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ILC2s – Development, Divergence, Dispersal

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Abstract

Over the last decade, we have come to appreciate group 2 innate lymphoid cells (ILC2s) as important players in host and tissue immunity. New studies of ILC2s and their precursors using novel reporter mice, advanced microscopy, and multi-omics approaches have expanded our knowledge on how these cells contribute to tissue physiology and function. This review highlights recent literature on this enigmatic cell, and we organize our discussion across three important paradigms in ILC2 biology: development, divergence, and dispersal. In addition, we frame our discussion in the context of other innate and adaptive immune cells to emphasize the relevance of expanding knowledge of ILC2s and tissue immunity.

Introduction

ILC2s share transcriptional regulomes and cytokines typical of Th2 cells, which accompany allergic and anti-helminth immune responses, but express these effector functions during basal states accompanying tissue homeostasis and physiologic remodeling [1]. As such, understanding the regulation of ILC2 activation can illuminate the 'ground state' of allergy, a compilation of responses such as itch, cough, mucus production and gut motility, that drives barrier clearance and is associated with dysregulated type 2 adaptive immune pathologies like atopic dermatitis, asthma and food allergy [2–4]. How does basal ILC2 wiring transform into disease-promoting morbidities of such widespread prevalence? Technical innovations have begun to outline the developmental pathways by which ILC2s adapt to residence in tissues, but also the effector trajectories launched in response to

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perturbations and ILC2 movement into the systemic circulation to achieve effects on distal tissues. Here, we review the recent literature on the development, divergence and dispersal paradigm used by resident ILC2s, which may be relevant to other immune cells in both the innate and adaptive arms of lymphocyte biology.

Development

Innate lymphoid cells, or ILCs, are compartmentalized by transcription factor profiles and cytokine outputs that mirror Th1, Th2 and Th17 helper T cell subsets. Thus, for ILC2s, expression of GATA3 and RORα and canonical outputs like IL-5 and IL-13 are defining features [5]. Fate-mapping tools coupling compound transcription factor reporters and flow cytometry with single-cell RNA sequencing, stromal cell cultures and adoptive transfer experiments have revealed common ILC precursors (ILCp) downstream of common lymphoid precursors (CLPs) that progressively extinguish alternative cell fates to become ILC2 precursors (ILC2p) and then ILC2s. Using 'polychromILC' mice to label 5 transcription factors, McKenzie and colleagues [6] identified early divergence of dual Bcl11b^{hi}, Gata3^{hi} ILC2 precursors (ILC2p) from multipotent ILC1/3/NK precursors in bone marrow from common ILCp characterized by expression of *Zbtb16*, *Gata3*, *Pdcd1* and *Tox*. Divergence of LTi cells using *Id2*- [7] and *Tcf7*-reporters [8] has been more difficult to resolve, but CRISPR/Cas9-edited multi-transcription factor reporters for Gata3, ROR$_\gamma$(t) and Tcf7 identified an earlier *Tcf7*-negative fetal liver precursor for LTi and ILCs that diverged based on expression of ROR$_\gamma$(t) or GATA3, and upstream of the early ILCp (eICLP; IL-7Ra$^-$) and cILCp (IL-7Ra$^+$) that later give rise to helper ILCs [*9].

Akin to tissue resident macrophages, ILCs first arise during fetal development – likely during late yolk sac and early embryonic hemogenic endothelial hematopoiesis – as precursors that seed tissues and differentiate *in situ* to finalize lineage- and tissue-scripted gene profiles [10,11]. Although abundant in human fetal small intestine by the second trimester, ILC2s become diluted by adaptive T cells by the time of birth [12]. Fetal, perinatal and adult waves of mouse ILC2 differentiation have been identified by temporal fate-mapping, demonstrating activation of adult tissue-specific gene programs that become apparent shortly after birth [*13,14]. Parabiosis experiments show adult ILC2s and ILCp are largely tissue-resident and few circulate under resting conditions, although circulating cells contribute over time and particularly during allergic challenges [15,16]. During early development, ILC2p depend on high IL-7 concentrations and intermittent Notch signals for differentiation [17], and their organization in tissues occurs in relation to unique populations of fibroblasts that express IL-33 and TSLP, and localize near IL-7$^+$ lymphatic endothelia [*18,19], driving much interest in the organizing principles of niche supporting cells [20] and the specialized fibroblasts underlying this process [21]. Understanding the basal turnover of ILCp to mature ILC2s remains incomplete, although temporal fate-mapping reveals replacement rates relative to tissue of origin, with slow turnover in lung and adipose tissue but relatively high turnover in constantly regenerating tissues like the gut, skin and bone marrow [*13]. Better understanding of the precursor pool size and its self-renewal and exogenous replacement capacities have been bolstered by lineage tracing and single-cell RNA sequencing, particularly as occurs during periods of stress driven by type 2-biased perturbations. Using an RORα lineage tracer, Takei and

