Group 2 innate lymphocytes at the interface between innate and adaptive immunity

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Abstract:

Group 2 innate lymphoid cells (ILC2) are innate immune cells that respond rapidly to their environment through soluble inflammatory mediators and cell-to-cell interactions. As tissue-resident sentinels, ILC2 help orchestrate localized type-2 immune responses. These ILC2-driven type-2 responses are now recognized both in diverse immune-processes, different anatomical locations, and homeostatic or pathological settings. ILC2-derived cytokines and cell-surface signalling molecules function as key regulators of innate and adaptive immunity. Conversely, ILC2 are governed by their environment. As such, ILC2 form an important nexus of the immune system and may present an attractive target for immune modulation in disease.

1. Introduction

In 2010, murine group 2 innate lymphoid cells (ILC2) were formally described as a novel innate lymphoid cell (ILC) population that promoted type-2 immunity[1-3]. ILC2 are tissue-resident immune cells important for orchestrating local type-2 inflammation, found to be enriched at mucosal and barrier surfaces[4]. Functionally, ILC2 are mainly described for their role in promoting protective immunity to helminth infections, tissue repair, metabolic homeostasis, as well as allergic inflammation[5-7]. ILC2 belong to a larger family of ILC that parallel the adaptive immune system in function, but lack antigen specificity (for a review of ILC-lineages, see ref. [8]).

ILC distinctively lack antigen-specific receptors and are activated in an antigen-independent manner. This enables ILC to rapidly respond to changes in their microenvironment without the need for adaptive immune cell priming[9]. More specifically, ILC express germline-encoded inhibitory or activating

receptors that regulate their activity, and enables prompt modulation of function. This central feature of innate immunity has put forth the hypothesis that ILC can instruct the direction of downstream innate and adaptive immune responses by providing early polarizing cues. Indeed, Natural Killer (NK) cells, in addition to their natural cytotoxic function, are critical regulators of innate and adaptive immune functions. NK cells possess rudimentary features of immune memory, and can help influence the development of type-1 adaptive immunity, but also limit inflammation[10,11]. Similarly, Group 3 ILC (ILC3) are known to drive downstream innate and adaptive immune responses[12]. Thus, it is clear that several ILC lineages have important immune-regulatory functions.

ILC2 were formally discovered in gut-associated tissues of mice[1-3], although their presence was previously hypothesized based on the observation that T and B cell deficient (recombination-activating gene, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$) mice were able to mount type-2 responses[13-15]. ILC2 were subsequently discovered at other barrier sites, such as the airways and skin, where they participated in type-2 inflammation. In contrast to T and B cells of the adaptive immune system, ILC2 are sparse in secondary lymphoid organs but enriched at mucosal and barrier sites. Moreover, ILC2 are also present in numerous other tissues under homeostatic conditions. Parabiosis experiments have shown ILC2 to be tissue resident cells that are maintained and expanded locally under physiological and pathological conditions[16,17]. The physical location of ILC2 suggests that they may be important in regulating local immune responses. In this review we will first summarise ILC2 development and identification (see ref. [18] for a comprehensive review), followed by a review of their regulation and effector function in different anatomical sites during homeostasis and disease.

2. ILC2 development and identification

ILC2 derive from bone marrow haematopoietic stem cells (HSC) in a differentiation pathway from common lymphoid precursors (CLP) or lymphoid primed multipotent precursors (LMPP). These early precursors are known to require ID2, as well as transient NFIL3 expression[19], for their development and give rise to early innate lymphocyte precursors (EILP), that can differentiate into all ILC subtypes, including natural killer (NK) cells[18,20]. Downstream of multipotent EILP are more lineage-restricted progenitors such as the common innate helper innate lymphoid precursors (CHILP) that further develop into ILC2-lineage restricted progenitors, under the transcriptional control of *Id2, Zbtb16, Tcf7, Tox,* and *Bcl11b*[18,20-23]. Current data is consistent with a model in which multipotent progenitor cells give rise to unipotent ILC precursors that have a more specific cell fate[18] (**Figure 1**). Recently it was observed that a loss of the transcription factor (TF) promyelocytic leukaemia zinc finger protein (PLZF) (encoded by *Zbtb16*) has long-term effects on the functional properties of mature ILC2[24]. So far, the origin of human ILC2 is still unclear, however it was shown that all human ILC subpopulations in secondary lymphoid tissues could be derived from a RORγt^{+} progenitor[25].

As described before and depicted in **figure 1**, proper development and function of ILC2 is dependent on several TFs, including *Gata3*[26,27], *Rora*[28,29], *Gfi1*[30], *Tcf7*[31,32], *Ets1*[33], and *Bcl11b*[34]. However, these TFs are also expressed by other lymphoid lineages, which rely on some for their function or

differentiation. How exactly these different TFs regulate ILC2 differentiation is incompletely understood, although individual TFs promote ILC2 reactivity to certain alarmins (*e.g.* IL-25 and IL-33) by upregulating their receptor expression and stimulating type 2 cytokine (IL-4, IL-5, and IL-13) production[35]. GATA3 in particular is recognised as a master regulator of type-2 effector genes, cell proliferation, and lineage stability[35]. Importantly, while critical for ILC2 development and function, GATA3 also has upstream functions in ILC and T cell development[35-37]. While less is known about the regulation of GATA3 in ILC2, its expression in Th2 cells is known to depend on IL-4 mediated STAT6 signalling, as well as TCR and/or IL-2-dependent pathways[35]. Moreover, *Gata3* in addition to *Tcf7* and *Bcl11b*, have been shown to be Notch-dependent targets, this might explain the strong impact of Notch signals in promoting ILC2 development and differentiation *in vitro*[28,31,38]. Phenotypically, mouse and human ILC2 can be identified by the absence of surface markers expressed by other haematopoietic lineages (lineage-negative), but positive staining for CD45 and CD127 (IL7Rα). Without a known ILC2-specific marker, additional identifying features must be incorporated for their reliable detection by flow cytometry or immunofluorescence microscopy. For mice these markers may include surface signalling receptors such as ICOS, KLRG1, CD25, CD117, ST2 (*Il1rl1*, IL-33 receptor component) and IL-17RB (IL-25 receptor component), or intracellular targets associated with ILC2 function such as GATA3, IL-5, or IL-13. In mice, gene-reporter animals provide an additional invaluable means to identify ILC2. In humans, ILC2-associated markers include CD117, CD161, and CRTH2[39,40], the latter being the receptor for prostaglandin D2, as well as GATA3 (See ref. [41] for a more exhaustive list).

