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Thinking differently about ILCs—Not just tissue resident and not just the same as CD4⁺ T-cell effectors

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Abstract

Innate lymphoid cells (ILCs) resemble adaptive T lymphocytes based on transcription factor expression, cytokine production, and their presumptive roles in immunity, but are activated for effector function through cytokine signaling and not antigen-specific receptors. The prevailing view is that ILCs adapt to specific microenvironments during development and operate as tissue-resident cells in co-operation with antigen-specific T cells to provide host protection and contribute to tissue maintenance. In particular, conventional models equate the activity of different ILC subsets with CD4⁺ effector T-cell types based on corresponding transcription factor expression and a potential for comparable cytokine production. Based on recent data from our laboratory, we suggest that these views on tissue residence and parallel functioning to CD4⁺ T cells are too restrictive. Our findings show that ILC2s can be mobilized from the gut under inflammatory conditions and contribute to distal immunity in the lungs during infection, whereas gut-resident ILC3s operate in a quite distinct manner from Th17 CD4⁺ effector cells in responding to commensal microbes, with important implications for control of metabolic homeostasis. In this review, we discuss the recent advances leading to these revised views of ILC inter-organ trafficking and the distinct and complementary function of ILCs with respect to adaptive T cells in establishing and maintaining a physiologic host environment.

Keywords

host-pathogen interaction; innate lymphoid cells; mucosa

1 | INTRODUCTION

Two major mechanisms contribute to preventing pathologic colonization of a host by micro-organisms, namely the physical barriers of skin and mucous membranes and the soluble as well as cellular effector components of the immune system. Among the latter, CD4⁺ and

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CONFLICT OF INTEREST

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CD8⁺ αβ T cells have for many years been considered the *sine qua non* of host cell-mediated immunity. Using somatic recombination, these lymphocytes generate an enormous number of clonally distributed antigen-binding receptors (TCR) able to detect a wide array of ligands. The relevant antigens were first identified as proteins and later discovered to be recognized in the form of peptides bound to surface major histocompatibility complex-encoded molecules. Over the years, this understanding of αβ TCR recognition of antigen has been expanded to lipid ligands presented by CD1 molecules to NK T cells and bacterial vitamin-related molecules presented to mucosal-associated invariant T (MAIT) cells.^{1–3} In addition, γδ T cells combine canonical use of TCR gene segments and limited recognition of Ig-superfamily molecules in the butyrophilin family with a more expansive repertoire and a capacity for recognition of as yet poorly identified ligands presented in the context of non-classical MHC class I molecules.⁴

For αβ T cells bearing diverse receptor specificities, maturation and selection for receptors depleted of overt self-reactivity occurs in the thymus, which is then followed by circulation between different secondary lymphoid tissues to surveil for cognate antigens. Upon appropriate TCR engagement, T cells undergo clonal expansion and differentiation to become effector cells that home to the primary site of infection for pathogen clearance. A fraction of the activated cells develops into long-lived memory cells that mount a superior response upon re-infection. The diversity, specificity, and memory attributes of these T cells are cardinal features of the adaptive immune system.⁵ However, while the development of such adaptive responses plays a critical role in host defense against a diverse array of pathogens, it takes several days for rare T lymphocytes to find antigen, become fully activated, proliferate to adequate numbers, and develop effector functions. During this early phase of infection, myeloid cells and lymphocyte subsets with more immediate responses to a limited set of stimuli, including NK T cells, γδT cells, and MAIT cells, play critical roles in suppressing pathogen replication and dissemination.

The field now recognizes the important roles of yet another set of lymphocytes that contribute to host defense during this critical time window but lack the rearranging, clonally distinct receptors that characterize αβ and γδ T cells, namely innate lymphocytes or ILCs. ILCs emerge from common lymphoid progenitors (CLPs) and are functionally diverse, with an array of effector phenotypes that resembles that of polarized T-cell subsets.^{6–9} Conventional natural killer (cNK) cells are prototypic members of the ILC family that were described decades ago and possess effector functions similar to those of CD8⁺ cytotoxic T cells. More recently, several groups have independently described three major additional ILC populations of helper-like ILCs (ILC1, ILC2, and ILC3). Each of these ILC populations expresses a particular lineage specific transcription factor that promotes a distinct gene expression profile, which supports a selective capacity for cytokine production that enables these lymphocytes to assist host defense against a large constellation of pathogens. ILC1 cells express the T-box transcription factor T-bet, produce IFN-γ, and promote cellular immunity against intracellular microorganisms. GATA-3 is the master transcriptional regulator in ILC2 cells, which produce IL-5 and IL-13 that help mediate responses to expel helminthic parasites. ILC3 cells develop in a RORγt-dependent manner and are critical for controlling fungi and extracellular bacterial infection by producing IL-17 and IL-22. Another member of the ILC family is the lymphoid inducer (LTi) cell that is not only

essential for the development of peripheral lymph nodes and Peyer's patches during embryonic life but also contributes to protective immunity during infection.^{10–12}

In naive conventional $\alpha\beta$ T cells, the genetic loci encoding the key transcription factors characteristic of differentiated effector subsets are in a repressed state and epigenetically remodeled after TCR-dependent activation in specific cytokine environments.¹³ Using an assay for transposase-accessible chromatin sequencing (ATAC-seq), which defines the chromatin regions as regulomes including enhancers, repressors, or silencers, the accessibility of regulatory elements to transcriptional activators that control signature cytokine expression in ILCs was found to develop during early differentiation of these lymphocytes.¹⁴ Only with such remodeling can these cells exhibit the prototypic effector cytokine production or cytotoxic activities that are essential for their roles in host defense, together with the chemokine receptor display necessary to guide these cells into parenchymal tissues for their anti-infective action. In striking contrast, ILC progenitors upregulate the master transcription factors and remodel their cytokine gene loci in a “programed” manner during development and directly preposition themselves within tissues without infection-dependent activation. These features have led to the widespread view of ILCs as having two defining characteristics: (i) they function as highly localized, tissue-resident cells responding to local signals, rather than circulating throughout the body and “searching” for the proper infection site at a distance from the tissue in which they are activated, a behavior specifically associated with activated adaptive T cells and (ii) they provide effector functions that parallel rather than complement their $CD4^+$ $\alpha\beta$ T-cell counterparts, with shared master regulator transcription factor expression equating to shared functionality.

