DIFFERENTIATION AND MECHANISMS OF ACTIVATION OF INNATE LYMPHOID CELLS

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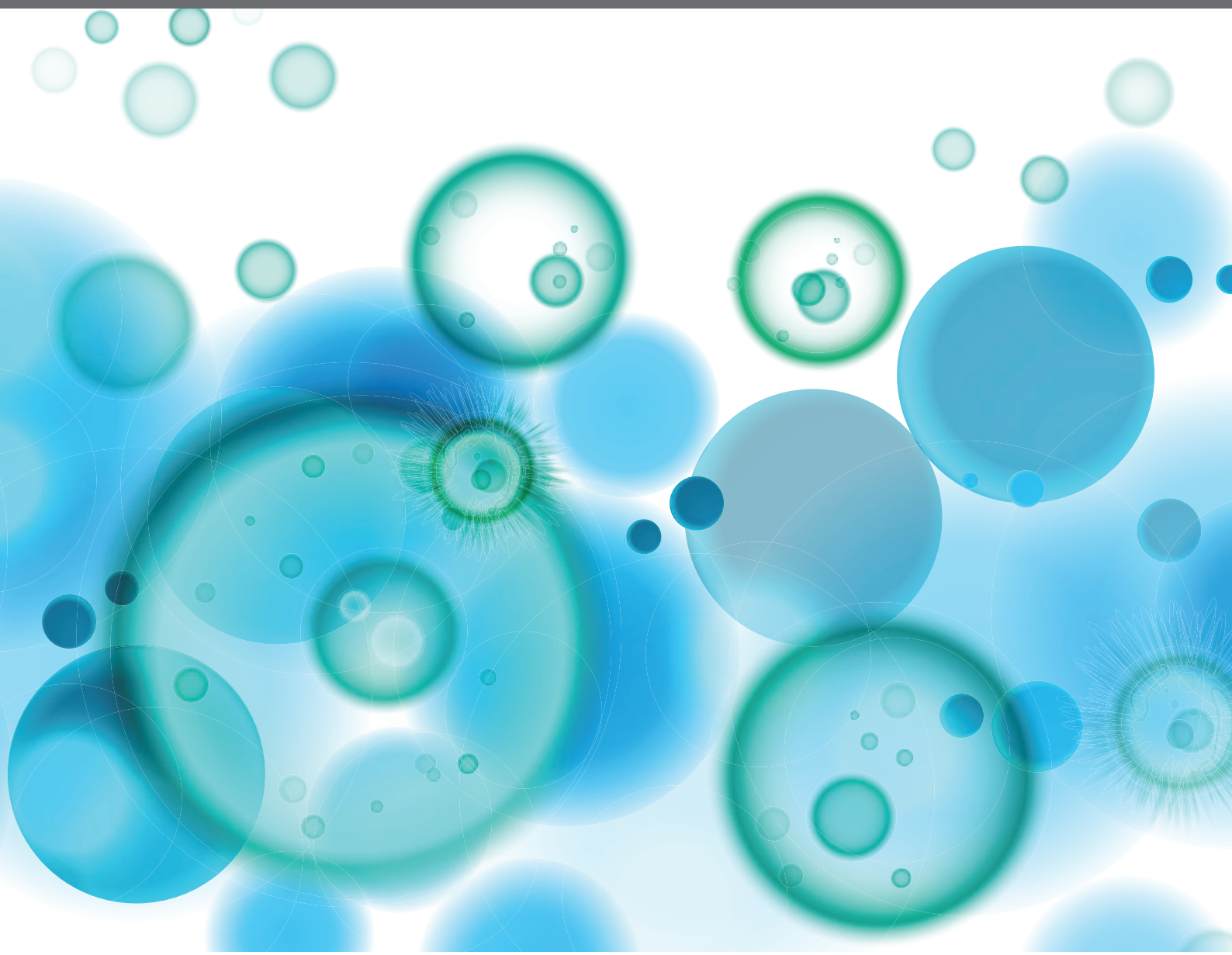

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DIFFERENTIATION AND MECHANISMS OF ACTIVATION OF INNATE LYMPHOID CELLS

Topic Editors:
Marina Cella, Washington University School of Medicine in St. Louis, United States
Chiara Romagnani, Deutsches Rheuma-Forschungszentrum (DRFZ), Germany

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REVIEW
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Group 2 Innate Lymphoid Cells in Pulmonary Immunity and Tissue Homeostasis

Barbara C. Mindt[1,2,3], *Jörg H. Fritz*[1,2,3,4*†] *and Claudia U. Duerr*[1,2,3,5*†]

[1] Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada, [2] McGill University Research Centre on Complex Traits (MRCCT), McGill University, Montreal, QC, Canada, [3] FOCiS Centre of Excellence in Translational Immunology (CETI), McGill University, Montreal, QC, Canada, [4] Department of Physiology, McGill University, Montreal, QC, Canada, [5] Institute of Microbiology and Infection Immunology, Charité – University Medical Centre Berlin, Berlin, Germany

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Edited by:
Chiara Romagnani, Deutsches Rheuma-Forschungszentrum (DRFZ), Germany

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***Correspondence:**
Jörg H. Fritz
jorg.fritz@mcgill.ca;
Claudia U. Duerr
claudia.duerr@charite.de

†These authors have contributed equally to this work.

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Group 2 innate lymphoid cells (ILC2) represent an evolutionary rather old but only recently identified member of the family of innate lymphoid cells and have received much attention since their detailed description in 2010. They can orchestrate innate as well as adaptive immune responses as they interact with and influence several immune and non-immune cell populations. Moreover, ILC2 are able to rapidly secrete large amounts of type 2 cytokines that can contribute to protective but also detrimental host immune responses depending on timing, location, and physiological context. Interestingly, ILC2, despite their scarcity, are the dominant innate lymphoid cell population in the lung, indicating a key role as first responders and amplifiers upon immune challenge at this site. In addition, the recently described tissue residency of ILC2 further underlines the importance of their respective microenvironment. In this review, we provide an overview of lung physiology including a description of the most prominent pulmonary resident cells together with a review of known and potential ILC2 interactions within this unique environment. We will further outline recent observations regarding pulmonary ILC2 during immune challenge including respiratory infections and discuss different models and approaches to study ILC2 biology in the lung.

Keywords: group 2 innate lymphoid cells, respiratory tract, innate immune responses, lung physiology, pulmonary microenvironment

INTRODUCTION

Group 2 innate lymphoid cells (ILC2) have been identified less than a decade ago and constitute a new member of the family of innate lymphoid cells (1–3). From an evolutionary aspect, ILC2 are thought to be a rather old cell type with ancestor populations proposed in lamprey and bony fish (4). The indication of an innate lymphoid cell population linked to a type 2 immune response was first made in the 2000s: in mice, an IL-25-inducible non-T non-B cell population was reported to release large amounts of IL-5 and IL-13 (5), and in humans, a $CD34^+$ population expressing both, IL-33R and TSLPR, was identified and shown to secrete type 2 signature cytokines upon stimulation with IL-33 or in combination with TSLP (6). In 2010, ILC2 were eventually described in detail and were discovered to reside in distinct tissues in mice and termed nuocytes, natural helper cells, or innate helper cells (3, 7, 8). Despite the identification of ILC2 in various anatomical sites such as adipose tissue, liver, mesenteric lymph nodes (LNs), and the small intestinal lamina propria,

common characteristics of ILC2 became apparent from these early studies. Those include their cytokine receptor expression profile, signature cytokines that are released, as well as their characteristic transcription factors. Thus, nomenclature of ILC2 was unified rather soon after their discovery (9). Until now, ILC2 have been described in mice and humans at varying body sites but with many overlapping characteristics: ILC2 share the ability to produce large amounts of type 2 cytokines within a short period of time and have common basic phenotypic characteristics regarding maintenance, regulation of surface molecule (e.g., CD127 and CD25) and transcription factor (e.g. GATA3) expression (9). However, it was also observed quickly that some variations in their typical phenotypic profile exist depending on the anatomical site and/or maturation level (e.g., expression of c-kit) (10). The location of ILC2 is not limited to mucosal sites, but due to their innate character and fast and immediate action, ILC2 are thought to be especially important at barrier surfaces. For example, in pulmonary immunity, ILC2 play a non-redundant role and are able to trigger airway hyper-reactivity even in the absence of adaptive immunity (7). However, it is clear that ILC2 are not isolated in their action and that their function and physiological role is regulated by and regulates other pulmonary hematopoietic and non-hematopoietic cells. In contrast to T helper 2 (Th2) cells, their adaptive counterparts, current evidence supports the notion that ILC2 are tissue resident and thereby act as key players within their corresponding tissue microenvironment (11, 12). Although all ILC types can be found at the mucosal surface of the small intestine, ILC2 are the predominant ILC population in the lungs at steady state. The reason for this skewed distribution of ILC populations in the lungs is not yet fully understood. However, the lungs provide without a doubt a unique microenvironment for ILC2.