In terms of epigenetic regulation, ILC2 effector genes are in a constitutive open chromatin conformation that, like their adaptive $CD4^+$ T helper 2 (Th2) counterparts, is distinct from other ILC or T helper cell lineages[9]. It has recently been shown that the lysine methyltransferase G9a promotes expression of ILC2 genes, implicating epigenetic regulation in their lineage commitment[42]. Similarly, post-transcriptional regulation by micro-RNA (miRNA) 155 was shown to be important for proper ILC2 function in a model of allergic airway inflammation[43]. More recently, it was described that miR-17~92 has important implications for ILC2 homeostasis, additionally, miR-17~92-deficient ILC2 were shown to be defective in cytokine expression upon IL-33 or thymic stromal lymphopoietin (TSLP) induced allergic airway inflammation. Moreover, the miR-17~92 cluster member miR-19a was found to specifically promote IL-5 and IL-13 production, by inhibiting expression of signalling inhibitors, including SOCS1 and A20[44]. Nevertheless, no transcription factor or other gene is known to be exclusively expressed by ILC2, thus complicating genetic targeting strategies.

3. Regulation of ILC2 activation

As mentioned in the previous section, tissue resident ILC2 do not express antigen-specific receptors and are regulated by germline-encoded receptors. As such, ILC2 are positioned to integrate local cues from the epithelium, the microbiota, other immune cells, and pathogens in their environment to modulate inflammation. Non-haematopoietic cell derived type-2 alarmins,

including IL-25, IL-33 and TSLP are essential for the activation of ILC2[2,45,46]. These alarmins, together with other co-regulatory molecules, govern the function of ILC2 (see **figure 2**).

IL-25

IL-25 belongs to the IL-17 cytokine family, and signals via IL-17RB (in conjunction with the shared IL-17RA receptor) and is strongly associated with type-2 inflammatory responses. In the airways, epithelial cells have been suggested to be able to produce IL-25[47], although other cellular sources may be present. Administration of IL-25 intranasally induced an IL-25 responsive ILC2 population expressing large amounts of the activation marker killer cell lectin-like receptor G1 (KLRG1) and IL-17RB, but not ST2. These KLRG1$^+$ inflammatory ILC2 are phenotypic distinct from the tissue-resident conventional ILC2, proliferated in response to IL-25, but not IL-33, but can develop into ST2$^+$ natural ILC2[48].

IL-25 plays a more prominent role in activating gut associated ILC2[49,50], where it is particularly produced by epithelial Tuft cells. Although the specific stimuli that prompt IL-25 release from Tuft cells are unclear, it is known that IL-25 driven activation of ILC2 plays a central role in anti-helminth parasite immune responses. Interestingly, steady-state ILC2 in the gut produce IL-13, enhancing the epithelial-stem-cell compartment to promote a basal level of Tuft cell development. In turn, Tuft cells produce IL-25, maintaining ILC2 activation in the steady-state gut[51,52]. During primary helminthic infection, like *Nippostrongylus brasiliensis*, as well as with exposure to the key fungal component chitin, the ILC2-Tuft cell-axis is enhanced by Tuft cell chemosensory signalling molecule Trpm5 and IL-13 produced by ILC2[53,54]. In this model IL-13 enhances Tuft cell differentiation from epithelial precursors in the intestine, indicating a positive feedback mechanism between ILC2 and the gut epithelium. This further stimulates intestinal goblet cell hyperplasia and mucous secretion that is important for parasite expulsion[55,56]. These data provide clear evidence of the importance of IL-25-activated ILC2 primary helminth infection and worm expulsion in the gut.

IL-33

IL-33 is a member of the IL-1 cytokine family but, unlike IL-1β and IL-18, it does not require processing by caspases for it to be biologically active[57]. IL-33 is sequestered in the nucleus of cells, and can be released in a passive manner during necrotic cell death, acting as an alarmin. However, under conditions of apoptotic cell death IL-33 in rendered inactive by proteolytic cleavage by caspase-1 and/or caspases-3 and -7[58]. In non-exacerbated asthmatics, IL-33 is expressed with deletion of exons 3 and 4 (Δ exon 3,4), conferring its cytosolic localization and facilitating secretion. This splicing variant form of IL-33 is strongly associated with type 2 immunity, whereas full-length IL-33 is not[59]. Moreover, the activity of full-length IL-33 can be further increased through alternative proteolytic cleavage after secretion into the extracellular milieu[60]. Although IL-33 is constitutively expressed in the nucleus of many cell types, expression can be further increased during inflammation. Upregulation of IL-33 protein has been observed in alveolar type II epithelial cells upon exposure to ovalbumin, chitin, or *Alternaria alternata*[61-63].

IL-33 is known to activate ST2^{+} ILC2, initiating an innate type 2 inflammatory response, through the production of IL-5 and IL-13. In the lung several allergens, like house dust mite (HDM), *Alternaria alternata*, and papain have also been reported to stimulate lung ILC2 via the release of IL-33[64-66]. IL-33 stimulation induces NF-κB p65, JNK, and p38 MAPK, the latter which subsequently induces phosphorylation of GATA-3 in ILC2. Phosphorylation of GATA3 by p38 strongly increased GATA3 binding to the *Il5* and *Il13* promotors, but not IL-6, and interestingly p38 MAPK inhibition during early phase of stimulated significantly suppressed IL-33-induced production of IL-5 and IL-13[67]. Recent work in mice has revealed that in the very early post-natal stages type 2 immunity is mediated by elevated IL-33 expression in the lung, which is dependent on ILC2 but not CD4^{+} T cells[68]. In adult mice, IL-33 and T cell-derived IL-2 expanded ILC2 populations in the lung in a model of *N. brasiliensis* infection that led to larval damage[69]. The release of IL-1β primes ILC2 to further increase their responsiveness to IL-33 and IL-25[70,71]. Recently, ATP-activated mast cells in the gut were found to increase ILC2 activation in part through increased IL-33 production[72]. In an indirect mechanism, IL-33 activated mast cells in the lung may activate ILC2 through production of lipid mediators, like leukotriene D4 and prostaglandin D2, as discussed in more detail below[73].