Based on new observations from our laboratory, we suggest in this review that revision is needed to this existing model of ILC and T-cell function in host defense and tissue homeostasis. In particular, our findings are consistent with a more expansive view of where ILCs are activated versus where they mediate effector function and the extent to which ILCs and $CD4^+$ T cells are complementary rather than similar in the effector functions they mediate in specific tissue settings. We first provide a condensed summary of known features of ILCs that support the existing paradigm, then discuss the new findings that we believe warrant a change in this understanding.

2 | TISSUE-RELATED CONTROL OF ILC MATURATION AND FUNCTIONALITY

The widely accepted notion that ILCs are highly adapted to local environments and serve as resident effector cells derives in part from insights into the maturation of these cells in distinct tissue settings. ILCs can be found widely distributed in both lymphoid and non-lymphoid tissues such as adipose tissue, liver, and especially barrier and mucosal surfaces, including the skin, gastro-intestinal tract, lungs, and urogenital tract.^{15,16} ILC progenitors first populate various tissues during the mid-to-late stages of fetal development or at the perinatal stage and then undergo further maturation along with the development of the relevant tissue. The best example involves LT α i cells, which first arise as early as embryonic

day (E)12.5–13.5 in the mouse. $LT\alpha 1\beta 2^+$ LTi cells circulate through the vascular system until they bind to $LT\beta R^+$ mesenchymal organizer cells in an integrin $\alpha 4\beta 1$ and VCAM-1 dependent manner. Binding of $LT\alpha 1\beta 2$ to $LT\beta R$ induces further upregulation of adhesion molecules including VCAM-1 and ICAM-1 and production of the chemokines CXCL13 and CCL19, which amplify LTi cell recruitment due to expression of the chemokine receptors CXCR5 and CCR7 on these cells. This positive-feedback loop leads to the accumulation of stromal and hematopoietic cells that complete the formation of lymphoid organs.^{12,17} Besides LTi cells, $CD56^+ CD3^- NKG2A^+$ NK cells are also present in several human fetal organs including liver, lung, and spleen as early as gestational week 15. These cells are highly responsive to cytokine stimulation and antibody-coated target cells but hyporesponsive to HLA class I-negative target cells, which suggests that fetal NK cells protect the host against infection, while remaining hyporesponsive to allogeneic cells of the mother.¹⁸ In addition, a $Lin^- CD127^+$ ILC that expresses chemoattractant receptor homologous molecule expressed on Th2 lymphocytes (CRTH2) and natural killer cell marker CD161 populates the fetal gut and responds to IL-25 and IL-33 to produce type 2 cytokines.¹⁹ In mice, fetal gut Arginase-1⁺ Id2⁺ ILC precursors have the capacity to differentiate into ILC1s, ILC2s, and ILC3s, and the ILC2 development is supported by mesenteric platelet-derived growth factor receptor α ($PDGFR\alpha$)⁺ glycoprotein-38 (gp38)⁺ mesenchymal cells.^{20,21} In a recent study, Koning and colleagues identified a $Lin^- CD7^+ CD127^- CD45RO^+ CD56^+$ population by mass cytometry in human fetal intestine could develop into $CD45RA^+$ NK cells and $CD127^+ ROR\gamma t^+$ ILC3s.²² All these reports suggest that fetal ILC progenitors populate the peripheral tissue in the prenatal period and give rise to different ILC subsets in the tissue microenvironment to provide appropriate protective functions during early life in the absence of antigen-specific adaptive lymphocytes.

In accord with this concept of ILC specialization during their development within specific organ sites, mature ILCs show clear differences in proportional representation among tissues. cNK cells are mainly found in the bone marrow, spleen, lymph nodes, lungs, and liver where they play a dominant role in tumor and viral infection surveillance.²³ ILC3 cells are mostly concentrated in the gut, whereas ILC2 cells localize in most barrier tissues including gut, lung, and skin. ILC2 cells also play a critical role in thermogenesis through a process termed “beiging” of adipose tissue.^{24,25} These and other emerging data indicate that different ILC subsets are strategically localized in specific tissues in a manner that relates to their roles in maintaining normal tissue function while contributing to protection against pathogens with a predilection for infection in those sites.²⁶

This theme extends to further specification of even more finely distinguished ILC subsets after progenitor seeding of various tissues. In the mouse small intestine, at least four subsets of ILC3 cells have been reported, including $CD4^{+/-} NKp46^- CCR6^+ ROR\gamma t^+$ LTi-like cells, $CCR6^- NKp46^- ROR\gamma t^+$ ILC3 cells, T-bet⁺ $NKp46^+ CCR6^- ROR\gamma t^+$ ILC3 cells, and a potential ILC3-ILC1 transitional subset that has downregulated $ROR\gamma t$, upregulated T-bet and NK cell receptor NK1.1 and gained the capacity to produce IFN- γ (“ex-ILC3 cells”) (Figure 1A). Although both $CCR6^+$ LTi-like cells and $NKp46^+$ ILC3s express $ROR\gamma t$, there is no precursor-progeny relationship between these cell types.²⁷ At birth, $CCR6^+ CD4^{+/-} ROR\gamma t^+$ ILCs are the dominant intestinal ILC3 population with very few $CCR6^-$ T-bet⁺

ROR γ ⁺ cells,²⁸ and both express the aryl hydrocarbon receptor (AHR), which drives their proliferation in response to endogenous or naturally occurring ligands such as dietary carotenoids and tryptophan derivatives.^{29,30} Like fetal LTi cells, CCR6⁺ LTi-like cells aggregate to form cryptopatches in the intestine. Together with activation of nucleotide-binding oligomerization domain containing 1 (NOD1) on epithelial cells by peptidoglycan from Gram-negative commensal bacteria, cryptopatches recruit B cells and mature into isolated lymphoid follicles (ILFs).³¹ Meanwhile, a proportion of CCR6⁺ ROR γ ⁺ ILC3s upregulate T-bet expression in a Notch-dependent manner.^{32,33} T-bet instructs the further differentiation of NKp46⁺ CCR6⁺ ROR γ ⁺ ILC3s and supports generation of “exILC3” cells, which produce INF- γ and are required to protect the epithelial barrier against *Salmonella* infections.²⁸

