated with viral infection. In these results, LAMA and TLR7 agonists are considered to act on the innate immune network, leading to the indirect inhibition of ILC2-dependent airway inflammation *via* basophils and macrophages (**Table 1**).

Neuropeptides, Lipid Mediators, Sex Hormones, and Angiotensin II

In addition to cytokines, various factors, including lipid mediators, neuropeptides, sex hormones, also regulate ILC2s and are directly involved in airway inflammation. Lipid mediators are produced by various cells including immune cells, such as neutrophils, mast cells,

TABLE 1 | Summary of drugs targeting cells interacting with ILC2 in murine model.

Drugs	Targets	Target cell	Effect on airway inflammation			
			BALF eosinophils	BALF ILC2s	Cellular infiltration around airway	Mucus secretion
Tiotropium	M3R	Basophils	↓	↓	↓	↓
R848	TLR7	Interstitial macrophages	↓	↓	↓	↓

BALF, bronchoalveolar lavage fluid; ILC2, group 2 innate lymphoid cell; M3R, muscarinic M3 receptor; TLR7, toll-like receptor 7.

basophils and macrophages (54, 55). However, the major source of lipid mediators acting on ILC2 has not been well understood. Cysteinyl leukotriene receptors (CysLTR) are expressed on ILC2. In an *Alternaria* species-induced murine model, leukotriene D_4 (LTD_4) activates ILC2 to rapidly produce high levels of IL-4, IL-5, and IL-13 (56). Further, PGD_2, which are produced by mast cells, causes human and mouse ILC2 chemotaxis and type 2 cytokine production *via* CRTH2 in the lungs (22, 57, 58). In the IL-33-induced murine model, PGE_2 results in decreased eosinophils numbers and type 2 cytokine production, and ameliorated ILC2-mediated lung inflammation (59). In the study of mouse and human ILC2s, PGE_2 has a suppressive effect on IL-33-induced ILC2 proliferation and type 2 cytokine production *in vitro* (59, 60). PGI_2 also has an inhibitory effect on ILC2s. In an *Alternaria* species-induced murine model, PGI_2 suppresses IL-5 and IL-13 protein expression, and IL-5- and IL-13-expressing ILC2 numbers in lungs. Moreover, in the study of human ILC2 stimulated with IL-2 and IL-33, PGI_2 inhibits IL-5 and IL-13 production (61). Regarding neuropeptides, ILC2s express neuromedin U receptor 1 (NMUR1) and calcitonin gene-related peptide (CGRP) receptor (CALCRL and RAMP1) (62, 63). Neuromedin U is expressed on the neurons in the thoracic dorsal root ganglia, which contain afferent sensory neurons (62), and CGRP is produced by pulmonary neuroendocrine cells (PNECs) that reside near ILC2s at airway branch points (63). Neuromedin U and CGRP induce ILC2-mediated allergic lung inflammation. ILC2s also express α7nAChR, a nicotinic acetylcholine receptor. Acetylcholine including nicotine is derived from parasympathetic nerves (64). GTS-21, an α7nAChR agonist, is associated with reduced airway inflammation and AHR in the IL-33-induced murine model (65). Sex hormones such as androgen regulate the development and function of ILC2s. ILC2 progenitors (ILC2P) express androgen receptors. In male mice, HDM or IL-33-induced airway inflammation is less prominent than those in female mice (66). Further, ILC2 numbers in the blood of asthmatics are higher in women compared to men (67). In a recent study, ILC2s are shown to express AT1a, angiotensin II receptor. Angiotensinogen, renin, and angiotensin-converting enzyme (ACE), which are the enzymes essential for angiotensin II production, are expressed on airway epithelial cells (68). Thus, angiotensin II may be derived from airway epithelial cells. Angiotensin II induces ILC2 responses in a cell-intrinsic and IL-33-dependent manner, leading to airway inflammation. Furthermore, the levels of angiotensin II in plasma positively correlate with the abundance of blood ILC2s and disease severity in asthmatics (68). These findings suggest that neuropeptides, lipid mediators, sex hormones, and angiotensin II and their receptors might be potential therapeutic targets for asthma and ILC2-related diseases.

THE EFFICACY OF BIOLOGICS AND THE POTENTIAL OF ILC2-TARGETED THERAPY IN ASTHMA

Biologic therapies target specific inflammatory pathways involved in the pathogenesis of asthma, particularly in patients with an endotype caused by type 2 inflammation (12). These therapies have been required in patients with uncontrolled severe asthma despite optimal treatment. Currently, available biologics compose anti-IgE, anti-IL-5, anti-IL-5 receptor α (IL-5Rα), and anti-IL-4 receptor α (IL-4Rα). These biologics reduce asthma exacerbation rates and improve asthma symptoms and lung function. Additionally, randomized controlled trials have recently shown the efficacy of biologics for targeting epithelial-derived cytokines, such as TSLP and IL-33, in patients with severe asthma (**Table 2**).

Anti-IgE

Omalizumab, a humanized anti-IgE monoclonal antibody (mAb), prevents IgE from binding to high-affinity IgE receptors (FcεRI) on mast cells and basophils, which inhibits the release of proinflammatory mediators, such as histamine and leukotrienes. Clinical trials have demonstrated that omalizumab reduces asthma symptoms and the frequency of asthma exacerbation (69, 70). The three biomarkers—FeNO, peripheral blood eosinophil counts, and serum periostin—were associated with the risk of asthma exacerbation (71). However, in asthma patients, the frequencies of ILC2s and ILC2-derived cytokine production were not influenced by omalizumab (72).

Anti-IL-5/IL-5Rα

Mepolizumab, a humanized anti-IL-5 mAb, binds to IL-5, preventing IL-5 from binding to its receptors on eosinophils (12). Among patients with uncontrolled eosinophilic asthma who have increased sputum or peripheral blood eosinophil counts, mepolizumab has been shown to reduce the frequency of asthma exacerbation, improve lung function and asthma control, and reduce the oral corticosteroid (OCS) dose (73–75). In the study compared treatment responses of weight-adjusted intravenous reslizumab, another humanized anti-IL-5 mAb, among patients previously treated with subcutaneous mepolizumab, the blood ILC2 numbers were significantly but modestly decreased. However, ILC2 numbers in blood or sputum were not decreased by reslizumab (76). On the other hand, benralizumab, a humanized anti-IL-5Rα mAb, prevents IL-5 from binding to IL-5Rα on eosinophils. The clinical trial revealed that benralizumab had the same effect as mepolizumab in asthmatics with elevated peripheral blood eosinophil counts

TABLE 2 | Summary of approved and new potential biologics for severe asthma.

Biologics	Targets	Effect on inflammatory biomarker			Effect on asthma control				OCS-sparing effect
		FeNO	Blood eosinophils	Sputum eosinophils	Exacerbation	Symptom	QOL	FEV_1	
Approved biologics									
Omalizumab	IgE	↓	↓	↓	↓	↓	↑	↑	No data on RCT
Mepolizumab	IL-5	→	↓	↓	↓	↓	↑	↑	50% reduction
Reslizumab	IL-5	No data	↓	↓	↓	↓	↑	↑	No data on RCT
Benralizumab	IL-5Rα	No data	↓	↓	↓	↓	↑	↑	75% reduction
Dupilumab	IL-4Rα	↓	↑	No data	↓	↓	↑	↑	70% reduction
New potential biologics									
Tezepelumab	TSLP	↓	↓	↓	↓	↓	↑	↑	No significant difference [#]
Itepekimab	IL-33	↓	↓	No data	No data	↓	↑	↑	No data
Astegolimab	ST2	→	↓	No data	↓	→	↑	→	No data

OCS, oral corticosteroid; FeNO, fractional exhaled nitric oxide; QOL, quality of life; RCT, randomized controlled trial, [#], unpublished data.

(77–79). Additionally, in patients with severe steroid-dependent asthma, benralizumab reduced the blood IL-5Rα$^+$ ILC2 numbers but not the numbers of total ILC2s (80).

Anti-IL-4Rα

Dupilumab, a humanized anti-IL-4Rα mAb, inhibits the signaling of both IL-4 and IL-13 by binding to IL-4Rα. Dupilumab has been shown to reduce the rate of asthma exacerbation and improve lung function and asthma control in patients with moderate-to-severe uncontrolled asthma (81, 82). Among patients with a peripheral blood eosinophil count ≥300/μl or FeNO ≥50 ppb, the effect of dupilumab on the asthma exacerbation rate was greater (82). Moreover, dupilumab had an OCS-sparing effect while decreasing the asthma exacerbation rate and improving lung function (83). In a murine model, IL-4 might promote cytokine production from ILC2s through IL-4R (45, 46). In the study of human ILC2s cultured with IL-2, IL-7, IL-25, and IL-33, the addition of IL-4 to the ILC2s also further enhanced the production of IL-5 and IL-13 *in vitro* (84). Treatment with dupilumab reduced ILC2 numbers in the peripheral blood of asthmatics. Furthermore, dupilumab repressed the expression of IL-5 and IL-13 mRNA in ILC2s. Thus, these results demonstrate that the blockade of IL-4Rα by dupilumab suppressed ILC2 response directly or indirectly in asthmatics and might be involved in the reduced frequency of asthma exacerbation (84). Human ILC2s have been shown to express IL-4Rα (85), and dupilumab can be a novel treatment targeting ILC2s.

Anti-TSLP

Recently, biologics targeting upstream type 2 inflammation have been developed, including anti-TSLP, anti-IL-33, and anti-ST2 therapies in addition to dupilumab. Tezepelumab, a humanized anti-TSLP mAb, prevents TSLP from interacting with its heterodimeric receptor. In a phase 2 study, tezepelumab regulated the rate of asthma exacerbation in patients with moderate-to-severe uncontrolled asthma (86). A recent study also demonstrated that tezepelumab suppressed type 2 inflammatory biomarkers, such as blood eosinophil counts, FeNO, serum IgE, type 2 cytokines, periostin, thymus and activation-regulated chemokine (TARC); there were positive correlations between these biomarkers at the baseline (87). Further, tezepelumab's suppression of asthma exacerbation was observed regardless of blood eosinophil counts (86). In a phase 3 study involving severe uncontrolled asthmatics, tezepelumab not only reduced the frequency of asthma exacerbation but also improved lung function and asthma control, regardless of blood eosinophil counts (88). So far, available biologics have not been shown to suppress asthma exacerbations in asthmatics with type 2-low endotype. Therefore, tezepelumab is expected to have an effect on asthmatics with type 2-low endotype.

Anti-IL-33/ST2

Genome-wide association studies have indicated that IL-33 and IL1RL1 (encoding ST2), which have obvious links to ILC2 biology, are associated with asthma susceptibility (89–91). Itepekimab is a human IgG4P mAb against IL-33. In a phase 2 trial involving patients with moderate-to-severe asthma, itepekimab led to improved lung function and fewer events indicating a loss of asthma control. Further, itepekimab also reduced blood eosinophil counts and FeNO levels (92). Astegolimab is a human IgG2 mAb that blocks IL-33 signaling by targeting ST2. In a phase 2b study involving patients with severe asthma, astegolimab suppressed annualized asthma exacerbation rates. Furthermore, the reduction of the asthma exacerbation rate for patients with low blood eosinophil counts was also comparable to the reduction in the overall population. In regard to biomarkers, astegolimab reduced blood eosinophil counts but not FeNO levels (93). ILC2-expressed IL-4R, ST2, and TSLPR; and IL-4, IL-33, and TSLP are required for ILC2 activation and expansion, as described above. Thus, suppressing these pathways that induce ILC2 responses might lead to improved asthma control.

CONCLUDING REMARKS

This review has highlighted current asthma treatment strategies for type 2 airway inflammation and described the therapeutic potential for targeting ILC2s (**Figure 1**). In recent studies, various pathways have been identified for regulating ILC2

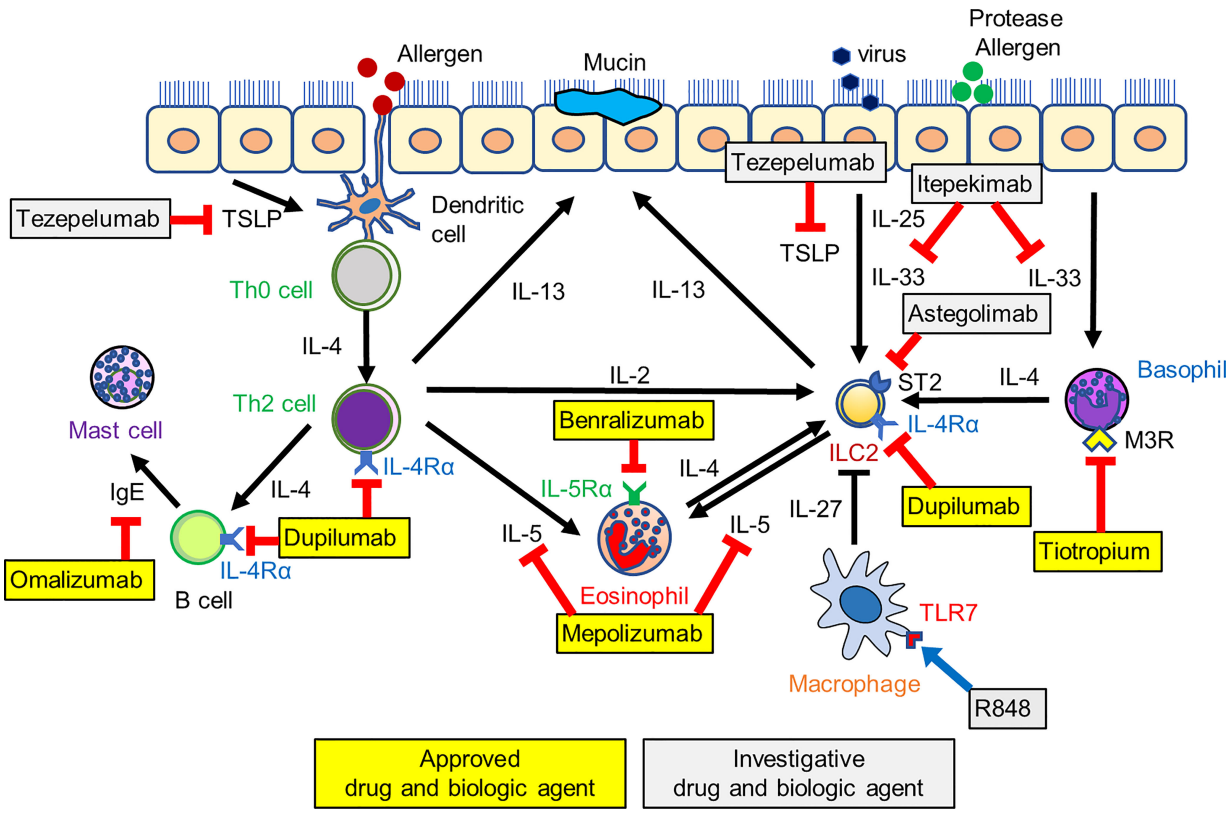

FIGURE 1 | The network between ILC2s and cells of the innate and adaptive immune system and a schematic of investigative and approved biologic therapies. Biologics target IgE, IL-5 and its receptor, IL-4 receptor, and alarmins such as TSLP and IL-33, leading to the suppression of asthma exacerbation and improved asthma control. Tiotropium suppresses basophil-derived IL-4 production, and R848, a toll-like receptor7 (TLR7) agonist, induces macrophage-derived IL-27 production. These pathways also indirectly induce the suppression of ILC2-mediated airway eosinophilic inflammation. ILC2, type 2 innate lymphoid cell; M3R, muscarinic M3 receptor.

function, and the network between ILC2s and the cells of the innate and adaptive immune system has been characterized. Some therapeutic agents, including tiotropium, attenuate ILC2-mediated airway inflammation indirectly by inhibiting cytokines derived from various immune cells. The regulation of the ILC2-mediated innate immune network also has an essential role in asthma control and can be linked to the potential therapeutic target.