ILC2 engagement by IL-33 induces the activation of ERK signalling pathway, which is negatively regulated by Sprouty-related Ena/VASP homology 1 domain-containing protein 1 (Spred1). Spred1 suppresses both secretion of type-2 cytokines as well as proliferation apoptosis of ILC2 via the stabilization of GATA-3. As Spred1 is highly expressed in IL-33 activated lung ILC2, Spred1 may be involved in a negative feedback loop affecting IL-33-activated ERK signalling pathway[74]. Induction of IL-33 and TSLP by lung epithelial cells after chitin exposure or helminthic infection has further been reported to induce an interferon regulatory factor 4 (IRF4)-IL9 program in ILC2, that promotes rapid IL-5 and IL-13 production required for optimal epithelial responses[75]. Moreover, constitutive expression of IL-9 by ILC2 after IL-33 (and TSLP) exposure can act in an autocrine manner to prolong survival of ILC2 in the tissue and prevented apoptosis of these cells, thereby enhancing ILC2-induced type 2 immune responses[76,77]. In general, IL-33 is recognized as the main cytokine activating ILC2, however it becomes increasingly clear that there are many other soluble and non-soluble factors implicated in ILC2 activation.

TSLP

TSLP is suggested to be expressed mostly by epithelial cells and keratinocytes in the skin, gut, and lungs, although DCs, mast cells, and basophils may also provide a cellular source for this cytokine[78]. A recent study revealed a subpopulation of tuft cells as another potential source of TSLP in the small intestinal epithelium[79]. TSLP is linked to multiple type-2 related diseases, such as atopic dermatitis, asthma, and allergic responses. TSLP is an IL-7-like cytokine and binds the TSLP receptor, which forms a heterodimer with IL-7Rα, and is expressed on ILC2, Th2 cells, DCs, and mast cells[80]. In contrast to the ILC2 populations in the lung and gut, which are critically dependant on IL-25 and IL-33 for their activation, skin and skin draining lymph node ILC2 were found to be independent of these canonical cytokines, but instead were activated primarily by TSLP[81]. Recent studies have suggested that TSLP can

contribute to ILC2 activation promoting corticosteroid resistance in the context of IL-33 induced allergic airway inflammation[82,83].

In humans TSLP-stimulated ILC2 were shown to upregulate GATA-3 and produce type-2 cytokines IL-4, IL-5, and IL-13[27]. Moreover, treatment of mild allergic asthmatics with an anti-TSLP monoclonal antibody (AMG 157) resulted in a significant reduction of allergen-induced airway eosinophils and bronchoconstriction[84]. These results indicate that TSLP serves an important role for ILC2-activation; however, it remains uncertain whether TSLP is a master switch in ILC2 activation or it is part of a concerted action by several parallel pathways.

Other cytokines that regulate ILC2

Apart from the canonical ILC2 activating cytokines, IL-25, IL-33, and TSLP, other cytokines are also capable of inducing ILC2 responses, such as IL-2, IL-4, IL-7 and IL-9. IL-2 is known to be important for ILC2 survival and serves as a major co-factor in regulating ILC2 function during type-2 immunopathology in the lung. Indeed, while IL-2 alone was found to be insufficient to increase *in vivo* IL-13 production by ILC2, synergy between IL-2 and IL-33 did augment *Il-13* expression by ILC2[85]. Nevertheless, *in vivo* IL-2/anti-IL2-mAb treatment is commonly used to expand ILC2 in mice. It is likely that T cell derived IL-2 synergises with type-2 alarmins to promote ILC2 activation[86]. Similarly, IL-7 can synergize with IL-33 to promote ILC2 activation *in vitro*[27,64]. Moreover, mice defective in IL-7 signalling are ILC2-deficient, indicating the importance of this cytokine for either ILC2 development and/or homeostasis[1,87,88]. IL-9 also has a co-stimulatory function on ILC2, enhancing their survival[77]. Lastly, basophil or eosinophil derived IL-4 is known to influence ILC2 by enforcing ILC2 lineage stability, proliferation, and function[70,89]. Importantly, these cytokines require the common gamma (c_γ) chain as part of their receptors, and c_γ chain-deficient mice or humans[90] lack ILC2. Importantly, ILC2 function can also be negatively influenced by both type I and type II interferon, as well as IL-27 signalling[17,70,91-93]. Suppression of ILC2 function by these cytokines has been shown to rely on the transcription factor STAT1[17,94]. Finally, ILC2 lineage plasticity and trans-differentiation can be induced by IL-1β, IL-12 and IL-18 [70,71,95,96].

Receptor-ligand interactions

In addition to the soluble factors that regulate ILC2, a number of receptor-ligand interactions have been shown to mediate ILC2 activation. Tumor necrosis factor member TL1A (*Tnfsf15*) is expressed by endothelial cells and myeloid cells and binds DR3 expressed by T cells and ILC2. TL1A-DR3 interaction leads to ILC2 proliferation and their expression of IL-5 *in-vivo* and *in-vitro*[97,98]. The presence of IL-7 was shown to enhance this response, but well below that induced by IL-33. Like the mouse system, also in humans TL1A is less potent than IL-33 and IL-25 but acts synergistically with both factors to drive an optimal ILC2 response. Human ILC2 have further been described to express NKp30 that upon interaction with its cognate activating ligand B7-H6 induces rapid production of type-2 cytokines. Interestingly, higher levels of B7-H6 expression where observed in lesions of AD patients[99].

In the lungs of allergic mice, ILC2 were found to express ICOS (as well as ICOS-ligand), interaction of ICOS with its ligand was observed to promote cytokine production, proliferation, and survival of ILC2 through STAT-5

signalling[100,101]. In contrast, KLRG-1 expression and its binding to E-cadherin have been shown to inhibit human ILC2 function[102].