In this review, we therefore focus on the pulmonary microenvironment, its physiology regulated by non-hematopoietic resident cells, and how ILC2 functionality is embedded within. We will further discuss different pulmonary infection and cytokine challenge models in which ILC2 effector functions have been shown to play important roles. We will only briefly address plasticity of ILC2 and their regulation since both have been recently discussed in an excellent review (13).

LUNG PHYSIOLOGY AND THE PULMONARY MICROENVIRONMENT OF ILC2

Group 2 innate lymphoid cells have been isolated from and were described in various tissues of the respiratory system. These reports include ILC2 from both mouse and human lungs (3, 7, 14–16) and adjacent sites such as human nasal and tonsil tissues (17). We will therefore give a short overview of our respiratory system and the main populations of tissue-resident cell populations focusing mainly on non-hematopoietic cells (**Figure 1**).

Our respiratory system can be divided into the upper and lower airways. The upper airways span from the nose (nasal cavity/nasopharynx) to the pharynx (oropharynx and laryngopharynx) and further down to the trachea and bronchi. The bronchi branch into the smaller terminal and subsequently into the respiratory bronchioles from which the alveolar ducts are generated and end in the alveolar sacs consisting of several alveoli. The alveolar ducts and the alveoli compose the lower airways. The main responsibility of the respiratory system including these different parts is to ensure efficient gas exchange and thereby provide oxygen to the organism in exchange for carbon dioxide. To accomplish this, air is inhaled at a rate of several liters each minute in the resting state and delivered to this network of branching tubes. The blind-ended alveoli are the place of efficient exchange of carbon dioxide to oxygen. All other parts of the airways can be envisioned in an oversimplified way as a conduit, which serves for the airflow to reach the alveoli. However, the delivery of inhaled air through this conduit, which may be loaded with potential antigens, allergens, and pathogens, is controlled by mechanical filters, physiological barriers, active expel, and immune defense mechanisms (18). These tasks are accomplished by the interaction and interplay of approximately 40 distinct cell populations in the lungs (19). The well-organized and delicate structure of our respiratory immune system and its immediate surrounding is therefore a unique microenvironment for ILC2.

The Upper or Conducting Airways

The upper airways span from the nose and trachea to the bronchi (primary, secondary, and tertiary) and further down to the bronchioles (terminal and respiratory). The lungs start at the first branching point of the bronchi from the trachea. The character of the tissue of the bronchi still resembles the trachea with a pseudostratified, ciliated columnar epithelium including goblet cells, mucus glands, and cartilage. The cells of the epithelial layer are tightly interlinked *via* adhesion junctions composed of tight junctions, adherens junctions, and desmosomes, thereby establishing a firm physical barrier (20, 21). Throughout the branching of the tubular network, the epithelium changes its composition from the characteristic ciliated columnar epithelium in the trachea and larger bronchi to a mix of non-ciliated and ciliated cells in the bronchioles and terminal bronchioles to the respiratory bronchioles with rare occurrence of ciliated cells (18). Several different epithelial and non-epithelial lung resident cells will be discussed below.

Goblet Cells

Goblet cells are defined by their goblet or cup-like shape that is acquired by their inner cellular structure of secretory granulae filled with mucins (22). Goblet cells are present in humans in the trachea and bronchi as well as in the submucosal glands at steady state and absent in the smaller branching of the airways. However, they can be induced upon infection or challenge. In the non-challenged laboratory mouse, goblet cells are mainly restricted to submucosal glands but can be induced upon challenge in trachea, larger bronchi, and even in the bronchioles. The distribution of goblet cells is an important difference between mouse and human physiology (23). IL-13 is key for goblet cell hyperplasia and function such as mucus secretion (24, 25) and is also one of the signature cytokines that is produced and released by ILC2 (26).

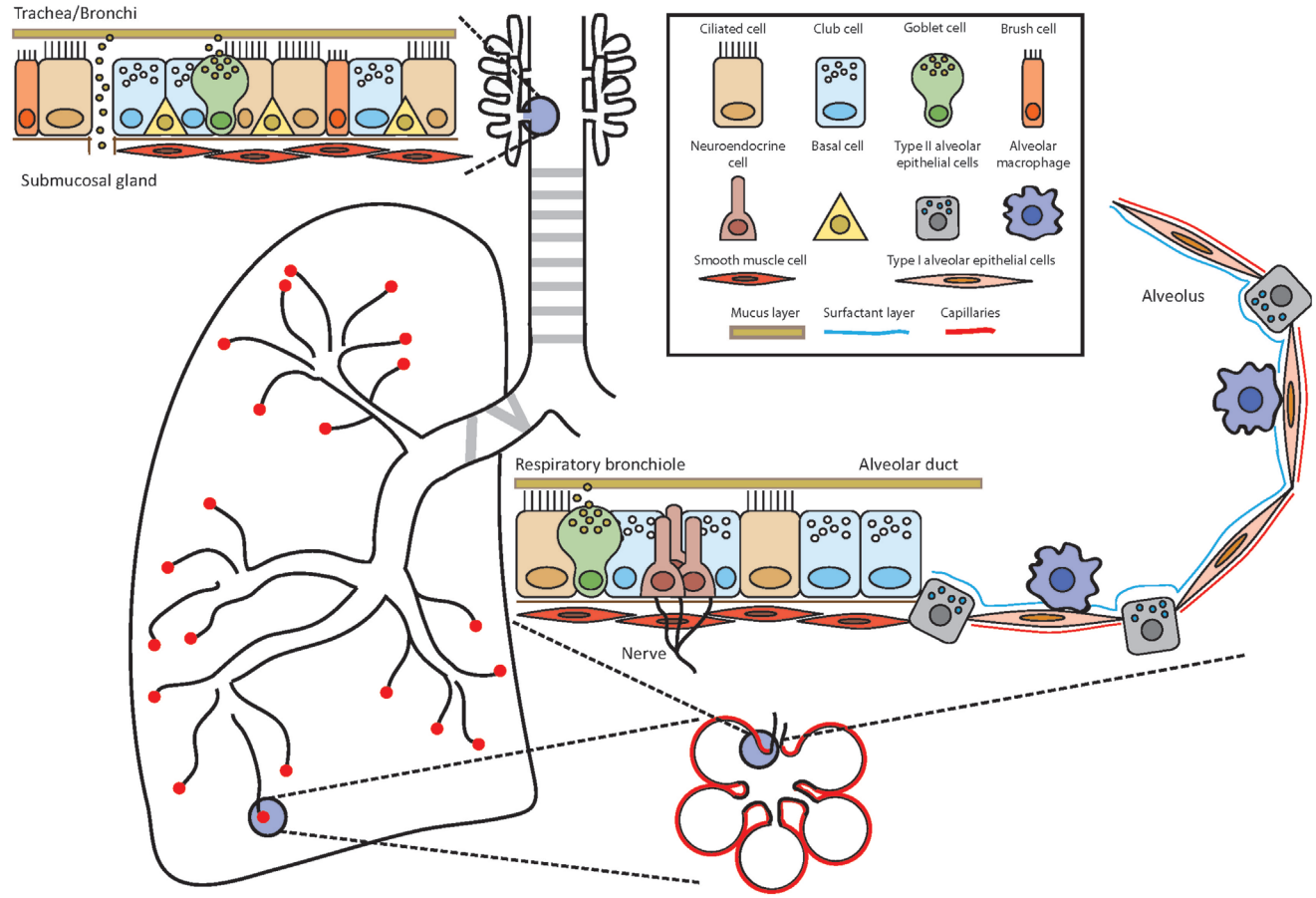

FIGURE 1 | Anatomy of the airways. Throughout the airways, the cell composition of the epithelium changes to ensure optimal and efficient gas exchange as well as maintenance of lung integrity and defense against potential pathogens and allergens. Depending on the location, the epithelium is composed of several different cell types such as ciliated cells, club cells, brush cells, goblet cells, airway smooth muscle cells, neuroendocrine cells, type I and type II alveolar epithelial cells, and alveolar macrophages. Basal cells have the potential to differentiate into several lineages and serve as stem cells.