Currently available biologics reduce asthma exacerbation and improve asthma control by blocking specific type 2 cytokines directly. However, these fail to prevent asthma exacerbation completely. This may be because the biologics inhibit pathways downstream in an immunological cascade, while leaving other cascades still active. On the other hand, tezepelumab blocks the TSLP pathway alone, leading to the sufficient suppression of type 2 inflammation. In addition to tezepelumab, itepekimab and astegolimab, which inhibit "alarmins," also have a positive effect on asthma outcomes. Therefore, blocking alarmins, which are upstream mediators, inhibits broad type 2 inflammatory response. The observation that ILC2s are activated by alarmins suggests that ILC2-targeted therapies are expected to be effective in the initial phase of airway inflammation. However, the association between biologics and ILC2s is limited. As IL-4Rα is expressed on ILC2, dupilumab has been shown to reduce not only type 2 inflammatory biomarkers (e.g., type 2 cytokines and FeNO) but also ILC2 numbers. There are no data that tezepelumab, itepekimab, and astegolimab reduce ILC2 numbers or ILC2-derived type 2 cytokine production. Therefore, further studies are required to clarify the effects of alarmin-targeted biologics on ILC2s. These studies will be relevant for the further development of therapeutic agents for targeting ILC2s in asthma.

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Waves of layered immunity over innate lymphoid cells

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Innate lymphoid cells (ILCs) harbor tissue-resident properties in border zones, such as the mucosal membranes and the skin. ILCs exert a wide range of biological functions, including inflammatory response, maintenance of tissue homeostasis, and metabolism. Since its discovery, tremendous effort has been made to clarify the nature of ILCs, and scientific progress revealed that progenitor cells of ILC can produce ILC subsets that are functionally reminiscent of T-cell subsets such as Th1, Th2, and Th17. Thus, now it comes to the notion that ILC progenitors are considered an innate version of naïve T cells. Another important discovery was that ILC progenitors in the different tissues undergo different modes of differentiation pathways. Furthermore, during the embryonic phase, progenitor cells in different developmental chronologies give rise to the unique spectra of immune cells and cause a wave to replenish the immune cells in tissues. This observation leads to the concept of layered immunity, which explains the ontology of some cell populations, such as B-1a cells, γδ T cells, and tissue-resident macrophages. Thus, recent reports in ILC biology posed a possibility that the concept of layered immunity might disentangle the complexity of ILC heterogeneity. In this review, we compare ILC ontogeny in the bone marrow with those of embryonic tissues, such as the fetal liver and embryonic thymus, to disentangle ILC heterogeneity in light of layered immunity.

KEYWORDS

ILC, layered immunity, prenatal development, thymic origin, heterogeneity

Introduction to ILC biology

The terminology innate lymphoid cells (ILCs) includes a wide range of cell types, such as natural killer (NK) cells, which were discovered in 1975 and has a damaging activity against virus-infected cells and tumors (1), and lymphoid tissue inducer (LTi) cells, which were discovered in 1997 and play essential roles in the formation of secondary lymphoid tissue, such as Peyer's patch (2), ILC3 which serves for innate mucosal immune defense with Th17 cytokine production such as IL-22 (3–6), and non-T/non-B cells that induce type 2 responses in an IL-25-dependent manner, which was

reported in 2001 (7) and turned out to play a pivotal role in allergic conditions by producing Th2 cytokines in 2010 (8–10). Thus, various names had been assigned to these cells during decades of ILC history. The nomenclature and terminology regarding mouse and human ILCs have recently been updated (11) and now include five groups: cytotoxic NK cells, group 1, 2, and 3 innate lymphoid cells (ILC1, ILC2, and ILC3), and LTi cells. ILC1 produce type 1 cytokines (12). ILC2 is characterized by the expression of GATA-3, a master regulator of Th2 differentiation, and secretes type 2 cytokines, such as IL-5 and IL-13 (13). The expression of RORγt, a critical transcriptional factor for Th17 differentiation, allows ILC3s to produce Th17-related cytokines IL-17A and IL-22 (14). Murine experiments indicated that LTi cells similarly express RORγt in addition to AHR, RUNX3, Notch, and Arg1 to promote secondary lymphoid tissue formation prenatally (11, 15–19). NK cells harbor similar characteristics to ILC1 but, unlike ILC1, express granzymes and perforin and has cytolytic activity. These characteristics make them analogous to T-cell subsets, such as $CD4^+$ Th1 cells with ILC1, $CD4^+$ Th2 cells with ILC2, ILC3 with Th17 cells, and NK cells with $CD8^+$ cytotoxic T cells. ILCs harbor the characteristic nature of the tissue residency. The parabiosis experiment with mice demonstrated that most ILCs were not replenished by blood supply (20). Other unique characteristics include unstable and elusive phenotypes, e.g., ILC1 can convert into ILC3 or vice versa (21). Furthermore, phenotypes of ILCs also differ according to anatomical site, e.g., the phenotypical difference between lung ILC2 and skin ILC2 (22). These characteristics of ILCs synergistically result in the significant heterogeneity in ILCs and bring a daunting difficulty in understanding the whole picture of ILC biology [for review (23)]. However, recent scientific progress provides ILC biology with an aspect of developmental biology. Here, we update ILC ontology in different niche sites. In addition, we introduce the concept of "layered immunity" which helps us to comprehend the heterogeneity of ILC biology.

Variation of the ontogeny of ILCs

Since progenitor cells of ILCs were discovered in mouse bone marrow (BM) (24), ILC ontology has been intensively studied with BM in adult mice (Figure 1A). Mouse ILC ontogeny originates from common lymphocyte progenitor cells (CLPs), which can give rise to all lymphocyte subsets, including ILCs (25). Nfil3 is a basic leucine zipper transcription factor essential for ILC lineage commitment (26, 27). Previous studies showed that mice lacking Nfil3 were deficient in all mature ILCs, including NK cells (26, 28). By genome analysis of *in vivo*-derived cells, α-lymphoid precursor cells (αLPs), which were defined as $CXCR6^+\alpha4\beta7^+IL\text{-}7R^+$ cells, were demonstrated to differentiate from mouse CLPs (27, 29). A phenotypically functionally combined approach with fate mapping with the Tcf7 gene, a T cell-specific transcription factor downstream of Notch/Wnt signaling (30, 31), showed that early innate lymphoid progenitors (EILPs) were also differentiated from CLPs (32). A recent study clarified the relationship between αLPs and EILPs (32). In the study, a reporter mouse system expressing $Tcf7^{EGFP}$ in addition to fate-mapped $CD127^{YFP}$ were used to demonstrate that GFP^+ EILPs were CD127 fate map positive. This observation suggested that EILPs reside downstream of αLP (33). It is of note that differentiation from CLPs toward EILPs *via* αLPs requires TOX, a transcriptional factor essential for the CD4 lineage program, since TOX regulates the gene expression of Tcf7 (34, 35). A reporter mouse experiment for Id2, a central hub in controlling helper-like ILC identity, showed that ILCs and NK cells differentiate from EILPs *via* branching to common helper-like ILC progenitors (CHILPs) and NK progenitors (NKPs) (32, 36–40). EILPs also harbor the potential to differentiate into dendritic cells (DCs) (41). CHILPs are divided into two groups depending on the expression of a transcription factor, promyelocytic leukemia zinc finger protein (PLZF which is coded in the Zbtb16 gene), which induces the maturation of various ILC subsets such as ILC2, ILC3, and some ILC1, but not NK cells or LTi cells (42–44). The $PLZF^+$ group called ILC precursors (ILCPs) differentiate into NK cells and ILC1/2/3s that are characterized by the expression of T-bet, GATA-3, or RORγt, respectively (43, 45, 46). Contrarily, the $PLZF^-$ population differentiates into LTi-like ILC3 (43, 45). Furthermore, CHILPs also retained NK potential (45). By definition, CHILPs generate only helper-like subsets of ILCs but not NK cells. Thus, CHILPs with NK potential need further study for proper categorization in the developmental path. It is of note that lymphoid-primed multipotent progenitors (LMPPs) serve as the progenitor of CLP in the BM. However, a recent study suggested that LMPPs bypass CLP to generate downstream lineages (24, 47). Thus, it is implied that ILC ontogeny may differ according to spatiotemporal context.

Although it is known that BM seeds ILCs into peripheral tissue *via* circulation, most ILCs are long-lived and tissue-resident with minimal turnover (48). Thus, ILCs are considered to differentiate and propagate in the niches of various tissues. In addition, it is yet to be fully clarified how circulating ILCs contribute to tissue-resident ILCs. Human ILCs are most studied in the tonsil (Figure 1B). It is of note that the similarity between mouse and human ILC developmental pathways is observed (Figures 1A, B). Their development begins with CLP (49). CLPs differentiate into early tonsillar progenitors (EToPs) which are subdivided into two groups, EtoP1 (defined as $Lin^-CD34^+CD10^+KIT^-$) then EtoP2 (defined as $Lin^-CD34^+CD10^-KIT^+$) (50, 51). Both populations are multipotent and can generate T cells and DCs *in vitro* (52). IL-1R1 expression further subdivides the EtoP2 population (52). $IL\text{-}1R1^-$ EtoP2 differentiates into not only mature ILCs but also T cells and DCs (52). In contrast, $IL\text{-}1R1^+$ EtoP2 cells are known to

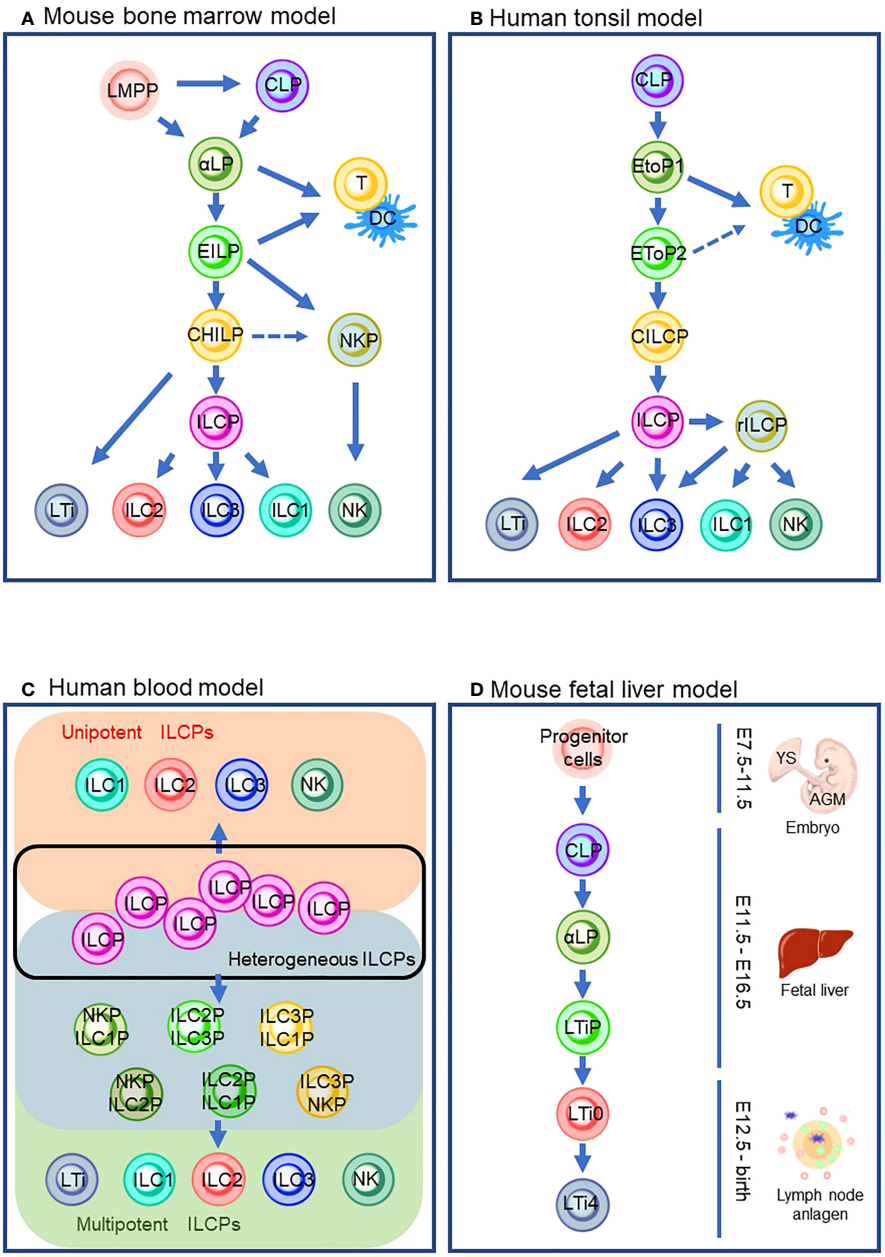

FIGURE 1
ILC differentiation pathways in the different models that are currently proposed. **(A)** Mouse bone marrow model. **(B)** Human tonsil model. **(C)** Human blood model which describes the heterogeneous ILCP group (inside a black line), ILC subsets from unipotent ILCPs (orange background), and ILC subsets from multipotent ILCPs (green background) *via* its intermediates (blue background). **(D)** Mouse fetal liver model. Solid lines indicate the differentiation pathway which is considered the main pathway. Dashed lines indicate the alternative pathways that are demonstrated by the experiments in specific conditions. LMPP: lymphoid primed multipotent progenitors; CLP, common lymphoid progenitor; αLP, α-lymphoid precursor cells; EILP, early inmate lymphoid progenitor; DC, dendritic cells; CHILP, common helper-like innate lymphoid progenitor; NKP, natural killer cell progenitor; ILCP, innate lymphoid cell progenitor; ILC1/2/3, group 1/2/3 innate lymphoid cell; NK, natural killer cell; LTi, lymphoid tissue inducer; EToP1/2, early tonsil progenitor 1/2; CILCP, common innate lymphoid cell progenitor; YS, yolk sac; AGM, aorta–gonad–mesonephros.

differentiate exclusively toward ILCs and are considered as committed common innate lymphoid progenitors (CILCPs) (24). CILCPs with limited differentiation potential, defined as $Lin^-CD34^-CD7^+IL\text{-}7R^+KIT^+$ cells, were found to differentiate exclusively into ILCs in various tissues (53). Thus, this cell type was annotated as ILCPs in human. Downstream of ILCPs, $CD34^-KIT^+CD56^+$ cells, which are called restricted ILCPs (rILCPs), encompass a further restricted differentiation potential (52). NK cells, ILC1/3s, but not ILC2, were produced from rILCPs (54).