Lipid mediators

Although generally involved in metabolism, bioactive lipids can influence key physiological mechanisms and cell signalling in mice and humans[103]. The first lipid mediator described to regulate ILC2 function was Leukotriene D_4 (LTD_4) which has very high affinity for the receptor CysLT1R. As ILC2 endogenously express this receptor, LTD_4 induction, by *Alternaria alternata* challenge, promoted accumulation, enhanced proliferation, and robust production of IL-4 and IL-5 by ILC2[104]. Similarly, Leukotriene C_4 (LTC_4) given in combination with IL-33 has been shown to directly potentiate IL-5 and IL-13 production by ILC2, via activation of the CysLT1R[105]. Interestingly, human ILC2 also express the pro-resolving receptor ALX/FRP2 which binds the resolvin lipoxin A_4. Lipoxin A_4 has anti-inflammatory properties and inhibits IL-13 production by human peripheral blood derived ILC2 co-cultured in the presence of IL-2, IL-25, IL-33, and PGD_2[106].

The eicosanoid prostaglandin D_2 (PGD_2) is the key prostaglandin driving type-2 inflammation and its receptor (CRTH2) is selectively expressed on Th2-cells and human ILC2. PGD_2-CRTH2 signalling was shown to induce ILC2 migration and IL-4 production, as well as potentiate IL-25 and IL-33-mediated responses[107]. In mice, PGD_2 was also shown to drive ILC2 function and accumulation in the airways[73]. Notably, the inhibitory prostaglandin I_2 (PGI_2), or prostacyclin, has been suggested to attenuate allergic inflammation when ligated to its receptor (IP)[108]. PGI2 has been shown to inhibit human and mouse ILC2 responses, as IP ligation blocked ILC2 proliferation and induced apoptosis. Furthermore, IP-deficient mice displayed higher IL-5 and IL-13 expressing ILC2 in the lung after *Alternaria alternata* challenge[109].

Recently, a study by Von Moltke and colleagues reported that, although largely dispensable for homeostasis, leukotrienes, and especially LTB_4 and cysteinyl LTs, play a nonredundant role for in activating the ILC2 which was shown to be dependent on NFAT signalling and worked in synergy with IL-33[110]. Interestingly, mucosal neuron derived neuromedin U (NMU) also results in the activation of ILC2 and immediate production of type 2 cytokines downstream of the NFAT signalling cascade[111]. Showing the wider importance of NFAT signalling in ILC2 activation.

Hormones

Transcriptional profiling of innate lymphoid cells has revealed that the androgen receptor (AR) is a prototypical ILC2 signature gene in tissue-resident ILC2[112]. This may suggest a role for hormones in the regulation of ILC2 function. Recent data has revealed that the development of ILC2 is greatly influenced by expression of the AR on progenitor ILC2. Engagement of the AR by its ligands, the male hormone androgen, inhibits differentiation of ILC2 progenitors into mature ILC2[113]. These data demonstrate that that androgen-signalling can regulate ILC2 development, however, as very few ILC2 develop from progenitors in adult mice[16], it may well be that AR-signalling imprints on steady-state numbers of ILC2.

Other hormones, like estrogen, have also been linked to airway hyperreactivity, although only in steady-state as no differences were observed

between wild-type and ERα$^{-/-}$ mice after allergen challenge[114]. However, this study does not specifically target ILC2. In all, very little is known about the hormonal control of ILC2.

Neuronal regulation
Neuronal control of ILC2 activation presents another dimension of immune-regulation. Recently it was found that the neurotransmitter Adenosine, an ATP catabolite binding the Adenosine receptor, has recently been suggested to negatively regulate type-2 cytokine production by ILC2[115]. Others have described that ILC2 selectively express neuromedin U receptor 1 (Nmur1) and ILC2 in the mouse gastrointestinal tract, as well as the lung, co-localize with cholinergic neurons that express the neuropeptide NMU[111,116]. Very similar to data obtained in ILC3, where neurons were shown to interact with ILC3 and regulate their cytokine expression[117]. Interestingly, single-cell RNA sequencing data from IL-25 and IL-33 stimulated lung ILC2 revealed the Nmur1 was preferentially expressed during steady state and after IL-25 stimulation[118]. It was shown that NMU controls ILC2s downstream of the activation of calcineurin and NFAT[111]. *In vitro*, as well as *in vivo* stimulation of ILC2 with NMU induced rapid cell activation, proliferation, and secretion of type-2 cytokines, resulting in accelerated clearance of nematode *Nippostrongylus Brasiliensis*[111,116]. Additionally, the circadian synchronizer vasoactive intestinal peptide (VIP) activates ILC2 by binding its receptor VPAC, stimulating IL-5 release and linking eosinophilic inflammation with metabolic cycling[87] These studies very elegantly present evidence of a selective mechanism by which enteric neurons can provide neuro-immune crosstalk and promote rapid type-2 immune responses at mucosal barriers.

4. Effector molecules produced by ILC2

As tissue-resident innate immune cells, ILC2 are positioned to rapidly respond to stimuli. Upon activation ILC2 can express various soluble or membrane-bound effector molecules. These include the well-established IL-4, IL-5, IL-9, IL-13, and amphiregulin (see **figure 2**), but also others inflammatory molecules such as IL-6, IL-8, IP10, methionine-enkephalin (MetEnk)[27,119]. Moreover, ILC2 express surface-bound molecules such as MHC class II, CD80/86, ICOSL and OX40L that may participate in immune-regulatory functions.