Unlike the diversity of ILC3 cells within the same organ environment, ILC2s from different tissues show phenotypic variability and respond to distinct cytokines depending on their local milieu (Figure 1B). Lung ILC2s express IL-33 receptor ST2 but low level of the IL-25 receptor IL-17RB. As a result, intranasal administration of IL-33 but not IL-25 induces lung ILC2 activation and the protease allergen papain induces allergic lung inflammation by activation of ILC2s in an IL-33 dependent manner.³⁴ In contrast, small intestinal ILC2s express IL-17RB and higher level of killer cell lectin-like receptor subfamily G member 1 (KLRG1) but not ST2. In the case of helminth infection, intestinal tuft cells sense the helminth infection by a TRPM5-dependent taste chemosensory pathway and secrete IL-25 to induce ILC2 activation, which is critical for helminth expulsion.^{35–37} Besides lung and intestine, ILC2s are also the dominant ILCs in mouse and human skin and are found to be highly enriched in lesional skin of patients with atopic dermatitis (AD).

Once they have developed from precursor cells and taken up residence as mature cells in a tissue, ILCs contribute to homeostatic regulation and pathogen responses by sensing specific cues in these diverse environments. ILCs are rapidly activated by myeloid or epithelial cells-derived cytokines, alarmins, and inflammatory mediators, such as IL-12 and IL-18, for ILC1 cells³⁸; IL-25, IL-33, TSLP, IL-2, TL1A, prostaglandin D₂, and Leukotriene D₄, for ILC2 cells^{39–43}; and IL-23, IL-1 α and β , TL1A, and prostaglandin E₂, for ILC3 cells.^{44–47} Besides these canonical immunological mediators, neurotransmitters can also be sensed by ILCs and induce inflammatory responses. In recent studies,^{48–50} intestinal and pulmonary ILC2 cells were shown to selectively express neuropeptide neuromedin U receptor 1 (NMUR1) and colocalize with neuromedin U (NMU)-producing cholinergic neurons. In response to parasite infection or exposure to allergens, neuropeptide NMU release led to a rapid and potent activation of ILC2 cells and induction of type 2 protective or inflammatory responses. In addition to NMU, calcitonin gene-related peptide (CGRP) derived from pulmonary neuroendocrine cells (PNECs) can enhance production of ILC2 cytokines in the presence of IL-33 and elicit a full type 2 immune response.⁵¹ Moreover, intestinal glial cells sense microbial-derived signals in a MyD88-dependent manner to control the production of neurotrophic factors, which in turn are able to activate adjacent ILC3 cells to produce IL-22 through RET signaling. ILC3-specific Ret ablation led to decreased IL-22 production, impaired epithelial activity, and increased susceptibility to bowel inflammation and microbial infection.⁵² ILC2-derived IL-5 and IL-13 are both necessary and sufficient for the development of pathology in the MC903-induced mouse skin inflammation model.

Interestingly, the cytokine responsible for skin ILC2 activation varies depending on the mouse genetic background: IL-25 and IL-33 are the predominant ILC2-activating cytokines in BALB/c mice, whereas thymic stromal lymphopoietin (TSLP) is more important in C57BL/6 mice.^{39,40,53} Thus, when ILC2 cells populate peripheral tissues they adapt their surface cytokine receptors expression to their environment to enable rapid responses to damage or pathogen infection. But how tissue signals regulate different cytokine receptor expression on ILCs is not yet resolved.

3 | THE MIGRATORY RESPONSES OF ILCs

Given the preceding evidence for the localized, tissue-relevant development of ILCs from progenitors and the crucial role of local factors in activating these resident cells to express a selected set of effector molecules, it has become a generally accepted view that ILCs operate as the innate equivalents of T resident memory (Trm) $\alpha\beta$ T cells. Further evidence for stable tissue residence of ILCs comes largely from parabiotic models. In these experiments, mice with distinguishable congenic markers are surgically attached via the skin, a procedure that induces connections to be formed between the vasculature of the two animals. Cells that regularly circulate in the bloodstream, such as T cells, B cells, neutrophils, and monocytes, readily exchange between the animals in the steady state, whereas ILC1s, ILC2s, and ILC3s do not.⁵⁴ The latter results suggest that the ILCs self-renew in the adult without substantial replenishment from bone marrow progenitors or other organs, although a basal level of constitutive trafficking of ILCs from intestine to the draining mesenteric lymph nodes was observed in a photoconvertible reporter mouse model.⁵⁵

This behavior distinguishes ILCs from most adaptive T cells, with the exception of Trm cells and subsets of $\gamma\delta$ T cells. Likewise, in humans, peripheral blood contains very rare ILC progenitors and ILC2s, while ILC3s or non-NK ILC1s are barely detectable.^{19,56} In mouse, ILC progenitors or mature ILC subsets are rare in peripheral blood in the steady state. These distinctions in the apparent circulatory behavior of ILCs and adaptive T lymphocytes are reminiscent of the recently recognized differences between fixed tissue macrophages and macrophages derived from circulating monocytes. Tissue-resident macrophages originate from yolk sac or fetal liver distribute into tissues at early stages of development and are largely self-maintained in the adult and do not exchange in parabiotic animal pairs, whereas monocytes from the adult bone marrow circulate in the blood, exchange between parabiotic hosts, and are able to migrate into the tissue and generate monocyte-derived macrophages throughout adult life.⁵⁷

However, a substantial change to this view of ILCs as locally acting tissue-resident cells comes from recent studies in our laboratory. *Nippostrongylus brasiliensis* or systemic IL-25 administration induces an ILC2 population in the lung, liver, spleen, and lymph nodes that is characterized by high expression of KLRG1 but low expression of the IL-33 receptor ST2 (KLRG1^{high} ST2^{low} ILC2s). In contrast to natural ILC2s (nILC2s), which reside in the lungs in the steady state, KLRG1^{high} ST2^{low} ILC2s are clearly inflammatory ILC2s (iILC2s) that appear in the lungs only after type 2 immune responses induced by infection or experimental stimuli.⁵⁸ The surprising aspect of studies in *N. brasiliensis*-infected parabiotic mice was that infection of one of the conjoined animals resulted in iILC2s in the lungs of each mouse

that were derived from both partners. This contrasted with the nILC2 population, which during the acute phase of the response remained host derived.