Since a unique ILC population with $CD127^+CD117^+$ phenotype resembles tonsil ILC3, the cells with this phenotype circulating in the human blood have long been regarded as ILC3 (55). Nevertheless, in the recent report, $CD7^+CD127^+CD117^+$ cells in the peripheral blood which resemble tonsil ILC3 were demonstrated to express IL-17A and RORγt only at the traceable level (53). An *in vitro* culture experiment showed that the cells generated ILC subsets, including NK cells, but not other hematopoietic lineages. The single-cell RNA sequence (scRNA-sec) showed that human ILCPs are a heterogeneous population that was composed of multipotent and unipotent ILCPs (Figure 1C). Thus, $CD7^+CD127^+CD117^+$ cells are regarded as a circulating ILC subset with characteristics of human ILCPs. The group also discovered that IL-1β is a potent growth factor for ILCs and triggered ILCP development along with IL-2 and IL-7 *in vitro* (56). The requirement of IL-1β in human ILCP differentiation leads to a stepwise model of ILC development in peripheral tissue: (1) circulation of ILCPs in the blood, (2) ILCPs' response to increased IL-1β in inflamed tissue, (3) locoregional proliferation of ILCPs, and (4) differentiation into mature ILC subsets (53, 57, 58). It is suggested that the blood-circulating ILCPs could provide "on-demand" replenishment of ILC subsets in the inflamed sites as their niche to differentiate and propagate, which makes a clear contrast to naïve T cells in the lymph node (LN). However, ILCPs harbor differentiation potential into ILC subsets (e.g., ILC1/2/3 with T-bet/Gata-3/RORγt expression functionally reminiscent of Th1/2/17) and a quiescent state with reduced glycolysis and mitochondrial activity, without effector cytokine production or extensive proliferation, which all associate with the characteristics of naïve T cells (59). This tempts us to speculate that the biological significance of the blood-circulating ILCPs may be an innate counterpart of naïve T cells, which differentiate into helper and cytotoxic T cells of the acquired immune system.

LTi cells can be produced postnatally in ILC ontogeny in mouse BM (60, 61) (Figure 1A). However, much effort has been made to clarify the embryonic ontogeny of LTi cells since LTi cells are associated with the formation of the secondary lymphoid organs in the embryonic phase (60). Previous studies revealed that the fetal liver (FL) serves as a niche for ILC development. The extra-embryonic yolk sac (YS) and aorta–gonad–mesonephros (AGM), an intra-embryonic hematopoietic site derived from methoderm, are known to produce hematopoietic progenitors during embryonic gestation days 7.5 (E7.5)–E9 and E8.5–E11.5, respectively (62–64) (details are described in Section 5). Hematopoietic progenitors from YS and AGM migrate toward FL around E12.5 (65) and differentiate into CLPs (66). Subsequently, CLPs differentiate into αLPs which express Id2. Id2 is known to repress E2A, a member of the E protein family which transcriptionally regulates many developmental processes, including B- and T-cell differentiation (67). Notably, a recent study demonstrated that Id2 prevents chromatin remodeling to differentiate toward naïve CD8 T cells, implying that Id2 might maintain the chromatin state essential for ILC lineages (68). Instead of expressing PLZF to differentiate into ILCPs, αLPs in FL differentiate into LTi precursors (LTiPs) that express CXCR5 and CXCR6, in addition to Tcf7 (69). These chemokine receptors expressed in LTiPs facilitate migration to the LN anlagen, where the expression of RORγt triggers further differentiation into mature LTi4 which expresses LTi potential marker, CD4 *via* $CD4^-$ LTi0 (33, 70).

Although the variation of ILC ontogeny between human and mouse may be due to the difference in species, the ontogenic variation among tissues in the same species may suggest that hematopoietic progenitors in different niches can produce distinct ILC subsets according to locoregional biological processes. In the following sections, we summarize experimental findings about ILCs in the embryonic thymus as an example of another prenatal ontogeny of ILCs. Furthermore, we introduce the concept of layered immunity to explain the phenomena where phenotypically different ILC subsets collectively exert robust immunity.

Embryonic development of T-cell lineage in the thymus

As described in the previous section, ILCPs were discovered in BM. Furthermore, early studies characterizing ILCs with Rag-deficient mice demonstrated that ILCs could be generated without Rag expression, raising the possibility that ILCs are not of the T-cell lineage (71). Thus, the association of ILCs to the T-cell lineage had been overlooked. Nonetheless, in addition to the resemblance of ILCPs to naïve T cells in recent studies, we discuss in the following section that the T-cell developmental pathway in the thymus plays an essential role in ILC ontology. T-cell differentiation is unique compared to other hematopoietic lineages since it requires the maturation process in the thymus before its colonization into peripheral tissues. The differentiation pathway of the T-cell lineage is commenced from either CLPs or LMPPs (Figure 2) (72–74). Upon reaching the thymus, $CD4^-CD8^-$ thymus seeding progenitors (TSPs) enter the double-negative (DN) phase of T-cell differentiation. The

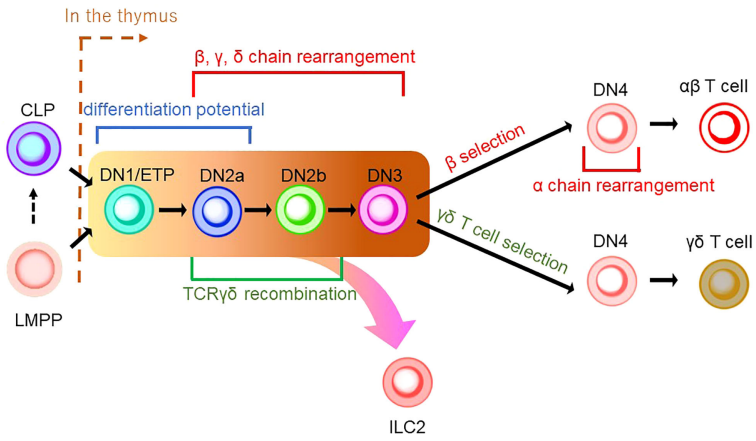

FIGURE 2
A schematic model of thymic development of T-cell lineages. CLP or LMPP is seeded into the thymus to become a thymocyte (the right side of a brown dashed line indicates thymic development). Thymocyte develops from a series of double-negative (DN) cells. αβ T cells and γδ T cells share the same developmental pathway from DN1/ETP to DN3 (indicated by the background in yellow to brown). DN1/ETP and DN2a harbor the differentiation potential as EILP in mice and EToP1/2 in humans (see Figure 1). T-cell receptor (TCR) recombination occurs during DN cell phases. Specifically, DN2a/b and DN3 undergo recombination of β, γ, and δ chains, while DN4 cells in the β-selection pathway do that of the α chain. TCRγδ recombination is known to occur in the phase between DN2a/b. In DN4 stage, γδ T-cell selection induces γδ T-cell development, while β-selection leads to αβ T-cell differentiation. ILC2 may be derived from the transition between the DN1/early thymic progenitor (ETP) and DN3 in the γδ T-cell development pathway.

developmental phases of DN thymocytes are classified as DN1/early thymic precursor (ETP), DN2a, DN2b, DN3, and DN4 according to expression levels of CD24, CD25, CD44, and KIT and the status of the TCR reconstitution (72, 73). DN1 cells have the ability and pluripotency to differentiate into myeloid and lymphoid lineages. However, the Notch-induced genetic program is activated to induce differentiation toward the T-cell lineage after the migration of DN1 cells into the corticomedullary junction (72, 73). As they progress to the DN2 stage, they localize to the subcapsular zone of the thymic cortex. They initiate the site-specific recombination of TCRβ, γ, and δ loci *via* Rag1/2 which cleaves DNA at conserved recombination signal sequences of the TCR locus (75). The proliferation and differentiation of DN2 require IL-7 (76). DN2 is subdivided into DN2a and DN2b according to the status of lymphocyte-specific protein tyrosine kinase (Lck) expression and differentiation potentials to NK cells, myeloid cells, and DC progenitors. It is of note that the differentiation potential toward DC, which EILPs in mouse and EToP1/2s in human harbor, is seen in DN1/ETPs and DN2a cells, but not in DN2b cells (Figures 1A, B and 2) (77). In the DN3 population, only cells expressing functional γδ or preTα/β (preTCR) chains go on to survive after the rearrangement of the TCR β, γ, and δ loci. The generation of a functional TCRβ chain serves as a checkpoint, which is termed "β-selection". A successful rearrangement of the TCRβ chain leads to preTCR formation (78). PreTCR$^+$ DN3 moves to the DN4 stage, where thymocytes return to the medulla and initiate TCRα gene reorganization by preTCR signaling. After expressing functional CD4 and CD8 receptors, thymocytes become double-positive (DP) thymocytes. Then, DP cells are positively selected for reactivity with MHC, resulting in CD4 or CD8 single-positive (SP) cells. Subsequently, the surviving SP cells undergo negative selection with autoreactivity to become mature naive T cells as a consequence of the αβ T-cell developmental pathway (Figure 2) (72, 73).

γδ T cells are the first T cells to develop in the thymus during fetal/neonatal life (79). γδ T cells go through the same developmental pathway as αβ T cells. TCR rearrangement starts from the γ and δ chains before the β chain during DN2a and DN2b developmental phases (80). γδ precursor cells express the TCRγδ/CD3 complex on the plasma membrane, like the pre-TCR, and initiates intracellular signaling pathways. This TCRγδ signal induces the process referred to as "γδ-selection" which triggers cell fate commitment of γδ T cells with the expression of functional TCRγδ (Figure 2) (78). Correspondingly, a previous study asked how Notch signaling influences developmental stages of γδ T cells and revealed that loss of Notch signaling led to a severe decline in γδ T-cell progenitor potential from DN2 cells, but not DN3 cells (81). In parallel, β-selection occurs in the DN3 stage, as mentioned above. Thus, these observations collectively suggest that the DN3 stage serves as an obligatory checkpoint at which the expression of the pre-T cell receptor (pre-TCR) or the γδ TCR determines the fate of the γδ or αβ cell lineages (78, 82, 83). According to the Vγ locus usage of reconstituted TCR, γδ T cells are subclassified into seven subtypes which are known to play biologically different roles in distinct anatomical areas. γδ T cells accomplish long-term

persistence by characteristic abilities, including tissue residency and self-renewal capacity (82). From the ontological point of view, these subtypes are derived from two distinct hematopoietic progenitors, the E13 ETP which generates the first wave at E13 and the E18 ETP which does the second wave at E18 (84). After the first wave of the E13 ETP in mouse embryo, Vγ5$^+$ appears on E15 and Vγ6$^+$, and Vγ7$^+$ follows. Subsequently, Vγ1$^+$, Vγ2$^+$, and Vγ4$^+$ emerge through the second waves by E18 ETP (Figure 3) (82, 84–87). This observation suggests that two distinct hematopoietic progenitors give rise to different subsets of the γδ T-cell lineage, although they seem to undergo the same differentiation pathway.

The rearrangement of the T-cell receptor locus in ILCs

While TCR gene expression is an indispensable feature of T-cell development, the seminal characteristics of ILCs include a lack of functional TCR expression. However, an unexpected discovery linked origins between T cells and ILCs in the research on the TCR gene in ILC progenitors. Previous studies used a Tcf7 reporter mouse system to cell-sort EILPs and ILCPs to propagate in cell culture. They showed that EILPs and ILCPs derived from adult BM were detected with high levels of unreconstituted TCR transcripts in RNA sequencing data. The observation suggested that EILPs and ILCPs express TCR transcripts in mRNA, although they lack TCR expression as proteins. These findings tempted the researchers to speculate a very close relationship between ILCs and T cells (32). Subsequently, an scRNA-sec analysis of cecum ILC subsets and lung ILC2 showed that transcripts of Cβ, a constant region of TCRβ, were expressed in all ILC subsets. In contrast, transcripts of other TCR chains, i.e., Cα, γ, and δ, were differentially expressed in each ILC subset. Genomic analysis revealed a genetic rearrangement of TCRγ but not in TCRβ in lung ILC2, which resembled the characteristics of mature Vγ2$^+$ γδ T cells. Deletion of at least one allele of TCRδ and frameshift mutations in the Vγ2-Jγ1 rearrangement were frequently seen in ILC2 (88). Based on the fact including transcripts of the highly expressed constant region of the TCR and the non-sensical recombination of TCRγ and δ gene without VDJ recombination which should be completed to assemble the variable region of TCRγδ before the DN3 stage, it is suggested that ILC2 originated from the developmental phase between the DN1/ETP stage and DN3 stage in the γδ T-cell developmental pathway (78) (Figure 2). The scientific evidence indicative of the failure of reconstitution at the Vγ2-Jγ1 locus in tissue-resident ILC2 may be a sign of "failed" Vγ2$^+$ γδ T-cell differentiation (Figures 2 and 3) (73). Based on the observations, including the presence of ILCs in the embryonic thymus and their dependence on early T cell transcription factors (89), it is noteworthy that ILC1 in human blood with and without the expression of EOMES, a master regulator of the development of ILC1 and NK cells, was reported to retain the expression of the T cell-related gene repertoire (90, 91). It was also asked whether EOMES$^{+/-}$ ILC1s express TCR transcripts without surface TCR expression. The results showed that blood EOMES$^+$ ILC1 express reconstituted αβ chains and that blood EOMES$^-$ ILC1 rearranged all four TCR chains (90). Another report showed that splenic NK cells revealed a rearrangement of the TCRγ locus but not of TCRβ. On the other hand, unlike ILC2, NK cells have been reported to express rearranged TCRγ. Furthermore, a reconstituted TCRδ locus (Vδ4-Jδ1) was detected only in neonatal NK cells, suggesting that, as with ILC2, at least one allele may have been deleted due to failure of gene rearrangement at the TCRα/β locus (92).

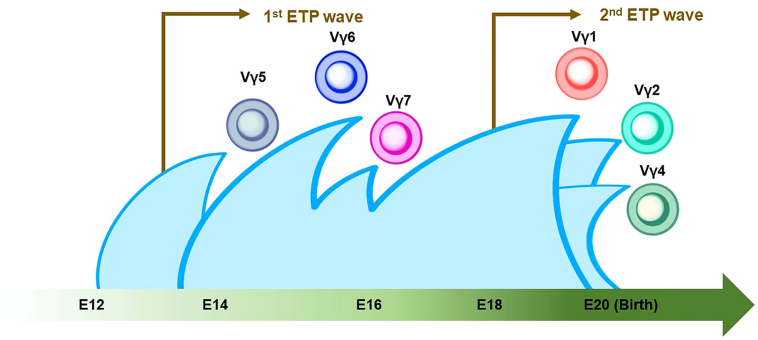

FIGURE 3
Thymic developmental waves of γδ T-cell subsets. The green arrow at the bottom indicates the developmental chronology of γδ T cells in the definitive wave. Vγ5$^+$/Vγ6$^+$/Vγ7$^+$ γδ T cells emerge between E14 and E16, followed by the Vγ1$^+$/Vγ2$^+$/Vγ4$^+$ γδ T cell around birth. Note that the first and second waves of early thymic progenitors (ETPs) come at E13 and E18 (indicated by brown arrows). The Vγ2$^+$ γδ T cell shares a similar genomic signature with ILC2.