Cytokines
Initial identification of ILC2 was made possible by their expression of type-2 effector cytokines[1-3]. While *Il4-egfp* reporter activity marks naïve ILC2, protein expression is observed only in activated ILC2[3,104]. Nevertheless, ILC2-derived IL-4 plays a role in polarising Th2 cells during helminth infection[120], and may also block the generation of allergen-specific Treg cells, thereby promoting food allergy[121]. In comparison to relatively scant production of IL-4, ILC2 are a potent cellular source of IL-13. Rapid secretion of IL-13 by ILC2 influences both immune and non-immune cells. Myeloid cells including macrophages and dendritic cells are activated by IL-13 to become polarised to a M2 or type-2 chemokine secreting phenotype respectively. ILC2-derived IL-13 also activates endothelial and epithelial cell-types to express adhesion molecules or enhance the secretion of mucous from goblet cells. Importantly, IL-4 and IL-13 share

common signalling components, and the overlapping roles of ILC2-derived IL-4 and IL-13 is not yet understood (for more details, see review on IL-4/13 by Wynn *et al.*[122]). ILC2 are the main innate source of IL-5, a growth factor essential for eosinophil homeostasis[87]. The transcript for *Il5* is constitutively expressed by a significant proportion of ILC2 in many anatomical locations, making the *Il5-tdTomato* reporter mouse an attractive model for ILC2 identification.

Although ILC2 can respond to IL-9, they are also an important source of this cytokine, leading to an autocrine signalling module, as depicted in **figure 2**) that enforces IL-5 and IL-13 production[76]. While this feed-forward axis is essential for lung inflammation, IL-9 producing ILC2 were found to resolve inflammation in a model of allergen-induced arthritis, through the activation of Treg cells[123]. This implies that IL-9$^+$ ILC2 can yield different outcomes depending on their location and the inflammatory microenvironment. Although very little is known about ILC2 Treg cell interaction, ILC2/Treg crosstalk has also been observed in studies focussing on helminth expulsion[124]. Furthermore, within the visceral adipose tissue and lung tissues ILC2 and Treg cells were shown to localize to similar areas under homeostatic conditions, as well as in multiple other tissues after IL-33 stimulation[91]. Interestingly, after IL-33 administration or *N. brasiliensis* infection ICOS-L$^+$ ILC2 and ICOS$^+$ Treg cells were found to accumulate in tissues, whereas Treg expansion in ICOS-L-deficient mice or after treatment with ICOS-L monoclonal antibodies was impaired[91]. Suggesting that ICOS-L expression by ILC2 could promote Treg cell accumulation and provide a mechanism for ILC2 to communicate with Treg cells. Interestingly, a novel gut-resident ILC subset that secretes IL-10 has recently been described[125], while another recent study identified IL-10 production in alternatively activated ILC2[126].

ILC2 act mainly through secreting soluble mediators. Given the fact that ILC2 are tissue-resident cells, their release of effector molecules in the microenvironment might be just the desired response for efficient signal transduction and amplification within the tissue. Apart from the classical type-2 cytokines, other ILC2 effector molecules have been described. For example, ILC2 are a main cellular source of amphiregulin, important for post-infection wound repair as well as for restoring epithelial integrity and lung function[127]. Additionally, there is MetEnk that was suggested to be an ILC2 effector mechanism to regulate metabolic homeostasis by eliciting beiging of white adipose tissue after IL-33 stimulation[119]. Lastly, ILC2 are also able to produce IL-2[85], which as with IL-9, may function in an autocrine or paracrine fashion.

In all, the effector molecules produced by ILC2 will in part be overlapping with T cells; however, there will also be unique functions for ILC2 secreted molecules that need to be further defined using specific ILC2 depletion strategies.

Immune-regulatory cell-surface molecules

In addition to soluble mediators, ILC2 can interact with other cells via surface bound molecules. ICOS and KLRG1 are among the few molecules that can interact with surface-bound ligands. KLRG1 has, in the context of atopic dermatitis, been shown to act as an inhibitory receptor on ILC2, reducing proliferation and expression of *GATA3,* as well as *IL5* and *IL13* gene transcripts upon E-cadherin ligation[102]. Interestingly, both ICOS and ICOS-L are co-

expressed on ILC2 and cognate interaction between ICOS-ligand on ILC2 and ICOS on T cells, after IL-33 stimulation, has been proposed to mediate Treg accumulation[91]. Additionally, ICOS/ICOS-L interaction on ILC2 were found to influence STAT5 signalling, promoting ILC2 proliferation and function in a model of allergic airway inflammation[100]. IL-33 has further been shown to upregulate OX40 ligand (OX40L) expression on ILC2, which was suggested to promote adaptive immunity by co-stimulation of T cells[128]. Additionally, OX40-OX40L ligation has further been shown to enhance Th2 cell expansion and survival[129,130]. Other surface molecules include programmed death 1 (PD-1) receptor, its ligand PD-L1, and MHCII. PD-1 has recently been found to be upregulated on activated ILC2, and depletion of these PD-1$^+$ ILC2 reduced papain induced lung inflammation[131]. Intriguingly, ILC2 can also express PD-L1, which functions to promote CD4$^+$ Th2 cell responses[132]. The expression of MHCII, and CD80 and CD86 by mouse ILC2 allows for cellular interaction with CD4$^+$ T cells. MHCII$^+$ ILC2 have an ability to induce Th2 cell differentiation *in vitro* from naïve CD4 T cells, while MHCII-deficient ILC2 experiments indicate a role for enhancing Th2 cell-driven helminth expulsion *in vivo*[86,128].