Because these data were at odds with the prevailing concepts of ILC2 function and residence, we undertook a series of studies to further explore the basis for these observations. The parabiosis results in infected mice made it unlikely that the ILC2s arose locally from the nILC2 population already present in the lung at the time of infection but the possibility of exchange of precursor cells from the bone marrow that seeded the lungs where they could undergo rapid differentiation into iILC2s could not be ruled out. But the involvement of both the lungs and intestine in this helminth infection, along with evidence of ILC2 existence in gut tissue, made the possibility of migration of differentiated ILC2 between tissues an alternative explanation. To test the capacity of cells from the lung itself, from bone marrow, and from the small bowel to generate lung iILC2s, cells from congenically marked donors were transferred into animals that were subsequently given IL-25. Remarkably, nearly all lung iILC2s came from small intestinal donor cells, not from lung donor cells, and very few from bone marrow precursors. These findings suggested that iILC2s were unlikely to have derived from either nILC2s or progenitor cells if any already in the lung at the time of administration of the activation stimulus, which would have been the expectation from the tissue resident model.

If the small bowel was truly the origin of the iILC2s found in the lungs, they would need to access the blood circulation to move between organ sites, and indeed, the majority of lung iILC2s generated by IL-25 injection were rapidly labeled by anti-CD45 antibody injected intravenously. In contrast, nILC2s remained unstained using this method, consistent with the localization in the lung parenchyma. Likewise, iILC2s derived from an IL-25-injected member of a parabiotic pair appeared in substantial numbers in the lungs of the parabiotic partner. Based on these data, a direct study of the localization and potential change in tissue distribution of iILC2s using advanced imaging methods was undertaken. These imaging studies revealed that iILC2s arise from resting ILC2s residing in the intestinal lamina propria. IL-25 induces rapid proliferation and blastogenesis of the intestinal ILC2s. These activated cells then appear within the lumen of villus lymphatics, suggesting they access the blood via the connection between these lymphatics and the circulatory system. From there they could emigrate into distant tissue sites such as the lung or liver to mediate host protective effects.

What promotes the movement of the KLRG1⁺ activated iILC2s from the lamina propria into the lymphatics so that they can circulate to other organs of the host? It is well established that receptors for sphingosine-1-phosphate (S1P), a phospholipid chemoattractant, guide the migration of activated mature adaptive lymphocytes from secondary lymphoid tissue sites across lymphatic endothelium and into the efferent lymph for drainage into the blood circulation.^{59,60} Following the downregulation of CD69 on activated T cells, which inhibits S1P-induced signaling through receptors such as S1PR1, T cells leave S1P^{low} lymph nodes and move into S1P^{high} lymph. For helper-like ILCs, the expression of S1PRs is barely detectable on intestinal ILC2s and ILC3s in the steady state; in contrast, IL-25-or helminthic infection-induced iILC2s express S1P₁ and S1P₄, and iILC2s in mesenteric lymph nodes also express S1P₅.⁶¹ Given these receptor expression data, the imaging data showing the

movement of activated iILC2s across the lymphatic endothelium, and additional flow cytometry data revealing that steady-state CD69 expression by the resting ILC2s in the lamina propria is reduced after IL-25 exposure, it seemed quite likely that a mechanism similar to that used by adaptive lymphocytes exiting secondary lymphoid tissue guided the iILC2s into the circulation via the lymph. To test this possibility, animals were exposed to *N. brasiliensis* and also treated with the drug FTY720, which antagonizes functional S1P-based signaling and migration. This treatment almost completely prevented the appearance of the small bowel-derived iILC2s in the blood, liver, or lungs, with a markedly reduced number making the journey to the nearby mesenteric lymph node. Consistent with an important function of this S1P-dependent migration of iILC2s in host defense, treatment with FTY720 led to the death of infected mice lacking adaptive lymphocytes (RAG KO animals), due to unresolved worm infection and tissue damage in the lungs. It is important to note that a key early study in the field that also used a parabiotic model to assess ILC exchange during *N. brasiliensis* infection. In that study, 10% of total lung ILC2s were partner derived late in infection but only 2%–3% partner-derived ILC2s were detected in the lungs at early times postinfection.⁵⁴ The discrepancy in frequency and in timing of ILC2 recruitment between this earlier analysis and the data we have recently published may be due to an important technical difference between the two sets of experiments. Gasteiger et al kept their mice on antibiotic treatment, whereas we did not and preliminary data in our laboratory suggest that the microbiome may play an important role in regulating the migratory capacity of IL-25-exposed gut ILC2s.

FTY720 (fingolimod, Gilenya) is a US Food and Drug Administration approved drug for treatment of multiple sclerosis. Other in-pipeline drugs targeting S1P receptors shows promise to colitis and psoriasis.⁶² The immunosuppressive effects of these drugs have been attributed to the prevention of activated adaptive T cells from exiting the lymphoid organs and accessing inflamed tissues. However, given our discovery of S1P regulation of iILC2 migration, and data revealing that S1P₅ is expressed on cNK cells and is required for the mobilization of NK cells to inflamed organs,⁶³ this presumptive mode of immunosuppressive action of FTY720 and similar drugs may need to be reassessed, especially as it is currently unknown if other ILC subsets such as ILC1 and ILC3 can show similar migratory behavior when the relevant activation stimuli / infectious agents are present.