Nonetheless, it has been disputed if the expression of TCR transcripts in ILCs might be due to experimental noises. A previous report showed that 7% of ILC1 upregulated the surface expression of TCRα/β following a 7-day culture and turned into the T cell-like phenotype (93). Another group demonstrated that a small proportion of ILC1 which transcriptionally expressed CD4, CD8A, and rearranged TCRα/β chains exhibited a partial clonal overlap with TCRα/β-rearranged T cells (90). These observations suggested that the current experimental design contaminated a small fraction of ILCs by misclassifying T cells, albeit with leading-edge technologies. Nonetheless, T-cell contaminants in an ILC population are a minor population. Therefore, ILCs are still likely to be derived from T-cell progenitors in the thymus (Figures 2, 3), based on the evidence of the genetic signature and mRNA expression in the TCR locus in ILC. However, an improved methodology that controls the experimental noises may be necessary to conclude this question.

Layered immunity models

The characteristic nature of ILC includes tissue residency and long-term persistence in specific anatomical sites, which resembles the characteristics of γδ T cells. Correspondingly, genomic sequence analysis showed similar patterns in the Vγ locus between ILC2 and γδ T cells. Other scientific evidence in the previous sections also suggested that a certain proportion of ILC may be derived from the embryonic thymus. On the other hand, intensive research revealed that postnatal BM produces at least some part of ILC. Thus, this raises the possibility that different progenitor cells with different natures in spatiotemporally distinct niches play a role as an ILC progenitor and collaboratively exert immune functions. This notion fits the concept of layered immunity, which was postulated in the research of innate-like lymphoid cells, such as γδ T cells and B-1a B cells (Figure 4) (94). Each progenitor cell during the embryonic phase produces a wave that expands an immune cluster with unique cell members as one layer in a stage- and site-specific manner. Thus, the more waves the progenitors produce, the more complex the immune network becomes with multiple layers of immune cells. The resultant layered immunity orchestrates the robust immune function. Research about the hematopoietic system in the embryo influenced the concept of layered immunity (95). On the one hand, the embryo requires mass production of differentiated red blood cells which supply oxygen to the rapidly growing body. On the other hand, undifferentiated hematopoietic stem cells need to be maintained throughout life. To achieve radically different goals, two types of fetal hematopoietic stem cells play roles in fetal development (Figure 5) (95). Early studies demonstrated that hematopoiesis occurs in two independent sites, YS and AGM. This led to a two-step model of the hematopoietic program: 1) a primitive wave with limited lineage potential independent of c-Myb, a hematopoietic master regulator gene, 2) a c-Myb-dependent definitive wave that gives rise to the first hematopoietic stem cells (HSCs) with long-term repopulation potential (96). The following studies which aimed to clarify the origin of HSCs discovered intermediate multipotent progenitors without long-term repopulation potential and assigned them as a transient definitive wave of hematopoiesis. Furthermore, recent scientific progress revealed that the concept is

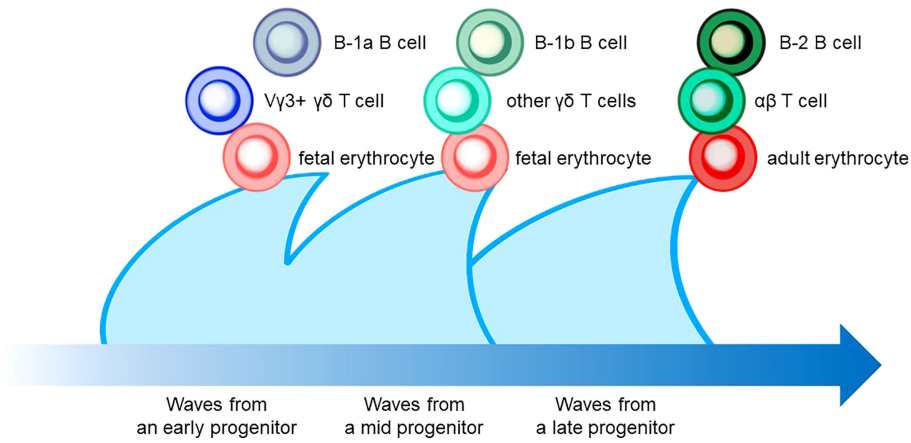

FIGURE 4
The layered immunity model which was proposed by Herzenberg et al. in 1992. A blue arrow at the bottom indicates the developmental chronology. In the concept, a hematopoietic progenitor during embryonic development generates the expansion of a unique cluster of blood and immune cells, which is described as a wave. Multiple waves generated by various progenitors compose multilayers of immune cell subsets, which collaboratively orchestrate the "organized" immunological function. Examples include B-cell, T-cell, and erythrocyte lineages that are derived from hematopoietic progenitors in different phases.

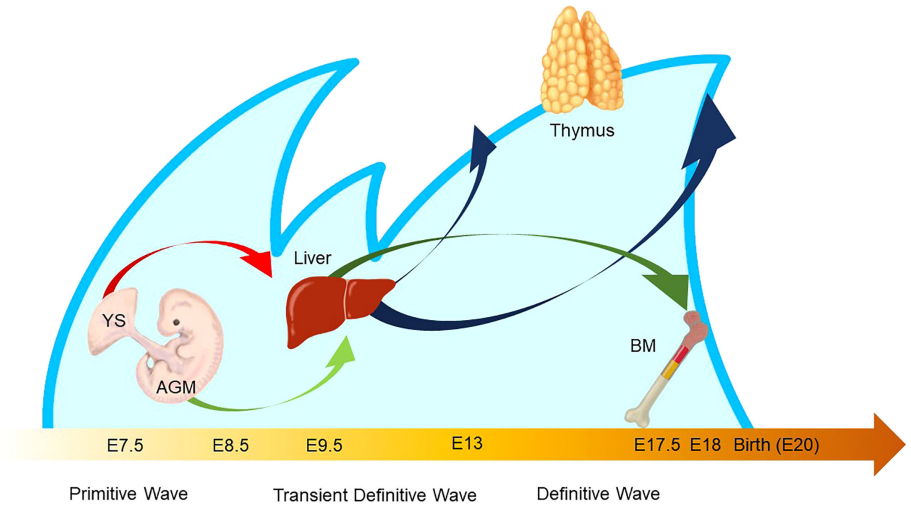

FIGURE 5
Waves of layered immunity during embryonic development in a mouse model. The blue waves indicate primitive, transient definitive, and definitive waves which start at E 7.5, E8.25, and E8.5, respectively. The blood islands in the yolk sac (YS) and aorta–gonad–mesonephros (AGM) independently give rise to their own hematopoietic progenitor cells. After the establishment of blood circulation, hematopoietic progenitors appear in the fetal liver at E 9.5. Then, the fetal liver turns into the main site for fetal hematopoiesis. Next, the waves of early thymic progenitors (ETPs) reach the thymus at E13 and E18. Subsequently, the hematopoietic progenitor cells are seeded into the bone marrow at E17.5.

applicable to other cell types, such as tissue-resident macrophages (95). The primitive wave begins in the blood islands of the YS at E7.5 in mice (Figure 5) (65). During the primitive wave, precursor cells in the YS produce early hematopoietic progenitors (97). The first intra-embryonic hematopoietic progenitors are formed in AGM (65). The YS- and AGM-derived progenitors are locally confined until E8.5/E9.0. The establishment of blood circulation leads to seeding HSCs into the FL at E12.5 (65). The FL is known to be the major hematopoietic organ for erythropoiesis. The switch from the YS to the FL as the major site of hematopoiesis occurs when the primitive wave switches to the definitive wave (98). It is of note that two waves of hematopoietic progenitors which differentiate into ETPs and the following descendants, such as DN cells, αβ T cells, and γδ T cells in the thymus, emerge at E13 and E18 (Figure 5). In the approximately same phase, myelolymphoid progenitor cells are colonized in BM and become the primary site of hematopoiesis from E17.5 onward (Figure 5) (65). All myelolymphoid cells derived from AGM, YS, FL, and fetal BM disseminate to lymphoid and non-lymphoid tissues (63, 99, 100).

Recently, one group reported a seminal study to ask if ILC ontology may fit the layered immunity model (87). They investigated the difference in differentiation potentials toward innate immune cells between the first (E13) and second waves (E18) of ETPs in the developing thymus by using the mouse model with the fate-mapping technique. Inhibition of the first wave of ETP by anti-IL7Rα injection results in a significant reduction in thymic LTi cells, Vγ5$^+$ γδ T cells, and mature mTEC expressing the autoimmune regulator (AIRE), suggesting that the first wave of the ETP contributes to the thymic development and establishment of immune tolerance (87). In addition, *in vitro* cell-culture assays demonstrated that the E13 ETP has a limited differentiation potential compared to the E18 ETP. Accordingly, the E13 ETP produced only LTi cells and T cells, while the E18 ETP produced B cells, ILC1/2/3s, and myeloid cells, indicating that the formation of the different immune layers is temporally regulated in the thymus. Correspondingly, data from a single-cell RNA sequence (scRNA-seq) exhibited the limited differentiation potential of E13 ETP which was transcriptionally primed toward the LTi cells and invariant T-cell profiles. In addition, the maturation of Vγ5$^+$ γδT cells in thymic tissue, Vγ6$^+$ γδ T cells in LNs, and medullary thymic epithelial cells (mTEC) in the thymus is dependent upon the first wave derived from the E13 ETP (87). Thus, these data can be interpreted as that E13 ETP mainly produces LTi cells, while E18 ETP gives rise to a wide range of ILCs. Furthermore, the data correspond to the previous studies that revealed that ILC2 harbored the genomic signature similar to Vγ2$^+$ γδ T cells which emerge in the late stage of embryonic development (Figures 3 and 5).

The findings showed that E13 and E18 ETPs generated distinct ILC subsets that consist of unique cell members, which are also different from the ILC subset in adult BM. Thus, ILC ontogeny was consistent with the concept of layered immunity. Therefore, this suggests that many layers of ILC

subsets synergistically exert a robust immunity function. Thus, it is necessary to comprehend how ILC subsets function and to which layer of the immune system the ILC subset belongs.

Layered immunity suggests the origins of ILC subtypes

Despite accumulating findings of heterogeneity of ILCs, most previous studies have failed to reveal the biological significance of heterogeneity of ILC subsets. In this section, we introduce the ILC subtypes whose phenotypical differences might support the concept of layered immunity.

A mouse model of *Nippostrongylus brasiliensis* (*N. brasiliensis*) infection provided a clue to classify a subset of ILC2 with different responsiveness to epithelial alarmins, such as IL-25 (101). IL-25-responsive ILC2 was absent in the lung at a steady state but was found to accumulate in the lungs of mice 5 days after *N. brasiliensis* infection and disappear within 12 days. The different cellular kinetics delineate the existence of two subtypes of ILC2 in mouse: 1) the tissue-resident IL-33-reactive ILC2 which is referred to as natural ILC2 or nILC2 and 2) a circulating IL-25-responsive subtype which is termed as inflammatory ILC2 or iILC2. iILC2 harbors a high expression of KLRG1 and a low expression of CD90, whereas nILC2 harbors a low KLRG1 but high CD90 expression (102). As suggested by the responsiveness to specific cytokines, iILC2 preferentially expresses more IL-17rb, a subunit of an IL-25 receptor, while nILC2 does more ST2, a component of the IL-33 receptor on their surface (101, 103). Additionally, Arginase 1 (Arg1) was identified to discriminate between two subsets, although ILC2 and its progenitor cells shared the expression of Arg1, previously (18, 104, 105). In the experiment with Arg1 reporter mice, iILC2 hardly expressed Arg1 signals while nILC2 showed high Arg1 signals (103). On the other hand, BM-derived ILC precursors in the mouse do not express Arg1, while embryonic ILCPs express Arg1 (18). However, both IL-33-reactive nILC2 and IL-25-responsive iILC2 are known to coexist in the small intestine (106). One seminal research demonstrated that iILC2s migrate from the intestine to the lungs through the blood circulation under the influence of microbiota in the parabiosis model (106). Furthermore, they showed that the majority of iILC2 from the intestine turned into a tissue-resident nILC2-like phenotype, while the minor proportion was kept in circulation (106). Thus, it is suggested that the intestine can be a source of tissue-resident nILC2, at least in the lung and minor population of circulating iILC2. On the other hand, another research focused on the circulating iILC2 with the atopic dermatitis model, in combination with photoconvertible KikGR which change the fluorescent color from green to red by UV irradiation (107). They first characterized the circulating ILC2 in the draining LNs and the skin-resident ILC2s by scRNA-seq (107). Subsequently, ILC2 in the skin was tracked by KikGR after UV irradiation, which revealed that skin- (red) and LN-derived ILC2 (green) shared consistent gene expression patterns. These data suggest ILC2 in LN is KLRG+ iILC2 which goes back and forth between the skin and LN, while ILC2 in the skin consists of both circulating KLRG+ iILC2 and tissue-resident CD103+ ILC2 (107). In line with a previous finding that BM is the major source of KLRG$^+$ iILC2 (108), this study suggested that iILC2 is a major population of circulating ILC2 which is derived from BM. Furthermore, recent studies revealed that the AP-1 superfamily transcription factor BATF provides the clue to comprehend the regulation mechanism of the ILC2 phenotype (103). BATF-deficient mice showed a significant reduction in migrating IL-25-responsive KLRG1^{high} ARG1^{low} iILC2 in the small intestine and complete loss of the population in the lung under *N. brasiliensis* infection. Thus, BAFT is considered to regulate the phenotypical change of nILC2 to iILC2. whose phenotypical differences might support the concept of layered immunity. Another group analyzed the BAFT function in nILC2 in an influenza virus infection model with BAFT-deficient mice (109). The group demonstrated that nILC2s lose their immune-protective properties and acquire pathogenic ILC3-like functions in the absence of BATF (109). These findings suggest that Arg1$^+$ nILC2 retains a tissue-resident phenotype although nILC2 in the intestine and the lung can convert to the other phenotypes. On the other hand, it is also yet to be concluded if circulating iILC2 from BM can phenotypically convert into tissue-resident nILC2. A simple speculation is Arg1$^+$ ILC2 is tissue-resident which can transiently convert into other subtypes, while KLRG1$^+$ ILC2 is circulating.