5. ILC2 immune function

Adipose tissue

ILC2 are enriched in adipose tissue, where they were initially identified in murine white adipose fat-associated lymphoid clusters[1,133]. White adipose tissue (WAT) ILC2 can regulate eosinophil and alternatively activated macrophages (AAMs) responses[134]. AAMs produce norepinephrine in response to eosinophil derived IL-4, which leads to a beneficial caloric balance and beiging of WAT[135]. More recently, Artis and colleagues showed that IL-33 administration resulted in elevated numbers of ILC2 in WAT, which was associated with an increase in beige adipocytes, improved glucose tolerance, and increased weight loss[119]. Concurrently, it was shown that ILC2 and beige adipocyte numbers were reduced in WAT of *Il33*$^{-/-}$ animals, while reconstitution of alymphoid mice with ILC2 was sufficient to promote WAT beiging, independent of the adaptive immune system, eosinophils, or IL-4 receptor signalling. Although recently others have demonstrated that AAMs do not synthesize relevant amounts of norepinephrine in response to IL-4 and subsequently failed to induce expression of thermogenic genes, like *Ucp1*, in adipocytes[136]. Therefore, the role of AAMs in adipocyte metabolism or adaptive thermogenesis is still subjected to some controversy. ILC2 were further reported to produce MetEnk peptides, leading to the upregulation of *Ucp1* by adipocyte (*in vitro*) and promoting beiging *in vivo*[119]. MetEnk treated animals' upregulated the beige adipocyte marker *Ucp1* in an IL-4 and IL-13 independent manner, as neither cytokine expression changed upon MetEnk administration[119]. However, using tissue specific deletion of IL-4Rα, Chawla and colleagues showed that IL-4 could act directly on PDGFRα+ adipocyte progenitors to regulate genes associated with beige adipocyte differentiation, such as *Ucp1, Klhl13, Tnfrsf9,* and *Tmem26* bypassing the AAM/norepinephrine axis[137,138]. They also found that ILC2 derived IL-13, in synergy with eosinophil derived IL-4, might directly act on adipocytes, through IL-4Rα signalling, to promote beige adipocyte development[137]. These studies

suggest that ILC2 can interact with various cell types within the adipose tissue to support metabolic homeostasis.

Interestingly, there is also evidence that the neuropeptide VIP, which is regulated by caloric intake, increased IL-5 production by ILC2 enhancing eosinophil responses associated with a lean state in mice[87]. Additionally, the ability of ILC2 to sense vitamin A deficiency suggest that ILC2 can respond to dietary factors in maintaining metabolic homeostasis[139]. In all, these studies offer important insights in the role of ILC2 in homeostasis, as well as their potential to be involved in metabolic disease.

Airways

Type 2 immune responses are typically seen as the hallmark of allergic airway inflammation[140]. Genome-wide association studies of asthmatic patients have identified many ILC2 associated genes, such as: *RORA, IL-33, IL1RL1, and IL-13*[141]. While ILC2 are the most prevalent innate lymphoid cells in the lungs of naïve mice, they remain a relative rare population among immune cells[127].

The emerging role of ILC2 in the development of allergic airway inflammation has been studied in various mouse models, using extracts of HDM[64,142] (more recently in combination with polutants[143]), protease containing allergens (papain[64] and *Alternaria alternata*[49]), ovalbumin[144], or chitin[75]. Early studies have linked ILC2 to the pathogenesis of allergic airway inflammation as treatment of *Rag1*$^{-/-}$ mice (which lack adaptive immune cells) with papain induced airway inflammation accompanied by eosinophilic infiltration and mucus overproduction[64], a feature previously primarily associated with Th2 cells. Moreover, *A. Alternata* exposure has been reported to cause IL-33 dependent exacerbated disease in mice sensitized with an alternative allergen[145], which together with work on ILC2-memory[146] suggests that antigen-independent activation of ILC2 can contribute to type-2 airway diseases. Similarly, administration of chitin or infection with the intestinal nematode *Strongyloides venezuelensis* triggered the production of IL-33, which lead to the activation of ILC2 in the lung and caused pulmonary eosinophilia[147].

Thus, ILC2 are indirectly activated by allergens via airway epithelial cells[64,148] (or other sources of the prototypical type-2 alarmins: IL-33, IL-25, and TSLP[49,64,144]). While administration of these alarmins has the ability to induce ILC2-driven lung inflammation, IL-33 appears to be the most prominent physiological stimulus in the airways[149,150], whereas IL-25 is regarded to be a more prominent ILC2 activator in the gastrointestinal tract[2]. Interestingly, intranasal administration of recombinant IL-25 promotes the emergence of an IL17RB^{+}KLRG1^{hi} ILC2 population in the lung[48]. Although these cells are at first ST2^{-}, they may convert into ST2^{+} ILC2 in the lung, suggesting that the tissue-specific microenvironment may strongly influence lung ILC2 regulation.

Lung ILC2 immune regulation

While ILC2 are the primary source of innate IL-5 and IL-13, the relative contribution of ILC2 versus that of Th2 cells in asthma has remained elusive. In wild-type mice, ILC2 can constitute over half of the cytokine producing immune cells after HDM or OVA exposure[144], while other studies have reported that ILC2 are redundant in the presence of Th2 cells[45]. Furthermore, the findings that effector Th2 cells are readily activated by type-2 alarmins in the absence of antigen[151], and that ILC-deficient patients experienced no increase in

disease[90], provokes discussion about the importance of ILC2. Nevertheless, studies using ILC2-targeted mouse models are revealing mechanisms by which ILC2 can modulate adaptive immune function in the airways.

ILC2-deficient mice, generated by bone marrow transplantation using *Rora*$^{sg/sg}$ (*Rora*$^{-/-}$) donors, revealed that ILC2 activity was essential for efficient Th2 cell differentiation following airway exposure to allergen[29,86,142]. Several mechanisms contribute to interactions between ILC2 and CD4$^+$ T cells; direct antigen-presentation by MHCII$^+$ ILC2 to naïve CD4$^+$ T cells and cell-to-cell co-stimulation promote Th2 cell activation and differentiation[86,128,152,153]. ILC2-derived IL-13 also indirectly influences Th2 cell differentiation by promoting LN homing of lung dendritic cells[65]. Recruitment of Th2 cells into the lung tissue also influenced by ILC2/DC crosstalk-driven CCL17 chemokine production[154]. In addition to regulating Th2 cells, ILC2 can also modulate ICOS-L$^+$ Treg cells in the lung through induction of ICOS expression[91]. Conversely, Treg cells may also suppress ILC2 cells to limit allergic airway inflammation[155]. Importantly, novel reagents that more selectively target ILC2 are essential to understand their precise function as immune-regulatory cells[45,86,154]. Nevertheless, it is clear that ILC2 are able to work in concert with adaptive immune cells, although the relevance of these interactions in human disease will need to be established.