These studies of the role of S1P signaling in ILC positioning place these innate cells in an interesting relationship with adaptive lymphocytes. Our findings show that iILC2s act very much like antigen-receptor-activated adaptive T cells in secondary lymphoid tissues. In both cases the cells undergo metabolic transformation and proliferation in the tissue, during which the expression of CD69 is temporally regulated to achieve a properly timed capacity to migrate across the lymphatic endothelium and enter the blood, allowing transit to a distant site of tissue infection. A similar reciprocal action involving the transcriptional loss of S1pr1 and the expression of CD69^{64,65} also plays a central role in the localization and migration arrest of Trm. Depending on one's view of whether ILCs arose before or after adaptive lymphocytes, it is intriguing to speculate that this mechanism may have evolved within the innate lymphoid system and was then later grafted onto the emerging adaptive system rather than being a late development of the T-cell adaptive immune system. Overall, these new

results shift our understanding of ILC biology by revealing an unanticipated capacity of tissue-resident ILCs to undergo interorgan migration and to provide immune protection not only locally but also in distal sites (Figure 2).

While our analyses have focused on a key role of the S1P chemoattractant in guiding interorgan migration of ILC2s, these findings raise the larger question about other forms of location control of these innate lymphocytes. Chemokines and chemokine receptors play central roles in determining cell positioning during development and under infectious and inflammatory conditions.^{66,67} With respect to innate lymphocytes, ILC2 precursors in bone marrow and mature cells in the intestine express CCR9,⁶⁸ while ILC2s in the skin mainly express CCR10 and have variable expression of CCR4.⁵³ ILC3s highly express CXCR6, especially the NKp46⁺ subset, while CCR6 is expressed by the majority of NKp46⁻ cells.^{28,69} The expression of CCR7 and CCR9 have also been observed by all ILC3 subsets.^{55,70} In the liver, a tissue-resident population of ILC1s expresses CXCR6 and CXCR3. To date, these observations relate only to ILC development and homeostasis and the field lacks insight into whether chemokines can direct ILC migration to and accumulation within a specific tissue site in the presence of inflammation or infection.

4 | DISTINCTIVE, NOT SHARED, CYTOKINE PRODUCTION BY GUT ILC3 AND TH17 CD4⁺ T CELLS

As noted in the Introduction, a central theme in ILC biology is the shared master regulator transcription factors (TF) in ILC subsets and CD4⁺ effector $\alpha\beta$ T cells. In vitro studies indicate that ILC and CD4⁺ T cells with such TF sharing also have the potential to make similar if not identical cytokines. In vivo studies provide evidence that at least in some conditions, ILCs with a given TF and cytokine production profile act as antigen-unspecific effectors with parallel functions to adaptive CD4⁺ T cells. However, despite the widespread acceptance of this model of ILC function, our recent research has uncovered a much more complex relationship between ILC3 and their putative CD4⁺ T-cell counterpart, Th17 cells.⁷¹

The starting point for the experiments that uncovered this new relationship initiated with analyses of ILC3 activity in RAG-deficient mice. These mutant animals are frequently used for assessment of ILC function because the absence of adaptive T cells allows exploration of ILC function without the confounding issue of ongoing activities of adaptive T cells that might mediate similar effector functions. As the development of ILC depends on the common cytokine receptor γ -chain, but not RAG recombinase, the immune phenotypes of RAG-deficient versus RAG γ c double-deficient mice are often compared in different disease models to further assess the specific roles of ILC in host defense and inflammatory or allergic diseases. However, the interpretation of experiments in these model systems is not as straightforward as it superficially seems. Studies with RAG fate-mapping mice show that RAG genes are transiently expressed by cNK, ILC2, and ILC3 cells during development and *Rag2*^{-/-} NK cell exhibit hyperresponsiveness under steady state but reduced survival during viral infection,^{72,73} so whether the function of ILCs other than NK cells is also influenced in

the RAG-deficient animals is unknown and might impact the results and hence, the interpretation of such experiments.

That said, we decided to study ILC3 activity in gut of RAG-deficient mice, using an advanced multiplex imaging method developed in our laboratory termed histo-cytometry.⁷⁴ We had previously used this method to study regulatory T cells and autoreponsive Tconv cells in relationship to IL-2-induced pSTAT5 generation⁷⁵ and considered the possibility that it might be very informative to use this method to assess pSTAT3 in the ileum, given that many of the cytokines involved in putative ILC3 activation and function, namely IL-23 and IL-22, induce phosphorylation of this transcription factor. We developed a method for reliable staining of pSTAT3 in large segments of ileal tissue in combination with antibody stains for epithelial and lymphocyte markers. In the steady state, RAG KO mice exhibited a strong pSTAT3 signatures in nearly all villus epithelial cells, along with a pSTAT3⁺ population of ROR γ t⁺ CD3⁻ ILC3 cells in both cryptopatches and villi. This pSTAT3 signature was lost in mice treated with broad spectrum antibiotics and in germ-free mice, indicating that the activation of the ILC3s was dependent on the microbiota. Further analysis showed that the previously described circuit of IL-23 activation of ILC3s to make IL-22 which then stimulated the epithelial cells was the origin of the pSTAT3 signals in the ILC3 and parenchymal cells, respectively. This was in accord with other data indicating that, in RAG-deficient mice, ILC2 and ILC3 cells are not only increased in number, but also are activated and producing effector cytokines in the steady state.^{40,71,76–78} The unexpected result was that these pSTAT3 signatures were absent in WT mice. We would have anticipated the same pSTAT3 epithelial cell staining as is the RAG KO mice, either because the microbiota-induced ILC3 activation would still occur or because, according to prevailing wisdom, adaptive ROR γ t⁺ Th17 or Th22 cells stimulated by the microbiota would make the same pSTAT3-inducing cytokines as the ILC3s.