Club Cells

Club cells or bronchiolar exocrine cells have characteristic short microvilli, a dome shape, and are present in bronchioles (terminal to respiratory). Club cells secrete surfactant proteins (surfactant protein A, B, and D) and express Clara cell 10 kDa protein (CC10, *Scgb1a1*), which can bind to surfactant lipids. Club cells are able to self-renew but also to differentiate into ciliated cells to regenerate the epithelium (27) as well as into goblet cells (28).

Pulmonary Neuroendocrine Cells (PNECs)

Pulmonary neuroendocrine cells are a rare cell population, which represent only approximately 1% of the airway epithelium. PNECs are present in clusters, termed neuroepithelial bodies, and are located at airway branching points. They are an innervated epithelial cell population (29), sense changes in oxygen levels, and release neuropeptides (calcitonin gene-related peptide), neurotransmitters (serotonin), and bombesin-related peptide (Neuromedin B) (30). In addition, PNECs play a role in the modulation of smooth muscle tonus. Since PNECs transmit environmental signals *via* the rich network of neural fibers (postganglionic parasympathetic neurons and the vagus nerve) to the central nervous system, they serve as a link between the nervous and endocrine system. A role of PNECs in immune responses and tissue remodeling has been recently reported (31) and deregulated PNECs are associated with different respiratory diseases such as chronic obstructive pulmonary disease (COPD) (32) or asthma (33).

Brush (Microvillous) Cells

The role of brush cells (Tuft cells, caveolated, multivesicular, and fibrilovesicular cells) in normal airways and alveoli is poorly understood yet, albeit their existence has been known for some time (19). Brush cells are pear or flask-like shaped cells (wide base and narrow microvillous apex) with a tuft of blant and broad, squat microvilli. They have first been described in the airway epithelium and later in alveoli (alveolar lining) as the third pneumocyte in addition to type I and type II airway epithelial cells (34). In humans, brush cells exist from the nose to the alveoli, but are only present in alveoli in disease states (35). In the mouse, brush cells are abundant in the trachea (36). Interestingly, brush cells express (bitter) taste receptors and are able to regulate breathing by signal transmission to neurons of the vagus nerve (36, 37). Brush cells

are not only present in the mucosa of the respiratory tract but also in the small intestine. Here, brush cells are termed tuft cells and have recently been shown to constitute an important source of the ILC2-stimulating cytokine IL-25 (38–40). Furthermore, IL-13 secreted by ILC2 is able to induce tuft cell hyperplasia in the small intestine, indicating a positive feed forward loop (38–40). However, although IL-25 can be detected in pulmonary tuft cells as well, it is currently unknown whether a similar regulatory interaction between tuft cells and ILC2 is also of functional relevance in the lungs. Upon intranasal administration, IL-25 is significantly less potent in eliciting a pulmonary type 2 immune response compared with IL-33 and also does not increase pulmonary ILC2 (41, 42). The mode of IL-25 administration (intranasal vs systemic) is also key in eliciting different ILC2 populations in the lung (43, 44) and as such it is not known yet if and to what extent brush cells are involved in induction of different pulmonary ILC2 subgroups.

Nerve Cells (Neurons)

Neurons have a very characteristic shape due to their function: their cell body is surrounded by dendrites to carry (electrical) impulses to the cell and a long axon is used to transfer impulses to other cells. Impulses are transmitted *via* the synapse, a gap between the axon of one neuron and the dendrites of another neuron. The upper and lower airways are innervated with neurons of nerves such as the vagus nerve (pneumogastric nerve, cranial nerve X) emerging from the base of the brain to the chest and lungs, and then passing the heart to the colon. The vagus nerve belongs to the parasympathetic division of the (peripheral) autonomic nervous system, which controls involuntary processes such as the regulation of the heart and respiratory rate (*via* smooth muscle cells) as well as the orchestration of glands and mucous secretion (45). The axons of the vagus nerve's neurons branch off into all parts of the airways. Vagus nerve stimulation leads to constriction of the airways and is essential for actual breathing (46). Spinal nerves transmit impulses between the spinal cord and the body. Several different spinal nerves are present in the body amongst them are the twelve pairs of thoracic nerves, which emerge from the thoracic vertebrae and are further divided into proximal and distal branches. Clusters of neuron cell bodies (soma) make up a ganglion and can be identified as swellings along nerves. In addition, impulses for defense mechanisms are initiated such as coughing, which is often deregulated and/or dysfunctional in airway diseases (47, 48). The neuro-immune axis is increasingly becoming the central focus of current pulmonary research and a link between ILC2 function and neurons has been described just recently (see below).

Airway Smooth Muscle Cells (ASM)

Airway smooth muscle cells cover the complex branching network of the bronchial tree in a circumferential fashion and perpendicular to the axis of the tube (49). At the start of the trachea, ASM lie in an almost complete sheet of smooth muscle in the posterior wall of the trachea at the opening of the C-shaped cartilage. This almost complete ring of ASM breaks down in the main bronchi between the ends of the U-shaped cartilage rings. In the subsequent bronchi, ASM form discontinuous bands between outside extremities of each cartilage ring. These muscular walls with cartilage rings extend from the larynx over the primary bronchus to the smaller bronchi in humans. From the bronchioles onward, the smooth muscle fibers lie between adventitial tissue and epithelium and the cartilage decreases with the presence of these incomplete muscular walls. In mice, no cartilage plates are present in the lower airways, and ASM muscle bundles describe helical patterns from the medium bronchi to the alveolar duct. Thereby, ASM build an important support structure for the lungs and prevent its collapse. Moreover, ASM are key for the contraction of the airways. But ASM may also play an important immunological role in the lungs with the expression of immune mediators and cytokines or their expression of (bitter) taste receptors (50). The implication of ASM in asthma has been reported almost a century ago since ASM mass is increased in asthma patients (51). However, the exact contribution and function of ASM in respiratory diseases, their cytokine secretion, induction of proliferation, as well as their potential interaction or competition with other pulmonary cell populations still needs to be elucidated in detail.

Stem Cells

The lungs show a low regenerative rate at steady state in contrast to other mucosal tissues such as the small intestine. However, upon infection or challenge, the lungs are able to initiate a high-regenerative turnover program (52). Basal cells have been described as the putative stem cells, are shared between bronchioles and alveoli, and have been suggested to generate ciliated, mucous, and neuroendocrine cells (23). In 2005, these basal cells were termed bronchioalveolar stem cells and their importance for lung regeneration but also for cancer was described (53). Recently, two reports identified two slightly different stem cell populations in mice upon influenza virus infection (54, 55). These stem cells have epithelial characteristics with the formation of Krt-5$^+$ cell clusters (pods) as an important and characteristic feature. Interestingly, these cells are able to migrate from a distal position to the injured tissue area, where they serve as progenitors for different cellular lineages such as type I and type II pneumocytes (54, 55). However, repair mechanisms in human lungs are far less understood than in mice.

Submucosal Glands

Mucus glands are present in the lamina propria of the trachea and bronchi but absent in the distal tubular network (18). Their lamina propria is a loose and collagen rich structure, which supports the pseudostratified columnar epithelium of the trachea and bronchi.