Another group directly asked if multilayered waves of ILC2 may contribute to innate immunity. The group performed experiments of time-controlled phylogenetic tracking of ILC2 by the fate-mapping approach combined with an Arg1- and Id2-driven ILC2 reporter mice (110). The study demonstrated that ILC2 emerged in multiple organs during late pregnancy (110), which corresponds to the timing of emergence of Vγ2$^+$ $\gamma\delta$ T cells that may share the same developmental pathway (Figures 2 and 3). During the postnatal phase, most of the peripheral ILC2 pool was generated by BM hematopoiesis, whose putative progenitors were not labeled in the Arg reporter system (Figure 6). On the other hand, the authors observed that Arg1$^+$ ILC2 was seeded during the embryonic phase. Furthermore, prenatal and perinatal ILC2 were replaced throughout the tissue with age. Nonetheless, tissue-resident ILC2 was notably increased after helminth infection by the local proliferation but not due to *de novo* generation by BM hematopoiesis (110). These results indicated that ILC2 pools in the mouse are replenished in a temporally distinct manner and that ILC2 from postnatal BM behaves differently in response to external stimuli compared to embryonic ILC2. As mentioned above, the progenitor cells from AGM originate postnatal ILC2 from BM while E18 ETP

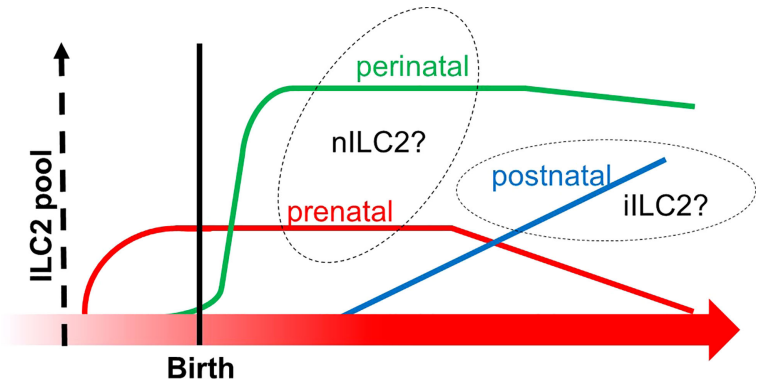

FIGURE 6
The transition of ILC2 pools from prenatal to postnatal phases. The X-axis indicates the developmental chronology, and the Y-axis shows the cellularity of the ILC2 pool. Three subgroups of ILC2 pools are depicted (prenatal in red, perinatal in green, and postnatal in blue). Prenatal and perinatal subgroups might be derived from the thymus since they increased before the bone marrow fully commenced hematopoiesis in the postnatal phase. This graph was created based on ref (110).

produces ILC2 during the embryonic phase, as discussed in Section 5 (Figure 5) (87). In addition, BM is known as the major pool of KLRG1$^+$ iILC2 (108). Thus, these observations fit the notion alongside layered immunity, that is, ILC2 progenitors from BM produce circulating KLRG1$^+$ ILC2 postnatally, while ILC2 progenitors do tissue-resident Arg1$^+$ ILC2 prenatally. This report further suggests that prenatal and perinatal ILC2 pools can to be distinguished since the cellular kinetics between them makes a clear contrast (Figure 6). Thus, it is also possible that another subset of innate lymphocytes in the embryonic thymus or FL could be a progenitor cells of prenatal ILC2 pools apart from the E18 ETP.

The ontogeny of LTi cells supports the concept of layered immunity. As described in Sections 2 and 5, FL and embryonic thymus serve as prenatal niches for LTi development. In addition, embryonic LTi cells were shown to be replaced by BM-derived LTi cells in adult mice (61). Notably, a research group asked if progenitors of LTi cells in FL are derived from YS or AGM (Figure 1D) (61). Since AGM but not YS expresses CXCR4 which exserts multiple functions, such as vascular, hematopoietic, and neural development (60, 111), they used the fate-mapping approach with the Cxcr4-CreErt2 mouse and demonstrated that progenitor cells of LTi cells were derived from the AGM region (Figure 1D) (61). Although the ontogeny of LTi cells can be well explained by the layered immunity model, intriguing biological questions remain: 1) if LTiPs in FL are the direct progenitor of E13 ETPs in the thymus or if they are distinct progenitors to give rise to different groups of LTi cells, 2) what the biological significance of BM-derived LTi cells is, and so on.

LTi cells were previously categorized as a member of a heterogeneous ILC3 group, based on dependency on the transcriptional differentiation program by RORγt in addition to overlap of phenotypical similarity. Nonetheless, ILC3 progenitors were shown to require PLZF for differentiation in both human and mice (112, 113). Nonetheless, LTi cells were still observed in the Zbtb16 knockout mice (43). Therefore, these data raised a possibility that LTi cells are non-ILC3 lineages (66). ILC3s consist of two major populations with or without natural cytotoxicity receptors (NCRs) that consisted of NKp46, NKp30, and NKp44 (114). ILC3 is known to promote intestinal immune and metabolic homeostasis. In fact, approximately 70% of ILCs are NCR$^+$ ILC3 in the small intestinal tract, while 15% of them are NCR$^-$ ILC3 (115, 116). NCR$^+$ ILC3 primarily express IL-22, but less IL-17, while NCR$^-$ ILC3 primarily express IL-17, but less IL-22 (117). *In vitro*, NCR$^-$ ILC3 can switch to NCR$^+$ ILC3 in the presence of IL-1β and IL-23 (118). NCR$^-$ ILC3 includes another unique subpopulation termed LTi-like ILC3. As the name of LTi-like cells suggests the cell harbors the close gene expression profile to LTi cells, except that LTi-like cells express OX40L and CD30L (119). Nevertheless, LTi-like ILC3 is not capable of maintaining secondary lymphoid tissue, unlike LTi cells. On the other hand, it is demonstrated that LTi-like ILC3 is required for the postnatal development of tertiary lymphoid tissues, such as cryptopatches (114). Previous reports showed that LTi-like ILC3 (NCR$^-$) was present in the fetal gut, while NCR$^+$ ILC3s are largely absent (120, 121). Furthermore, they are known to be replenished postnatally by BM progenitors (120). However, the annotation of specific progenitor cells and a detailed differentiation pathway are yet to be investigated. As discussed in Section 2, PLZF$^+$ ILCP produces ILC1, ILC2, and NCR$^+$ ILC3, but not LTi-like ILC3, which suggests that PLZF$^+$ ILCP have lost the capacity to generate LTi-like ILC3 progeny (43). A recent study annotated Arg1$^+$ fetal ILCP (ftILCP) in the fetal intestine at E13 which generates ILC1/2/3 *in vitro*. Thus, these observations indicate that ILC3 is derived from various progenitors. Notably, ftILCP is apparently different from E18

ETP described in the previous section. Thus, it is of intrigue how the TCR transcript in ftILCP looks like since the modes of VDJ recombination in TCR could suggest the origin of ftILCP, as we observed the similarity between ILC2 from E18 ETP and γδ T cells.

NK cells have long been studied with the circulating NK cells in the blood since they were discovered in 1975 (1). The conventional NK cells are derived from BM driven by the expression of EOMES (91). On the other hand, tissue-resident NK cells are also known to exist in various organs and tissues (122, 123). Recent studies revealed that the cells derived from E8.5 YS in mice harbored a potential to differentiate NK cells (122, 124). Moreover, ILC1 in the liver, which some researchers regard as tissue-resident NK cells, was intensively studied to clarify if the origins of ILCs and NK cells are different or not. One seminal study with the PLZF-fate mapping mouse system demonstrated that tissue-resident NK in the liver was not derived from EILP or CHILP, but from unique PLZF-expressing ILCPs (113). The research group showed that the developmental pathway of tissue-resident NK cells in the liver was similar to that of conventional NK cells, although the progenitor cells were distinct (113, 122). These findings again tempt us to speculate the existence of at least two subtypes of NK cells: tissue-resident NK cells that harbor an ILC1-like phenotype and are possibly derived from YS *via* FL, and circulating, conventional NK cells that are derived from BM.

The contrastive examples between tissue-resident and circulating subsets of ILCs imply that tissue-resident ILCs are seeded during prenatal to perinatal phases while circulating ILCs are derived from postnatal BM. Further study is needed to clarify if the concept of the layered immunity model can be generalized to much broader cell types by the characteristic feature of tissue residency.

Concluding remarks

Here, we introduced the scientific evidence for different sites of ILC ontogeny, the similarity of ILC to γδ T cells, and the possible classification of ILC subtypes based on the layered immunity. This notion may enable the delineation of the accurate figures of elusive ILCs. Although it could be oversimplified, ILCs might be described in light of the concept of layered immunity as follows. 1) Tissue-resident ILCs are primarily seeded during the embryonic phase. 2) At least a part of tissue-resident ILC2 undergo the same differentiation pathway as γδ T cells and are branched from DN cells in the thymus. 3) Circulating ILCs are derived from BM and replace tissue-resident subtypes in the various tissue postnatally. 4) Multipotent and unipotent ILCPs that give rise to circulating ILC can stochastically propagate into any subtypes of ILCs. 5) Circulating ILCPs provide an "on-demand" supply of ILC subsets in the inflammatory sites.

It has just begun that the layered immune concept has been applied to account for some parts of the biological significance of ILCs. However, the concept of layered immunity showed the great potential to disentangle the daunting complexity of heterogeneity of ILCs. Therefore, further studies for ILC-biology in light of layered immunity are necessary.

Author contributions

TK wrote the first draft of this manuscript. GE, TN, and KK contributed to editing the manuscript. All authors contributed to the article and approved the submitted version.

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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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ILC2 require cell-intrinsic ST2 signals to promote type 2 immune responses

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The initiation of type 2 immune responses at mucosal barriers is regulated by rapidly secreted cytokines called alarmins. The alarmins IL-33, IL-25 and TSLP are mainly secreted by stromal and epithelial cells in tissues and were linked to chronic inflammatory diseases, such as allergic lung inflammation, or to resistance against worm infections. Receptors for alarmins are expressed by a variety of immune cells, including group 2 innate lymphoid cells (ILC2s), an early source of the type 2 cytokines, such as IL-5 and IL-13, which have been linked to atopic diseases and anti-worm immunity as well. However, the precise contribution of the IL-33 receptor signals for ILC2 activation still needs to be completed due to limitations in targeting genes in ILC2. Using the newly established *Nmur1*$^{iCre\text{-}eGFP}$ mouse model, we obtained specific conditional genetic ablation of the IL-33 receptor subunit ST2 in ILC2s. ST2-deficient ILC2s were unresponsive to IL-33 but not to stimulation with the alarmin IL-25. As a result of defective ST2 signals, ILC2s produced limited amounts of IL-5 and IL-13 and failed to support eosinophil homeostasis. Further, ST2-deficient ILC2s were unable to expand and promote the recruitment of eosinophils during allergic lung inflammation provoked by papain administration. During infection with *Nippostrongylus brasiliensis*, ILC2-intrinsic ST2 signals were required to mount an effective type 2 immune response against the parasite leading to higher susceptibility against worm infection in conditional knockout mice. Therefore, this study argues for a non-redundant role of cell-intrinsic ST2 signals triggering proper activation of ILC2 for initiation of type 2 immunity.

KEYWORDS

ILC2 - group 2 innate lymphoid cell, IL-33 and ST2, type 2 immune response, mucosal immunity, innate immunity

Introduction

Barrier surfaces allow for vital exchange with the environment but are also exposed to a broad array of stressors and are exploited by pathogens for invasion. In case the physical, chemical and biological measures to protect the epithelium from invasion do not prevail, the penetration of the pathogen triggers the secretion of danger signals, such as Interleukin (IL)-33, IL-25 and thymic stromal lymphopoietin (TSLP). Such 'alarming' signals activate tissue-resident immune cells and initiate the combat against the pathogen (1). Alarmins are produced to a large degree by specialized epithelial, endothelial or stromal cells in tissue. Recent research has exposed stromal cells as the predominant source of IL-33 in tissues, but dendritic cells, mast cells, endothelial and epithelial cells have been described as IL-33 producers as well (2–6). IL-33 has a chromatin binding motif and nuclear localization sequence, which targets the cytokine in the nucleus. Cell death and cellular stress appear to trigger the release of IL-33 from the nucleus, but the entire process is poorly understood (7). Further processing of full-length IL-33 by various proteases, including allergic proteases, significantly increases its biological activity (8).

Expression of the IL-33 receptor was reported from many immune cells, including innate lymphoid cells type 2 (ILC2s), $CD4^+$ and $CD8^+$ T cells, basophils, mast cells, eosinophils and macrophages (3, 7, 9–13). The IL-33 receptor is a member of the IL-1 receptor family, which in addition includes IL-1, IL-18 and IL-36 receptors. The binding of IL-33 to the ST2 chain (*Il1rl1*) enables the association with the IL-1rap chain, recruitment of the Myd88 adapter for signal transduction and triggering of a signaling cascade which ultimately leads to the activation of NF-κB as the main transcription factor promoting inflammation (7).

Tissue-resident immune cells include innate lymphoid cells, which are enriched at barrier surfaces and which have a similar functional diversity to T cells in terms of lineage-specifying transcription factors and effector functions. The transcription factors GATA-3, GfI-1, RORα and Bcl11b are crucial for ILC2 development and function in general (14, 15). In addition, ILC2 require the lymphoid cytokine IL-7 to develop (9) and ILC2 activation is regulated by a variety of cytokines, inflammatory mediators, neuronal factors, metabolites and hormones. Upon stimulation, ILC2s secrete IL-5, IL-9, IL-13 and amphiregulin as main effector cytokines to regulate the type 2 immune response in tissues (14, 15).

Single nucleotide polymorphisms (SNPs) in the IL-33 gene have been linked to allergic asthma and chronic inflammatory diseases (16, 17). IL-33 plays an essential role in triggering type 2 immune responses in mouse models of lung inflammation (18, 19). However, since ST2 is expressed by many type 2 effector cells, the precise contribution for ILC2 activation and the initiation of type 2 inflammation remains incompletely understood. This study provides evidence for a cell-intrinsic requirement of ST2 in ILC2s in steady state, as well as models of allergic lung inflammation and anti-worm immunity. By genetically ablating ST2 in ILC2s using *Nmur1*$^{iCre\text{-}eGFP}$ mice, we establish a conditional knockout model to investigate the role of ST2 in ILC2 biology. ST2-deficient ILC2s produced limited amounts of type 2 cytokines and, therefore, did not provide sufficient support for proper eosinophil homeostasis. Furthermore, *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ failed to recruit eosinophils during allergic lung inflammation provoked by papain treatment and mount a fully protective type 2 immune response against *Nippostrongylus brasiliensis* (*N. brasiliensis)* infection. Taken together, our data demonstrate a cell-intrinsic and non-redundant role for ST2 as a regulator of type 2 immunity *via* activation of ILC2s.