In seeking to understand the difference between the WT and RAG KO animals in terms of pSTAT3 generation, we explored whether adaptive $\alpha\beta$ T cells, B cells, or both contributed to the apparent suppression of ILC3 mediated signaling. Again, we were surprised that neither TCR α -deficient nor μ MT animals, lacking $\alpha\beta$ T cells and conventional B cells, respectively, showed the pSTAT3 signature in epithelial cells and ILC3s. However, given the evidence that the microbiota played an essential role in the ILC3 activation, we co-housed the TCR α KO and the μ MT mice with the RAG KO animals showing strong pSTAT3 activation, and found that the TCR α KO but not the μ MT animals then showed pSTAT3 staining in most epithelial cells in the ileum. Further analysis revealed that segmented filamentous bacteria (SFB), known to be able to promote Th17 CD4⁺ T cell differentiation in a selective manner,⁷⁹ were the causative agent in our animal colony in promoting this ILC3-epithelium activation circuit. In the small intestine of mice that lack CD4⁺ T cells, SFB constantly induces IL-23 secretion from CCR2⁺ monocytes and monocyte-derived dendritic cells that results in persistent activation of ILC3 cells and production of IL-22. The IL-22 stimulates the epithelial cells to produce anti-microbial peptides and promotes glycan fucosylation to protect the tissue from microbial damage (Figure 3A).

The absence of the strong pSTAT3 signature in WT mice and its presence in the co-housed TCR α KO animals indicated that $\alpha\beta$ T cells played a dominant role in preventing ILC3s

activation by IL-23 and subsequent production of IL-22. In agreement with related data from a previous study,⁷⁶ adoptive transfer experiments showed that polyclonal CD4⁺ T cells could eliminate the pSTAT3 signature in recipient RAG KO, SFB-positive hosts, confirming a dominant suppressive role of this $\alpha\beta$ T-cell population on the ILC3 response. Additional analyses established that both Tregs, through interference with the production of IL-23 from CCR2⁺ myeloid cells (macrophages and dendritic cells), and Th17 CD4⁺ T cells, through suppression of SFB colonization that limits the stimulation of IL-23 production by these bacteria, contribute to suppression of ILC3 activation and the elimination of persistent pSTAT3 generation.

These findings raise important questions about the relationship between adaptive T-cell immunity and ILC action. Instead of the usual model in which ILC and adaptive T cells sharing TF expression (in this case ROR γ t) make the same cytokines and mediate nearly identical effector activities stimulated via cytokine signaling or antigen, respectively, we now have evidence for antagonistic interactions between these cell types and a clear distinction in cytokine production. The ILC3s made IL-22, not IL-17, whereas the Th17 make mostly IL-17 and little IL-22. The question then became what was the physiologic basis for the evolution of this unexpected dichotomy and antagonism in function of the two lymphocyte subsets. One possibility was suggested by data on pathogen infection, specifically studies employing *Citrobacter rodentium*, which found that infection with this organism provoked sequential waves of IL-22-producing ILC3 and CD4⁺ T cells that were both critical to host defense during a primary infection.^{10,80,81} Such temporally ordered activity of innate and then adaptive immune cells follows the general model of immune defense in which innate mechanisms come into rapid action while the rare precursors involved in adaptive immunity become activated and clonally expand to mediate useful function at a subsequent time. Translating this paradigm to the ILC3 / CD4⁺ T-cell behaviors we saw, it seemed likely that there would be an ontogenic ordering of early ILC3 activity that then was extinguished due to dominance of antigen-induced Treg and Th17 responses later in life (Figure 3B). Indeed, this is what was observed. Newborn mice, WT or RAG KO, both lack the pSTAT3 signature in ileal epithelial cells due to limited SFB colonization that is suppressed by lactoferrin and maternal antibodies.⁸² At the time of weaning, as full microbiome colonization of the gut begins, both WT and RAG KO mice show the pSTAT3 signature because adaptive responses specific to SFB are lacking and ILC3 activation proceeds unimpeded. After a few weeks, the presence of an expanded pool of specific Tregs and Th17 cells in the WT but not RAG KO animals reduces IL-23 production and extinguishes the high level of IL-22 production from ILC3s that drives the strong pSTAT3 response.⁷¹

This early life activation of ILC3 has multiple functions. First, the anti-microbial peptide production and epithelial fucosylation, which are induced by IL-22, play critical roles in controlling expansion of SFB and providing gut microbiota stability.^{83–85} Deficiency of ILC3 in *Ahr*^{-/-} mice allows specific expansion of SFB and result in increased Th17 cell induction, which further indicate that ILC3 activation is essential for maintaining physiologically appropriate microbial colonization and gut homeostasis.⁸⁶ Second, ILC3-derived IL-22 regulates epithelial NFIL3 expression, which is an important regulator of a circadian lipid metabolic program in enterocytes and promotes lipid uptake into intestinal

epithelial cells.⁸⁷ Third, early ILC activation can also modulate development of adaptive immunity. Epithelial serum amyloid A proteins 1 and 2 (SAA1/2) production induced by IL-22 influences Th17 polarization and enhances local IL-17 generation.^{79,88} With the maturation of intestinal Th17 cells, these adaptive T cells specifically constrain SFB abundance and thus prevent ILC3 activation.⁷¹ Taken together, establishment of homeostatic gut microbiota involves the sequential activity of innate and adaptive lymphocytes, and adaptive immunity is a dominant force in maintaining the stability of commensal microbiota.