Lower or Respiratory Airways

The alveoli of the lower airways are the place where oxygen is drawn and carbon dioxide is discarded. Whereas no cartilage is present in the alveolar duct and alveoli, smooth muscle cells are quite rare in the alveolar duct and absent in the alveoli. The delicate organization of the alveoli provides the perfect physiological anatomy to ensure this exchange. The alveoli are lined by type I and type II alveolar epithelial cells (pneumocytes). Type I cells are flat and spread out and thereby cover approximately 90% of the alveolus' surface area even if they are not the most

frequent cell population in numbers. Thereby, they provide a thin cellular layer, which serves as an optimal respiratory surface. Importantly, type I cells are not able to divide but can be restored by type II cells differentiating into type I cells (56). Type II cells are of cuboid shape and have characteristic microvilli on their apical surface and lipid rich lamellar bodies containing surfactant proteins. Surfactant proteins cover the alveolus surface in a thin layer and are important to prevent a collapse of the alveoli during breathing but also exert antimicrobial activity (57). Type II cells also produce cytokines such as TNFα (58). Alveolar macrophages are situated in close proximity to type I or type II cells lining the alveolus as well as in the alveolar space. Moreover, type I cells are in close contact to the endothelial cells of the alveolar capillary which are key for gas exchange. The alveolar capillaries are circumventing alveoli with a network so that every capillary is lining two alveoli, one from each side, thereby an optimal gas exchange is guaranteed. The alveolar capillaries are present within the septae, which separate the alveoli from each other. Interestingly, each alveolus is not isolated but connected to adjacent alveoli by discrete holes, termed the pores of Kohn or interalveolar connections (59). These alveolar pores have been proposed to play a role in ensuring that the lungs do not collapse and equalize pressure. They are important in collateral ventilation and ensure minimal ventilation even if the lung partially deflates. However, other observations suggest that these pores are not empty (60), which could indicate that these interalveolar connections allow spreading of small particles and other infectious material such as bacteria or viruses. Moreover, it has been suggested that immune cells such as alveolar macrophages are able to migrate through these pores (61). It is tempting to speculate that upon challenge pulmonary ILC2 might use these pores as a passageway to get from one alveolus to another and thereby survey the tissue.

The organization of human and mouse lungs is very similar in general; however, several important differences exist, and some of them have been already mentioned in this review. For the sake of completeness, we would like to point out that unlike in humans, the mouse lung consists of five lobes: the left lung, and the right lung with the superior, middle, inferior, and post-caval lobes. By contrast, the human lung is composed of the two lobes (upper and lower lobe) of the left lung, and three lobes of the right lung (upper, middle, and lower). In addition, the branching pattern of the airways is less complex in mice compared to humans (62, 63), with the predominant cell types of the conducting airways being ciliated cells in humans, whereas more secretory cells are present in mice (64, 65).

DEFINITION AND CHARACTERISTICS OF PULMONARY ILC2

The definition of pulmonary ILC2 was shaped by their first identification in cytokine reporter mice. Here, pulmonary ILC2 were defined by their respective reporter signal in IL-4 (4get) and IL-13 (YetCre-13) reporter animals (3). Later on, ILC2 were described in the lungs of non-reporter and wild-type mice. Although the gating strategies vary, ILC2 are in general defined as lineage negative cells, which are positive for Thy1, Sca-1, GATA-3, T1/ST2, inducible T cell costimulator (ICOS), CD44, CD25, CD127, KLRG1, and c-kit low to positive (7, 14, 66–68). These characteristics of pulmonary ILC2 also are consistent with ILC2 at other mucosal sites such as the gut (2, 67, 69). To the best of our knowledge, there is so far no sole known (surface) marker or characteristic that defines ILC2 at one site but is not present or inducible at any other site. This is supported by the current concept that ILC2 represent one group of innate lymphoid cells, but that this group is not always homogenous. Single-cell sequencing analysis of ILC2 cells at steady state in the small intestine showed that ILC2 are indeed heterogeneous and several subgroups with different gene expression patterns were identified (70). Moreover, an approach using signature type 2 cytokine reporter mice revealed that pulmonary ILC2 exhibit different mRNA expression patterns of these cytokines when compared with small intestinal ILC2. *Il5* is expressed at steady state in both pulmonary and intestinal ILC2, whereas *Il13* is continuously expressed in small intestinal ILC2, but can be induced in pulmonary ILC2 upon challenge (71). In the lungs, inflammatory (i) and natural ILC2 (nILC) have been reported upon systemic administration of IL-25 or IL-33. Although these ILC2 subgroups are well defined by their slightly different surface markers, namely high killer cell lectin-like receptor subfamily G member 1 (KLRG1) expression for iILC2 and ST2 expression for nILC2, a transition has been noted between iILC2 and nILC2, highlighting a strong relation of these two populations (43). These reports underline that ILC2 belong to one group within the ILC family but that ILC2 biology and characteristics are highly influenced and shaped by their respective microenvironment, its immunological profile at steady state as well as upon immune challenge.

Our knowledge of human pulmonary ILC2 is limited at the moment due to the fact that the accessibility of human lung samples is highly restricted. The information about human ILC2 originates from deceased patient material (organ donors) or from surgeries of the lungs such as routine bronchoscopy upon lung transplantation. In those tissues, ILC2 can be detected and defined as lineage negative, $CD127^+$ $CD25^+$ and $ST2^+$ (14) and thereby share important characteristics with mouse ILC2. In lungs of idiopathic pulmonary fibrosis and COPD patients, ILC2 were identified as lineage negative, $CD127^+$ $ST2^+$ ILC2, or lineage negative, $CD127^+$ $CRTH2^+$ ILC2. $ST2^+$ ILC2 show high expression of arginase 1, which was identified in this elegant study as an important characteristic for ILC2 functionality (15). Human ILC2 obtained from patients undergoing lung tumor surgery can be identified as $CD45^+$ and $CD127^+$ cells upon excluding lineage positive cells. Interestingly, compared with ILC2 from nasal polyps of chronic rhinosinusitis patients, these pulmonary ILC2 exhibit differences in their pattern of CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) and c-kit expression (16), which might be due to their distinct microenvironment. Furthermore, CRTH2 was recently identified to play a role in ILC2 accumulation in the lungs of human and mice, and differences in CRTH2 expression between human peripheral blood (high) and pulmonary (low) ILC2 ($lineage^-$ $CD127^+CD45^+ST2^+$) may as such determine tissue localization and function (72). These data underline that human and mouse ILC2 share biologically important similarities.

TISSUE RESIDENCY AND LOCAL DISTRIBUTION OF PULMONARY ILC2

In the lungs, the presence of hematopoietic cells at steady state is relatively low compared with other mucosal sites such as the small intestine. However, B and T cells [including T regulatory (Treg) cells], alveolar and interstitial macrophages, different types of dendritic cells, and ILC can be detected in addition to resident, non-hematopoietic cells (described above). Interestingly, ILC2 are the predominant ILC population in the lungs at steady state (14). Although the localization and migration capacity of ILC2 determine their (potential) interaction partners, we are only just beginning to understand these dynamics.

Based on observations made by two recent studies using a parabiosis approach, ILC2 are regarded as tissue-resident cells. No significant numbers of ILC2 of the parabiont host were found in the lungs within 4 months of parabiosis (11). Strikingly, even within one week post *Nippostrongylus brasiliensis* infection, only host ILC2 were detected in the lungs, underlining the tissue residency of ILC2. However, two weeks post-infection a significant increase of donor-derived ILC2 were detected in lung, small intestine, and mLN, while no changes were observed for ILC3 in the small intestine. This might reflect the limited local proliferation of the small and insignificant number of donor ILC2 during the infection. In another elegant study, Koyasu and colleagues analyzed parabiotic pairs after more than one month of shared circulation for pulmonary T cells, ILC2, and eosinophils at steady state, or up to one week post intratracheal IL-33 challenge. Whereas T cells and eosinophils are present from donor and host in this parabiotic pair, ILC2 of the parabiotic donor could not be detected (12), underlining that ILC2 remain local and do not migrate into the parabiotic host. Both reports elegantly show and strengthen the current notion that ILC2 are tissue-resident cells that primarily retain their tissue residency at steady state as well as during immune challenge. Interestingly, ILC2 have been shown to accumulate in the mouse lungs during ontogeny with ILC2 numbers peaking at about two weeks after birth (73, 74). The ILC2 of these immature mice exhibit increased levels of intracellular cytokines when compared with ILC2 in adult animals and are to some extent dependent on IL-33. Upon challenge with house dust mite (HDM), more IL-33 protein can be detected in lungs of immature mice compared to adult mice accelerating innate as well as adaptive type 2 immune responses (73, 74). However, further research will be needed to better understand how ILC2 are directed to find their respective tissue niches during ontogeny. Moreover, since ILC2 are observed in lungs at steady state and upon challenge, the question remains where within the tissue these cells are exactly located, how they take part in tissue surveillance and which cellular interactions with other resident and infiltrating cells are established, supported, and needed for maintenance and function in acute and chronic lung diseases.