colleagues identified Gata3^{int}IL18r1[+]ST2^{−}Kit[+] multipotent ILCp in the lung that could differentiate to subsets of mature ILC2s producing type 2 cytokines or amphiregulin [*22]. Using different methods, Gasteiger and colleagues independently identified intravascular or circulating bone marrow-like multipotent ILCp in the adult and postnatal mouse lung that transitioned to a tissue resident ILC2p characterized by an IL18r1^{+}CD103^{+} phenotype before acquiring the IL18r1^{−}ST2^{+} phenotype typical of most mature lung ILC2s [**23]. In mouse skin, ILCp were characterized by expression of repressive transcriptional marks important in maintaining pluripotent stem-like states that differentiated in fluid 'cloud-like' states of interchanging phenotypes that included canonical IL-5^{+} ILC2s and activated IL-13^{+} ILC2s, suggesting distribution within domains amenable to deployment of effector cytokines mediating different functions during alterations of basal tissue physiologic states [*24].

Divergence

In early life ILC2s expand in virtually all mouse tissues, including skin, intestine, lung, adipose tissue, central nervous system meninges, liver, and secondary lymphoid organs, and begin to express tissue-specific programs. Using scRNAseq and cytokine reporter analysis, early postnatal day 14 ILC2s in the mouse lung, small intestine, and skin displayed an activated signature, marked by proliferative genes and increased cytokine expression, but already co-clustered with their respective adult ILC2s [*13]. Tissue imprinting manifested through a plethora of changes, including upregulating of IL-25R in the intestine, IL-18R in the skin, and IL-33R (ST2) in adipose and lung tissues, likely tuning cells to discrete microenvironmental activation signals [14]. As discussed below, ILCp and mature ILC2s entering from circulation can also contribute to tissue-resident mature ILC2s (Figure 1). The functional consequences of these recent ILC2 tissue immigrants is not well-understood, although they may ultimately adapt similar tissue programs, functions, and niches as native ILC2s. For example, using scRNAseq, parabiosis, and shielded bone marrow chimeras, Gasteiger and colleagues found that blood-derived ILCp and ILC2s in naïve mice, or after infection with *Nippostryongylus brasiliensis*, ultimately adopted lung transcriptomes that were indistinguishable from lung resident cells [**23]. Among ST2^{+} adipose and lung ILC2s, effector responsiveness to IL-33 relied on enhanced expression of PPAR$_\gamma$, reflecting metabolic linkage with tissues of residence [25]. Similar tissue adaptation likely occur in human ILC2s, where blood ILC2s/ILC2p demonstrated a migratory phenotype and lung ILCs displayed a continuum from a basal CRTH2 (PGD2 receptor)[+] phenotype to an activated CRTH2^{−} phenotype associated with expression of effector cytokines such as IL-13 in response to alarmin activation [26,27]. Indeed, tissue-specific adaptations are conserved across multiple lymphocyte subsets, including unconventional T cells [28] and Treg subsets [29].

Of note, similar paradigms exist for tissue resident macrophages, where macrophages from the yolk sac and fetal liver become variably supplemented with adult monocyte-derived macrophages that ultimately become virtually indistinguishable from resident macrophages. However, monocytes and monocyte-derived macrophages perform unique functions after recent trafficking and during periods of post-acute inflammation. Whether recent ILC2 immigrants or de novo ILCp-derived ILC2s have similar unique, transitory function(s) is

possible but remains undefined, and whether these transiting populations interact uniquely with other transiting or resident populations is also unexplored.