ILC2-derived IL-13 is known to influence innate type-2 lung inflammation by promoting eosinophilia and macrophage activation. Interestingly, recent data using these mice suggests that IL-13 production by IL-33 activated ILC2 contributes to asthma pathogenesis by increasing bronchial epithelial tight-junction barrier leakiness[156]. Moreover, work on neonatal mice revealed a profound increase in lung IL-33 after birth, which was associated with ILC2 proliferation and IL-13-mediated M2 macrophage polarisation[68,157,158]. These observations suggest that in early postnatal stages, type 2 immunity is established by IL-33-driven recruitment of inflammatory cells, in part via ILC2. Interestingly, exposure to antigens during this neonatal period resulted in the development of allergic airway inflammation, which was minimally dependent on Th2 cells, confirming an important role T-cell-independent activation of lung inflammation by ILC2[68]. Another recent paper highlighted the importance of ILC2 cell-intrinsic metabolism. In this study both mouse and human ILC2 were shown to express arginase-1 and arginine metabolism was found to control ILC2 proliferation and their production of type-2 cytokines. Ultimately suggesting that arginine metabolism within ILC2 is able to promote type 2 allergic lung inflammation[159].

In patients, ILC2 have been detected in blood and sputum of mild and severe asthmatics, where they could promote the persistence of eosinophilic inflammation[160]. However, frequencies of circulating ILC2 in PBMCs were not found to be different between allergic patients and non-allergic controls[161]. Interestingly, higher numbers of circulating ILC2 observed in patients that presented with signs of allergic asthma compared to non-asthmatic allergic patients[162]. Moreover, the frequency of IL-13 producing ILC2 was found to be significantly higher in patients with uncontrolled asthma, whereas it was decreased dramatically when these patients had their symptoms controlled by treatment[163]. Apart from identifying ILC2 as being possible predictors of asthma control, several studies have suggested these cells as possible therapeutic target. Recent data has shown that dexamethasone exposure attenuated the

production of type-2 cytokines by ILC2 and Th2 T cells after IL-33 stimulation[164]. Although others have reported allergen induced TSLP production makes ILC2 resistant to steroid-induced cell death through STAT5 activation[82] and indeed neutralization of TSLP has been shown to be effective in mild asthmatics[84]. Finally, the DP_2 receptor (CRTH2) inhibitor, fevipiprant, has been shown to reduce eosinophilic airway inflammation in patients with moderate-to-severe asthma, despite inhaled corticosteroid treatment[165].
Taken together, these data present an important role for ILC2 not only in the initiation of allergic airway inflammation, but also in fostering a network of cellular responses and interactions with many other cell in the lung to propagate the allergic immune response.

Role of ILC2 in viral induced infection
Besides their role in allergic lung inflammation, ILC2 also contribute to airway hyper responsiveness in response to respiratory viruses, like influenza, rhinovirus (RV), or respiratory syncytial virus (RSV) infection in adult and neonatal mice and humans, respectively[166,167]. Viral infections can damage the airway epithelium and induce type-2 immune responses via the activation of epithelial cells and their subsequent production of IL-25 and IL-33. RV infection induced epithelial IL-25 production has been shown to augment allergen-induced IL-25 production in mice and subsequently mediate RV-induced asthma exacerbations[168]. In addition, influenza studies report that activation of ILC2 during pandemic H1N1, or H3N1 influenza A viral infection results in a rapid development of airway hyper responsiveness (AHR) in an IL-33-dependent manner[166,169]. Others have shown that enhanced ILC2 activity, in the absence of IFNγ[17,93], characterized by increased production of IL-5 and amphiregulin, exhibited decreased susceptibility to lethal infection with H1N1-influenza[93]. Studies in immature mice have shown that IFNγ inhibits ILC2 expansion and IL-13 production thereby attenuating RV-induced goblet cell metaplasia and demonstrating the antagonistic function of IFNγ on ILC2 expansion and gene expression[170]. Moreover, deficiency in signalling through the type I interferon receptor has been shown to lead to elevated activation of ILC2[92]. This type I interferon-mediated inhibition of ILC2 proliferation and cytokine release was further suggested to be dependent on the heterotrimeric ISGF3 complex consisting of STAT1, STAT2, and IRF9[17,92,94]. Interestingly, influenza-virus-induced inflammation in a model of COPD exacerbation initiates ILC2 plasticity. The observed conversion of ILC2 was accompanied by a significant reduction in GATA-3 expression and the exposure to microenvironmental cues, such as IL-12 and IL-18[96].

During RSV infection, early TSLP-dependent activation of ILC2 resulted in the production of IL-13 and the subsequent development of AHR and mucus accumulation, in the absence of eosinophilia[171]. However, as in other models, IL-33 was also elevated in the lungs during RSV-infection and airway inflammatory responses were reduced in IL-33 KO mice, clearly showing that both TSLPR and IL-33R signalling pathways play an important role in the viral induction of type-2 immunity[172].

Interestingly, following acute influenza viral infection, accumulation of ILC2 is not only associated with immunopathology, but also with tissue repair and restoration of tissue integrity. Tissue repair functions of ILC2 were IL-13 independent, but relied on their production of amphiregulin[127]. Together these

studies show that although ILC2 may have immunopathological roles during viral infection, they could seemingly induce opposite pathways restoring lung homeostasis and tissue repair after lung injury.

Skin

Atopic dermatitis (AD) is an inflammatory skin disease characterized by epithelial barrier dysfunction and often associated with a type 2 immune response to common environmental allergens, as shown by the high levels of IL-4 and IL-13 found in skin lesions of AD patients[173]. Genes linked to susceptibility to atopic disease, such as *TSLP*, *IL-4*, *IL-5*, and *IL-13*, are also associated with ILC2 responses[174]. Most data regarding the role of ILC2 in atopic dermatitis has been derived from mouse models and these have shown that skin-resident ILC2 responses can be elicited by TSLP, IL-25, and IL-33, the vitamin D analog calcipotriol, and complexes of IL-2 with anti-IL-2 mAb[81,102,175,176]. Moreover, TSLP activates a population of skin-resident ILC2 in healthy mouse and human skin, which are also enriched in dermal lesions of human AD patients, independent of IL-33R or IL-25R signalling[81]. Others have shown that mice expressing IL-33 under the keratin 14 promoter develop a spontaneous AD-like inflammation of the skin, associated with the infiltration of IL-5^{+} ILC2[176]. suggesting that depending on the specific micro environmental cues ILC2 get differentially activated to produce type-2 cytokines and cooperate in AD pathogenesis. However, besides their pathological role in dermal inflammation, IL-33 activated ILC2 have been shown to harbour a protective tissue reparative role during cutaneous wound healing[177].