In addition, it will be of great interest to understand how and under which circumstances ILC2 egress from the bone marrow as well as how they migrate between distinct peripheral tissues. A recent study by Stier and colleagues sheds light on the migratory ability of ILC2. They observed that IL-33 is key in regulating ILC2 egress from the bone marrow (75) since ILC2P in IL-33-deficient mice show similar fitness to wild-type mice but are retained in the bone marrow due to higher expression of CXCR4. In addition, by using an elegant combination of parabiosis and tissue injury, they show that ILC2 are capable of migration after sublethal irradiation and thereby repopulating the empty ILC2 niche. This report demonstrates that under specific circumstances ILC2 are indeed able to traffic, even if mainly regarded as a tissue-resident population. In this report, ILC2 are identified by their expression of the IL-25 receptor chain (IL-25R or IL-17RB), which identifies ILC2 progenitors in the bone marrow (76) but limits detection to iILC2 (43) as well as memory ILC2 (77) in peripheral tissue. If this holds true for ILC2 in general including nILC2, which are in contrast identified based on lower levels of KLRG1 and alternatively by GATA3 expression, still needs to be elucidated. Another elegant report using parabiotic mice identifies that iILC2 are able to migrate from the small intestine to the lungs upon systemic (i.p.) IL-25 administration or helminth infection (78). This study further shows that especially intestinal iILC2 have the potential to migrate and that this migration is regulated in a S1P-dependent manner. Interestingly, upon systemic IL-25 challenge, nILC2 reside in the alveolar space in contrast to iILC2, which are confined to the vascular space.

In addition, S1PR1 (*S1pr1*) and L-selectin (CD62L, *Sell*) expression was detected in naïve lung ILC2 and decreased upon stimulation with IL-33 (77). The adaptive counterpart of ILC2, T cells are guided by S1PR1, which binds to S1P1, to exit secondary lymphoid organs and the thymus (79). Moreover, ILC2 have been detected in the mesenteric LN upon systemic challenge (2) but also in the mediastinal LN of the lungs upon intranasal administration of IL-33 or papain (77). This might be due to proliferation of local tissue-resident ILC2 or active migration. However, the ability of homing of ILC2 from distal sites, such as the lung or small intestinal tissue, to the respective draining LNs is only beginning to be understood. In an elegant study using *Kaede* transgenic mice in which distinct cell movements can be tracked *via* a photoconvertible fluorescent protein, Mackley and colleagues show that all ILC populations constitutively traffic from the small intestine to the mLN (80). Interestingly, this migration is only dependent on the CCR7 receptor for LTi-like ILC3, but not for ILC1 or ILC2 (80). Moreover, opposite trafficking from the mLN to the small intestine is dependent on CCR7 for ILC1 and ILC3 and their further migration to the small intestine is regulated by a retinoic acid regulated homing receptor switch in contrast to ILC2 (81), further indicating that different homing programs exist for the different ILC populations. Importantly, immunofluorescence analysis revealed that ILC2 and ILC3 are located in close proximity to each other, within the interfollicular space, in the mLN (80). Whereas both of these ILC populations as well as ILC1 are present in all investigated LNs, this close proximity of ILC3 and ILC2 was only observed in the mLN (80). Interestingly, ILC3 are most prominent in wild-type mice in the mLN, but the ratio is shifted to higher levels of ILC2 in Rag-deficient mice (80). How this imbalance is caused has not been fully understood but a different activation and metabolic state in ILC3 in Rag1-deficient mice compared with wild-type mice has been recently reported (82)

and this might in addition interfere with the trafficking ability of ILC3. However, whether communication between ILC2 and ILC3 in the special microenvironment of the mLN and in draining LNs exists and if this is a general phenomenon still needs to be determined.

Thus, ILC2 might support immune reactions not by circulating through the blood to reach peripheral tissues, but by traveling rather short distances to a respective LN. It is tempting to speculate that ILC2 might have a special function within the elicited immune response at a defined location similar to what has been described for natural killer (NK) cells (83). However, the importance of the microenvironment for ILC2 in the mediastinal LN and the regulatory mechanisms of homing to and from it as well as to the lungs still need to be elucidated.

In contrast to mice, ILC2 have been identified in the blood of humans since their detailed description (16). Lombardi et al. investigated patients with allergic rhinoconjunctivitis with or without asthma and healthy, non-allergic individuals (84). Surprisingly, the number of ILC2 as well as cytokine production profile is similar between healthy donors and allergic subjects. However, ILC2 are slightly increased in asthmatic patients compared with non-asthmatic within the group of allergic subjects. However, this trend is also observed with peripheral ILC3. mRNA sequencing comparing allergic and non-allergic subjects revealed an increase of genes involved in the activator protein 1 molecule and suggests a higher activity of its related pathway. Importantly, surface expression of the CCR10 receptor on ILC2 was significantly elevated in allergic compared with non-allergic individuals and expression of CCR10 can be correlated to increased severity of asthma. However, no change was observed in CCR4 expression in peripheral blood of allergic and non-allergic individuals (84).

Since ILC2 are the dominant population of ILC in the lungs, it is important to determine their location(s) to understand their biology, cellular interaction partners, as well as the influence of the pulmonary microenvironment on their function. The first report on the localization of pulmonary ILC2 identified them in collagen rich structures in close proximity to the airways at steady state by using an IL-5 reporter mouse (71). This location was further defined to be close to the epithelium and small conducting airways (8). Upon IL-33 challenge, pulmonary ILC2 are often situated in clusters that are located in the vicinity to the airway epithelium as well as in the alveolar space. The formation of these ILC2 clusters is not limited to cytokine administration and has been also detected in alveolar spaces of helminth-infected mice (*N. brasiliensis*, two weeks post-infection) (8). Interestingly, clusters of ILC2 are situated close to infected foci/cells of bronchioles and alveoli upon influenza infection (ST2-GFP-reporter mice) (85). Upon pneumectomy, ILC2 are induced and have been identified in the conducting airways and in the alveolar space using IL-5 reporter mice (86). Recent reports further identified that ILC2 are located in the vicinity of (enteric) neurons (41, 87).

In summary, ILC2 are located at peripheral and central sites of the lungs at steady state and are present in clusters within affected cellular foci upon challenge. The appearance of these ILC2 clusters is interesting and raises the question of whether ILC2 actively proliferate at these locations due to stimuli provided by their infected microenvironment or/and if they migrate from more peripheral locations toward these infected foci. Moreover, it will be of interest to decipher whether pulmonary ILC2 are able to keep the lung tissue under surveillance similar to skin ILC2 (88), and thereby actively contribute to the homeostasis of the lungs. In addition, passing and migrating through the pores of Kohn would be a huge advantage to reach alveoli as speculated above and as was proposed for macrophages (61). However, how and if these migration activities of ILC2 are directed still remains elusive.