In addition to tissue-specific adaptation, it is increasingly apparent that ILC2s within single mouse and human tissues are not homogeneous, and display a spectrum of transcriptomic/ epigenomic states and functional capacities. In the skin, epidermal ILCs express high levels of IL-18R while dermal/subcutaneous subsets express higher ST2. The epidermal ILCs are supported by IL-7 and TSLP and localize to sebaceous glands, where they can function to repress sebaceous hyperplasia and limit antimicrobial lipid production [**30].

In virtually all tissues, ILC2 subset(s) localize near 'adventitial fibroblasts', a stromal cell subset which line the boundary regions of larger vessels, ducts, and airways and produce high levels of IL-33 and variable TSLP. Adventitial ILC2s express high levels of ST2 and can feedback to regulate fibroblast function via IL-13 [*18,19,31,32]. Adventitial fibroblasts likely cooperate with neuronal, lymphatic and interstitial macrophage-derived signals that also localize to these adventital microenvironments. In the intestinal lamina propria, IL-25-producing epithelial tuft cells control IL-25R$^+$ ILC2 activation, which promotes tuft cell and goblet cell differentiation from intestinal stem cells. This feed-forward loop is further driven by intestinal pathosymbionts such as *Tritrichomonas* and promotes a secretory gut state [33]. A similar feed-forward cycle may occur in skin, where colonization by commensal *Demodex* mites drives epidermal ILC2 activation and IL-13 production that limit hypercolonization; interruption of this circuit can lead to skin inflammation and ultimately hair loss and follicular senescence [34]. Highlighting the critical role of intestinal tuft cells in ILC2 regulation, prostaglandin D2, a well described positive regulator of ILC2 trafficking and function, was shown to be produced by tuft cells and act as a brake on intestinal type 2 immunity by promoting epithelial cell proliferation and inhibiting differentiation towards goblet cells [35]. In contrast, tuft-cell derived leukotrienes cooperated with IL-25 and promoted the feed-forward circuit, leading to further intestinal ILC2 activation and anti-helminth immunity [36]. In the stomach, ST2$^+$ gastric ILC2s are driven by commensal bacteria-induced IL-7 and IL-33, and can be further activated by *H. pylori* to drive protective IgA production by B-cells [37]. In adipose tissue, sympathetic activation of PGDFRα$^+$ stromal cells release glial-derived neurotrophic factor (GDNF), which activated adipose ILC2s to release cytokines enhancing adipocyte energy expenditure, thus outlining a neural-mesenchymal-immune circuit integrating body energy balance [**38]. Loss of adipose ILC2s with aging contributed to thermogenic failure of the adipose tissue [39]. Whether similar spatially discrete ILC2 niches exist in human tissues to reinforce local multicellular networks of communication remains poorly defined, although technologic improvements in multiplexed imaging should assist these efforts [40].

Although ILC2s display a basal state of activation in all tissues, activating cues can drive them to adapt *de novo* inflammatory states. Using scRNAseq and ATACseq, the Flavell group demonstrated that skin challenges (such as IL-23 and imiquimod – models for psoriasis) drive a continuum of quiescent and active ILC2s towards a hybrid, pathogenic ILC2/ILC3 'emergency' state [*24]. Skin ILC2s, in addition to canonical type 2 cytokines, elaborated IL-6 as mediated by expression of TLR2 and NOD2 [41]. Mice with conditional deletion of the transmembrane endopeptidase ADAM10 in interferon-responsive cells are

