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## Type 2 innate lymphoid cells in the induction and resolution of tissue inflammation

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### Summary

Type 2 immunity against pathogens is tightly regulated to ensure appropriate inflammatory responses that clear infection and prevent excessive tissue damage. Recent research has shown that type 2 innate lymphoid cells (ILC2s) contribute to steady-state tissue integrity and exert tissue-specific functions. However, upon exposure to inflammatory stimuli, they also initiate and amplify type 2 inflammation by inducing mucus production, eosinophilia, and Th2 differentiation. In this review, we discuss the regulation of ILC2 activation by transcription factors and metabolic pathways, as well as by extrinsic signals such as cytokines, lipid mediators, hormones, and neuropeptides. We also review recent discoveries about ILC2 plasticity and heterogeneity in different tissues, as revealed partly through single-cell RNA sequencing of transcriptional responses to various stimuli. Understanding the tissue-specific pathways that regulate ILC2 diversity and function is a critical step in the development of potential therapies for allergic diseases.

### Keywords

disease resolution; innate immunity; innate lymphoid cells; mucosal immunology; neuroimmunology; type 2 inflammation

## 1 | DISCOVERY AND FUNCTION OF ILC SUBSETS

Mucosal surfaces are exposed to an array of environmental stimuli, some of which are beneficial, such as commensal microbes or nutrients present in food, while others are potentially harmful, such as pathogenic bacteria, helminths, viruses, allergens, and other noxious substances. The resident innate immune system therefore constantly monitors

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mucosal environments to both promote epithelial barrier function and rapidly recognize potential pathogens. Recently, an innate immune cell type, termed innate lymphoid cells (ILCs), was discovered in mice and humans, and shown to play crucial roles in both tissue homeostasis and the coordination and amplification of innate immune responses.<sup>1-3</sup>

While ILCs develop from a common lymphoid progenitor and express the receptor for interleukin (IL) 7 (CD127), ILCs lack expression of common lineage markers, including those typical of T cells.<sup>1,4</sup> In contrast to T cells, which express antigen-specific receptors, ILCs instead recognize tissue damage via inflammatory cytokines and mediators released by different cell types in response to stress or injury (Figure 1).<sup>1,5-7</sup> Because they are similar to T-helper cells in terms of key transcription factors and effector cytokines, ILCs have been classified analogously into T-bet<sup>+</sup> type 1 ILCs, GATA3<sup>+</sup> type 2 ILCs, and RORγt<sup>+</sup> type 3 ILCs.<sup>8,9</sup> Type 1 ILCs, which consist of conventional NK cells and less cytotoxic ILC1s, produce IFNγ and are implicated in tumor and viral immunity, as well as the defense of intracellular pathogens.<sup>1,10</sup> Type 2 ILCs, or ILC2s, produce IL-5 and IL-13 and are crucial for the expulsion of helminths, while also promoting the development of allergic inflammation.<sup>6,11,12</sup> Type 3 ILCs, which include lymphoid tissue inducer cells (LTi cells)<sup>13,14</sup> and ILC3s<sup>15-17</sup>, produce IL-17 and IL-22, and play a critical role in defense against extracellular bacteria and control of fungal infections.<sup>17-19</sup>

Although ILCs are predominantly found at mucosal barriers, including the lung<sup>20,21</sup>, gut<sup>21-23</sup>, skin<sup>24-29</sup> and uterus<sup>30</sup>, they are also present in other tissues, such as adipose tissue<sup>5,31-33</sup>, liver<sup>34</sup> and meninges<sup>35-37</sup>. The relative proportions of different ILC types are distinct in each tissue, likely reflecting the unique homeostatic requirements of the tissue. In settings of inflammation and tissue damage, however, ILCs demonstrate a degree of plasticity, whereby ILCs of one subset preferentially expand,<sup>38</sup> or partially adopt the phenotype of another subset (eg, ILC2s can become ILC1-like or ILC3-like cells).<sup>39-44</sup> ILC plasticity may support a rapid, flexible immune response and promote tissue integrity, but it could also conceivably lead to alterations in tissue homeostasis, resulting in persistent organ dysfunction. By understanding the tissue-based cues that regulate ILC function, we can better assess the changes in ILCs that play a role in chronic disease.

While all three types of ILCs contribute to tissue homeostasis, for the purposes of this review, we focus on ILC2s, which play both protective and pathogenic roles in type 2 inflammatory responses in the lung and intestine. ILC2s were the first tissue-resident ILC subset to be described: a population of cells producing type 2 cytokines was noted in mice that lack T and B cells in studies examining the function of the cytokine IL-25.<sup>45-47</sup> However, the cells were not further phenotypically characterized, and no function was shown. In 2010, several seminal studies identified ILC2s as critical for mounting an innate type 2 immune response against helminths.<sup>5,6,11</sup> ILC2s are largely tissue-resident cells that do not migrate between organs,<sup>48,49</sup> except for a subpopulation of IL-17-producing ILC2s, called “inflammatory ILC2s”, that appear to migrate from the gut to other organs during helminth infection.<sup>44,50</sup>

The tissue microenvironment is shaped by interactions between multiple cell types, including stromal cells, epithelial cells, immune cells, and neurons, as well as by the

presence of cytokines, inflammatory mediators, and environmental exposures. Recent evidence has highlighted that ILC2s integrate both pro- and anti-inflammatory signals to promote host defense and epithelial barrier function.<sup>6,20</sup> Moreover, although all ILC2s apparently arise from the same precursor and produce the signature type 2 cytokines IL-5 and IL-13,<sup>51</sup> ILC2s from different tissues can differ significantly in terms of surface markers, resting transcriptional state, and response to certain stimuli.<sup>35,38,50,52,53</sup> This suggests that the tissue context influences the function of ILC2s, which in turn adapt themselves to tissue-specific homeostatic requirements. Below, we highlight the signals that alter ILC2 function and phenotype, both at steady state and during inflammatory responses, and discuss the diversity and plasticity of ILC2s under homeostatic and inflammatory conditions in different tissues.

## 2 | ACTIVATION OF ILC2S BY EXTRINSIC SIGNALS

ILC2s are activated by the cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), so-called alarmins, which are released into the local tissue environment by epithelial or other cells in response to cellular stress, damage or nonprogrammed cell death.<sup>54,55</sup> In addition to alarmins, other mediators, such as neuropeptides and lipid mediators, and co-stimulatory molecules, including ICOS, can also activate ILC2s, and will be discussed in detail below (Figure 2).

### 2.1 | Activation of ILC2s by alarmins

IL-33 belongs to the IL-1 cytokine family and is localized at steady state in the nucleus of epithelial cells and other cell types, including endothelial and fibroblastic reticular cells.<sup>54</sup> Upon release, IL-33 binds to its receptor ST2 (*Il1rl1*) and, in conjunction with the IL-1 receptor accessory protein (IL-1RAcP), activates intracellular signaling pathways that promote type 2 immune responses.<sup>54</sup> In murine and human ILC2s in vitro, IL-33 signaling induces ILC2 expansion and promotes production of the type 2 cytokines IL-5 and IL-13.<sup>5,6,56–58</sup> IL-33 is also a potent activator of ILC2s in vivo, as IL-33 promotes ILC2 responses in the liver in a mouse model of hepatic fibrosis,<sup>34</sup> causes lung inflammation upon nasal administration,<sup>59</sup> and induces atopic dermatitis in mice, with elevated frequencies of IL-5- and IL-13-producing ILC2s and eosinophilia.<sup>27</sup>

The alarmin IL-25 (IL-17E), expressed by epithelial cells and leukocytes, is part of the IL-17 family, with IL-17RA and IL-17RB forming the IL-25 receptor.<sup>60,61</sup> Binding of IL-25 activates different signaling pathways, including that of the nuclear factor NF- $\kappa$ B.<sup>60,61</sup> By promoting expression of the type 2 cytokines IL-4, IL-5, and IL-13 in vivo, IL-25 elicits eosinophilia, mucus production, and elevated serum levels of IgA, IgE, and IgG1.<sup>45,46</sup> In the absence of IL-25, mice are less efficient in clearing the helminth *Nippostrongylus brasiliensis* (*N. brasiliensis*), indicating that endogenous IL-25 is important for rapid worm expulsion.<sup>47</sup> Recently, tuft cells, an epithelial chemosensory cell type, were identified as the major IL-25 producers during helminth infection and following colonization with protozoa.<sup>62–64</sup> Using IL-25-reporter mice, von Moltke et al<sup>62</sup> also demonstrated that during *N. brasiliensis* infection, there is substantial expansion of tuft cells, which is reciprocally dependent on IL-13 produced by ILC2s. Indeed, IL-13 directly induces tuft cell hyperplasia,

as shown by the increased frequency of tuft cells in epithelial organoid cultures in response to IL-4 or IL-13 stimulation.<sup>62–64</sup> Other helminths, such as *Heligmosomoides polygyrus* and *Trichinella spiralis*, as well as the protozoa *Tritrichomonas muris* also promote tuft cell amplification.<sup>64</sup>

While IL-33 and IL-25 both activate ILC2s and promote type 2 cytokine production,<sup>56</sup> these cytokines vary in their efficiency in inducing specific responses or expanding distinct cell populations. In the skin, both IL-33 and IL-25, as well as thymic stromal lymphopoietin (TSLP), were shown to be important for inducing ILC2 responses and inflammation, using a mouse model of atopic dermatitis induced by calcipotriol (MC 903).<sup>24,26</sup> In a mouse model of ovalbumin (OVA)-induced airway inflammation, *Il1rl1*-deficient mice developed significantly less airway hyperreactivity in response to methacholine challenge than wildtype (WT) mice.<sup>65</sup> In contrast, the airway hyper-reactivity of *Il17rb*-deficient mice was only slightly decreased compared to WT mice, suggesting that IL-33 signaling contributes more to the development of allergen-induced airway inflammation than IL-25 signaling.<sup>65</sup> In accordance with those observations, more IL-13 expressing cells were detectable in the lungs of mice treated with IL-33 compared to IL-25.<sup>65</sup>

Moreover, it was recently shown that IL-33 and IL-25 promote the expansion of two distinct lung ILC2 populations, termed natural ILC2s (nILC2s) and inflammatory ILC2s (iILC2s), respectively.<sup>44</sup> Both populations are lineage negative and positive for IL-7R $\alpha$ , but nILC2s are ST2<sup>+</sup> Thy1<sup>hi</sup> KLRG1<sup>int</sup> IL-17RB<sup>lo</sup>, while iILC2s are ST2<sup>-</sup> Thy1<sup>lo</sup> KLRG1<sup>hi</sup> IL-17RB<sup>hi</sup> and capable of jointly producing the cytokines IL-17 and IL-13, as well as participating in immune responses against the fungus *Candida albicans*.<sup>43,44</sup> In vitro, iILCs develop into ST2<sup>+</sup> cells with a surface phenotype similar to nILC2s and produce IL-5 and IL-13 after stimulation with IL-33 but not IL-25.<sup>44</sup> In vivo, these cells also convert into ST2<sup>+</sup> nILC2-like cells after adoptive transfer into RAG2<sup>-/-</sup>  $\gamma_c$ <sup>-/-</sup> mice and are able to reduce worm burden during *N. brasiliensis* infection.<sup>44</sup> While these data point to the possibility that iILC2s may be a progenitor population of ILC2s that contributes to clearance of helminth infection, iILC2s could also represent an induced ILC3-like ILC2 population that reverts to a more classical type 2 phenotype without appropriate signaling. The latter scenario is consistent with the finding that Notch signaling is required for the emergence of iILC2s and induces the conversion of mature nILC2s into iILC2s, apparently by upregulating *Il17rb* mRNA expression and downregulating *Il1rl1* mRNA expression.<sup>43</sup>

Subsequent to the identification of iILC2s, Huang et al<sup>50</sup> used parabiotic mice to show that iILC2 progenitors are located in the intestinal lamina propria at steady state. Upon intraperitoneal injection of IL-25 or infection with *N. brasiliensis*, these progenitors give rise to proliferating iILC2s that migrate to other organs, including the lung and liver. In contrast, nILC2s do not circulate in response to IL-33. The authors observed that treatment with the inhibitor FTY720 prevented IL-25-induced migration of iILC2s, suggesting that their migration depends on chemotaxis mediated by sphingosine 1-phosphate (S1P). Consistent with this, the S1P receptors S1PR1 and S1PR4 were detected on IL-25-induced iILC2s. The study further shows that RAG1<sup>-/-</sup> mice were unable to contain *N. brasiliensis* infection when lymphocyte migration was inhibited by FTY720 treatment and succumbed to worm infection. However, adoptive transfer of iILC2s into these mice before FTY720

administration significantly increased the survival rate, suggesting that circulating iILC2s from the intestine play an important role in *N. brasiliensis* expulsion in the absence of adaptive immunity.<sup>50</sup>