PLASTICITY OF PULMONARY ILC2

Group 2 innate lymphoid cells have been originally identified in different organs under different conditions and with slightly different characteristics. The key characteristics of ILC2, which have led to their standardized nomenclature were mainly the expression of the transcription factor GATA3, the expression of surface receptors such as ST2, and the production of type 2 signature cytokines. However, it has been observed that ILC2 are able to adapt due to different challenges and microenvironments. This plasticity of ILC2 changes the typical ILC2 characteristics such as transcription factor expression, cytokine expression, and surface receptor expression or a combination thereof. Upon IL-25 challenge, iILC2, which have migrated from the small intestine, are observed in the lungs (43, 78). iILC2 express both GATA3 and RORγt and have the potential to express IL-17 besides IL-13 in *ex vivo* culture experiments, thereby iILC2 can as well support antifungal immune responses. In addition, iILC2 exhibit plasticity by transitioning to nILC2 characterized by ST2 instead of IL-17RB surface expression under certain conditions (43).

Importantly, ILC2 downregulate the key transcription factor GATA3 upon respiratory challenges of viral or bacterial origin as well as environmental pollution such as cigarette smoke. These converted ILC2 are able to secrete IFN-γ in response to IL-12 and IL-18 (85). Of note, human ILC2 are able to adapt to this ILC1-like phenotype when cultured with IL-12 (89). Moreover, the induction of this conversion in human ILC2 is initiated by IL-1β (89, 90). Interestingly, IL-1β in combination with IL-2 is a strong activator of human peripheral blood ILC2 and signals through NF-κB (89, 90). Thus, ILC2 plasticity is of high importance regarding the development of therapeutics to counter pulmonary type 2 immunopathologies.

CELL SURFACE RECEPTOR–LIGAND INTERACTIONS OF ILC2 WITH OTHER CELLS OF THE PULMONARY ENVIRONMENT

Group 2 innate lymphoid cells express various surface molecules that can interact with their respective ligands on other cells. However, only a small number of all possible cell–cell contacts of ILC2 with other lung populations have been studied so far, as depicted in **Figure 2**, and will be further discussed below.

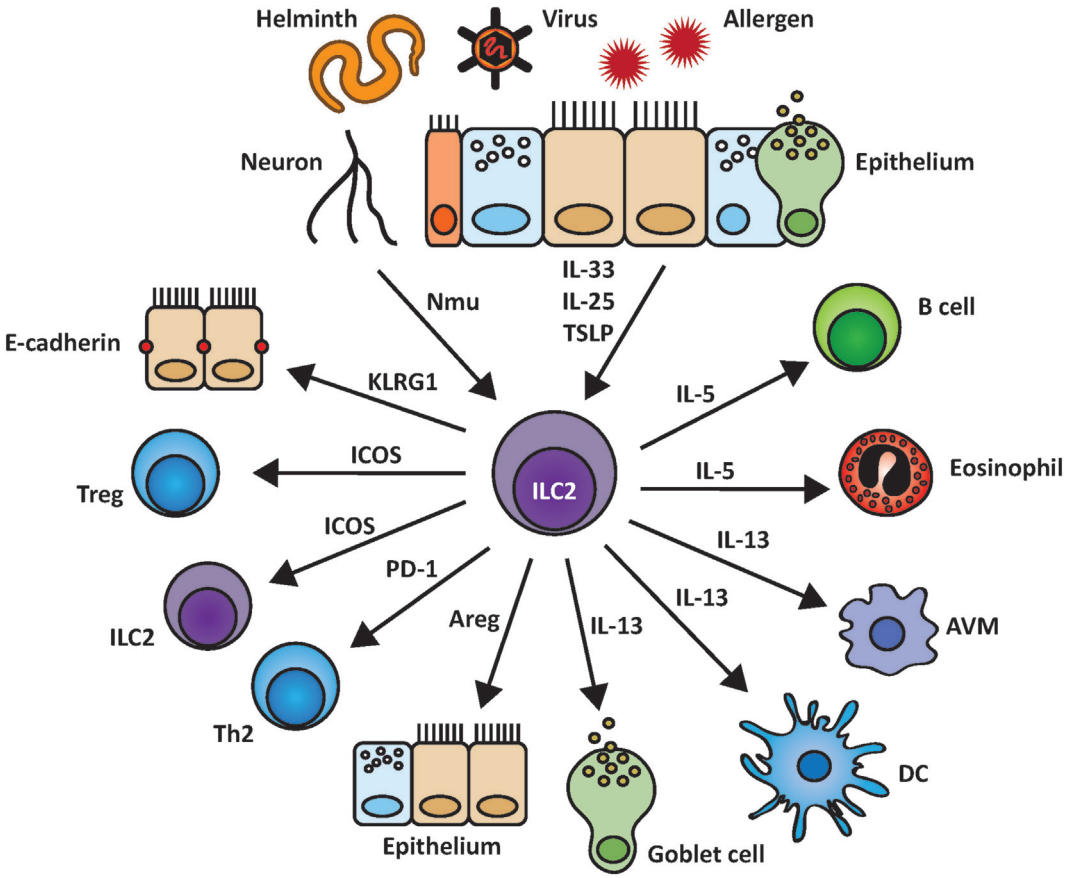

FIGURE 2 | Group 2 innate lymphoid cells (ILC2) and their interactions within the pulmonary environment. ILC2 are induced by epithelial-derived cytokines as well as by other biological mediators such as the neuropeptide neuromedin U (NMU). Upon activation, ILC2 are able to stimulate and communicate with other cell populations by indirect cytokine based or cell–cell mediated interactions including the release of IL-5, IL-13, and amphiregulin (Areg) and binding of KLRG1 to E-cadherin.

ICOS–ICOS-L

Inducible T cell costimulator (CD278) belongs to the CD28 family and is an important molecule in T cell signal transduction (91, 92). ICOS is expressed by mouse and human ILC2 independent of their location, at steady state but also upon stimulation. Deficiency of ICOS or its ligand ICOS-L leads to reduced numbers of ILC2 in the lungs (and small intestine) in combination with lower surface expression of KLRG1 (93). Moreover, ICOS and ICOS-L-deficient mice show reduced levels of pulmonary ILC2 upon intranasal IL-33 stimulation (93, 94). ICOS deficiency in ILC2 in the lungs has also been linked to reduced survival, cytokine production, and pSTAT5, all key for efficient function and signal transduction of ILC2 (94). In the lungs, both, Treg cells and ILC2 are rare at steady state, but can be located at similar sites (8). Interestingly, Tregs have been reported to suppress ILC2 function *via* ICOS and ICOS-L interaction. This interaction appears to take place independent of their location since it was observed in the lungs as well as in visceral adipose tissue (8). However, it is not yet known which signals direct the colocalization of Tregs and ILC2 *in vivo*. Another report investigated the potential of ILC2 suppression by Tregs *via* ICOS–ICOS-L interaction (95). Interestingly, major differences of the suppressive potential of inducible Tregs (iTregs), and natural Tregs (nTregs) were observed. Only iTregs but not nTregs suppressed ILC2 and lead to reduced cytokine secretion by ILC2 (95).

Programmed Death 1 PD-1-PD-L1/PD-L2

Programmed death 1 (CD279) is another member of the CD28 family like ICOS and has been described as an important negative regulator of T cells (96). Recently, PD-1 treatment has been successfully used in cancer therapy (96). Both ligands for PD-1, PD-L1, and PD-L2, are expressed on various immune and non-immune cells (96). Importantly, ILC2 biology has recently been shown to also be influenced by PD-1 and its ligands. PD-1 is a key factor for ILC2 development and function and is expressed on mature ILC2 as well as on ILC2 progenitors (76, 97). PD-1 negatively regulates KLRG1^{+} ILC2 by inhibiting STAT5 phosphorylation, leading to reduced ILC2 proliferation and cytokine production (97). Deficiency in or blocking of PD-1 results in enhanced ILC2 effector function (97). PD-1 and PD-L1 are upregulated on ILC2 upon immune challenge including IL-33 stimulation (97) or *N. brasiliensis*

infection (98). The latter study identified that ILC2 interact *via* PD-L1 with PD-1 on Th2 cells and promote their type 2 effector function (98). Thereby another important regulatory mechanism of ILC2 and their adaptive counterpart, the Th2 cells was identified. Moreover, the importance of the induction of PD-1 or PD-L1 on ILC2 by probably local cells is key to trigger type 2 immune responses.