susceptible to dysbiosis and inflammatory destruction of hair follicles driven by ILC2s [42]. IL-13 secreted predominantly by ILC2s in skin provides a homeostatic signal that maintains a distinct population of CD11b^{lo} type 2 conventional DCs (cDC2s) that inhibits inappropriate inflammation and supports allergic sensitization [43]. Mouse intestinal ILC2s produced the neuropeptide acetylcholine (ACh) in response to helminth infection that served as a feedback enhancer for type 2 cytokine output [44,45]. After initial activation, ILC2s display epigenomic and functional modifications consistent with "trained immunity", showing a predilection for more robust responses upon subsequent challenges [46]. In contrast, chronic ILC2 activation can upregulate suppressive programs that limit excessive type 2 inflammation, including increased expression of PD-1 [**23]. In the intestine, it was previously proposed that 'ILCregs' can limit excess inflammation via IL-10 production; however, recent data demonstrates that ILC2s are the dominant source of ILC-derived IL-10 [47,48], a phenotype that was driven by activation with certain cytokines and neuromedin but inhibited by TL1A. ILC2s also have cell-intrinsic pathways to limit hyperexcitability, expressing A20 (TNFAIP3) and CISH that restrict a hyperactive ILC2-tuft cell intestinal circuit [49], and tristetraprolin (Zfp36) to destabilize *Il5* mRNA and regulate intestinal eosinophils [50]. Whereas ILC3s were shown to produce IL-2 to support intestinal Tregs [51], activated ILC2s could support local 'type-2' Tregs via multiple contact-dependent pathways [52]. Together, these data demonstrate tissue perturbation can drive ILC2s into tolerant/repair states involving effectors like IL-10 and amphiregulin, but also pathologic states characterized by IL-9, GM-CSF and IL-17A. Whether these 'subsets' represent divergent stable states or arise from distinct precursors will require better fate-mapping, particularly given the presence of multipotent precursors in most tissues studied, and remain important areas for further research in hopes of discovery of methods for exogenous 'programming' to alleviate inflammation and restore tissue homeostasis.

Dispersal

Initially described as tissue-resident with predominant local contributions to immunity, ILC2s activated within tissues can proliferate, upregulate SIP receptors *(i.e.* S1PR1, S1PR4) and enter the circulation, where they can affect tissue function at distant sites. Based on prior studies identifying 'inflammatory' ILC2s (iILC2s) that appear in lung during infection with the migratory helminth *N. brasiliensis*, Germain and colleagues showed these cells originate from small intestine and can contribute to defense of the lung barrier [**53]. The appearance of iLC2s in the lung is dependent on the AP-1 family basic leucine zipper transcription factor (BATF), as infection of BATF-deficient mice with *N. brasiliensis* showed a selective defect in IL-25-mediated helminth clearance and a corresponding loss of iILC2s in the lung [54]. Migratory behavior was also seen in response to gut-limited parasites, such as *Heligmosomoides polygyrus*, where activated gut circulatory ILC2s mediated enhanced mucin production in the lung and conjunctiva months after terminating infection [55]. Although *H. polygyrus* molts transiently in the gut submucosa, adult *N. brasiliensis* elicited comparable migratory behavior of ILC2s from the small intestine when gavaged orally into mice or when allowed to develop *in situ* after migration of larvae from the lung, thus demonstrating that local activation alone sustains ILC2 dispersal [56]. Indeed, migratory, lung-traversing *N. brasiliensis* also drives lung-derived ILC2s into the

circulation, as shown by fate-mapping, and skin-derived ILC2s appear in blood after a variety of cutaneous inflammatory conditions including psoriasis, atopic dermatitis, and drug reactions with eosinophilia [34,57–59]. Withers and colleagues used Kaede mice containing a photo-convertible fluorescent cell tag to illuminate peripheral lymph node ILCs, which were mostly ILC1s/NK cells that turned over more slowly than adaptive T cells [60]. After cutaneous inflammation, however, activated, proliferating skin ILC2s appeared in draining lymph nodes from the circulation rather than via afferent lymphatics, as occurs among migratory gut ILC2s [**53,61], and raising the possibility for biasing lymph node adaptive immune responses. Studies using parabiosis show that tissue-evicted ILC2s can re-enter distal tissues [13,23,53], and adoptively transferred ILC2s from one organ can enter and acquire tissue-specific metabolic markers used by local ILC2s [*62]. Human blood ILC2s rely on oxidative phosphorylation, revealing a metabolic state comparable to other tissue resident immune cells, that can become dichotomous by channeling glycolysis to sustain IL-13 production while relying on oxidative phosphorylation for survival and proliferation [63]. These findings argue for some capacity for ILC2 expansion and malleability among differentiated ILC2s, although the relative contributions of ILCp versus mature ILC2s to ongoing ILC2 maintenance and function in tissues remains unclear. Studies of NK/ILC1s [64] and resident adaptive T cells [65] hint at similar 'retrograde' migratory pathways that allow 'tissue-resident' lymphocytes to re-enter circulation and enable durable yet adaptable maintenance of tissue reservoirs for surveillance and homeostasis across the lifespan [66].