Using massively parallel droplet-based single-cell RNA sequencing (scRNA-seq), we recently characterized the transcriptional landscape of lung-resident ILCs, both at steady state and in response to the alarmins IL-25 and IL-33.<sup>38</sup> While both IL-25 and IL-33 upregulated production of type 2 effector cytokines in ILC2s, they also induced subpopulations with distinct profiles (discussed further below).<sup>38</sup> For example, IL-33 induced a much more proliferative response, consistent with previous findings.<sup>38,56,57</sup> We also identified a population of primarily IL-25 induced cells that were phenotypically similar to iILC2s, eg, expressing very high levels of *Klrg1* but only low levels of *Il1rl1*.<sup>38</sup> However, this population represented a minority of all IL-25-activated ILC2s,<sup>38</sup> in contrast to other reports,<sup>44,50</sup> apparently due to differing routes of IL-25 administration, as intranasal administration induces a much smaller population of iILC2s than intraperitoneal administration.<sup>50</sup>

The alarmin cytokine TSLP promotes type 2 inflammation, both by activating mouse and human ILC2 responses<sup>24,58</sup> and by regulating the function of CD4 T cells, dendritic cells, basophils and mast cells.<sup>66</sup> TSLP is expressed by epithelial cells and keratinocytes during inflammation and binds to a heterodimeric receptor consisting of the TSLP receptor chain and IL-7R $\alpha$ .<sup>58,66,67</sup> TSLP signaling phosphorylates signal transducer and activator of transcription 5 (STAT5) and induces expression of GATA3, the key transcription factor of ILC2s and Th2 cells.<sup>58</sup> In vitro, human ILC2s isolated from blood or nasal polyps secreted type 2 cytokines in response to IL-2 and TSLP, though at lower levels than in response to the combination of IL-2 and IL-33.<sup>58</sup> The combination of IL-2, TSLP, and IL-33 synergistically stimulated high levels of type 2 cytokine production.<sup>58</sup> In mice, TSLP is sufficient to activate ILC2s in the skin, as demonstrated by intravenous injection of a TSLP-encoding cDNA plasmid, but TSLP induced fewer ILC2s in skin-draining lymph nodes (LNs) from IL-33-deficient mice than it did in WT mice, indicating again that IL-33 and TSLP synergize to activate ILC2s in vivo.<sup>24</sup> Slightly contradictory findings were reported regarding the role of TSLP in a model of calcipotriol-induced atopic dermatitis-like skin inflammation. In this mouse model, ILCs contribute significantly to the development of skin inflammation, demonstrated by the fact that RAG1<sup>-/-</sup> mice treated with anti-CD25 or anti-CD90.2 to deplete ILCs develop less skin inflammation than isotype control-treated mice.<sup>24</sup> One study reported that TSLPR-deficient mice, but not IL-33-deficient or IL-17RB-deficient mice, had lower frequencies of ILC2s in skin-draining LNs and a reduced severity of dermatitis compared to C57BL/6 wildtype mice, suggesting that activation of ILC2s in this model requires TSLP but not IL-33 or IL-25.<sup>24</sup> However, another study reported that in Balb/c mice, IL-33 and IL-25 signaling contributed more than TSLP to the ILC2 response during skin inflammation in this model, and noted that in their hands IL-33 and IL-25 also seem to be important for the development of skin inflammation in C57BL/6 mice.<sup>26</sup>

In summary, IL-25 appears to preferentially promote intestinal ILC2 responses, while IL-33 promotes lung ILC2 responses and TSLP seems to facilitate skin ILC2 responses. There is clearly overlap, however, as recent data indicate that all three alarmins appear to have

partially redundant roles in the induction of an immune response to *N. brasiliensis* infection.<sup>68</sup> While WT mice are able to clear intestinal worms by day 10 after infection, a high worm burden is still detectable in ST2/IL-25/TSLPR-triple deficient KO mice.<sup>68</sup> Mice singly deficient in ST2, IL-25 or TSLPR have persistent intestinal worms at day 10, but fewer than in the triple KO, indicating that the cytokines can compensate for the loss of each other to some extent.<sup>68</sup> IL-33 and IL-25 signaling seemed to contribute more than TSLP signaling to worm clearance, supporting the idea that these alarmins are critical for lung and intestinal ILC2 activation.<sup>68</sup> However, another study reported that after respiratory syncytial virus infection, lung-resident ILC2 responses were dependent on TSLP,<sup>69</sup> suggesting that the type of pathogen, in addition to the tissue environment, influences the dependence of ILC2 activation on specific cytokines.

## 2.2 | Activation of ILC2s by other cytokines

In addition to IL-33, IL-25 and TSLP, other cytokines also play important roles in regulating ILC2 responses. ILC2s express IL-2R $\alpha$  (CD25), suggesting a role for IL-2 in their function.<sup>25,70</sup> Treatment of RAG1<sup>-/-</sup> mice with IL-2 promotes ILC2 expansion, resulting in greatly elevated IL-5 and IL-13 levels in the lungs and skin and the concomitant development of type 2 inflammation in both tissues.<sup>25,70</sup> IL-2 does not appear to directly induce IL-5 or IL-13 production, but rather, in combination with IL-33, it results in higher frequencies of IL-13-positive ILC2s and enhances IL-13 production on a per cell basis<sup>70</sup>, suggesting that IL-2 both directly induces ILC2 proliferation and functions as a co-stimulatory signal that promotes cytokine production by ILC2s.

IL-4 also promotes ILC2 responses in type 2 inflammation. In distinct mouse models of atopic dermatitis-like skin inflammation and papain-induced allergic airway inflammation, basophil-derived IL-4 is required for the expansion of ILC2s and development of type 2 inflammation in the skin or lung, respectively.<sup>71,72</sup> In addition, IL-4 promotes ILC2 proliferation<sup>72</sup> and induces the production of type 2 cytokines and CCL11, a chemokine implicated in eosinophil recruitment, in ILC2s.<sup>71</sup> However, with the exception of iILC2s, which express little IL-5, ILC2s do not generally express IL-4, while highly expressing IL-13 and IL-5.<sup>6,38</sup> Given the contrast with Th2 cells, which express all three type 2 cytokines, and the role of IL-4 in promoting ILC2 responses, the generally low production of IL-4 by ILC2s may represent a regulatory mechanism to prevent excessive ILC2 activation and expansion via autocrine or paracrine responses to IL-4.

Finally, ILC2s are also activated by TL1A (TNFSF15), a member of the tumor necrosis factor family.<sup>73,74</sup> TL1A is expressed in the endothelium and myeloid cell compartment in response to inflammation and has been implicated in promoting CD4 T-cell function by inducing cytokine production and proliferation.<sup>73</sup> Murine and human ILC2s express DR3, the receptor for TL1A, and stimulation with TL1A promotes type 2 cytokine production and ILC2 expansion.<sup>73,74</sup> In the absence of TL1A signaling, ILC2 numbers are reduced in mice challenged with papain.<sup>73,74</sup> In addition, DR3-deficient mice develop less airway inflammation, suggesting that TL1A signaling promotes type 2 immune responses through expansion of ILC2s.<sup>73,74</sup>

### 2.3 | Activation of ILC2s by lipid mediators

Besides their activation by cytokines, ILC2s can also be activated by lipid mediators, often in synergy with alarmin cytokines. Leukotrienes are lipid mediators that are synthesized from arachidonic acid and have a well described role in promoting type 2 airway inflammation.<sup>75</sup> Recent work suggests that leukotrienes mediate this pro-inflammatory effect in part by acting upon ILC2s in mice and humans.<sup>76–78</sup> ILC2s express cysteinyl leukotriene receptor 1 (CysLT1R) and 2 (CysLT2R), which bind leukotriene C<sub>4</sub> (LTC<sub>4</sub>) and leukotriene D<sub>4</sub> (LTD<sub>4</sub>).<sup>76,77</sup> Stimulation of ILC2s with LTC<sub>4</sub> or LTD<sub>4</sub> elicits type 2 cytokine production in a Cyslr1-dependent manner.<sup>76,77</sup> In vivo, administration of LTD<sub>4</sub> to *Alternaria* exposed RAG2<sup>-/-</sup> mice promotes IL-5 production and expansion of ILC2s, as well as eosinophilia.<sup>76</sup> Moreover, lack of Cyslr1 signaling results in reduced frequencies of IL-13-producing ILCs during airway inflammation or helminth infection, indicating that leukotrienes contribute to ILC2 responses.<sup>77</sup> While the transcription factor nuclear factor of activated T cells (NFAT) is observed in the cytoplasm of unstimulated ILC2s, it is detected in the nucleus after addition of LTC<sub>4</sub>, indicating that LTC<sub>4</sub> signaling promotes nuclear translocation of NFAT.<sup>77</sup> NFAT translocates into the nucleus upon dephosphorylation by calcineurin.<sup>79</sup> Inhibition of calcineurin by cyclosporin A dampens LTC<sub>4</sub>-induced IL-13 expression, suggesting that LTC<sub>4</sub> signaling promotes cytokine production via activation and translocation of NFAT.<sup>77</sup> In T cells, TCR engagement activates different signaling pathways that result in activation of NFAT, NF- $\kappa$ B and activator protein 1 (AP-1).<sup>79</sup> Von Moltke et al<sup>77</sup> propose that in ILC2s, concomitant stimulation with leukotrienes and IL-33 mimics TCR signaling, as leukotrienes activate NFAT, whereas IL-33 activates NF- $\kappa$ B and AP-1.

Other lipid mediators have also been shown to modulate ILC2 responses. For instance, prostaglandin D<sub>2</sub> (PDG<sub>2</sub>), produced by mast cells, is another derivate of arachidonic acid that activates ILC2s.<sup>80</sup> Stimulation of human ILC2s with PDG<sub>2</sub> and IL-2 induced expression of IL-13, which was dramatically amplified if IL-25 and IL-33 were added to the culture.<sup>81</sup> PDG<sub>2</sub> also promotes production of multiple cytokines, including IL-4, IL-5, and GM-CSF, as well as expression of ST2, and these effects are dependent on activation of CRTH2, a receptor for PDG<sub>2</sub>.<sup>80</sup> PDG<sub>2</sub> expression is elevated in patients with severe asthma, and PDG<sub>2</sub> promotes migration of murine and human ILC2s in vitro suggesting that it might mediate the accumulation of CRTH2<sup>+</sup> ILC2s at the site of inflammation.<sup>80,82–84</sup> Consistent with this, significantly more wildtype ILC2s than CRTH2-deficient ILC2s were found in the lungs of mixed bone marrow chimeras several weeks after infection with *N. brasiliensis*.<sup>82</sup> These findings suggest that lipid mediators can act in concert with alarmins to synergistically enhance ILC2 responses.

### 2.4 | Activation of ILC2s by sex hormones

ILC2s are resident in the uterine mucosa and accumulate there in response to IL-33, but not IL-25, treatment, which also promotes their production of IL-5 and IL-13.<sup>30</sup> ILC2s in the uterus express estrogen receptors  $\alpha$  (ER $\alpha$ ) and  $\beta$  (ER $\beta$ ), and respond transcriptionally to in vitro activation with 17 $\beta$ -estradiol.<sup>30</sup> Intriguingly, uterine ILC2s are greatly reduced after surgical removal of ovaries, while treatment with the combination of 17 $\beta$ -estradiol and progesterone is sufficient to restore their numbers, indicating that female sex hormones are required for ILC2 homeostasis in the uterus.<sup>30</sup> On the other hand, removal of the ovaries and

subsequent treatment with hormones did not affect ILC2 numbers in the lung, suggesting that the local tissue environment shapes ILC2 responsiveness to such signals.<sup>30</sup>

A recent study of experimental autoimmune encephalomyelitis (EAE) gave evidence that, partly through their effect on ILC2s, sex hormones modulate EAE pathogenesis.<sup>85</sup> This study shows that while female mice from the SJL strain develop EAE in response to immunization with proteolipid peptide, male SJL mice develop no disease. After immunization, male mice have increased frequencies of ILC2s and Th2 cells and reduced frequencies of Th17 cells in their CNS, pointing to the possibility that an increased type 2 response might prevent EAE development. Mast cells from immunized male SJL mice express more IL-33 and in vitro stimulation of bone marrow derived macrophages with testosterone promotes IL-33 expression. Since IL-33 activates ILC2s, the authors suggest that IL-33 could prevent EAE in part via the induction of ILC2 responses. Supporting this hypothesis, treatment of female mice with IL-33 during EAE induces elevated ILC2 and Th2 frequencies and improves functional outcome, though additional work is needed to address the specific role of ILC2s.<sup>85</sup>