Results

Genetic ablation of ST2 using *Nmur1*$^{iCre\text{-}eGFP}$ renders ILC2 unresponsive to IL-33

In order to investigate the importance of the IL-33 receptor for ILC2 regulation *in vivo*, we aimed to target the ST2 chain of the IL-33 receptor (encoded by the *Il1rl1* gene) in ILC2 using the Cre-loxP system. To this end, we crossed the recently developed BAC-transgenic *Nmur1*$^{iCre\text{-}eGFP}$ mouse, which allows gene targeting in ILC2 (20, 21), to *Il1rl1*$^{flox/flox}$ mice (22). Indeed, *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ lacked ST2 mRNA and protein expression in ILC2s of the small intestine, lung, adipose tissue, mesenteric lymph node, and skin similar to *Il1rl1*$^{-/-}$ mice but not in $CD4^+$ T cells, $CD8^+$ T cells, eosinophils, basophils or mast cells (Figures 1A–G; Supplementary Figures 1A–E).

To validate whether ST2-deficient ILC2s lost the capacity to sense IL-33, we sort-purified ILC2s from the small intestine, which have low ST2 expression, and from the bone marrow, which have high ST2 expression (Figure 1D), and cultured the cells with IL-7 alone to provide a survival signal, or IL-7 + IL-33 to induce activation. While control ILC2s massively expanded, blasted and secreted type 2 effector cytokines upon IL-33 stimulation, ST2-deficient ILC2s failed to respond to IL-33, indicating efficient deletion of ST2 and unresponsiveness to react to the ligand (Figures 2A–C; Supplementary Figure 2A). Next, we aimed to test if ILC2 still properly responded to stimulation with other alarmins despite the conditional deletion of ST2. To this end, we performed a similar *in vitro* assay with sort-purified ILC2s from the small intestine but now exposed the cells to IL-7 or IL-7 + IL-25. ST2-deficient ILC2s expanded and blasted to a comparable degree to ST2-sufficient ILC2s upon IL-25 stimulation (Figures 2A, B). We also exposed ILC2s to combinations of TSLP together with IL-7 or IL-25 and IL-33 and assessed blasting and cytokine production of ILC2s (Supplementary Figures 2B–D). However, ST2-deficient ILC2s could still respond to TSLP stimulation. Taken together, these data suggest that ILC2 were selectively unresponsive to IL-33 but could still respond to other alarmins, such as IL-25 and TSLP.

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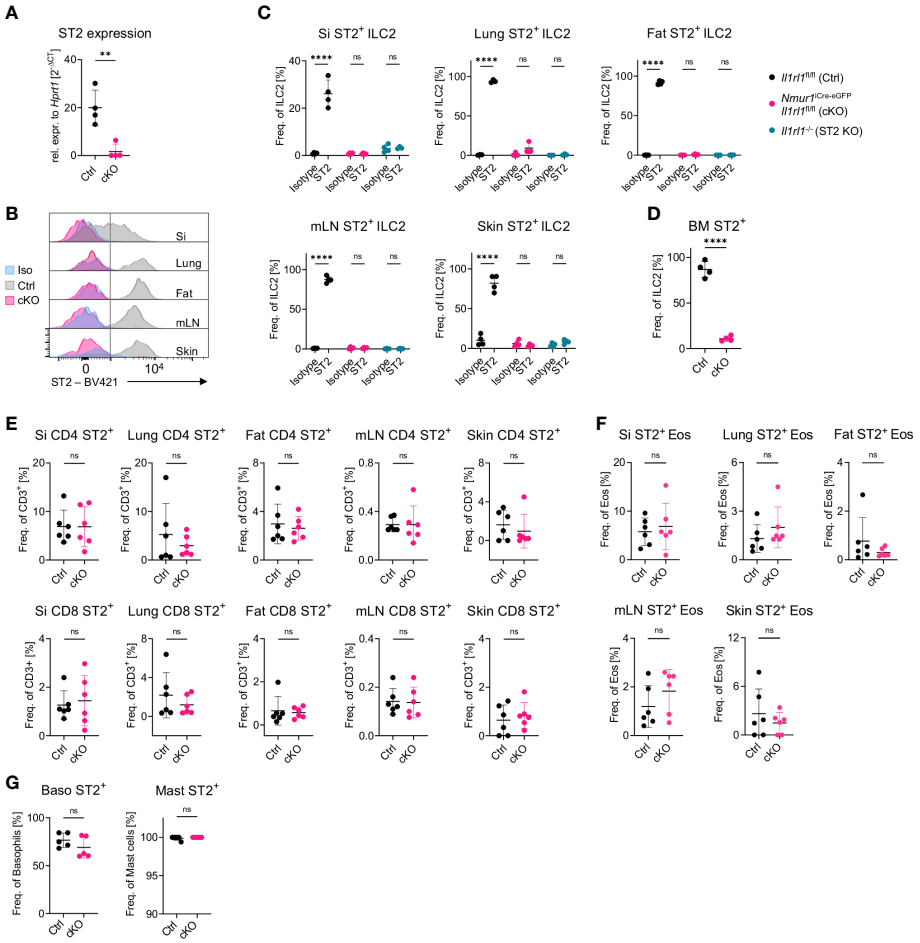

FIGURE 1
A model for ILC2-specific genetic ablation of ST2. **(A)**, Expression of the *Il1rl1* gene in *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) sort-purified ILC2s. **(B)**, Flow cytometric histograms of ST2 expression in ILC2s of *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice across organs, including isotype control on *Il1rl1*$^{flox/flox}$ (Iso) in the steady state. ILC2s were gated on live CD45$^+$ Lin$^-$ (CD3, CD5, CD19, Ly6G, Fcεr1), CD127$^+$ and KLRG1$^+$. **(C–G)** Percentage of ST2$^+$ expression in *Il1rl1*$^{flox/flox}$ (Ctrl), *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) **(D–G)** and *Il1rl1*$^{-/-}$ (ST2 KO) mice including isotype control **(C)** across different organs in the indicated immune cells. Basophils from spleen and mast cells from the peritoneal lavage. For **(C–G)**: Each symbol represents data from one mouse, data are representative of two experiments with four to six mice per group. Mean +/- SD, Student's t-Test. ns, not significant, **p < 0.01, ****p < 0.0001.

ILC2s develop in comparable proportions without ST2 but have reduced PD-1 expression despite limited phenotypic changes

Consistent with previous reports investigating mice with germline mutations of ST2, ILC2 were not reduced in frequencies or absolute cell numbers in *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice, suggesting that IL-33-ST2 signals are not essential for proper expansion and survival of ILC2s (23, 24) (Figure 3A). Similar results were obtained for bone marrow ILC2 progenitors, indicating that lack of ST2 signals did not lead to overt developmental defects (Figure 3B) (25).

To test whether deletion of ST2 altered the heterogeneity of ILC2s in tissues, we performed multicolor phenotyping of ILC2s by flow cytometry. Overall, the heterogeneity of ILC2s in tissue was not perturbed and most surface markers used for ILC2 phenotyping, in particular IL-25R or TSLPR were expressed equally in ILC2s from *Il1rl1*$^{flox/flox}$ and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (Figure 3C). In contrast, the marker PD-1 was consistently downregulated in ST2-deficient ILC2s (Figures 3C–E; Supplementary Figure 3). Since PD-1 was described as an activation marker on ILC2s (26), these data suggest that ILC2 fail to terminally mature without ST2 signals. In summary, *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice are a suitable model to investigate the role of ST2 for ILC2 function *in vivo*.

Loss of IL-33 signals on ILC2s results in decreased production of effector cytokines and reduced eosinophil counts

Several publications have highlighted the essential role of ILC2 and ILC2-derived IL-5 for eosinophil homeostasis (21, 27). Additional evidence suggests that IL-5 secretion is under the control of alarmin signals (23, 28) and that PD-1$^+$ ILC2 are potent cytokine producers (26). Therefore, we asked if ST2 signals

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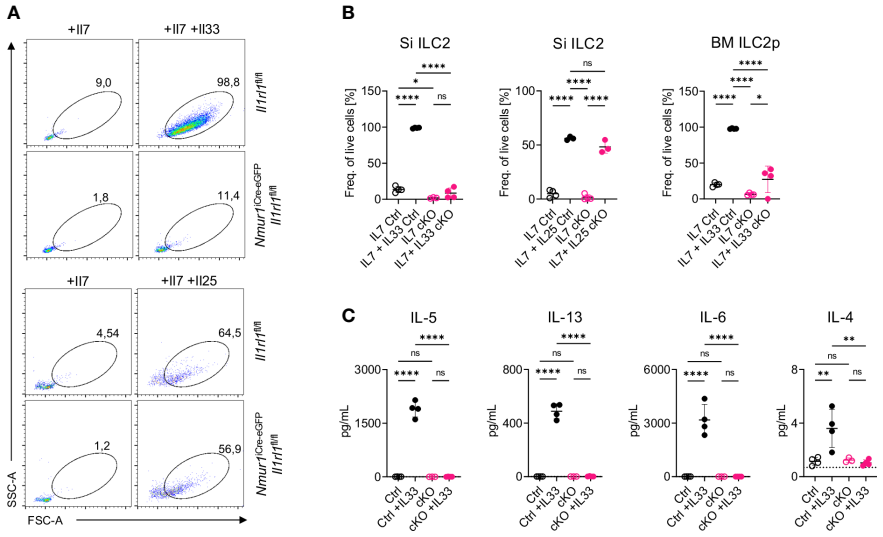

FIGURE 2
ILC2 of *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice are unresponsive to IL-33. **(A)**, Flow cytometric plots of small intestine ILC2s from *Il1rl1*$^{flox/flox}$ and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice after stimulation with IL-7, IL-7 and IL-33 or IL-7 and IL-25. ILC2s were gated on live CD45^{+} Lin^{-} (CD3, CD5, CD19, Ly6G, Fcer1), CD127^{+} and KLRG1^{+}. **(B)**, Quantification of ILC2 activation from **(A)** and bone marrow ILC2s of *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice after a three day culture with cytokines IL-7 or IL-7, IL-33 or IL-7, IL-25. **(C)**, Concentration of the indicated cytokine as determined in the cell culture supernatant 3 days after stimulation as in **(A, B)**. For **(B, C)**: Each symbol represents data from one mouse, data are representative of two experiments with four mice per group. Mean +/- SD, One-way ANOVA with multiple comparison. ns, not significant, *p < 0.05, **p < 0.01, ****p < 0.0001.

are required for type 2 cytokine production by ILC2. Indeed, IL-5 and IL-13 mRNA levels were significantly reduced in sort-purified ILC2s from *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ compared to control ILC2s (Figure 4A). Next, we assessed the number of eosinophils in *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice at steady state due to the IL-5 cytokine being crucial for eosinophil homeostasis (29, 30). Consistent with reduced IL-5 levels in ILC2s, we detected a significant reduction of eosinophils in the lung, adipose tissue, mesenteric lymph nodes and skin, suggesting that ILC2-intrinsic sensing of IL-33 *via* ST2 is necessary for type 2 cytokine production regulating proper eosinophil homeostasis (Figures 4B, C).

ILC2-intrinsic ST2 controls eosinophil recruitment during allergic lung inflammation

Next, we tested how the lack of ST2 on ILC2s influences the type 2 immune responses in the context of allergic lung inflammation, which was provoked by the administration of the protease allergen papain. Papain administration resulted in expansion of ILC2s, which was significantly reduced in *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (Figures 5A, B). We also found a strong increase in eosinophil counts in lung and BAL fluid upon papain treatment (Figures 5C–E). This increase in eosinophil counts was drastically reduced in papain-treated *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (Figures 5C–E). A more detailed analysis revealed that both CD11b^{+} SiglecF^{int} tissue-resident and CD11b^{+} SiglecF^{high} inflammatory eosinophils (31) were reduced in *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice, suggesting that ST2 signals in ILC2s are required for recruitment and local proliferation of eosinophils (Figures 5C, F).

Nmur1$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice have a defective type 2 immunity to *N. brasiliensis* infections

To test whether ST2 is required in a cell-intrinsic manner on ILC2s to mediate resistance against helminth infections, we infected *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ and littermate control mice with the worm *N. brasiliensis*. Littermate control mice mounted a potent type 2 immune response upon worm infection, which was diminished in *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (Figure 6). The massive expansion of ILC2s upon infection was weakened in *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice compared to littermate controls (Figures 6A–C). Furthermore, eosinophil frequencies and tuft cell hyperplasia were reduced in conditional knockout mice, underlining the importance of ST2 signals for a proper type 2 immune response (Figures 6D–I). As a consequence, *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice had higher worm counts in the intestine (Figure 6J). Therefore, our data expose cell-intrinsic ST2 signals in ILC2s as a non-redundant stimulatory pathway for triggering protective type 2 immunity.

Discussion

Alarmins are important mediators of inflammation and molecules targeting these pathways have already been or are about to be tested in clinical trials (32–35). However, since alarmins are sensed by a variety of immune cells, the mechanism of how they modulate immune response is incompletely understood. In this study, we characterized the IL-33-ST2 signaling pathway for ILC2 activation in detail, which could not

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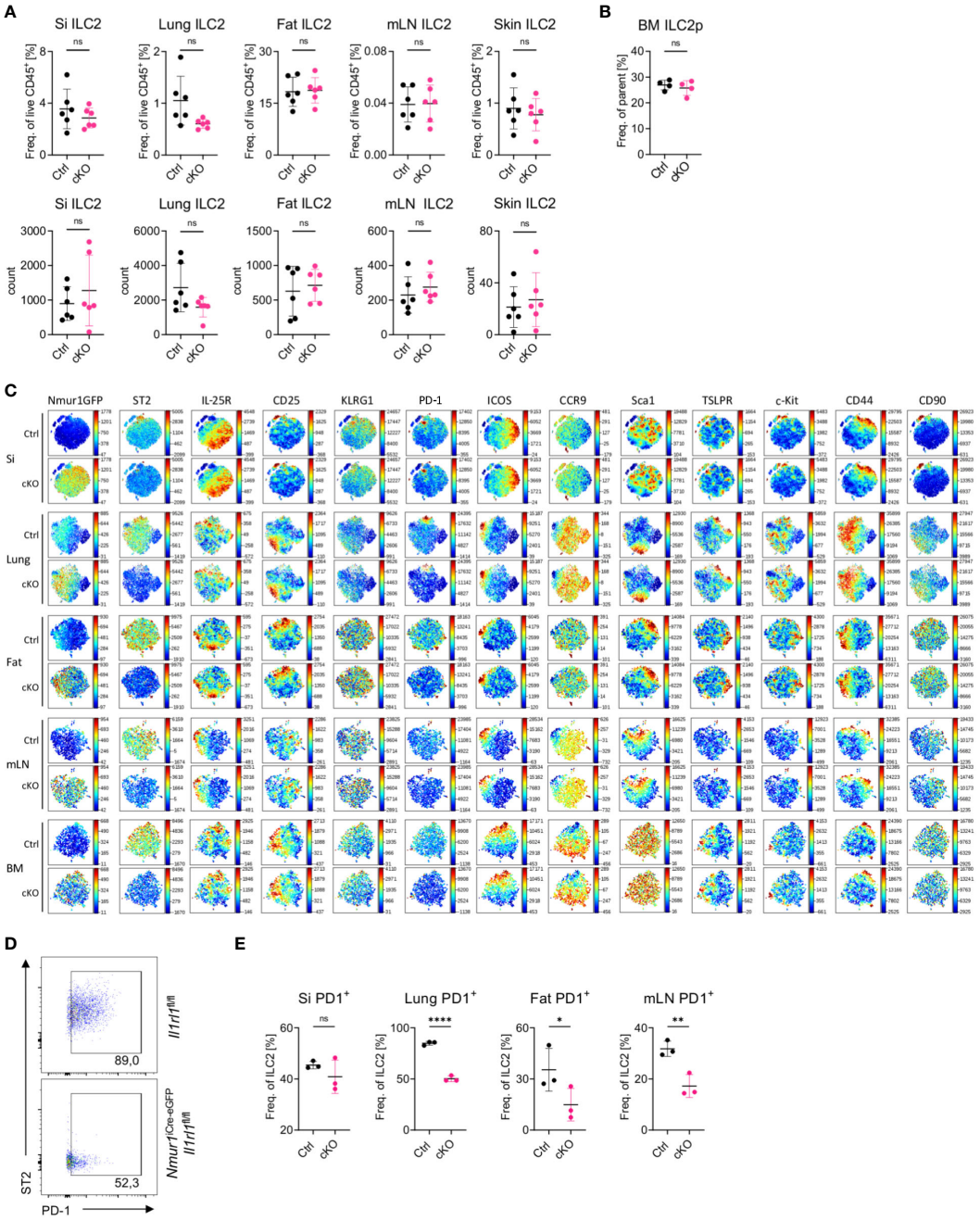