Regardless of the mechanism(s), the impact of activated and/or migratory ILC2s on tissues, whether mediated by their circulating cytokines or the cells themselves, are likely to affect multiple cell types with the potential to alter function in ways that might be beneficial or detrimental. When challenged by pathogens requiring type 1 and interferon-mediated confrontation, tissues containing recently activated ILC2s are vulnerable to substantial damage [67]. In contrast, loss of IFN_γ-producing NK cells exacerbates ILC2 activation in atopic dermatitis [68], and a child with genetic absence of Tbet (*Tbx21*), a transcription factor required for $IFN\gamma$ production, had elevated innate and innate-like lymphocytes that produced elevated IL-5 and IL-13 [48]. Indeed, IFN_γ acts cell intrinsically to restrict type 2 inflammation by confining ILC2s to adventitial niches, limiting their S1P-dependent trafficking program and enhancing cell death among non-adventitial ILC2s; this 'niche confinement' was necessary to survive infection by the type-1 skewing pathogen *Listeria monocytogenes* [69]. Such findings are contextual, and in cancer models, activation of tumor ILC2s with IL-33 or IL-18 restricted the growth of orthotopic and subcutaneous tumors, respectively, which was further augmented by blocking PD-1 expression on resident ILC2s [**70]. In a melanoma model, ILC2 infiltration and modulation with a combination of IL-33 and anti-PD-1 enhanced ILC2 GM-CSF production and anti-tumor immunity by promoting eosinophilia [71]. In transplant models of graft-versus-host disease, IL-10-producing ILC2s [47] prolonged the survival of islet allografts [72], and GM-CSF from bone marrow ILC2s accelerated hematopoietic stem cell recovery after 5-fluorouracil ablation [73]. ILC2s decreased in blood with age among a Gambian population [74] and aged lung ILC2s were unable to maintain tissue homeostasis in mouse models [75], whereas adoptive transfer of activated ILC2s improved cognition in aged mice [76]. In COVID-19 infection, circulating ILC2s were reduced in patients with severe disease [77]. Further studies are needed to

delineate the lifespan/healthspan of tissue ILC2s and their progenitors in humans, and to define the mechanisms by which immune cells sustain tissue resilience with age [78]. Their capacity for rapid adaptation to residence and functional support in multiple tissues suggests a potential role for exogenous ILC2s, perhaps following genetic manipulation, in therapeutic interventions for organ dysfunction and failure [79].

Concluding remarks

Deeply integrated into the daily physiology of tissues, ILC2s continue to yield new discoveries that enhance our understanding of organizational processes that transform disparate cell types into functional organs interconnected across the greater organism. The processes by which these activities come to be conscripted by pathologic adaptive Th2 cells remain unclear, though likely reflect aberrant adaptation of some of the underlying trajectories taken by innate ILC2s. Although much remains to be learned, continued study of these enigmatic lymphocytes is likely to have a bright future.

Acknowledgements

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Highlights

- scRNAseq analyses have expanded our understanding of ILC2 heterogeneity

- IL-18R expression on ILC2 precursors sheds light on their residence and maturation

- ILC2s continue to yield discoveries that enhance understanding of tissue physiology