## 2.5 | Activation of ILC2s by surface molecules

Besides soluble factors, ILC2 function is also regulated by cell-surface molecules. Inducible T cell costimulator (ICOS) and its ligand ICOS-L are both expressed on mouse and human ILC2s, and their interactions regulate ILC2 homeostasis and effector function.<sup>86–88</sup> Notably, the frequency of ILC2s is reduced in both the lung and intestine of ICOS and ICOS-L deficient mice.<sup>87</sup> Moreover, in vivo blockade of the ICOS/ICOS-L interaction with a monoclonal antibody against ICOS-L inhibited ILC2 proliferation at steady state.<sup>87</sup> To study the function of ICOS during inflammation in vivo, WT or ICOS-deficient ILC2s were transferred into RAG2<sup>-/-</sup>γ<sub>c</sub><sup>-/-</sup> mice and challenged with IL-33.<sup>86</sup> As suggested by the in vitro experiments, mice that received ICOS-deficient ILC2s had reduced ILC2 numbers and did not develop airway inflammation.<sup>86</sup> In addition, culture of ILC2s on a cell line overexpressing ICOS-L promoted IL-2/IL-33-induced type 2 cytokine production and this effect was dependent on ICOS/ICOS-L signaling.<sup>88</sup>

ILC2s also express major histocompatibility complex 2 (MHC2), which seems to be needed to mount an optimal type 2 immune response, since transfer of wildtype, but not MHC2-deficient, ILC2s to IL-13-deficient mice restored the ability to clear helminth infection.<sup>89</sup> The authors of that study suggest that MHC2-expressing ILC2s activate T cells to produce IL-2, which then enhances ILC2 expansion and IL-13 production, thus facilitating worm expulsion. Indeed, TCR-activated CD4 T cells produce IL-2 and induce ILC2 proliferation and type 2 cytokine production in vitro in an IL-2 dependent manner.<sup>89,90</sup> Accordingly, treatment of RAG2<sup>-/-</sup> mice with IL-2 complex increases ILC2 numbers and restores the ability to expel worms after *N. brasiliensis* infection.<sup>89</sup>

## 2.6 | Activation of ILC2s by neurons

Recent lines of inquiry support the idea that the nervous system may, in fact, directly respond to immune provocation. The sensory nervous system is well equipped to support the immune system, as it innervates surfaces that are in direct contact with the environment and



responds very rapidly to danger signals.<sup>91,92</sup> Noxious stimuli, such as heat, are recognized by sensory neurons, which in turn initiate reflexes to escape the harmful stimulus.<sup>91</sup> However, sensory neurons also express receptors usually found on immune cells, such as danger-associated molecular pattern receptors, Toll-like receptors, and cytokine receptors, that enable them to directly and in-directly sense pathogens.<sup>91</sup> In addition, nociceptor neurons can be directly stimulated by the bacterium *Staphylococcus aureus* via bacterial-derived pore-forming toxins or N-formylated peptides.<sup>93</sup> The type 2 cytokines IL-4 and IL-13, as well as the alarmin cytokine TSLP, were shown to promote itch by binding to their respective receptors on sensory neurons,<sup>94,95</sup> emphasizing the potential of sensory neurons to not only respond directly to bacteria but also to pathogen-or allergen-induced tissue inflammation. Upon activation, sensory neurons not only promote a muscular response, for example, coughing to remove the pathogenic stimulus, but also contribute to immune responses, for example, promoting skin inflammation, colitis, and OVA-induced airway inflammation.<sup>91,96–99</sup> While multiple immune cell types, including mast cells, invariant NKT cells, and macrophages, are regulated by the nervous system,<sup>100–105</sup> ILC2s appear to have particularly close interactions with the nervous system.

Neurons can also promote ILC2 responses through the production of neurotransmitters. Two studies found that vasoactive intestinal peptide (VIP), which is produced by sensory neurons, activates ILC2s.<sup>106,107</sup> ILC2s express VPAC1 and VPAC2, the receptors for VIP, and stimulation with either VIP or a pharmacologic VPAC2 agonist promotes IL-5 production.<sup>106</sup> Serum IL-5 levels and eosinophil frequencies were observed to correlate with circadian rhythm and the feeding cycle of the mice, suggesting that VIP secreted by neurons in response to caloric intake promotes ILC2 activation in vivo and thus regulates eosinophil homeostasis.<sup>106</sup> VIP is also released by sensory neurons during allergic airway inflammation in response to IL-5, and induces ILC2 and Th2 cell activation.<sup>107</sup> Treatment with an antagonist of VPAC2 during allergic airway inflammation diminishes IL-13 production and activation of ILC2s, as well as CD4 T-cell numbers in the lung, indicating that sensory neurons facilitate allergic airway inflammation, in part, via VIP/VPAC2 signaling.<sup>107</sup>

Three recent studies, including ours, identified another neuropeptide, Neuromedin U (NMU), as an amplifier of ILC2 responses that promotes allergic airway inflammation and helminth clearance.<sup>38,52,53</sup> Neuromedin U receptor 1 (Nmur1), the receptor of NMU, is expressed by ILC2s and not other ILCs, and NMU/Nmur1 signaling induces production of IL-5 and IL-13 by ILC2s in vitro and in vivo.<sup>38,52,53</sup> While NMU alone increases IL-5 and IL-13 expression dramatically in intestinal ILC2s, the effect is much smaller in lung-derived ILC2s.<sup>53</sup> However, the combination of IL-25 and NMU synergistically induces significant IL-5 and IL-13 production in lung ILC2s,<sup>38</sup> suggesting that the constitutive presence of IL-25 in the intestine might modulate the response of ILC2s to NMU. Indeed, type 2 cytokine responses to NMU were diminished in intestinal ILC2s from *Il1rl1/Il17rb* KO mice compared to WT mice.<sup>53</sup> In addition, IL-25-producing tuft cells expand in response to colonization with *Tritrichomonas muris*, a protozoan that is often part of the microbiota.<sup>64</sup> Thus, IL-25 from tuft cells in the intestine may pre-activate ILC2s, causing differential responses of lung-vs gut-derived ILC2s to NMU. Mechanistically, Nmur1 signaling activates G<sub>aq</sub> proteins<sup>52</sup> and induces ERK1/2 phosphorylation and calcium flux, which then activates calcineurin and promotes translocation of NFAT into the nucleus.<sup>53</sup> NMU is upregulated

after helminth infection,<sup>52</sup> and purified *N. brasiliensis* excretory and secretory products or recombinant IL-33 stimulate NMU expression in neuronal organoids in a process dependent on MyD88, an adapter protein crucial for a number of innate immune receptor signaling pathways, including ST2.<sup>53</sup> Furthermore, mice that lack MyD88 specifically in cholinergic neurons have reduced ILC2 activation in response to *N. brasiliensis* infection,<sup>53</sup> indicating that both alarmins and helminth-derived factors can directly stimulate NMU expression in neurons, which then leads to ILC2 activation and facilitates immune responses to allergens and helminths. These findings are particularly exciting since they highlight a novel pathway by which the immune system and the nervous system communicate with one another at mucosal surfaces, to promote rapid detection and response to dangerous stimuli.

The crosstalk between mucosal ILCs and neurons potentially co-evolved since both systems constantly monitor the environment to identify potential dangers and both can respond quite rapidly. Moreover, if either neurons or immune cells recognize danger, it could be useful to have one system activate the other, potentially saving energy and time, as well as providing a means of amplifying responses, as seen with NMU and IL-25. Furthermore, the nervous system can learn to avoid potentially dangerous situations. Mouse studies showed that OVA-immunized mice avoid sweetened OVA solution compared to nonimmunized mice, indicating that the immune system can potentially teach the nervous system what is potentially beneficial or harmful, although this may also lead to the development of allergies.<sup>108</sup> The communication of the nervous system and immune system has implications for the treatment of allergic diseases. Since the majority of therapies, such as corticosteroids or antihistamines, target the immune system, developing therapies that specifically target the nervous system might be of great benefit for asthmatics or patients with food allergies.

### 3 | INHIBITION OF ILC2S BY EXTRINSIC SIGNALS

Without tight regulation, ILC2 responses can drive exaggerated tissue inflammation. Several cytokines and mediators have been identified that negatively regulate ILC2 function (Figure 3).

#### 3.1 | Inhibition of ILC2s by interferons

Interferons boost immunity against viruses and bacteria through a variety of effects on different immune cells, including by promoting expression of antimicrobial mediators and pro-inflammatory cytokines and by enhancing the differentiation of effector T cells.<sup>109,110</sup> However, interferons have also been implicated as immunosuppressive in certain settings.<sup>109</sup> Three families of interferons exist: the large type I interferon family that includes IFN $\beta$ , the type II interferon family consisting only of IFN $\gamma$ , and the type III interferon family of IFN $\lambda$  molecules.<sup>109,110</sup> The receptors for IFN $\beta$  and IFN $\gamma$  are expressed on ILC2s at low levels at steady state and are upregulated upon activation.<sup>49,111</sup> Stimulation of ILC2s with either IFN $\beta$  or IFN $\gamma$  inhibited IL-33-induced proliferation and type 2 cytokine production in vitro, an inhibition that was dependent on the STAT 1 pathway.<sup>49,111,112</sup> The inhibitory effect of IFN $\beta$  and IFN $\gamma$  was also observed in vivo, as the severity of IL-33-induced airway inflammation was significantly reduced in the presence of IFN $\beta$  or IFN $\gamma$ .<sup>49,111,112</sup> In addition, in mouse models of influenza or helminth infection, elevated ILC2 numbers and

type 2 immune responses were observed in mice lacking either the type 1 interferon receptor or IFN $\gamma$  receptor 1 compared to control mice, indicating that interferons control ILC2-mediated immune responses.<sup>49,111</sup>

Supporting this, NK cells can inhibit ILC2 expansion and type 2 cytokine production through secretion of IFN $\gamma$ .<sup>113</sup> In mice challenged with papain in the absence of NK cells, ILC2 expansion was enhanced, and increased frequencies of IL-5- and IL-13-producing ILC2s were observed, resulting in increased type 2 immunopathology.<sup>113</sup> Moreover, in vivo, IL-33-induced ILC2 expansion was more pronounced in the presence of IFN $\gamma$ -deficient NK cells compared to wildtype NK cells.<sup>113</sup> IFN $\gamma$  promotes expression of T-bet in ILC2s, which inhibits ILC2 expansion and IL-9 production during inflammatory responses, thereby limiting IL-33-induced lung inflammation.<sup>114</sup> Similarly, a STAT1-dependent inhibitory function was also described for IL-27, which inhibited ILC2 responses in vitro and in vivo during *Alternaria alternata*-induced allergic airway inflammation and *N. brasiliensis* infection.<sup>49,111,115</sup>

### 3.2 | Inhibition of ILC2s by lipid mediators

Lipid mediators are also able to negatively regulate ILC2 function. Human ILC2s express ALX/FPR2, the receptor for lipoxin A4, and lipoxin A4 inhibits IL-13 production by ILC2s in response to IL-2, IL-25, IL-33, and Prostaglandin D2.<sup>81</sup> Another lipid mediator, Maresin-1, is upregulated in the lung during the sensitization and resolution phase of OVA-induced airway inflammation.<sup>116</sup> In vitro, Maresin-1 inhibits ILC2 cytokine responses both directly and by stimulating TGF $\beta$ -induced Treg differentiation.<sup>116</sup> In vivo, treatment with Maresin-1 during OVA-induced airway inflammation results in faster recovery from airway inflammation.<sup>116</sup> The frequency of Tregs was elevated in these mice and ILC2s produced less IL-5 and IL-13,<sup>116</sup> suggesting that Maresin-1 controls ILC2 responses both by directly modulating ILC2 function and also via induction of Tregs.