KLRG1–E-Cadherin

Killer cell lectin-like receptor subfamily G member 1 is expressed on T cells and NK cells and binds to cadherins (E, N, and R). In contrast to N- and R-cadherins, which are expressed by the nervous system, E-cadherin can be found on epithelial cells as well as on Langerhans cells of the skin (99). A suppressive effect of the interaction of E-cadherin with KLRG1 on human skin ILC2 has been shown, resulting in reduced proliferation and cytokine expression as well as downregulation of GATA3 (100). Pulmonary KLRG1$^+$ ILC2 may interact with E-cadherin expressed on lung epithelial cells, especially as part of the adherens junctions, which are located basolateral below the tight junctions and are part of the intercellular junctions formed by the lung epithelium (62, 101). Especially of interest is that high KLRG1 expression has been reported as a surface expression characteristic of iILC2 in the lungs (43). In humans, E-cadherin loss and presence in the sputum correlates with asthma severity (102). However, the exact mechanisms and the downstream signaling pathways within ILC2 upon KLRG1 ligation are not yet understood. Moreover, whether pulmonary ILC2 are able to interact with N- and R-cadherins expressed by the nervous system *via* KLRG1 still remains elusive.

MHC Class II (MHC-II)–TCR

Different populations of ILC, including ILC2, have been reported to express or upregulate MHC-II upon activation (2, 103, 104). However, the role of MHC-II activation for ILC2 function was just recently deciphered (105, 106). ILC2 express MHC-II upon systemic IL-33 activation to a various extent depending on their location (106): pulmonary ILC2 show less MHC-II expression compared with small intestinal ILC2. ILC2 express functional MHC-II and are able to process and present antigen (106). However, MHC-II expression on ILC2 is only stable *in vivo* and ILC2 loose MHC-II cell surface expression when cultured *ex vivo* for longer periods of time (106). The reasons for that are not yet understood. However, the importance of MHC-II on ILC2 was shown to be independent of the expression levels of MHC-II (106). In addition to systemic administration, intranasal administration of IL-33 induces MHC-II expression on a subpopulation of pulmonary ILC2 (106). Interestingly, despite its heterogeneous expression of MHC-II, the resulting ILC2 population is able to present antigen to T cells. Moreover, IL-2 secreted by T cells further supports type 2 cytokine secretion and proliferation of ILC2 in co-culture experiments (105, 106). Thus, MHC-II on intestinal ILC2 is important to amplify type 2 immune responses in intestinal infections (*N. brasiliensis*). Further research will elucidate whether this mechanism also comes into play for the regulation of beneficial and detrimental type 2 immune responses in the lungs.

INDIRECT INTERACTIONS OF ILC2 WITH OTHER CELLS OF THE PULMONARY ENVIRONMENT

In addition to direct cell surface receptor–ligand interactions of ILC2 with other non-hematopoietic and/or hematopoietic cells, ILC2 sense their microenvironment and can be regulated by certain cytokines including IL-25 and IL-33 and other immune mediators, which has been recently reviewed (107). However, expression, processing, and release of mediators regulating ILC2 and their cellular origin at steady state and upon immune challenge are poorly investigated and further research is needed to determine their detailed activation mechanism in the pulmonary environment (**Figure 2**). In addition, cells activating ILC2 are no longer limited to epithelial, endothelial or hematopoietic origin since neurons have been recently added as important regulators of ILC2.

Regulation of ILC2 by Neurons

Neuromedin U (NMU)

Three recent reports demonstrated that the neuropeptide NMU is a potent activator of ILC2 in the intestinal and respiratory tract (41, 87, 108). NMU is expressed by cholinergic neurons that use the neurotransmitter acetylcholine to transmit impulses. Cholinergic neurons are part of the enteric nervous system, the parasympathetic vagal nerve, and the thoracic dorsal root ganglia (described above). NMU signals *via* the receptors NMUR2, which is mainly expressed on nerve cells, and NMUR1, present on ILC2, their progenitors, and to a much lower degree on T cells (41, 87). NMU can be induced by *N. brasiliensis* excretory/secretory products (NES), alarmins (IL-33), and toll like receptor ligands (LPS) in a-MyD88-dependent manner (LPS) (87). Pulmonary ILC2 express NMUR1 at steady state and upon IL-25 stimulation. By contrast, NMUR1 was shown to be downregulated upon IL-33 stimulation (41). Interestingly, NMU and IL-25 act synergistically to upregulate IL-5 and IL-13 mRNA and protein levels when given intranasally over several days (41). NMU alone was shown to be more potent to induce IL-5 and IL-13 mRNA in intestinal than in pulmonary ILC2 in short-term *ex vivo* stimulations (87). NMU signals *via* ERK1/2 and induces Ca^{2+} influx followed by calcineurin and NFAT activation (87). NMU is also induced in the lung and gut during *N. brasiliensis* infection and NMUR1$^+$ ILC2 are superior to NMUR1$^-$ ILC2 in fighting off *N. brasiliensis* infection (108). Thus, NMU is an important activator of pulmonary ILC2 (41, 87, 108).

Vasoactive Intestinal Peptide (VIP)

The neuropeptide VIP has been initially described as a polypeptide isolated from the small intestine with diverse effects on different systems such as cardiovascular and respiratory systems (109). VIP is expressed by neurons of the central and peripheral nervous system and can be transmitted by VIP receptor type 1 (VPAC1) or VIP receptor type 2 (VPAC2), which are differently regulated dependent on cell type and activity state (110, 111). Interestingly, intestinal and pulmonary ILC2 express VPAC1 and VPAC2 and release IL-5 when they are cultured with IL-7 and

VIP- or VPAC2-specific agonist (71). An important link between afferent neurons, VIP, ILC2, and T cells was reported by Talbot and colleagues (112). IL-5 released by ILC2 stimulates nociceptors on afferent neurons and induces the release of VIP, which signals *via* VPAC2 and triggers ILC2 and subsequently T cells to induce more IL-5 and thus creating a type 2 inflammatory feed forward loop highly depending on the neuro-immune axis (112).

Mediators Released by ILC2

Group 2 innate lymphoid cells also actively shape their microenvironment by the release of several different cytokines mainly IL-5 and IL-13 but also IL-9, IL-10, and amphiregulin (Areg). The secretion of these immune mediators can be beneficial but also detrimental for the host and therefore a well-balanced and fine-tuned release by ILC2 cells is needed.

IL-5

Upon activation, ILC2 are able to secrete IL-5, which is important for eosinophil homeostasis (71) and B cell function (1). Both B-1 and B-2 cells are important and prominent populations in the lungs. The production of IgA upon co-culture of mesenteric ILC2 and splenic B cells has been shown to be IL-5 dependent (1). In addition, induction of proliferation as well as IgA, IgM, IgE, and IgG1 secretion by B-1 and B-2 cells by peripheral (peritoneal cavity, spleen) and pulmonary ILC2 was observed in *ex vivo* co-cultures (113). Moreover, pulmonary ILC2 trigger especially IgM secretion by B cells *in vivo* upon NP-Ficoll administration in an IL-5-dependent manner (113). However, how B cells and ILC2 interact within their pulmonary environment *in vivo* still needs further investigation. Moreover, $ICOS^+$ ILC2 play a beneficial role through their secretion of IL-5 in a bleomycin model in the lungs (114). Interestingly, especially the timing of IL-5 secretion appears to be crucial for the beneficial effect in this disease model, (114). However, future experiments will be needed to decipher the cellular and molecular cascade triggered by IL-5 to support tissue restoration and homeostasis.