Skin lesions of AD patients, but not psoriasis, have been shown to be enriched for ILC2 producing IL-5 and Il-13, in addition to amphiregulin (which mediates tissue repair)[178]. Furthermore, the discovery that E-cadherin-KLRG1 ligation on human ILC2 shows a clear reduction in IL-5 and IL-13 production and the fact that E-cadherin is characteristic of filaggrin insufficiency, suggests that both mechanisms may cooperate in AD immunopathogenesis[102]. However, it is to note that only half of the AD patients carry filaggrin mutations and of those 40% do not progress to allergic sensitization and the development of AD[179,180]. Even if filaggrin deficiency and its role in barrier dysfunction on its own may not be sufficient to cause AD lesions, the absence of additional E-cadherin ligation on ILC2 may favor type 2 immune responses and the development of AD skin lesions. Moreover, ILC2 have been demonstrated to be the predominant IL-13-producing cell population in the skin and elevated IL-13 levels in the skin predispose mice for the development of skin lesions[175,176]. Therefore, there has been great interest in blocking IL-13 as therapeutic intervention strategy. The blocking of IL-13 activity, in humans, through dupilumab has been shown to alleviate the symptoms of atopic disease[59].
Together data from GWAS of patients and data collected from human studies and mouse models suggest skin-resident ILC2 to play a prominent role in the pathogenesis of AD; although additional work is awaited to elucidate the exact role of ILC2 in the skin.

The role of ILC2 in other anatomical sites

It is becoming increasingly clear that ILC2 are not restricted to mucosal barrier sites, but are present in numerous other tissues. In the liver, ILC2 have been associated with IL-33-induced liver fibrosis. It is demonstrated that IL-33

released by stressed hepatocytes activates ILC2, which trigger the IL-13 dependent profibrogenic activation of hepatic stellate cells[181,182]. IL-33 is further elevated in models of viral hepatitis, however, in this model ILC2 have been suggested to limit liver injury[183].

ILC2 have also been found in the brain meningeal space, where they respond to spinal cord injury by the production of type-2 cytokines[184]. In addition, brain-resident ILC2 were found to polarize the immune response to cerebral malaria[185]. ILC2 in the nervous system may also play a role in multiple sclerosis. As ILC2 found to accumulate in the brain and draining lymph nodes of mice resistant to experimental autoimmune encephalitis[186].

Although far from exhaustive the implications of ILC2 in the diseases described above and their discovery in numerous mucosal and non-barrier tissues implicates the importance to consider these cells in an increasing number of organs and diseases.

6. Conclusion & future directions.

Since their discovery as an innate source of type-2 cytokines in the gut, ILC2 have been identified in an expanding number of other anatomical locations. Their tissue-residency reflects their sentinel function, and role in tissue homeostasis. It is becoming increasingly evident that ILC2 are intricately involved in regulating both immune and non-immune cell types. Conversely, ILC2 function is tightly modulated by diverse upstream factors. As such, it is likely that ILC2 are an important tissue-specific nexus between immune and non-haematopoietic cells in type-2 immunity. Importantly, findings of mouse ILC2 biology will need to be translated into humans to resolve their importance in disease.

Nevertheless, intriguing findings in murine models show that ILC2 are essential in the initiation and propagation of allergic type-2 immune responses from birth to adulthood. The identification of ILC2 and the further insight into their function in inflammation and tissue repair has highlighted the important role of this type of innate lymphoid cells in triggering immunity. Recent mouse and human data together allow us to become increasingly aware of the fact that ILC2 responses are greatly influenced by their interactions with microbiota, nutrients, metabolites, neurons, and stromal cells. However, specific microenvironmental factors mediating the plasticity of ILC2 have not been identified and the molecular mechanisms underlying ILC2 interaction with their environment remain poorly understood, although the use of transcriptomic datasets can further enable investigations into this issue[112]. It is expected that future studies will provide more detailed answers to the remaining outstanding questions and lead to the development of novel and effective therapy for ILC2-driven diseases.

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Figure legends

Figure 1. Innate lymphoid cell development from progenitors. Downstream of the haematopoietic stem cell (HSC) *Id2* gene activation leads to the development of the lymphoid primed multipotent precursor (LMPP) and common lymphoid progenitor (CLP) that are restricted to the lymphoid lineage. Further commitment to the ILC lineage is established by upregulation of $\alpha_4\beta_7$ and downregulation of FLT3. The early ILC progenitor (EILP) initiates additional expression of IL-7Rα (CD127) and TCF1, but has low levels of ID2. The EILP can still develop in all ILC subsets, as well as NK cells. ILC differentiation proceeds to the common helper-like ILC subset (CHILP) that is characterized by high ID2 expression. Two subsets of CHILP can be discriminated, based on their expression of PLZF and PD1. Early $PLZF^-PD1^-$ CHILP can give rise to all ILC subsets including LTi cells. Thereafter, CHILP cells upregulate the lineage-defining transcription factors of the various ILC lineages.

Figure 2. Regulation of ILC2 activation and effector functions of ILC2-derived cytokines. ILC2 integrate multiple signals and although they are primarily regulated by the alarmins IL-25, IL-33, and TSLP, ILC2 activation is also strongly influenced by γc-cytokines, lipid mediators, cell-cell interactions, and cell-surface molecules. Activated ILC2 are a prominent source of cytokines and other molecules, important for the regulation of inflammation and homeostasis. In red are the inhibitory receptor-ligand interactions. TSLP, thymic stromal lymphopoietin; VIP, vasoactive intestinal peptide; NMU, neuromedin; LXA_4, Lipoxin A_4; PGD_2, prostaglandin D_2; LTD_2, Leukotrien D_2, Areg, amphiregulin; Met-Enk, methionine-enkephalin.