### 3.3 | Inhibition of ILC2s by sex hormones

Laffont et al<sup>117</sup> showed that male sex hormones can suppress ILC2 responses. In this study reduced ILC2 frequencies and less airway inflammation were observed in male mice compared to female mice in a model of IL-33-induced airway inflammation. Castration restored both ILC2 frequencies and airway inflammation to levels observed in female mice, pointing to negative regulation of ILC2s by male sex hormones. Interestingly, even the development of ILC2s seems to be controlled, as ILC2 progenitors were diminished in the bone marrow of male mice. ILC2 progenitors expressed transcripts of the androgen receptor, and their differentiation to mature ILC2s was impaired in the presence of dihydrotestosterone, suggesting that testosterone is responsible for the observed differences between male and female mice. Indeed, during IL-33-induced airway inflammation, the differences in ILC2 frequency and severity of airway inflammation seen between male and female mice were abolished in bone marrow chimeras lacking the androgen receptor, indicating that androgen signaling inhibits the generation of ILC2s, which accounts for the reduced airway inflammation in male mice.<sup>117</sup>

### 3.4 | Inhibition of ILC2s by surface molecules

In mice, programmed death 1 (PD-1) is expressed on ILC2s and ILC3s and upregulated especially on ILC2s during inflammation, whereas in humans PD-1 is predominantly found on ILC2s.<sup>118,119</sup> Treatment of RAG1<sup>-/-</sup> mice with PD-1 antibody led to the loss of almost all PD-1<sup>hi</sup> ILC2s and resulted in a greatly diminished type 2 immunopathology in response to papain challenge.<sup>118</sup> Cell-intrinsic PD-1 signaling does not regulate ILC2 development but dampens IL-33-induced proliferation of mature KLRG1<sup>+</sup> ILC2s in a STAT5-dependent manner.<sup>119</sup> RAG2<sup>-/-</sup>γ<sub>c</sub><sup>-/-</sup> mice that received PD1-deficient ILC2s had elevated frequencies of cytokine-producing KLRG1<sup>+</sup> ILC2s and were better in clearing worms than mice that received WT ILC2s.<sup>119</sup> ILC2s also express elevated levels of the PD-1 ligand PD-L1 after administration of IL-33 both in vitro and in vivo.<sup>120</sup> Mice lacking PD-L1 were less efficient in clearing worms than WT mice, although ILC2 proliferation and cytokine production were unchanged.<sup>120</sup> Instead, ILC2s regulated the T-cell response via PD-L1 expression. Binding of PD-L1 to PD-1 expressed on T cells induced expression of GATA3 and IL-13 in Th2 cells, which facilitated worm clearance in a mouse model of helminth infection.<sup>120</sup>

### 3.5 | Inhibition of ILC2s by Tregs

Regulatory T cells are crucial for the prevention of exaggerated immune responses and inhibition of autoimmunity.<sup>121</sup> Several studies show that regulatory T cells (Tregs) can also inhibit ILC2 responses.<sup>116,122,123</sup> Morita et al<sup>122</sup> report that IL-33 activated mast cells to secrete IL-2, which increased the frequency of Tregs. Tregs, in turn, inhibited IL-33-induced ILC2 expansion via IL-10 production in vitro.<sup>122</sup> In a mouse model of IL-33-induced airway inflammation, mice deficient in mast cells developed more eosinophilia than wildtype mice, and this was rescued by transfer of wildtype, but not IL-10-deficient, Tregs, indicating that Treg-derived IL-10 controls type 2 inflammation via inhibition of ILC2s.<sup>122</sup>

Two other studies report inhibition of IL-13 production of ILC2s by Tregs.<sup>116,124</sup> As noted earlier, the pro-resolution lipid mediator Maresin-1 enhanced the inhibitory function of Tregs, which dampened the type 2 immune response in a model of OVA-induced allergic airway inflammation.<sup>116</sup> A subsequent study found that induced Tregs (iTregs) limited type 2 cytokine production by ILC2s, whereas natural Tregs (nTregs) were unable to inhibit them.<sup>123</sup> The suppressive effect was mediated by interaction of inducible T-cell costimulator ligand (ICOS-L) on ILC2s with ICOS expressed on Tregs and the Treg-derived cytokines IL-10 and TGFβ.<sup>123</sup> Humanized mice that received human ILC2s developed airway hyperreactivity in response to IL-33 treatment, while simultaneous transfer of iTregs decreased airway hyperreactivity.<sup>123</sup> However, if anti-ICOS-L antibody was added, the transferred Tregs were less suppressive, indicating that ICOS-L contributes to the inhibition of ILC2s by Tregs.<sup>123</sup>

Another study suggests a role for RORα-expressing Tregs in the regulation of ILC2 responses in atopic dermatitis-like inflammation.<sup>125</sup> In Calcipotriol-or OVA-induced skin inflammation, Treg-specific deletion of Rora resulted in more severe type 2 inflammation with increased frequencies of IL-5-producing ILC2s and Th2 cells.<sup>125</sup> Interestingly, expression of the TL1A receptor death receptor 3 (DR3) was reduced on skin-resident Tregs from these mice.<sup>125</sup> Previous studies showed that ILC2s also express DR3, and proliferate

and produce cytokines in response to TL1A stimulation.<sup>73,74</sup> Thus, the expression of DR3 on Tregs presumably decreases binding of TL1A to ILC2s, thereby limiting ILC2-driven skin inflammation.<sup>125</sup> However, a definitive role of ILC2s as mediators of this effect has not yet been shown.

### 3.6 | Inhibition of ILC2s by neurons

While we and others found that the nervous system promotes type 2 inflammation by positively regulating ILC2 responses,<sup>38,52,53,106,107</sup> an inhibitory role of the nervous system has been described more recently.<sup>126</sup> Moriyama et al<sup>126</sup> report expression of  $\beta_2$ AR (Adrb2), the receptor for norepinephrine, on ILC2s, as well as other ILC subsets, in both mouse and human, and used immunofluorescence microscopy to show that ILC2s were adjacent to adrenergic neurons. In this study, after helminth infection, mice treated with a  $\beta_2$ AR agonist had difficulties mounting an efficient immune response, and this effect was independent of CD4 T cells. These mice also had lower frequencies of Ki67<sup>+</sup> ILC2s and IL-5- and IL-13-producing ILC2s, suggesting that  $\beta_2$ AR signaling impairs both proliferation and type 2 cytokine production by ILC2s. Consistent with this, Moriyama et al observed exaggerated ILC2 expansion in mice in which  $\beta_2$ AR was conditionally deleted in IL-7R-positive cells, resulting in an increased efficiency of worm expulsion, even after depletion of CD4 T cells. The inhibitory effect of  $\beta_2$ AR signaling on ILC2 proliferation and function was also seen in lung-resident ILC2s in airway inflammation and occurred in a cell autonomous manner. While the level of norepinephrine did not change, ILC2s downregulated Adrb2 expression in the small intestine during helminth infection, showing that ILC2s can decrease their responsiveness to norepinephrine during infection to promote type 2 inflammation. These findings highlight the importance of ILC2s in integrating neuroimmune signals and are particularly clinically relevant, given the widespread use of  $\beta$ -adrenergic agonists in the treatment of asthma. Further research is required to elucidate the pathways that regulate  $\beta_2$ AR expression on ILC2s.

## 4 | INTRINSIC REGULATION OF ILC2S

### 4.1 | Development of ILC2s

All lymphoid lineage cells, including T cells, B cells, and ILCs, originate from a common lymphoid progenitor (CLP) in the mouse in fetal liver and bone marrow that expresses the cytokine receptor Flt3.<sup>127,128</sup> From this CLP, development moves to the  $\alpha$ LP stage, spurred by induction of the critical transcriptional regulator TOX by the transcription factor NFIL3.<sup>129,130</sup> Increased expression of the integrin  $\alpha 4\beta 7$ , followed by upregulation of the chemokine receptor CXCR6, is associated with loss of B- and T-cell potential, respectively.<sup>131</sup> In mice, the NK lineage, dependent on Eomes, separates from that of the other ILCs.<sup>132</sup> By directly regulating the transcriptional regulator *Id2*, NFIL3 then orchestrates the emergence of the IL-7R $\alpha$ <sup>+</sup>  $\alpha 4\beta 7$ <sup>+</sup> ID2<sup>+</sup> CD25<sup>-</sup> Flt3<sup>-</sup> common helper-like innate lymphoid progenitor (CHILP) from which all IL-7R $\alpha$ <sup>+</sup> ILCs (T-bet<sup>+</sup> Eomes<sup>-</sup> non-NK ILC1s, Gata3<sup>hi</sup> ILC2s, and ROR $\gamma$ t<sup>+</sup> ILC3s) are derived.<sup>132-134</sup> GATA3 is indispensable for the development of these ILC subsets.<sup>135,136</sup> After divergence of the ROR $\gamma$ t<sup>+</sup> lymphoid tissue inducer (LTi) lineage,<sup>133,137</sup> transient, high expression of PLZF characterizes the GATA3<sup>+</sup> TOX<sup>+</sup> ID2<sup>+</sup> PLZF<sup>hi</sup> ILC precursor (ILCP) of the remaining ILC lineages,<sup>133,138,139</sup> which is also marked

by high expression of PD-1.<sup>118,137</sup> ILC lineage polarization initiates with multi-lineage priming characterized by co-expression in ILC precursors of genes associated with different ILC types.<sup>133</sup> In immature ILC2s, the ILC3 potential is repressed by the lysine methyltransferase G9a, which, along with Bcl11b and Gfi1, maintain ILC2 identity and block expression of ILC3-associated genes in mature ILC2s,<sup>140–143</sup> further supporting the concept that ILC polarization occurs via restriction of alternative programs. Robust ILC2 differentiation is specifically dependent on Notch signaling in the CHILP, as well as on the downstream transcription factor TCF-1.<sup>133,144–146</sup> An early PD-1<sup>hi</sup>IL-25R<sup>hi</sup> stage of development is specific to ILC2s,<sup>118</sup> and depends critically on the transcription factors ROR $\alpha$ <sup>144,147</sup> and Ets1<sup>148</sup>, as well as the zinc-finger protein Bcl11b<sup>118,141,142,149</sup>.

#### 4.2 | Intrinsic regulation of ILC2s by transcription factors

The coordinate activity of a number of transcription factors is needed to support the differentiation and maintenance of ILC2s. Foremost among these is the transcription factor Gata3, which is important not only for ILC2 development but also for their function.<sup>58,135,136,150,151</sup> ILC2s, but not ILC1s or ILC3s, disappear in mice after tamoxifen-induced deletion of Gata3, suggesting that Gata3 is crucial for both the generation of ILC2s and maintenance of fully differentiated ILC2s.<sup>151</sup> The degree of Gata3 expression appears to determine whether common lymphoid precursors develop to ILC2s and also functionally regulates mature ILC2s.<sup>150</sup> Moreover, Gata3 expression in human CRTH2<sup>-</sup> ILCs promotes differentiation into an ILC2 phenotype characterized by the expression of CRTH2, ST2, and TSLPR, and the ability to produce IL-5 and IL-13 upon IL-33 and TSLP stimulation.<sup>58</sup>

The transcription factor ROR $\alpha$  is also important for the development of ILC2s from common lymphoid progenitors in a cell-intrinsic manner. Mice harboring a deletion of the ligand binding domain of ROR $\alpha$  have no goblet cell hyperplasia following IL-25 administration, and this is associated with a lack of increase in ILC2 frequency in the mesenteric LNs.<sup>144</sup> Moreover, both bone marrow chimeras reconstituted with ROR $\alpha$ -deficient bone marrow and mice with lymphocyte-specific conditional deletion of ROR $\alpha$  have impaired ILC2 development, resulting in a diminished ability to expel helminths and to develop allergen-induced airway inflammation.<sup>89,144,147</sup>

In contrast to GATA3 and ROR $\alpha$ , which directly promote the ILC2 developmental program, other transcription factors, such as Bcl11b and Gfi1, work in part by repressing expression of genes associated with other ILC subsets.<sup>140,142,149</sup> Chimeras generated with Bcl11b deficient fetal liver have severely decreased ILC2 progenitor frequencies and impaired clearance of helminths.<sup>142</sup> Interestingly, while the population of mature ILC2s is significantly reduced in the small intestine in the absence of Bcl11b, ILC3 frequencies are elevated.<sup>142</sup> Consistent with this, Bcl11b-deficient ILC2s produce Th17 cytokines and proliferate in response to IL-23.<sup>141</sup> Chromatin immunoprecipitation and retroviral transduction of ILCs with Gfi1 indicate that Bcl11b controls the type 2 cell fate by inhibiting expression of aryl hydrocarbon receptor, which promotes ILC3 lineage identity, and by inducing expression of Gfi1.<sup>141</sup> While Gfi1 promotes the development of ILC2s in the bone marrow, it also appears to regulate mature ILC2 responses by silencing genes associated with ILC3s, and enhancing ILC2 proliferation and promoting a type 2 transcriptional

program, including expression of Gata3, ST2 and IL-5.<sup>140</sup> Interestingly, while Gfi1-deficient cells lose their responsiveness to IL-33, they can still be activated by IL-25.<sup>140</sup>

While many transcription factors regulate both ILC2 development and maintenance of subset identity, alterations in hypoxia-inducible factor 1-alpha (HIF1 $\alpha$ ) levels by deletion of VHL appear to selectively impair the maturation of ILC2s in peripheral tissues.<sup>152</sup> The transcription factor HIF1 $\alpha$  is hydroxylated during steady state and subsequently labeled by VHL for proteasomal degradation.<sup>153</sup> However, during hypoxia, HIF1 $\alpha$  cannot be hydroxylated and translocates into the nucleus, where it induces expression of genes associated with cell metabolism, differentiation and function.<sup>153</sup> This process is mimicked in mice that lack VHL in common innate lymphoid progenitor cells, resulting in accumulation of HIF1 $\alpha$ .<sup>152</sup> While accumulation of HIF1 $\alpha$  did not influence ILC progenitor development, it did impair maturation of functional ILC2s, as VHL-deficient mice had a reduced capacity to mount an ILC2-dependent immune response after allergen challenge.<sup>152</sup> This appears to be in part related to alterations in ILC2 metabolism, as VHL deficiency resulted in increased glycolysis, which in turn led to different epigenetic modifications that reduced ST2 expression.<sup>152</sup>