Although ILCs and CD4⁺ T cells share same transcription factors and effector cytokines, they appear to preferentially adopt different strategies to execute their function and contribute to distinct outcomes. In a previous ILC transcriptional analysis, unlike ILC3 signature genes, including *Rorc*, *Ii23r*, and *Ii22*, *Ii17* is not significantly upregulated in any intestinal ILC3 subset as compared with other ILC populations.⁸⁹ In line with this finding, SFB-activated ILC3 cells do not produce IL-17, but rather secrete large amounts of IL-22, which stimulates epithelial cells to release antimicrobial peptides into the gut lumen. These antimicrobial peptides inhibit the growth of SFB and prevent formation of the characteristic long filamentous development form morphology but have no influence on the vegetative form, which is used to anchor the epithelial cells. Conversely, SFB-induced Th17 cells preferentially produce IL-17, but not IL-22, which is consistent with the absence of high levels of STAT3 phosphorylation in epithelial cells in immunocompetent SFB-bearing mice. IL-17 subsequently recruits neutrophils in a CXCR2-dependent manner.⁹⁰ In contrast to the effects of antimicrobial peptides, the IL-17-neutrophil pathway interferes with the SFB vegetative form adhering to epithelial cell without affecting the SFB developmental form maturing into long filaments. In *IL-23a*^{-/-} *Rag1*^{-/-} mice, which lack both the IL-22-AMP pathway and IL-17-neutrophil circuit, both developmental and vegetative forms of SFB dramatically expand.⁷¹ This indicates that ILC3 and Th17 cells exploit different cytokine pathways to control the composition of the microbiota. Such observations also raise the key question of whether other ILC populations also utilize distinct effector pathways from their T-cell counterparts as defined by TF expression.

Every coin has two sides and ILCs are not an exception. They are not only a major player in host defense, but are also involved in the physiological activity of the tissues with which they are associated. Our findings that adaptive CD4⁺ T cells shut down an ontologically early profound activation of ILC3 IL-22 production raised the issue of what was the evolutionary benefit of the shut down by CD4⁺ T cells. We suspected that persistent high IL-22 stimulation of epithelial cells must have a detrimental effect on long-term physiology of the host, and using transcriptional analyses of gut epithelial tissue discovered that the RAG KO SFB⁺ animals showed a marked reduction in transcripts for lipid transporters, accompanied by low serum triglycerides and free fatty acids, along with reduced adipose tissue. RAG RORγt DKO mice did not have this metabolic abnormality, consistent with IL-22 production by ILC3 as the origin of the dysregulation, and this was confirmed by studies in WT mice with prolonged overproduction of IL-22 from an adenoviral vector where lipid transporter expression and serum lipids were again depressed.⁷¹ Although low intensity and transient activation of ILC3 is required for maintaining gut lipid absorption by epithelial cells in immune-competent mice,⁸⁷ persistent ILC3 activation and IL-22 production disrupts host lipid metabolism in the absence of adaptive immunity regulation.

Given our findings⁷¹ and those of others^{47,91} that dys-regulated ILC activation can lead to inflammatory disorders and host defects, the intensity and duration of ILC activation must be tightly regulated in both an intrinsic and extrinsic manner. ILCs express several surface inhibitory receptors that modulate ILC activation, most of which have been well-studied in NK cells, including human killer-cell inhibitory receptor-L (KIR-L), mouse Ly69, T cell Ig, and ITIM domain (TIGIT), lymphocyte activation gene-3 (Lag-3), T-cell immunoglobulin-3 (Tim-3), programmed cell death protein 1 (PD-1), CD161, and KLRG1.^{92–95} Interestingly, PD-1 is also expressed on human and mouse ILC2 cells and engagement of this molecule regulates ILC2 cell proliferation and IL-13 production.⁹⁶ In addition, receptor activator of nuclear factor κ B ligand (RANKL) expressed by CCR6⁺ ILC3s negatively regulate ILC3 abundance and activity through interaction with its receptor RANK on adjacent ILC3s.⁹⁷ Besides these inhibitory receptors, signaling molecules such as Ikaros, A20, and microRNA miR17~92 have also been shown to control ILC3 and ILC2 homeostasis and effector functions under various conditions.^{98–100} Beyond these intrinsic control mechanisms, regulatory T cells extrinsically inhibit innate lymphocytes activation by various mechanisms. First, as both Treg cells and some ILCs including NK cells, ILC2, and CCR6⁺ ILC3 cells express the high affinity IL-2 receptor CD25, Treg cells can control the activity of NK cells by competing for T-cell-derived IL-2.^{101–103} Whether Treg also inhibit IL-2-dependent ILC2 and ILC3 cell activation is not known. Second, induced Treg cells suppress ILC2-derived IL-5 and IL-13 production by direct interaction with ILC2 cells through ICOS-ICOSL.¹⁰⁴ Third, Treg cells limit ILC-activating cytokine production from myeloid cells. In our study, we found that suppression of CCR2⁺ myeloid cell-derived IL-23 production by Treg cells prevented ILC3 activation by SFB.⁷¹ Fourth, Treg cells-derived cytokines such as TGF- β and IL-10 are important mediators of Treg cell suppressive function in controlling innate immunity.⁷⁷ These studies suggest that Treg cells are a potent regulator of ILC biology and are critical for maintaining innate immunity homeostasis.

These observations on ILC3 cells in the small bowel provide a clear model for how the interplay of the innate and adaptive lymphocyte populations work together to provide effective host defense, maintain tissue physiology, and support the development of a useful, non-damaging commensal microbiota. Early in ontogeny, ILC3 activity helps balance the growth of different bacterial species during early colonization of the gut through IL-22 production that promotes epithelial cell secretion of antimicrobial peptides. The high-rate production of IL-22 under these conditions, however, limits lipid uptake and the development of adipose tissue reserves, requiring a shift from this high-rate IL-22 production to a circadian low-level secretion pattern that supports physiological lipid transporter expression. This shift is mediated by the development of adaptive lymphocyte populations of IL-17 producing effector cells and the emergence of adequate number of Tregs that together limit the production of IL-23 and hence, the IL-22 secretion by ILC3s while also reducing the pro-inflammatory signals that can be induced by the expanding gut microbiota, either through direct suppression of chemokine production by myeloid cells or by inhibiting excess growth of pathobionts. The evidence from our work revealing the importance of the proper ILC—adaptive T-cell balance bears on recent work showing that metabolic diseases such as Kwashiorkor are associated with a dysregulated microbiome.^{105,106} Since the microbiome influences the activity of the lymphocyte populations and their

production of mediators such as IL-22 that can dysregulate molecules like lipid transporters that affect metabolism, it will be necessary to re-evaluate whether the microbiota alters metabolism by direct action on nutrients or the production of metabolites themselves, or indirectly through their action on immune cells that secondarily alter metabolic activities in the bowel.