FIGURE 3
ILC2 develop in normal proportions without ST2 but have reduced PD-1 expression **(A)**, Quantification of relative and absolute ILC2 numbers in *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice across different organs in steady state. **(B)**, Quantification of relative ILC2 progenitor numbers in *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice in the BM. **(C)**, t-distributed stochastic neighbour embedding plots showing the expression level of different ILC2 markers. ILC2s from naïve *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice are shown. Each dot represents a single cell. Data are representative of two experiments with four to five mice per group. **(D)**, Flow cytometric plots of PD-1 expression in lung ILC2s from *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ and *Il1rl1*$^{flox/flox}$. **(E)**, Quantification of PD-1 expression in *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice across different organs in steady state. For **(A, B, D)**: Each symbol represents data from one mouse, data are representative of two experiments with three to eight mice per group. Mean +/- SD, student's t-Test. ns not significant, *p < 0.05, **p < 0.01, ****p < 0.0001.

be addressed so far because of limitations in genetic targeting strategies, using a novel Cre deleter strain based on the *Nmur1* promoter.

IL-33 and the type 2 effector cytokines IL-4, IL-5 and IL-13 have been linked to atopic diseases in mice and humans (16, 17). Similarly, type 2 effector cells, such as ILC2, Th2, mast cells and basophils were linked to this disease entity and were reported to respond to IL-33 with the release of type 2 effector cytokines and are therefore a potential candidate to mediate the effects of IL-33 in the context of type 2 inflammation. In our study interrogating the role of ST2 on ILC2s, we found that conditional deletion of ST2 rendered ILC2s unresponsive to IL-33 and resulted in decreased

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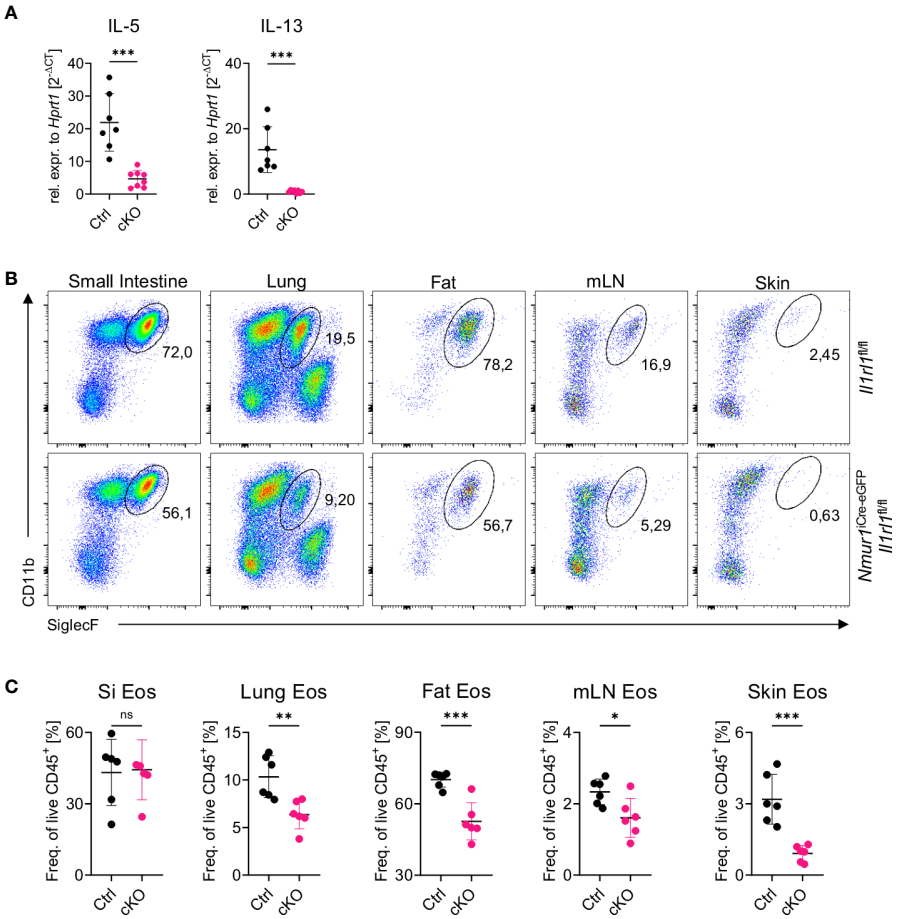

FIGURE 4
Loss of constant ST2 signaling results in reduced eosinophil counts. **(A)**, Relative expression of the cytokine IL-5 and IL-13 of *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ Il1rl1$^{flox/flox}$ (cKO) mice from the lung at steady state. **(B)**, Flow cytometric plots of eosinophils from *Il1rl1*$^{flox/flox}$ and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice across different organs in steady state. Eosinophils were gated on live CD45$^+$ Lin$^-$ (CD3, CD5, CD19), CD11b$^+$ and SiglecF$^+$. **(C)**, Quantification of **(B)** in *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice across different organs in steady state. For **(A, C)**: Each symbol represents data from one mouse, data are representative of two experiments with five to seven mice per group. Mean +/- SD, student's t-Test. ns, not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

production of the effector cytokines IL-5 and IL-13 directly *ex vivo*. In contrast, ILC2s developed in regular proportions without ST2. These findings are consistent with several studies, which found that the germline deletion of the alarmin receptors IL-25, IL-33 and TSLP did not result in decreased ILC2 numbers but diminished cytokine expression (23, 24). We further detected a reduction in eosinophil numbers at steady state in *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice, which is most likely the consequence of diminished IL-5 production by ILC2s, since ILC2s were described being the major source of IL-5 in tissues (27) and the IL-5–IL-5ra signaling was proven indispensable for proper development of eosinophils (29, 30). Finally, ILC2-deficient mice have defects in eosinophil development comparable to the deficiency described in IL-5 or IL5rα knockout mice (21, 29, 30). Of note, similar effects on eosinophil numbers were found in mice with germline deletion of ST2 combined with recombinant IL-33 administration (36). The results of our study allow to conclude that ILC2s are the cell type requiring IL-33 signals *via* ST2 in order to regulate eosinophil homeostasis at steady state and during allergic lung inflammation.

Despite the well-established role of alarmins in triggering allergic lung inflammation, the target cell types are only partly defined. IL-33 receptor was found to play a pivotal role in activating different effector cells in the context of type 2 inflammation including T_H2 cells, eosinophils, basophils and mast cells. The contribution of our study is the demonstration of an indispensable role for the IL-33-ST2 axis to stimulate ILC2 activation during allergic lung inflammation, which cannot be compensated by other signaling pathways or cell types. While we did not examine the role of conditional deletion of ST2 in other type 2 immune cells, our data suggest that IL-33 primarily activates ILC2 to initiate type 2 inflammation.

We used the *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mouse model to obtain genetic deletion of ST2 in ILC2s but not in other cell types investigated (Figures 1A–G). The *Nmur1*$^{iCre\text{-}eGFP}$ mice have reported highly efficient recombination capabilities (20, 21). To confirm the efficient deletion of ST2, we have compared ST2 levels to isotype control and mice with germline mutation of ST2. While very efficient deletion was obtained in most organs as validated by

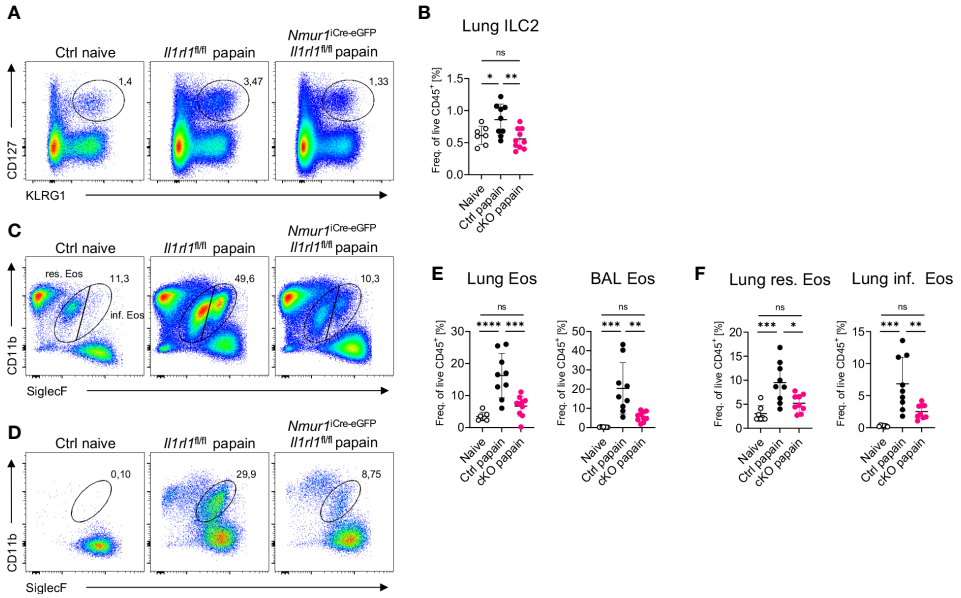

FIGURE 5
ST2-deficient ILC2s fail to recruit eosinophils in acute lung inflammation. **(A, C, D),** Flow-cytometric plots showing ILC2s **(A)** in the Lung, eosinophils **(C)** in the lung or BAL **(D)** of untreated Ctrl (naïve) animals and papain treated *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (cKO) and littermate controls (Ctrl). Data are representative of two experiments with four to six mice per group. **(B, E)**, Quantification of relative ILC2 **(B)** and eosinophil numbers from the lung or BAL **(E)** in the lung and BAL. **(F)**, Quantification of relative resident and inflammatory eosinophil numbers in the lung. For **(B, E, F)**: One-way ANOVA with multiple comparisons, ns, not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

flow cytometry, a minor fraction of cells remained formally ST2$^+$ in the lung of *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice, which might be explained by the limitations in the discriminatory power of the ST2 antibody staining (Figures 1B, C).

Our results with respect to ST2 signaling in ILC2s are comparable to published studies investigating the effect of conditional gene deletion of alarmin receptors in ILC2s. Kabata et al. could show that TSLP receptor is required on ILC2s to trigger allergic lung inflammation provoked by papain using *Il7r*$^{Cre/+}$ and *Il5*$^{Cre/+}$ and conditional alleles of TSLP receptor. The same study described a pivotal role of TSLP receptor on dendritic cells and CD4$^+$ T cells in the ovalbumin-induced airway inflammation model (37). Furthermore, Leyva-Castillo et al. conditionally deleted the IL-25 receptor (IL-25R) by crossing *Rora*$^{Cre/+}$ and *IL17rb*$^{flox/flox}$ mice. In a model of skin inflammation provoked by ova treatment, the symptoms were reduced in *Rora*$^{Cre/+}$ and *IL17rb*$^{flox/flox}$ mice, suggesting a crucial role for the IL-25R for ILC2 activation (38).

Previous studies reported a pivotal role for IL-25 and IL-33 in regulating worm expulsion of the parasites *N. brasiliensis* and *Strongyloides venezuelensis* (9, 28, 39–41). IL-25 together with other inflammatory mediators is secreted by Tuft cells and activates a subsets of IL-25R$^+$ ST2$^-$ ILC2s coined inflammatory ILC2s (42–46). IL-33 was also reported to stimulate the generation of inflammatory ILC2s *via* activation of the enzyme tryptophan hydroxylase in ILC2s (43, 47). A correlation of ILC2 frequencies and worm resistance during *N. brasiliensis* infection further supports the idea that ILC2s have a crucial function for worm expulsion, most likely activated by alarmin signals. Adoptive transfer experiments of ILC2s in lymphopenic or immunodeficient mice did rescue defects to expel the parasite, and therefore provide additional evidence for a pivotal role of ILC2s (9, 28). Complete depletion of ILC2 using *Nmur1*$^{iCre\text{-}eGFP}$ *Ild2*$^{flox/flox}$, *Nmur1*$^{iCre\text{-}eGFP}$ *Gata3*$^{flox/flox}$ or *Klrg1*^{Cre} *Gata3*$^{flox/flox}$ resulted in a severe defect in worm expulsion demonstrating the essential role of ILC2 for resistance to *N. brasiliensis* infection (21, 48). IL-25R and ST2 fulfill complementary functions to trigger ILC2 activation since genetic ablation of both receptors resulted in a comparable phenotype to ILC2-deficient mice characterized by strongly delayed worm expulsion and ongoing worm infection until day 14 - 20 post infection (28).

The *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mouse model allows us to more directly address the question of ILC2 redundancy and IL-33 signals without using adoptive cell transfer systems in combination with lymphopenic mice. Overall, our data from the *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice establish a cell-intrinsic and non-redundant role of ST2 for ILC2 function in the context of a type 2 immune response in lymphoreplete mice.