### 4.3 | Intrinsic regulation of ILC2s by metabolic pathways

The role of metabolism in regulation of ILC2 function is just beginning to be investigated. In addition to HIF1 $\alpha$ -mediated alterations in glycolysis, arginine metabolism also contributes to the regulation of ILC2 response. Arginase 1 (Arg1) is a metabolic enzyme that catalyzes the generation of ornithine and urea from arginine.<sup>154</sup> ILC2 progenitors and mature ILC2s express Arg1 at steady state and during type 2 inflammation.<sup>154,155</sup> While Arg1 appears to be dispensable for ILC2 development and homeostasis, it may be crucial for ILC2 expansion and effector cytokine responses. Conditional deletion of Arg1 in IL-7R $\alpha$ -positive cells did not change ILC2 frequencies at baseline, but it did lead to reduced ILC2 frequencies and impaired IL-5 and IL-13 production after papain challenge, resulting in diminished allergic lung inflammation.<sup>154</sup> This appeared to be, in part, due to decreased generation of the downstream metabolites of arginine in the absence of Arg1, many of which have been described to modulate cell proliferation and survival, while also dampening glycolytic function when Arg1 was pharmacologically inhibited.<sup>154</sup> However, an earlier paper reported that ILC2 function was not altered when Arg1 was deleted in IL-5-expressing cells using an IL-5-specific Cre, raising the question of how to reconcile these findings.<sup>155</sup>

A recent study reports that the relative proportion of ILC2s to ILC3s in the gut is controlled by retinoic acid, a derivative of vitamin A.<sup>156</sup> Mice on a vitamin-A-deficient diet, or treated with an inhibitor of the retinoic acid receptor, have elevated ILC2 frequencies and decreased ILC3 frequencies compared to control mice, an imbalance that is also observed in the absence of an adaptive immune system or microbiota. Mechanistically, retinoic acid compromises the generation of ILC2s, while promoting IL-22 production and increasing the number of ILC3s. The authors further show that infection of RAG1<sup>-/-</sup> mice with *Citrobacter rodentium* caused increased weight loss and colonic inflammation in the absence of retinoic acid signaling, suggesting that lack of vitamin A and the resulting decreased ILC3 frequency renders mice more susceptible to bacterial infection. In contrast, after helminth infection,

inhibition of retinoic acid signaling resulted in more efficient clearance of *Trichuris muris* worms in an IL-13-and ILC2-dependent manner. These data indicate that an increase in ILC2s confers protection against helminth infection in times of limited access to nutrients, for example, in the absence of vitamin A.<sup>156</sup> A subsequent study showed that ILC2s rely on fatty acids but not glucose during vitamin A deficiency, and that fatty acid oxidation contributes to IL-13 expression by ILC2s.<sup>157</sup> While ILC2s are independent of fatty acid metabolism at steady state, they require fatty acids during helminth infection to efficiently clear worms.<sup>157</sup>

## 5 | EFFECTOR FUNCTION OF ILC2S

ILC2s are part of a complex interplay of different cell types that promote type 2 immune responses to helminth infection and allergens. Activated ILC2s produce an array of cytokines and factors with unique functions, while also regulating other cell types (Figure 4).

### 5.1 | Key effector program of ILC2s

Murine ILC2s express transcripts for the type 2 cytokines IL-4, IL-5, and IL-13.<sup>5,151</sup> However, while activated ILC2s generally produce large amounts of IL-5 and IL-13,<sup>6,12,20,56,158</sup> IL-4 is typically barely detectable on a protein level,<sup>6,56,158</sup> except in specific inflammatory settings or disease models.<sup>25,38,44,120,124,159</sup> ILC2s in airway inflammation induced by OVA or house dust mite produce IL-5 and IL-13, and less IL-4,<sup>56,57</sup> and during helminth infection, ILC2s produce IL-13 but not IL-4.<sup>6,160</sup> Similarly, human IL-25-or IL-33-activated ILC2s produce significant amounts of IL-5 and IL-13, while IL-4 is only expressed to a lesser extent.<sup>21,58</sup>

IL-5 binds to the IL-5R, a heterodimer formed by IL-5R $\alpha$  and the common  $\beta$ -chain<sup>161</sup> and induces eosinophilia by promoting eosinophilopoiesis in the bone marrow and peripheral eosinophil survival,<sup>162,163</sup> and by recruiting eosinophils to the site of inflammation in conjunction with eotaxin.<sup>164,165</sup> IL-13 binds to the type II IL-4 receptor, composed of IL-4R $\alpha$  and IL-13R $\alpha$ 1, and the decoy receptor IL-13R $\alpha$ 2,<sup>166</sup> and promotes mucus production by goblet cells independent of IL-5 signaling.<sup>164</sup> IL-4 also binds to the type II IL-4 receptor, as well as to the type I IL-4 receptor, which is formed by IL-4R $\alpha$  and the common  $\gamma$ -chain.<sup>166</sup> Upon ligand binding, both IL-4 receptors signal via the signal transducer and activator of transcription 6 (STAT6) pathway.<sup>166</sup> Notably, monoclonal antibodies targeting both IL-5 and IL-4R $\alpha$  (thus inhibiting both IL-4 and IL-13) have recently been found to be effective in the treatment of eosinophilic and moderate to severe asthma, respectively, highlighting the importance of these pathways in allergic disease.<sup>167–169</sup>

Studies published before the discovery of ILCs show that IL-13 is crucial for the clearance of helminths<sup>170</sup> and contributes to the development of airway inflammation, even in the absence of an adaptive immune system.<sup>171,172</sup> The initial publications describing ILC2s reported type 2 cytokines and goblet cell hyperplasia in RAG2<sup>-/-</sup> but not ILC-deficient RAG2<sup>-/-</sup> $\gamma$ <sub>c</sub><sup>-/-</sup> mice during *N. brasiliensis* infection.<sup>5</sup> Transfer of ILCs in combination with IL-25 treatment restored the capability to induce eosinophilia and expel worms in



RAG2<sup>-/-</sup>γ<sub>c</sub><sup>-/-</sup> mice after helminth infection.<sup>11</sup> Moreover, transfer of wildtype but not IL-13-deficient ILC2s restored worm clearance in mice lacking the receptor for IL-25.<sup>6</sup> These findings demonstrate that ILC2-derived IL-13 plays a crucial role in the defense against helminth infection. ILC2s are also sufficient to induce eosinophilia and mucus production in response to papain challenge in the absence of an adaptive immune system.<sup>12</sup> After influenza virus infection, depletion of ILCs with anti-CD90.2 antibody in Rag2<sup>-/-</sup> mice impaired development of airway hyperreactivity.<sup>173</sup> Furthermore, adoptive transfer of wildtype, but not IL-13-deficient, ILC2s restored airway hyperreactivity in IL-13-deficient recipient mice, indicating that ILC2s are sufficient to induce airway hyperreactivity, even in the absence of T and B cells.<sup>173</sup>

IL-9 production can be transiently induced in ILCs during airway inflammation and is dependent upon IL-2 produced by adaptive immune cells.<sup>174</sup> IL-33, IL-25 and TSLP are unable to elicit IL-9 expression in ILC2s on their own, though the combination of IL-33 and TSLP can induce IL-9 production in ILC2s in an IRF4-dependent manner.<sup>174,175</sup> In vitro stimulation of ILCs with IL-9 promoted IL-5 and IL-13 expression, while neutralization of IL-9 during lung inflammation inhibited it, indicating that IL-9 facilitates type 2 cytokine production in ILC2s.<sup>174</sup> Following helminth infection, ILC2s are the major IL-9 producers and also express IL-9R, suggesting an autocrine loop.<sup>176</sup> Analysis of helminth-infected IL-9R-deficient mice revealed that IL-9 signaling is important for the survival and effector function of ILC2s, and facilitates worm clearance and tissue repair, in part by promoting eosinophil accumulation in the lung and alternative macrophage activation.<sup>176</sup> In a mouse model of cystic fibrosis, fungal infection induced the production of IL-9, which then activated mast cells to produce IL-2.<sup>177</sup> In response to IL-2, ILC2s proliferated and further activated Th9 cells, resulting in exaggerated lung disease.<sup>177</sup> In contrast, another study reports that in a mouse model of arthritis ILC2-derived IL-9 ameliorated inflammation by expanding ILC2s and promoting Treg function.<sup>178</sup> In this study, Tregs from mice lacking IL-9 were less suppressive than control Tregs. However, when Tregs from IL-9-deficient mice were activated with recombinant ICOS-L and anti-GITR, they ameliorated joint inflammation in the resolution phase of antigen-induced arthritis. IL-9 induced proliferation and expression of ICOS-L and GITRL on ILC2s, suggesting that via production of IL-9 and subsequent upregulation of these co-stimulatory ligands, ILC2s promote the suppressive function of Tregs. Indeed, transfer of ILC2s in IL-9-deficient mice resulted in less inflammation in a model of antigen-induced arthritis. In line with these findings, the authors observed more IL-9-expressing ILC2s in rheumatoid arthritis patients in the remission phase compared to patients with active disease.<sup>178</sup>

Amphiregulin (encoded by Areg), a member of the epidermal growth factor family of cytokines, is produced by lung ILC2s during influenza virus infection and contributes to tissue repair.<sup>20</sup> In lung ILC2s the expression of Areg seems to be regulated by IL-33 and IL-25.<sup>20,38</sup> Amphiregulin-expressing ILC2s are also found in the mesenteric LNs and lamina propria of the colon and are expanded in DSS-induced colitis.<sup>179</sup> Mice with impaired signaling of the epidermal growth factor receptor (EGFR), the receptor for amphiregulin, developed more severe colitis, similar to Areg-deficient mice, indicating that signaling of amphiregulin via EGFR induces tissue protective responses.<sup>179</sup> ILC2-derived amphiregulin is important for maintaining barrier integrity by inducing tight junctions and mucus

production during colonic inflammation.<sup>179</sup> Areg expression is also detected in human skin ILC2s and increased upon IL-33-induced ILC2 activation.<sup>26</sup>

## 5.2 | Interaction of ILC2s with CD4 T cells

Infection with parasites requires a fast and efficient type 2 response of the immune system to prevent dissemination and promote expulsion. As one of the main producers of IL-5 and IL-13, ILC2s are crucial for the initiation of type 2 inflammation. Along with inducing mucus hyperplasia and eosinophilia, ILC2s also communicate with other cell types to amplify inflammation and induce adaptive immunity and memory. Within hours of receiving alarmin signals, ILC2s induce type 2 inflammation via production of IL-5 and IL-13.<sup>12</sup> In contrast, antigen-specific effector T cells need several days to respond, as they must be activated by antigen-presenting DCs in the draining LNs, differentiate into Th2 cells, and migrate to the site of tissue inflammation.<sup>180,181</sup> Bridging the time until Th2 cells arrive, ILC2 activity goes beyond activation of innate immunity to communication with DCs and effector T cells. Th2 responses are diminished in mice lacking ILC2s, and, depending on the model of inflammation, this can be associated with reduced airway inflammation or impaired worm clearance.<sup>89,90,180,182,183</sup> In a papain-induced airway inflammation model, elevated numbers of Th2 cells were found in the mediastinal lymph nodes (mLNs) of mice reconstituted with wildtype bone marrow.<sup>180</sup> However, in ROR $\alpha$ -deficient bone marrow chimeras, which lack ILC2s, Th2 cell numbers were severely reduced in mLNs after papain treatment, indicating that ILC2s promote the differentiation of Th2 cells.<sup>180</sup> Subsequent experiments showed that during airway inflammation the frequency of activated DCs in the mLNs was also diminished in ILC2-deficient bone marrow chimeras and that administration of recombinant IL-13 restored DC numbers.<sup>180</sup> Moreover, migration of DCs toward the chemokine CCL21 was reduced in lungs from IL-13-deficient mice compared to WT mice.<sup>180</sup> These findings suggest that ILC2s enhance Th2 cell differentiation by production of IL-13, which facilitates migration of activated DCs to draining LNs.