5 | CONCLUDING REMARKS

Ongoing studies are constantly revealing new contributions of ILCs to various aspects of host physiology, including control of metabolic state, tissue integrity, anti-microbial defense, and useful commensalism. There is also increasing evidence that ILC can contribute to various disease states previously thought to be exclusively mediated by aberrant function of adaptive T lymphocytes. In this short review, we have emphasized how recent data from our laboratory have altered some of the fundamental assumptions about ILC biology. Rather than purely acting in a tissue-resident manner, we have shown that ILC2s are activated by IL-25 signaling in the lamina propria of the small bowel in a manner that facilitates the S1P-dependent egress through lymphatics into the blood circulation for delivery of effector function at distant sites such as the lungs. Such migration is critical to host defense against helminths. This raises the question of whether under specific infectious or inflammatory conditions, other ILC populations also undergo induced migration from their typical tissues of residence or if this is a peculiar feature of ILC2 because of adaptation to the multi-tissue lifestyle of helminths. Our studies of ILC3s have revealed that ILCs and adaptive $\alpha\beta$ CD4⁺ T cells do not show the parallel effector functions assumed for cells with expression of the same TF. Instead, ILC3s respond to commensal organisms with predominant IL-22 production, whereas CD4⁺ T cells expressing ROR γ t make mostly IL-17 family molecules and little IL-22. This dichotomy is important during ontogeny for the establishment of a non-inflammatory commensal population that does not disrupt lipid metabolic homeostasis through persistent IL-22 signaling of epithelial cells, revealing that ILCs play important and likely non-redundant roles in establishing immune balance from birth to adulthood.

Moving forward, it is apparent that generation of mouse models to selectively delete ILC subsets while preserving adaptive lymphocyte function will be critical in adding to our understanding of the role of these cells in a multi-layered immune system. Identification and study of ILCs and/or T cells in vertebrate ancestors would greatly help to understand the lineage relationship between innate and adaptive lymphocytes and which cell contributed what evolved mode of behavior to the other (for example, S1P-dependent trans-lymphatic migration). High resolution technologies, including advanced tissue imaging, can be applied to ILC investigations to provide additional insight into the spatial and dynamic aspects of their development, heterogeneity, function, and interaction with other cells. By challenging current orthodoxy, the new findings from our laboratory emphasize the need for an open mind about how the complex interactions of the many components of the immune system play out during development and organismal maturation. Applying an increasingly deep knowledge of ILC biology with an open mind to the possibility of many unexpected behaviors of these cells under various conditions will help reveal precisely how these cells contribute to immune-related diseases as well as open new opportunities for their manipulation for therapeutic purposes.

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FIGURE 1.

Tissue adaptation of ILCs. A, Intestinal $CCR6^{+}$ and $CCR6^{-}$ ILC3s develop separately from common helper-like innate lymphoid progenitor (CHILP) during fetal development or the perinatal stage, and proliferate in response to dietary AHR ligands after seeding in the gut. While $CCR6^{+}$ ILC3s accumulate to form cyrotopatches, $CCR6^{-}$ ILC3s differentiate into $NKp46^{+}$ ILC3 by upregulation of T-bet expression in response to Notch signaling. Meanwhile, a fraction of $NKp46^{+}$ ILC3 may further lose $ROR\gamma^{t}$ expression locally in the presence of inflammatory cytokines and differentiate into ILC1-like “ex-ILC3” cells. B, ILC2s from different tissues exhibit distinct cytokine receptor expression. Intestinal ILC2s express IL-25R and can be activated by Tuft cell-derived IL-25 during helminth infection, whereas lung and adipose tissue ILC2s express IL-33 receptor ST2, but not IL-25R, and mediate allergic inflammation and beige fat development in response to allergens and environment cold, respectively. In contrast, TSLP and IL-33 activate skin ILC2s to promote atopic dermatitis-like skin inflammation

FIGURE 2.

The local and migratory responses of ILC2s during helminthic infection. After subcutaneous inoculation, *Nippostrongylus brasiliensis* larvae quickly move to the lungs of mice and trigger epithelial cells and myeloid cells, such as dendritic cells (DCs), to secrete alarm cytokine IL-33, which activates lung-resident ST2⁺ nILC2 cells. Worm larvae then gradually migrate via the trachea to the small intestine, where Tuft cells sense the signals of infection and produce IL-25. Gut ILC2 (gILC2) cells respond to IL-25 to proliferate and migrate into the lymphatics in a S1P-dependent manner, with the downregulation of CD69 expression. These induced or inflammatory ILC2 (iILC2) cells circulate through the lymph to the blood stream, presumably via mesenteric secondary lymphoid tissues, and accumulate in the lungs where they cooperate with local nILC2 to contribute to worm clearance and tissue repair, providing early protection prior to Th2 cells emergence. In the later stage of infection, a portion of iILC2s in the lungs phenotypically converts to nILC2-like cells, while another portion homes back to the small intestine presumably in a CCR9-dependent manner.

FIGURE 3.

Dynamic activation of intestinal ILC3 by microbiota. In the absence of CD4⁺ T cells, SFB induce CCR2⁺ myeloid cell to produce IL-23, which persistently activates pSTAT3 in ILC3 to secrete high amounts of IL-22. IL-22 in turn induces STAT3 phosphorylation in epithelial cells and triggers anti-microbial peptide secretion to control SFB expansion. The persistent epithelial cell activation by IL-22 disrupts host lipid metabolism by downregulating lipid transporter expression. In immunocompetent mice, this IL-23-ILC3-IL-22-epithelial cell circuit is transiently activated during ontogeny at weaning before maturation of tissue adaptive immunity. Along with the development of intestinal CD4⁺ T cells, Treg cells suppress IL-23 production from CCR2⁺ myeloid cells, whereas Th17 cells constrain SFB abundance in a neutrophil-dependent manner, both of which are sufficient to terminate ILC3 activation and prevent disruption of intestinal lipid absorption. This sequential activity of

innate and adaptive lymphocytes is critical for supporting the non-inflammatory gut commensalism and host physiology

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