- ILC2 dispersal into the circulation can transmit effector functions to distant sites
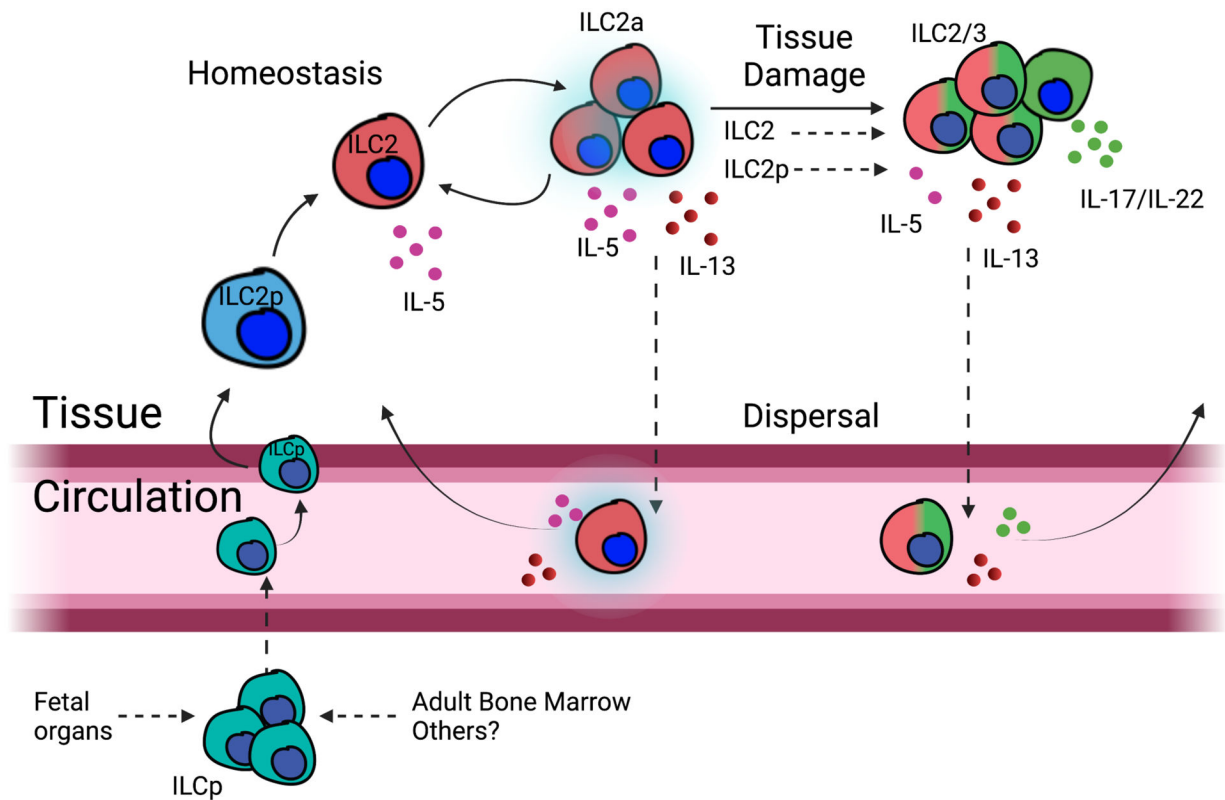
Fig. 1. ILC2 Life-cycle.
ILC precursors (ILCp) are produced in a continuum throughout life from hematopoietic tissues and differentiate to ILC2 precursors (ILC2p) and mature ILC2 that express transcriptomic signatures aligning with their tissue of residence. Based on tissues studied to date, ILC2s exist in equilibria among ILC2p and mature ILC2s transiting between resting (IL-5+ as assessed using reporter mice [*13]) and activated (IL-13+, ILC2a) states within distinct tissue microenvironments as driven by the physiologic demands of homeostasis. With perturbations leading to damage that requires recruitment of circulating cells, ILC2p and ILC2 undergo rapid proliferation and can take on features of ILC2/3 cells, with expression of IL-17A and IL-22. Rapid proliferation is accompanied by upregulation of S1P receptors and entry into the bloodstream, resulting in the systemic dispersal of cytokines, which become measurable in blood. The rapid deployment of the ILC2p pool may underlie the subsequent recruitment of immature ILCp and ILC2p that replenish the space, and provide additional cellular requirements in the face of ongoing injury. Mature circulating ILC2 and ILC2/3 can also enter depleted tissues, potentially providing further cellular replenishment.