ILC2s are also directly involved in the stimulation of effector CD4 T-cell responses. In vitro, ILC2s promote the proliferation of CD3/CD28-activated CD4 T cells.<sup>183</sup> In the absence of CD3/CD28-induced activation, naïve ILC2s are not sufficient to induce T-cell proliferation.<sup>89,90</sup> However, if ILC2s are pulsed with OVA peptide, they can induce proliferation of OVA-specific CD4 T cells.<sup>89,90,182</sup> T-cell proliferation is inhibited if an anti-MHC2 antibody is added to the co-culture or if MHC2-deficient ILC2s are used.<sup>89,90,182</sup> Curiously, if ILC2s were pulsed with OVA-protein instead of OVA peptide, they were not able to elicit CD4 T-cell proliferation, although ILC2s were able to process the protein.<sup>89,90,182</sup>

Besides MHC2, the co-stimulatory molecules CD80 and CD86 are also required for ILC2-induced T-cell proliferation.<sup>89</sup> Co-culture of ILC2s and T cells resulted in increased type 2 cytokine production, which was dependent on the cytokine IL-4 as well as cell-cell contact via MHC2, CD80, CD86 and OX-40L.<sup>89,90,182,183</sup> In vivo, OX40L expression on ILC2s was required for an efficient Th2 response during allergen-induced airway inflammation or *N. brasiliensis* infection.<sup>184</sup> Another study found that interaction of programmed death-ligand 1 (PD-L1) on ILC2s with PD-1 on CD4 T cells promoted Th2 differentiation and effector function in the context of type 2 inflammation.<sup>120</sup> PD-L1 is expressed by all ILC subsets,

but is specifically induced in ILC2s during *N. brasiliensis* infection and also by IL-2 or IL-33.<sup>120</sup> Worm clearance was enhanced in RAG2<sup>-/-</sup>γ<sub>c</sub><sup>-/-</sup> mice, and the type 2 immune response against *N. brasiliensis* was more robust, if CD4 T cells were transferred together with wildtype rather than PD-L1-deficient ILC2s.<sup>120</sup> After *Heligmosomoides polygyrus* infection, ILC2s are an important source of IL-4 and contribute to Th2 differentiation, presumably via IL-4 secretion.<sup>159</sup> ILC2s not only promote the differentiation of Th2 cells but also facilitate the recruitment of memory Th2 cells upon re-challenge to the site of allergen exposure.<sup>185</sup> When ILC2s were deleted after sensitization with papain and 2W1S-peptide, the numbers of Th2 cells were decreased in the lung upon allergen re-challenge.<sup>185</sup> The study further found that ILC2-derived IL-13 induced secretion of the chemokine CCL17 by CD11b<sup>+</sup> CD103<sup>-</sup> DCs, and CCL17 in turn promoted migration of memory Th2 cells to the tissue.<sup>185</sup>

Collectively, these data suggest that the interaction of ILC2s and Th2 cells via direct contact and cytokines, or via the regulation of DC migration and activation, is important for the development and amplification of type 2 inflammation. Due to their powerful inflammatory capacity, ILC2s must be tightly regulated to prevent uncontrolled or excessive immune responses. As already discussed, several studies show that Tregs contribute to ILC2 inhibition. However, recent evidence suggests that ILC2s also influence Treg function. While OX40L expression on ILC2s leads to increased Treg numbers during type 2 inflammation<sup>184</sup> and ILC2-derived IL-9 promotes the suppressive function of Tregs,<sup>178</sup> ILC2-derived IL-4 inhibits Treg differentiation and thereby facilitates development of food allergy,<sup>124</sup> indicating ILC2s can both promote and inhibit Treg function.

### 5.3 | Interaction of ILC2s with other cell types

Along with regulating effector and regulatory T cells, ILC2s can also promote B cell responses. ILC2s stimulated B1 cell proliferation in an IL-5-dependent manner in vitro and in vivo.<sup>5,186</sup> In addition, B cells activated with IL-4 and either anti-CD40 or LPS produced more IgM, IgG1, IgE, and IgA if they were cultured in the presence of ILC2s.<sup>5,186</sup> The elevated production of IgM was not observed in the presence of IL-5-deficient ILC2s or after the administration of an anti-IL-5 antibody, indicating that ILC2-derived IL-5 positively regulates IgM production by B cells in vitro.<sup>186</sup> Adoptive transfer of ILC2s into IL-7R-deficient mice increased antigen-specific IgM but not IgG production upon nasal administration of the protease bromelain and the thymus independent antigen NP-Ficoll.<sup>186</sup>

ILC2s also communicate with cells that are not of hematopoietic origin. Three-independent studies reported that ILC2-derived IL-13 promotes both Tuft cell differentiation and goblet cell hyperplasia, thus altering the intestinal and lung epithelial barrier.<sup>62-64</sup> Moreover, IL-5 induces secretion of VIP from sensory neurons, which then activates ILC2s and Th2 cells, thereby driving airway inflammation.<sup>107</sup> Although it is not shown that ILC2-derived IL-5 promotes VIP release, the findings suggest that ILC2s can regulate neurons since they produce high levels of IL-5 upon activation. ILC2s also potentially regulate neurons via other cytokines, as neurons express several cytokine receptors, including IL-13R, IL-33R, and IL-25R.<sup>107</sup>

## 6 | TISSUE-SPECIFIC EFFECTOR FUNCTION OF ILC2S

### 6.1 | Tissue-residency vs migration of ILC2s

ILC2s exert different functions depending on the tissue environment where they reside. To elucidate whether ILC2s are replenished by bone marrow progenitors or migrate between organs, experiments with parabiotic mice were performed. One month after parabiosis, almost all ILC2s in the lung, small intestine, salivary gland and mesenteric LNs were still host-derived, and 3 months later, only a small fraction of intestinal ILC2s were derived from the parabiotic partner.<sup>48</sup> The frequency of this donor-derived ILC2 population increased very gradually over time, as 6–8 months after parabiosis, approximately 10% ILC2s in the small intestine and 20% of ILC2s in the mesenteric LNs were donor-derived.<sup>50</sup> These experiments indicate that ILC2s are largely locally maintained, tissue-resident cells. To study whether ILC2s remain tissue resident during inflammation, different disease models were induced in parabiotic mice. During autoimmune disease and IL-33-induced airway inflammation, ILC2s did not enter the circulation, but during *N. brasiliensis* infection, a small portion of ILC2s exchanged between parabiotic partners.<sup>48,49</sup> During acute worm infection, ILC2s remained largely tissue-resident, with donor-derived ILC2s only observed 15 days after infection.<sup>48</sup> However, as mentioned previously, a recent study reported that KLRG1<sup>hi</sup> iILC2s, a population present in the lung during *N. brasiliensis* infection or after intraperitoneal injection of IL-25,<sup>44,50</sup> migrated during helminth infection in an S1P-dependent manner from the small intestine to other organs, including the lung, where it effected an immune response.<sup>50</sup> This study also reports a small percentage of donor-derived ILC2s in the lung 20 days after infection, though the authors suggest that these cells are former donor-derived iILC2s that became nILC2s.<sup>50</sup>

### 6.2 | Tissue-specific effector function of ILC2s in adipose tissue

ILC2s are present at mucosal barriers but are also located in adipose tissue.<sup>5,33,187</sup> Similar to ILC2s from other tissues, these ILC2s produce IL-5 and IL-13 in response to IL-33 treatment in vitro and in vivo.<sup>5,33</sup> In addition, they contribute to the homeostasis of eosinophils and alternatively activated macrophages (AAM) in adipose tissue, demonstrated by the decrease in eosinophil and AAM numbers caused by a lack of ILC2s.<sup>33</sup> IL-33 increases eosinophil and AAM frequencies in visceral adipose tissue in an ILC2-dependent manner, and eosinophil frequencies are also elevated in the presence of ILC2s in a model of helminth infection.<sup>33</sup> In the absence of IL-5 signaling or eosinophils, mice fed a high fat diet (HFD) were heavier and developed increased glucose intolerance, while oxygen uptake and heat generation were reduced, indicating that ILC2s might help suppress development of obesity via their regulation of eosinophil accumulation in adipose tissue.<sup>33</sup> Indeed, RAG1<sup>-/-</sup> mice lacking ILCs developed increased adiposity and glucose resistance on HFD, whereas adoptive transfer of ILC2s temporarily reduced weight in obese animals.<sup>187</sup> Reduced ILC2 frequencies are also observed in adipose tissue from obese mice and humans.<sup>31,33</sup>

IL-33 induced accumulation of murine ILC2s in white adipose tissue (WAT), and, while body weight was not influenced, percentage of body fat decreased and caloric expenditure rose.<sup>31</sup> Caloric expenditure is controlled by beige adipocytes, a special type of adipocytes that express uncoupling protein 1 (Ucp1).<sup>188</sup> The protein Ucp1 disconnects the proton

gradient generated by the mitochondrial electron transport chain from ATP synthesis, resulting in heat generation.<sup>31,188</sup> Thus, beige adipocytes limit development of obesity by increasing caloric expenditure. Indeed, ILC2s promoted generation of beige adipocytes, indicating that they negatively regulate obesity.<sup>31</sup> IL-33 treatment induced Ucp1 expression in white adipose tissue in an ILC2-dependent manner, as Ucp1 expression was only induced by IL-33 in RAG2<sup>-/-</sup>γ<sub>c</sub><sup>-/-</sup> mice after adoptive transfer of ILC2s.<sup>31</sup> Formation of beige adipocytes and expression of Ucp1 is regulated by ILC2s via production of methionine-enkephalin, for which adipocytes express the receptors.<sup>31</sup> Adipocytes upregulate Ucp1 expression in response to methionine-enkephalin, suggesting that ILC2s directly induce beiging of adipocytes.<sup>31</sup> Consistent with this, another study showed that IL-33 induced Ucp1 expression and beige adipocyte formation.<sup>32</sup> The authors of this study report that IL-33 promoted norepinephrine-induced oxygen consumption in an Ucp1-dependent manner. Adipocyte progenitors proliferated and expressed the beige adipocyte markers TMEM26 and CD137 upon IL-33 treatment in the presence of ILC2s. Eosinophils and the type 2 cytokines IL-4 and IL-13 are important for this proliferative response, as adipocyte progenitors proliferated less in mice lacking eosinophils or IL-4 and IL-13 signaling. Deficiency of IL-4Ra on adipocyte precursors, but not on myeloid cells, decreased the frequency of adipocytes precursors positive for the proliferation marker ki67, indicating that proliferation of adipocyte precursors depends on IL-4Ra signaling. Ucp1 expression and generation of beige adipocytes also depended on IL-4Ra signaling in adipocyte precursors, yet deficiency of IL-4Ra on mature adipocytes had no effect on the generation of beige adipocytes.<sup>32</sup>

A more recent study shows that, along with IL-13Rα1, the IL-13 decoy receptor IL-13Rα2 is also upregulated in WAT of mice placed on HFD.<sup>189</sup> The authors hypothesize that increased expression of IL-13Rα2 reduces IL-13 levels in WAT of obese mice, thereby promoting obesity. While mice lacking IL-13Rα2 respond to HFD like control mice do, additional treatment with IL-33 leads them to have more pronounced weight loss and decreased blood glucose levels.<sup>189</sup> However, IL-13Rα2-deficient mice on HFD treated with IL-33 also develop more diarrhea, thus, making it difficult to attribute the weight loss specifically to metabolic changes in response to IL-13Rα2 deficiency.<sup>189</sup> As use of the IL-4Ra antagonist dupilumab becomes more widespread, it will be interesting to see if it is also associated with weight gain in asthmatics.<sup>167</sup>

### 6.3 | Effector function of ILC2s in other tissues

ILC2s are also located in the brain and spinal cord meninges, where they expand in response to IL-33.<sup>35</sup> Meningeal ILC2s are transcriptionally distinct from lung ILC2s.<sup>35</sup> Spinal cord injury induced transcriptional changes in meningeal ILC2s, including the expression of IL-5 and IL-13, and IL-33 signaling contributed to ILC2 activation in the context of this injury model.<sup>35</sup> While ILC2s were largely absent in the spinal cord parenchyma at steady state, spinal cord injury induced accumulation of ILC2s in the parenchyma at the site of inflammation.<sup>35</sup> Locomotor dysfunction after spinal cord injury in ST2-deficient mice was ameliorated by the transfer of wildtype lung ILC2s into the cerebrospinal fluid.<sup>35</sup> Moreover, these mice exhibited smaller lesion size, indicating that ILC2s may promote recovery from spinal cord injury.<sup>35</sup>

In a wound healing model, ILC2s were significantly increased at the site of skin injury compared to healthy skin, while mice lacking IL-33 had reduced ILC2 numbers and demonstrated slower closure of cutaneous wounds.<sup>190</sup> In addition, diminished re-epithelialization and wound healing were also observed in Rag1<sup>-/-</sup> mice depleted of ILCs, suggesting that ILC2s contribute to wound closure.<sup>190</sup> However, it remains to be clarified whether ILC2s also promote cutaneous wound healing in the presence of an adaptive immune system and how ILC2s exert their effect.