Experimental procedures

Mouse strains

C57BL/6 mice were purchased from Janvier. *Il1rl1*$^{flox/flox}$ mice (22) (provided by the UC Davis/MMRRC repository), *Il1rl1*$^{-/-}$ mice (28) and *Nmur1*$^{iCre\text{-}eGFP}$ mice (20, 21) bred locally at Charité. Sex and age-matched animals were used for experiments if not otherwise indicated. We did not use randomization to assign animals to experimental groups. All animal experiments were

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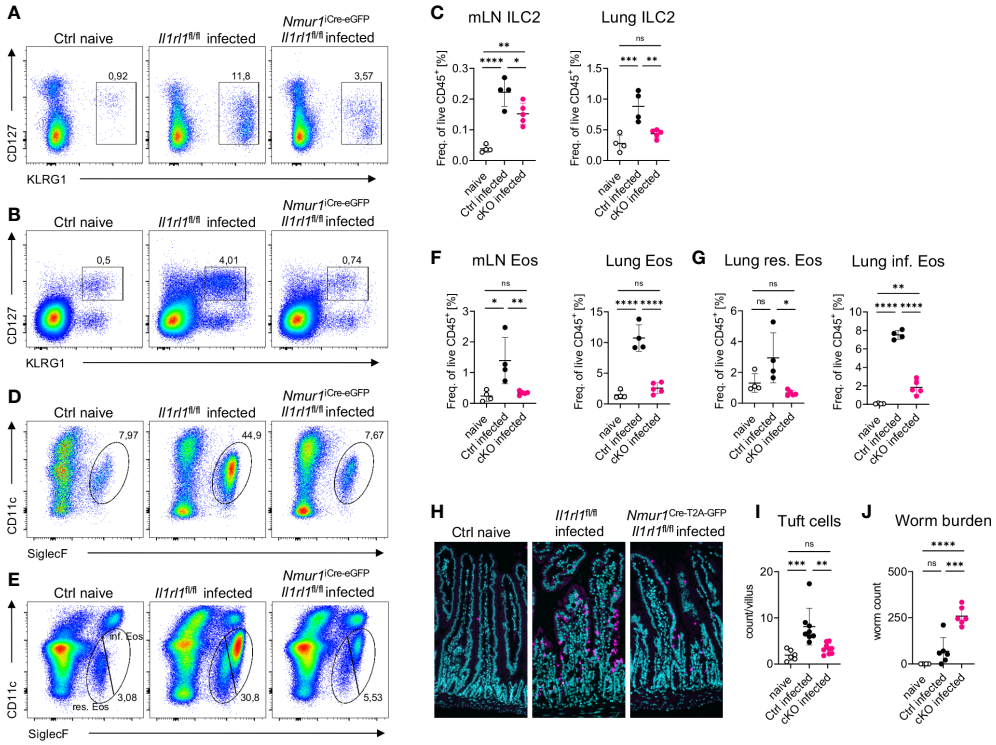

FIGURE 6
ILC2 intrinsic ST2 is required for immunity against *N. brasiliensis* infection. **(A, B)**, Flow-cytometric plots showing ILC2s in the mLN **(A)** and in the lung **(B)** of untreated Ctrl (naïve) animals and infected *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (cKO) and littermate controls (Ctrl). Mice were infected with *N. brasiliensis*. **(C)**, Quantification of **(A, B)**. **(D, E)** Flow-cytometric plots showing eosinophils in the mLN **(D)** and in the lung **(E)** of untreated Ctrl (naïve) animals and infected *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (cKO) and littermate controls (Ctrl). Mice were infected with *N. brasiliensis*. **(F)**, Quantification of **(D)** and **(E, G)**, Quantification of relative resident and inflammatory eosinophil numbers in the lung of untreated Ctrl (naïve) animals and infected *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (cKO) and littermate controls (Ctrl). **(H)**, Immunofluorescence migrographs of histological sections of the murine small intestine, tuft cells stained with DCLK1 (magenta), DAPI (blue). **(I)**, Quantification of **(H)**. Tuft cell numbers per villus. Data combined from two independent experiments. **(J)**, Worm burden in untreated animals (naïve) and infected *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (cKO) or littermate controls (Ctrl). For **(C, F, G, I, J)**: Data are representative of two experiments with four to six mice per group. One-way ANOVA with multiple comparisons, ns, not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

approved and are in accordance with the local animal care committees (Lageso Berlin).

Cell isolation

Small intestine was removed, cleaned from remaining fat tissue and washed in ice-cold PBS. Peyer's patches were eliminated, small intestine was opened longitudinally and washed in ice-cold PBS. Dissociation of epithelial cells was performed by incubation on a shaker at 37°C in HBSS (Sigma-Aldrich) containing 10 mM Hepes (Gibco) and 5 mM EDTA (Roboklon) two times for 15 min. After each step, samples were vortexed and the epithelial fraction discarded. Afterwards, remaining tissue was chopped into small pieces and enzymatic digestion was performed using dispase (0,5 U/ml; Corning), collagenase D (0,5 mg/ml; Roche) and DNaseI (100 µg/ml; Sigma-Aldrich). Leukocytes were further enriched by Percoll gradient centrifugation (GE Healthcare). Lungs were chopped and incubated in the enzyme cocktail described above for 40 min on a shaker at 37°C. The remaining tissues were mashed with a syringe plunger and single cell suspensions were filtered through a 70 µm cell strainer. Leukocytes were then further enriched by Percoll gradient centrifugation. Mesenteric lymph nodes were chopped and incubated in RPMI 1640 medium (Gibco) supplemented with 1% BSA (Sigma-Aldrich), collagenase II (1 mg/ml; Sigma-Aldrich) and DNaseI (100 µg/ml) for 20 min on a shaker at 37°C. Afterwards, cells were dissociated using a pasteur pipette, and filtered through a 70 µm cell strainer. Epididymal white adipose tissue was removed and incubated in the same digestion buffer for 45 min on a shaker at 37°C. After incubation, cells were dissociated using a Pasteur pipette, filtered through a 70 µm cell strainer, spun down and the adipocyte layer was aspirated. For skin preparation, ears were removed, split and put dermal side down in DMEM supplemented with Liberase TL (0,5 mg/ml; Roche) at 37°C for 1,5 h. The tissue was then mashed through a 70 µm cell strainer and the leukocytes further enriched by Percoll gradient centrifugation. For isolation of bone marrow cells, femur and tibia bone were crushed with a pestle, rinsed and cells were filtered through a 70 µm cell strainer. Red cell lysis was performed in ACK lysis buffer for 3 min.

Flow cytometry and cell sorting

Dead cells were routinely excluded with Fixable Aqua Dead Cell Stain or SYTOX Blue Dead Cell Stain (Thermo Fisher Scientific). Single cell suspensions were incubated on ice with anti-CD16/CD32 antibody and the following conjugated antibodies in PBS (Ca2+ and Mg^{2+}-free, Sigma-Aldrich). If indicated, lineage-positive cells were excluded by staining for CD3e (145-2C11 or 500A2), CD5 (53-7.3), CD19 (1D3 or 6D5), FcεRIα (Mar-1) and Ly6G (1A8). For surface staining the following antibodies were used: c-Kit (2B8), CCR9 (9B1), CD11b (M1/70), CD11c (N418), CD127 (A7R34), CD25 (PCG1.5), CD4 (GK1.5 and RM4-5), CD44 (IM7), CD45.2 (104), CD49b (DX5), CD64 (X54-5/7.1), CD8a (53-6.7), CD90.2 (53-2.1), F4/80 (BM8), ICOS(C398.4A), IL-25R (Munc33), KLRG1 (2F1 or MAFA), Ly6G (1A8), NK1.1 (PK136), PD-1 (29F.1A12), Sca-1 (D7), SiglecF (E50-2440), ST2 (RMST2-33), TSLPR (22H9), Rat IgG2b kappa Isotype (eB149/10H5). All antibodies used in flow cytometry were purchased from eBioscience, Biolegend or BD Biosciences if not otherwise indicated. All flow cytometry experiments were acquired using a custom configuration Fortessa flow cytometer and the FACS Diva software (BD Biosciences) and were analyzed with FlowJo V9.9.3 or V10.6.2 software (TreeStar) and for the t-SNE plots analyzed with the Cytobank Software using custom configurations (Backman Coulter) or sort-purified by using a custom configuration FACSAria cell sorter (BD Biosciences).

Quantitative real-time PCR

Sort-purified cells were homogenized in Trizol (Thermo Fisher Scientific) and stored at -80°C. RNA was extracted with chloroform and RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription of total RNA was performed using the High Capacity cDNA Reverse Transcription kit according to the protocol provided by the manufacturer (Thermo Fisher Scientific). Reaction was detected on a QuantStudio 5 Real-Time PCR (Thermo Fisher Scientific) using Taqman Gene Expression Assay (Applied Biosystems) with *Il5* (Mm00439646_m1), *Il13* (Mm00434204_m1) or SYBR Green Master Mix with *Il1rl1* (forward: 5'- GGGCACACAGGTCCTACTTG-3', reverse: 5'- ATGTAGTTGGTTCCATTCTCCG-3'). Gene expression was normalized to the housekeeping gene *Hprt1* (Mm00446968_m1) for Taqman and *Hprt1* (forward: 5'-GATACAGGCCAGACTTTGTTGG-3', reverse: 5'-CAACAGGACTCCTCGTATTTGC-3') for SYBR Green.

Helminth infection and allergic asthma induction

Third-stage larvae (L3) of *N. brasiliensis* were purified with a Baermann apparatus. After washing three times in PBS, larvae were counted and 500 purified larvae were injected subcutaneously in PBS. Mice were killed, organs were analyzed and worm burden was determined in the small intestine 7 days post infection.

For allergic asthma induction, 30 µg of Papain (Roche) in PBS were administered intranasally on three consecutive days. Mice were killed on day seven after initial administration, organs were collected and analyzed.

Cell culture

Sort-purified ILC2s (as live $CD45^+$, Lin^-, $NK1.1^-$, $CD127^+$ and $KLRG1^+$ for small intestine or $CD25^+$ for bone marrow) were incubated in DMEM with high glucose supplemented with 10% FCS, 10 mM Hepes, 1 mM sodium pyruvate, non-essential amino acids, 80 µM 2-Mercaptoethanol, 2mM Glutamine, 100 U/ml Penicillin and 100 µg/ml Streptomycin (all from Gibco) in 96-well U-bottom microtiter plates (Nunc) for three days at 37°C and 5% CO_2. As indicated, the culture was supplemented with a cocktail of IL-7 only, IL-7 and IL-33 or IL-7 and IL-25, IL-7 and TSLP or IL-7 and IL-33, IL-25, TSLP (Biolegend, TSLP from R&D, 20 ng/ml each).

Cytokine measurement

Cytokine concentrations in culture supernatants were determined by using the LEGENDplex murine T_H2 Panel V03 multiplex beads-based assay (Biolegend) according to the manufacture's protocol. Samples were recorded on a custom configuration Fortessa flow cytometer and the FACS Diva software (BD Biosciences) and the flow cytometry data files were analyzed using the Legendplex cloud-based analysis software suite (Biolegend).

Histology and immunofluorescence microscopy

For immunofluorescence staining, the tissue was fixed in 4% PFA at 4°C until tissue embedding. Paraffin-embedded sections were de-paraffinized and rehydrated. Sections were permeabilized with 0.5% Triton-X in PBS and blocked with PBS 0,5% Triton X-100 and 10% serum and stained with rabbit anti-DCLK1 (Abcam) followed by donkey anti-rabbit antibody coupled to Alexa Fluor 555 (Thermo Fisher Scientific). Nuclei were counterstained with DAPI (Thermo Fisher Scientific). Images were captured on a Zeiss Axio Observer 7 microscope and analysed with Zen software (Zeiss). For tuft cell numbers, five to ten representative villi were counted on four independent images per mouse using ImageJ.

Statistical analysis

Data is plotted showing the mean +/- standard deviation. P values of data sets were determined by unpaired two-tailed Student's t-test with 95% confidence interval. If equal variances could not be assumed, Welch test was performed. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to

analyze several groups. Normal distribution was assumed. Before mentioned statistical tests were performed with Graph Pad Prism V9 software (GraphPad Software, Inc.). (*p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 and ns, not significant).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material further inquiries can be directed to christoph.klose@charite.de.

Ethics statement

The animal study was reviewed and approved by Lageso Berlin.

Author contributions

PMT and ZAR carried out the experiments and analyzed the data. MOJ, PL, AS, AP and CSNK helped performing experiments. LMLT and KK helped with the t-SNE analysis. CUD and DA provided crucial input and tools for the study. CSNK and PMT conceived the project and wrote the manuscript with input from all co-authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DA has contributed to scientific advisory boards at Pfizer, Takeda, FARE and the KRF.

The remaining 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.

The handling editor HK declared a past co-authorship with the author DA.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1130933/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(related to Figure 1): Gating strategy for flow cytometry. **(A, B)**, Gating strategy of ILC2s and eosinophils from the small intestine, lung, mesenteric lymph nodes, fat, skin **(A)** and bone marrow **(B)**. Lineage: CD3, CD5, CD19, Ly6G, FcεRIα. **(C)**, Gating strategy of CD4+ and CD8+ T cells from the lung. **(D)**, basophils from the spleen and mast cells from the peritoneal lavage. Lineage: CD3, CD5, CD19, Ly6G. **(E)**, Geometric mean fluorescent intensity measurement of ST2 in ILC2s from *Il1rl1*$^{flox/flox}$ (Ctrl), *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) and *Il1rl1*$^{-/-}$ (ST2 KO) mice (indicated as ST2) with isotype control (indicated as Isotype) across different organs. For **(A-D)**: *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice (cKO). Mean ± SD. Student's t-Test. ns, not significant, **p < 0.01, ****p < 0.0001.

SUPPLEMENTARY FIGURE 2
(related to Figure 2): ST2-deficient ILC2s are unresponsive to IL-33 but not to other alarmins. **(A)**, Cytokine measurement from culture supernatants three days after stimulation as in (Figure 2C) (extending). **(B)**, Flow cytometric plots of ILC2s from the small intestine of *Il1rl1*$^{flox/flox}$ and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ mice after stimulation with IL-7, IL-7 and IL-33, IL-7 and Il-25, IL-7 and TSLP or IL-7 and IL-33, IL-25 and TSLP (alrmins) as indicated. ILC2s were gated on live CD45^{+} Lin^{-} (CD3, CD5, CD19, Ly6g, Fcer1), CD127^{+} and KLRG1^{+}. **(C)**, Quantification of ILC2 activation from **(B)** of *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice after a three day culture with cytokines IL-7, IL-7 and IL-33, IL-7 and Il-25, IL-7 and TSLP or IL-7 and IL-33, IL-25 and TSLP (alrmins) as indicated. **(D)**, Concentration of the indicated cytokine as determined in the cell culture supernatant three days after stimulation as in **(B, C)**. For **(A, C, D)**: Each symbol represents data from one mouse, data are representative of two experiments with four mice per group. Mean +/- SD. One-way ANOVA with multiple comparisons **(A)**, Student's t-Test **(C, D)**. ns not significant, * p<0.05, ** p<0.01, **** p<0.0001.

SUPPLEMENTARY FIGURE 3
(related to Figure 3): ST2 dependent changes in PD-1 expression. Geometric mean fluorescent intensity measurement of PD-1 in ILC2s from *Il1rl1*$^{flox/flox}$ (Ctrl) and *Nmur1*$^{iCre\text{-}eGFP}$ *Il1rl1*$^{flox/flox}$ (cKO) mice across different organs in steady state. Each symbol represents data from one mouse, data are representative of two experiments with three to four mice per group. Mean +/- SD, student's t-Test. ns not significant, * p<0.05, **** p<0.0001.

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