## 7 | TISSUE SPECIFICITY AND PLASTICITY OF ILC2S

### 7.1 | Transcriptional heterogeneity of ILCs

ILCs were initially characterized by flow cytometry using surface markers as well as subset-specific transcription factors and cytokines. However, this approach is limited by available antibodies and the limitations of flow cytometry, making it difficult to identify plasticity between ILC subsets, heterogeneity within each ILC subset, as well as ILC-specific functions that vary between tissues. To overcome these limitations, several studies have used RNA-sequencing (RNA-seq) to define the transcriptional landscape of ILCs.<sup>7,38,191,192</sup>

In addition to verifying expression patterns of genes previously associated with the major ILC subsets (eg, high levels of *Gata3* in ILC2s and *Ccl5* in ILC1s),<sup>7</sup> transcriptional studies using population RNA-seq identified ILC subset-specific genes that indicate possible effector functions and novel genes associated with each ILC subset. For example, the first study to characterize murine ILC subsets in the lamina propria of the small intestine (siLP) via population RNA-seq observed in ILC2s (defined as Sca-1<sup>+</sup> ST2<sup>+</sup> KLRG1<sup>+</sup> ILCs) unique expression of genes associated with lipid metabolism, such as *Dgat2*, *Pparg*, and *Lpcat2*, and of *Bmp2*, which is implicated in communication with enteric neurons<sup>7</sup>. Similarly, another study of intestinal ILC populations found specific expression of genes associated with sphingolipid and amino acid metabolism in ILC2s (defined as KLRG1<sup>+</sup> ROR $\gamma$ T<sup>-</sup> ILCs).<sup>192</sup>

Human tonsil-resident ILC2s analyzed by plate-based scRNA-seq, expressed common ILC2 marker genes, as well as genes associated with specific pathways, such as Notch signaling and prostaglandin generation and signaling.<sup>191</sup> This study sampled in an unbiased way all CD127<sup>+</sup> ILCs and also specifically gated on ILC1s and ILC2s to enrich these populations, which are rarer in the tonsils. Three clusters of ILC3s but only one cluster of ILC2s were identified, a finding that could stem from ILC2 homogeneity in that tissue, especially without a type 2 stimulus, or from a lack of statistical power due to the small number of cells sequenced.

Massively parallel scRNA-seq enabled a better powered, unbiased transcriptional assessment of murine CD127<sup>+</sup> ILCs isolated from the siLP and the lung.<sup>38,192</sup> Genes previously strongly associated with populations of ILCs from a single type turned out to have significant substructure in expression, depending on activation context and possibly on tissue. For example, in our study of murine lung ILCs, *Dgat2* was upregulated specifically in ILC2s from IL-25 treated mice, while *Lpcat2* and *Bmp2* were observed primarily in resting ILCs from control mice.<sup>38</sup> The single-cell data from the siLP ILCs suggests that, in addition to being expressed in ILC2s, *Dgat2* is expressed in some ILC3s, particularly in a cluster with

high MHC class II expression.<sup>192</sup> Even *Iir11* (ST2), *Klrg1*, and *Ly6a* (Sca-1), genes commonly used to identify murine ILC2s,<sup>7</sup> have distinct expression patterns that suggest association with particular ILC activation states. Regardless of ILC type, activation appears to upregulate *Ly6a* in lung ILCs, while *Iir11* (ST2) is broadly expressed in lung ILC2s, except in an IL-25-induced *Klrg1*-high group of iILCs that jointly produces *Iil3*, *Iil4*, *Iil7a*, and some *Gzma*, but little *Iil5*.<sup>38,44,50</sup> The iILC2s are either not present among these siLP ILCs, or they are mixed with other ILCs in the clusters, possibly contributing cells to the “ILC2d” cluster, which has high *Klrg1* and some *Iil7f* expression, and the “ILC1a” cluster, which has expression of *Gata3*, *Gzma*, *Iil7a*, and *Klrg1* (and very low levels of *Tbx21*). The gradation of expression of both *Ly6a* and *Klrg1*, in addition to *Gata3*, *Iil5*, *Areg*, and *Csf2*, across the ILC2 clusters in the siLP study suggests that a spectrum of activation may be partly driving the clustering.

There is increasing evidence that, at least in some contexts, ILCs may be better described by their positions on continuous spectra representing different aspects of immune response, rather than by discrete categories.<sup>41,150,193</sup> Clustering does capture transcriptional programs that are unique to certain cell subpopulations, such as expression of MHC2 genes in distinct clusters of human tonsil ILC3s, murine siLP ILC3s, and murine lung ILC2s.<sup>38,191,192</sup> However, responses to specific stimuli, changes in metabolism, and other biological processes can create convergent gene expression patterns in otherwise transcriptomically distinct groups of cells. For example, expression of *Odc1*, the rate-limiting enzyme in polyamine biosynthesis, is upregulated in certain clusters of siLP ILC3s, as well as in alarmin-induced clusters of lung ILC2s, which suggests that these groups may share specific metabolic regulation, despite differences in ILC type and tissue context. Similarly, treatment with antibiotics was shown to modulate the transcriptome and epigenome of ILC1 and ILC2 clusters in the siLP so they become transcriptionally and epigenetically more like ILC3s in a non-discrete way.<sup>192</sup> While a functional role of these changes has not been shown yet, the findings point to the tissue environment, in this case, the microbiome, as a major influence on ILC plasticity. A potential mechanism underlying ILC plasticity was identified in a study of chromatin accessibility in ILCs, in which some regulatory elements at the loci of lineage-determining transcription factors were found to be accessible in multiple ILC lineages.<sup>194</sup> For example, in ILC2s, the regulatory elements at the *Rorc* and *Iil7* loci are partially open, suggesting that inflammatory ILC2s are poised to produce IL-17 under specific conditions.<sup>44,194</sup>

On the other hand, despite functional plasticity, ILC lineages do apparently have distinct open-chromatin landscapes. Different types of ILCs from the same tissue had characteristic accessible regulatory elements that differed significantly more than the transcriptomes did, and which were not greatly changed by activation, especially compared to the activation-induced changes in T-cell chromatin.<sup>194</sup> At steady state, lung ILC2s and naive CD4 T cells had very distinct open-chromatin landscapes, yet nearly three quarters of the regulatory elements that became accessible in Th2 cells after *N. brasiliensis* infection were already accessible in ILC2s, indicating the existence of shared regulatory networks for type 2 lymphocyte responses that are poised or functioning even before activation in ILC2s.<sup>194</sup> While the comparison of transcriptional and epigenetic differences between ILC subsets suggests that the tissue environment primarily influences the transcriptome,<sup>194</sup> this requires

validation to be generalized to tissues outside the siLP. Future work is needed to directly compare ILC subsets from different tissues to determine how the tissue environment influences the transcriptome and effector functions of ILCs, ideally both at steady state as well in various models of tissue inflammation.

## 7.2 | Transdifferentiation of ILC2s

Each tissue has a unique composition of different ILC subsets. The lung, for example, harbors mostly ILC2s in mice,<sup>38</sup> which are poised to mount the type 2 immune response required to clear helminth infections. Yet, intracellular bacteria and viral infections provoke type 1 immune responses, which raise the question of how a type 1 immune response to a viral infection is mounted in the lung tissue when almost no ILC1s are present at steady state. This problem could be solved by either substantial expansion of a specific ILC subset, by conversion of one subset into another or by activation of another cell type with relevant effector function. Recent studies indicate that ILC2s can convert to ILC3-like ILC2s which express IL-17 along with IL-13<sup>43,44</sup> and ILC1-like ILC2s which acquire ILC1-like features, including expression of T-bet and IFN $\gamma$  production, while lowering expression of Gata3 and ST2.<sup>39,40,42,195</sup> The cytokines IL-12 and IL-18 induce differentiation of murine ILC2s to ILC1s in vitro, and adoptively transferred ST2<sup>+</sup> ILC2s seem to convert partly into ILC1-like ILC2s in Rag2<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> mice upon influenza infection.<sup>42</sup> Interestingly, ILC1-like ILC2s are not only induced by influenza virus but also by the bacterium *Staphylococcus aureus*, as well as long-term exposure to smoke, one of the major environmental stimuli underlying the development of chronic obstructive pulmonary disease (COPD).<sup>42</sup> Increased frequencies of ILC1s are observed in patients with COPD compared to non-smoking healthy individuals, suggesting that ILC2s might convert to ILC1s in humans in response to smoke exposure.<sup>42</sup>

In vitro experiments indicate that human ILC2s can also demonstrate plasticity and switch between subsets. Human ILC2s cultured with IL-12 together with alarmin cytokines or IL-1 $\beta$  convert to ILC1-like ILC2s, characterized by expression of T-bet and production of IFN $\gamma$ .<sup>39,40,42,195</sup> The cytokine IL-1 $\beta$  signals through IL-1R1 and the NF- $\kappa$ B pathway and promotes type 2 cytokine production and ILC2 proliferation.<sup>39,40</sup> Yet, IL-1 $\beta$  also contributes to the conversion of ILC2s into ILC1-like cells, as it triggers expression of IL-12R $\beta$ 2 and, together with IL-12, induces epigenetic modifications enabling the co-expression of type 1 and type 2 cytokines and transcription factors.<sup>40</sup> In a mouse model, adoptive transfer of ILC1s induced by IL-12, IL-18 and IL-33, resulted in significantly more weight loss during influenza infection than did transfer of ILC2s, indicating that ILC1s play an important role during influenza infection and could contribute to post-viral exacerbations in COPD patients.<sup>42</sup> The plasticity of ILC2s and ILC1s is bi-directional, as the cytokine IL-4 is able to switch ILC2-derived ILC1s back to ILC2s.<sup>39</sup> In humans, IL-4 is expressed by eosinophils and may contribute to transdifferentiation back to ILC2s, as co-culture of activated eosinophils with ILC2s promoted IL-5 production by ILC2s in an IL-4-dependent manner.<sup>39</sup>

## 8 | CONCLUSIONS AND FUTURE DIRECTIONS

Although ILC2s possess common core effector and transcriptional programs, they have diverse functions and phenotypes depending on the tissue environment. Their phenotype can



be modified by inputs from a diverse array of cell types, including immune, epithelial, neuronal, and endocrine. Their ability to integrate signals from a wide spectrum of cell types in turn allows ILC2s to both take on unique, tissue-specific effector programs, such as modulating adipose tissue function, and coordinate responses to tissue injury or stress in a manner appropriate to the tissue in which they reside. The tissue-specific signals that confer these properties are just beginning to be uncovered. Notably, while epithelial-derived alarmins play a crucial role in ILC activation, these appear to be somewhat redundant between tissues.<sup>68</sup> Rather, recent work has begun to suggest that signals derived from neurons and the endocrine system appear to be particularly important in inducing or modulating tissue-specific ILC phenotypes, although further work in this area is clearly needed.

The use of single-cell transcriptomics has begun to unravel the specific transcriptional programs of ILCs in different tissues, both in health and disease, as well as highlight the diversity of ILC2s both within and between tissues. Indeed, the work of our group and others has found that, while ILC2s within a given tissue are often largely transcriptionally homogeneous at steady state, inflammation can markedly alter ILCs within a tissue, resulting in transcriptionally distinct subgroups of cells, as well as expanded transcriptional heterogeneity along continuous dimensions. Crucial areas of future investigation include understanding the functional role of these changes, and determining whether induced transcriptional states are transient or represent discrete functional states.

Finally, the phenotype of ILC2s can change so dramatically in inflammatory settings that the cells appear to adopt some or all of the transcriptional profiles typical of ILC1s or ILC3s. Emerging data using ATAC-Seq and other methods to assay chromatin accessibility has highlighted the extent to which ILCs may be able to rapidly express effector cytokines typically thought of as characteristic of distinct ILC types. Understanding the mechanisms underlying disease-induced ILC plasticity can also help answer the question of which inflammation-induced alterations in ILC populations contribute to disease chronicity vs correlate with resolution. Indeed, given the degree to which ILCs integrate diverse signals, understanding the state of ILCs in a tissue may provide insight into the health of the tissue itself, while manipulating signals central to ILC regulation may offer a means to impact the course of chronic inflammatory disease.

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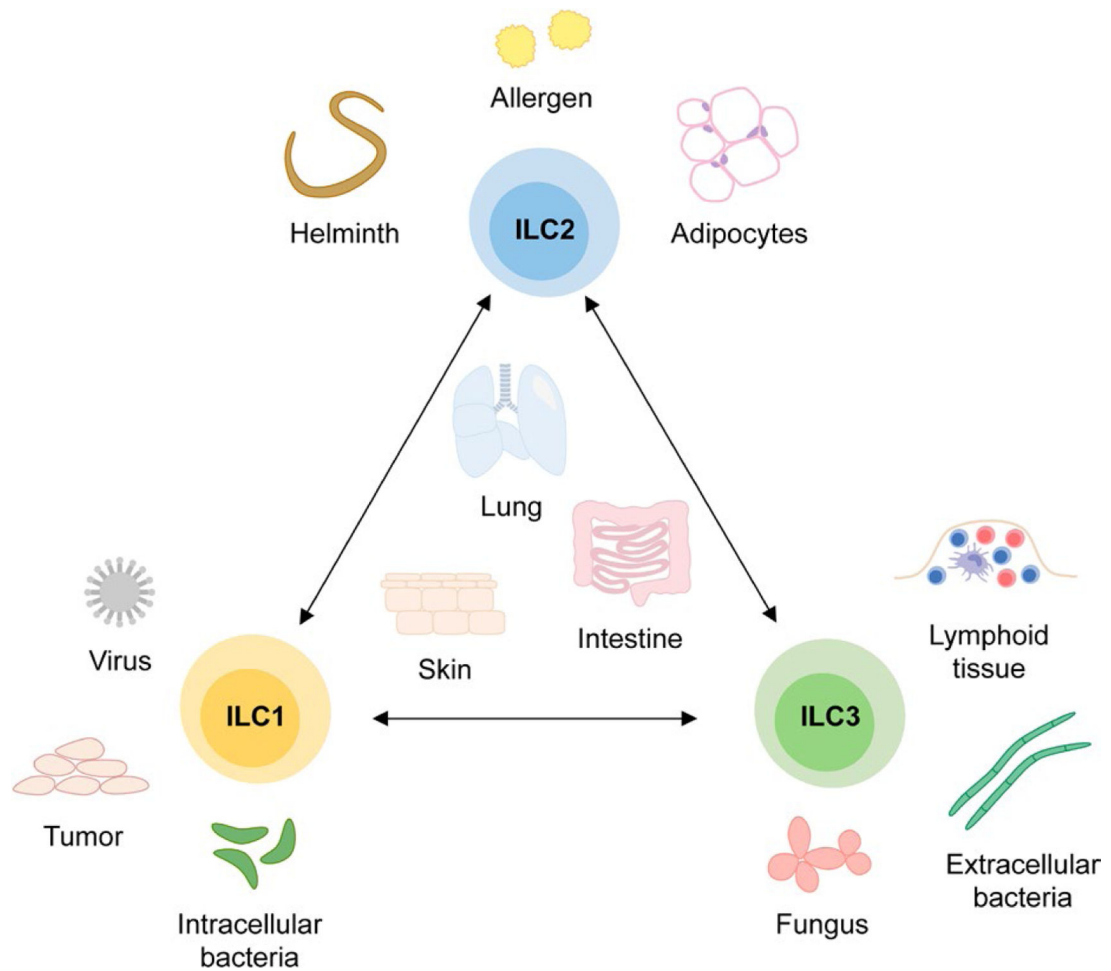
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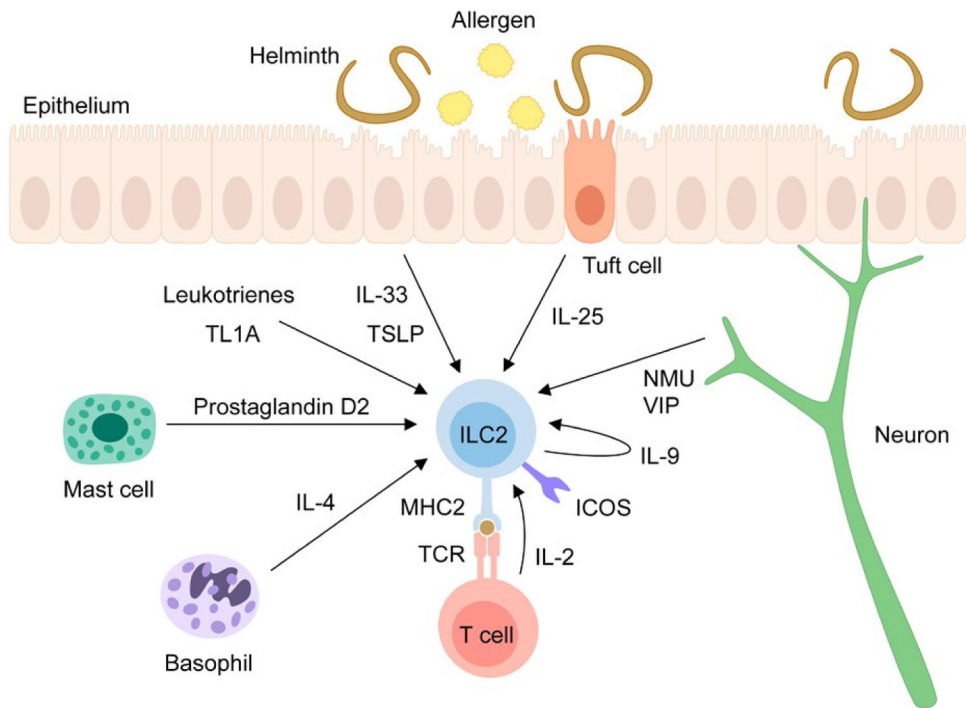
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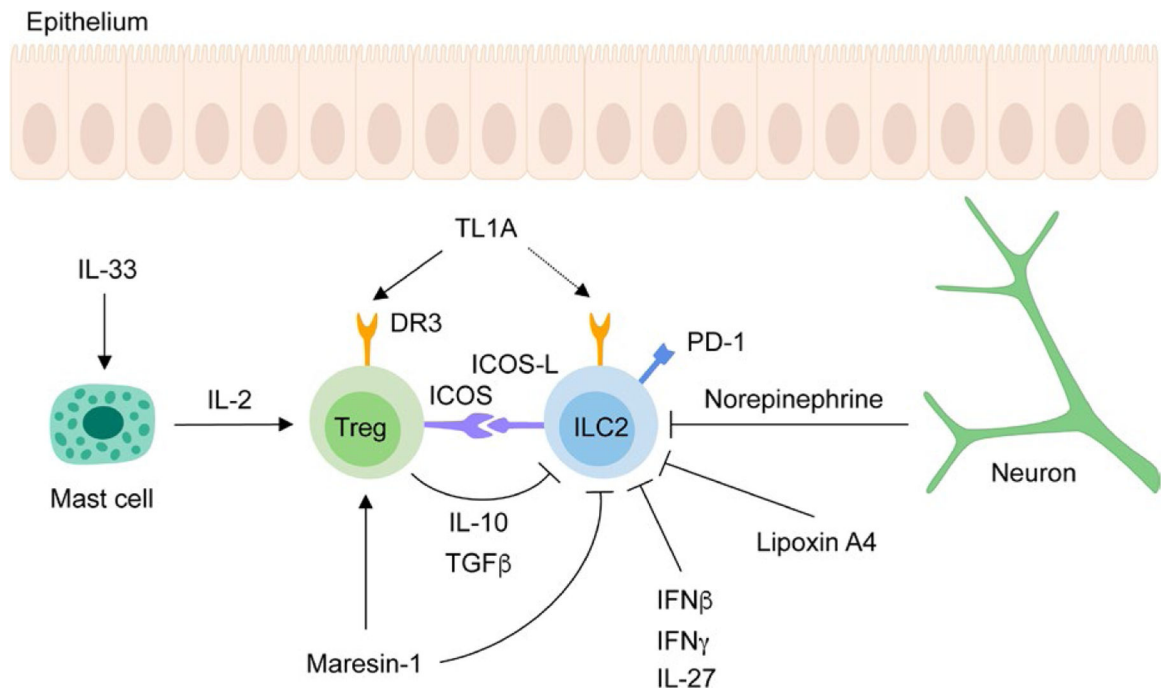
**FIGURE 1.**

Innate lymphoid cell (ILC) subsets and functions. ILCs consist of three subsets that play important roles in the immune response against distinct types of pathogens. While ILC1s are implicated in immunity against viral infections and intracellular bacteria as well as anti-tumor responses, ILC3s contribute to the development of lymphoid tissue and the defense of fungal infections and extracellular bacteria. ILC2s, however, are critical for the expulsion of helminths and play a protective role in the development of obesity but also contribute to the development of allergic inflammation. Importantly, this separation into different subsets is not stringent as each ILC subset can acquire phenotypic and functional features of other ILC subsets in response to the environment



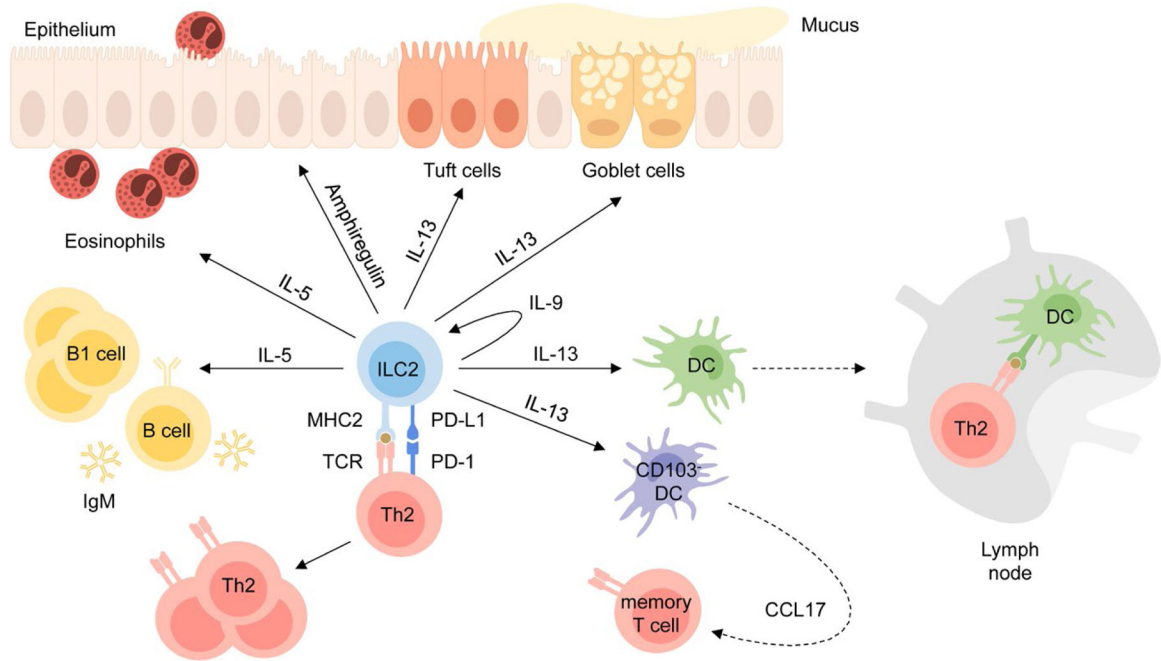
**FIGURE 2.**

Pathways controlling ILC2 activation. ILC2s are activated by different cytokines, such as the alarmin cytokines IL-33, IL-25 and thymic stromal lymphopietin (TSLP) as well as IL-2, IL-4, IL-9 and TL1A. While IL-33 and TSLP are released by epithelial cells in response to stress or damage, IL-25 is produced by tuft cells, which undergo expansion during helminth infection. IL-2 is released by Th2 cells upon interaction with ILC2s via the TCR and MHC2 complex and IL-4 is produced by basophils whereas IL-9 can be produced by ILC2s themselves. Lipid mediators such as leukotrienes and mast cell-derived prostaglandin D2, as well as the neuropeptides Neuromedin U (NMU) and vasoactive intestinal peptide (VIP) play also important roles in the activation of ILC2s. Surface molecules including ICOS also promote ILC2 responses upon interaction with ICOS-L and expression of MHC2 enables the interaction with T cells, which subsequently activate ILC2s via production of IL-2



**FIGURE 3.**

Pathways inhibiting ILC2 activation. ILC2s are negatively regulated by several pathways. Neuron-derived norepinephrine, the lipid mediator lipoxin A4 and the cytokines IFN $\beta$ , IFN $\gamma$  and IL-27 inhibit ILC2 responses. ILC2s express PD-1, which dampens ILC2 proliferation upon ligand binding. Tregs inhibit ILC2s via the production of IL-10 and TGF $\beta$ . The suppressive function of Tregs is promoted by maresin-1, mast cell-derived IL-2 and interaction of ICOS expressed on Tregs with ICOS-L expressed on ILC2s. Tregs also express DR3, the receptor for TL1A and compete for the binding of TL1A to DR3 expressed by ILC2s, thereby inhibiting their activation

**FIGURE 4.**

ILC2 effector mechanisms. ILC2s have tissue-protective and pro-inflammatory effector functions. ILC2s produce IL-13 which promotes tuft cell and goblet cell hyperplasia, activates DCs to migrate to the lymph nodes, where they promote Th2 differentiation, and induces CCL17 production by CD103-DCs which recruits memory T cells to the site of inflammation. ILC2s also induce eosinophilia, B1 cell expansion and production of IgM by B cells via production of IL-5 and produce IL-9 which leads to autocrine activation. Interaction with Th2 cells via MHC2 and PD-L1 promotes Th2 differentiation and effector function. While these cytokines and surface molecules contribute to the development of tissue inflammation, the epidermal growth factor amphiregulin was shown to be important for tissue homeostasis and repair