IL-9

The expression of the IL-9 receptor by ILC2 has been reported since their detailed description in 2010 (3), and the autocrine role of IL-9 on ILC2 function was deciphered by using IL-9 reporter and subsequently IL-9 fate mapping mice (115, 116). IL-9 reporter mice ($IL9^{Cre}R26R^{eYFP}$) were used to initially investigate the origin of this cytokine in an IL-33-dependent, papain-induced lung inflammation model where IL-9 production was restricted to innate lymphoid cells (defined as lineage negative, $CD45^+$, $Thy1^+$ with mixed expression of ST2, CD25, MHCII, and Sca-I). These ILC2-like cells expressed IL-9 only transiently and switched to produce type 2 signature cytokines IL-5 and IL-13. Moreover, IL-33 but not IL-25 induced IL-9 competent ILC. IL-9 production by ILC was induced by IL-2 provided by adaptive immune cells, highlighting again the close relationship between innate and adaptive lymphoid cells. In additional work, IL-9 fate mapping and IL-9R-deficient mice were investigated during *N. brasiliensis* infection and in the latter, less type 2 signature cytokines and Areg were detected indicating that IL-9 is key for efficient ILC2 cytokine production and effector functions. Moreover, IL-9-induced upregulation of BCL3 in these cells is important for their survival (116). Thus, IL-9 acts in an autocrine manner and is important for ILC2 biology. Importantly, IL-2 has been reported to act as a cofactor on ILC2 and increases cell survival and proliferation by triggering NF-κB activation and gene transcription *via* p65 translocation (117). Roediger and colleagues investigated the model of eosinophillic crystalline pneumonia, which spontaneously occurred in Rag-deficient mice in their animal facility. Since several challenges of Rag-deficient mice by IL-2 increased levels of ILC2 (88), $CXCR6^+$ ILC2 were investigated and identified as predominantly located in the perivascular in the lungs. Moreover, IL-2 alone did not affect cytokine expression but survival and proliferation and works synergistically with IL-33 to enhance type 2 cytokine expression. Interestingly, next to T cells, Lin^- $CD90^{hi}$ $CD2^+$ ILC, potentially ILC3, but no myeloid population including dendritic cells and eosinophils were identified as IL-2 producers in the lungs (117). IL-2 and IL-9 are closely interlinked in directing ILC2 biology and enhanced IL-9 expression is linked to an asthma-like phenotype in mice and humans underscoring the importance of these cytokines (118–121).

IL-10

The recently described population of regulatory ILC are able to produce IL-10 (122); however, IL-10 production by ILC2 has already been reported (2). Recently, IL-10-producing ILC2 have been observed upon IL-33 or papain challenge in IL-10 reporter mice (123). Interestingly, IL-10 production of ILC2 is transient and decreases after weeks. Although these IL-10-producing ILC2 have a unique profile, they keep ST2 receptor upregulated and do not express T-bet (123).

IL-13

With the release of IL-13, ILC2 target non-immune and immune cells in the lungs. Through IL-13 release, ILC2 can initiate mucus secretion but also goblet cell hyperplasia. Whereas controlled mucus secretion is needed to repel particles from the lungs, overproduction of IL-13 leads to goblet cell hyperplasia overexpression of mucus and can thereby also negatively affect tight junctions in the lungs (124). However, the exact pattern of IL-13 receptors on cells of pulmonary origin at steady state and during immune challenge is not yet fully understood. Moreover, IL-13 can induce smooth muscle contraction (125). In addition, ILC2 can prime alveolar macrophages during ontogeny by release of IL-13 into a type 2 immune cell phenotype (126). In addition, ILC2 can target dendritic cells and propagate their migration from the lungs to the LNs in an IL-13-dependent manner (127). Thus, ILC2 are able to influence and trigger innate and adaptive pulmonary type 2 immune responses through release of IL-13.

Amphiregulin (Areg)

Group 2 innate lymphoid cells contribute to pulmonary wound healing upon influenza infection *via* the secretion of Areg (14), which belongs to the family of epidermal growth factors (EGF) and signals *via* the EGF receptor (EGFR) (128). Although the detailed expression pattern of EGFR on pulmonary cells is not yet completely elucidated, EGFR has been reported to be expressed by non-hematopoietic and by hematopoietic cells (129). Whereas

wound healing and the initiation of mucus secretion is beneficial in some respiratory diseases, it may also be a disadvantage in diseases with enhanced, adverse, and overproduction of mucus (52). Moreover, enhanced EGFR stimulation or signaling has also been implicated to be detrimental in asthma (130). In addition to ILC2, pulmonary Tregs are also able to secrete Areg upon influenza virus infection independent of TCR signaling (131). As such, innate Areg expression by Tregs and ILC2 constitutes an important mechanism to promote wound healing and tissue homeostasis after pulmonary challenge.

LUNG ILC2 EXERT IMPORTANT EFFECTOR FUNCTIONS IN PULMONARY DISEASES

Respiratory Virus Infections

Lung infections are the most prevalent infections independent of the economic status of a country and thereby represent a significant disease burden (132). Respiratory virus infections have a high infection and mortality rate worldwide with influenza virus infections alone accounting for approximately 250,000 deaths each year (133). Infections with respiratory viruses can affect the upper [rhinovirus and respiratory syncytial virus (RSV)] as well as the lower respiratory tract [(para-)influenza virus] and often occur in combination with asthma and asthma exacerbations (134). Mice are often used to study respiratory virus infections and the virus is in general administered intranasally to anesthetized mice. Importantly, ILC2 have been shown to be induced upon respiratory virus infections in mice and humans (7, 12, 14, 135–138) and induce an asthma-like phenotype in mice even in the absence of adaptive immunity (7).

Influenza A Virus (IAV)

Influenza A virus infection has been shown to induce IL-33 and thereby elicit ILC2 (7, 14, 135). In an infection model using IAV (H3N1 strain), it was shown that ILC2 drive airway hyper-responsiveness (AHR) independently of the adaptive immune response. In this model, alveolar macrophages have been reported to be an important source of IL-33, while eosinophils have not been induced early in infection (7). Another report by Monticelli et al. observed an increase of ILC2 in combination with eosinophilia and identified the contribution of ILC2-derived Areg in wound healing upon infection with a mouse-adapted recombinant H1N1 IAV strain (14). We have recently shown that H1N1-PR8 IAV strain induced eosinophilia as well as neutrophil recruitment and pulmonary type 2 immunopathology. Furthermore, we observed that this immunopathology is significantly increased in interferon receptor 1-deficient animals and identified type I interferon as an important negative regulator to restrain ILC2 upon pulmonary viral infection (135).

Rhinovirus

Rhinovirus (RV-16) induces IL-25 mRNA and protein levels in asthmatic patients when compared with healthy individuals (137). The induction of a population of non-T non-NK cells with ST2 and ICOS expression was further reported, which probably corresponds to ILC2 (137). Moreover, rhinovirus (RV-16) infection has been shown to induce IL-33 in asthmatic patients together with a type 2 immune signature *in vivo*. In addition, human Th2 and ILC2 cells can be triggered to secrete type 2 cytokines upon *ex vivo* stimulation with infected bronchial epithelial cells (138). Moreover, rhinovirus (RV1B, propagated in HeLa cells) can induce IL-25, IL-33, and TSLP protein expression and release, thereby triggering ILC2 expansion and activation in young mice (136, 139). Exogenous IFN-γ decreases goblet cell hyperplasia, mucus production, and type 2 signature cytokine expression in the lungs through restraint of pulmonary ILC2 in immature mice (140). Thus, elicitation of ILC2 may depend on a combined induction of IL-25, IL-33, and TSLP in both mouse and humans upon rhinovirus infection.

Respiratory Syncytial Virus

Respiratory syncytial virus infections are often linked to asthma exacerbations especially in children. Increased numbers of ILC2 are highly dependent on IL-33 in young mice upon RSV infection (strain A2, propagated in Vero cells) (141). In adult mice as well, ILC2 are elicited upon RSV infection (strain 01/2-20, human isolate, propagated in Hep-2 cells) and the main source of IL-13 early in the infection leading to AHR, goblet cell hyperplasia, and increased mucus production (142). Furthermore, TSLP, probably released by epithelial cells, has been shown to be important for ILC2 activation in this model (142). Interestingly, RSV is able to trigger ILC2 and ILC3 accumulation in the lungs in STAT1-deficient animals resulting in a mixed type 1 and type 2 immune response (143), a phenotype, which is observed sometimes in human asthma (144).

Helminth Infections

Helminth infections are the most common infections worldwide with approximately 1.5 billion people affected, which equals approximately one-fourth of the world's population (145). Most helminth infections are not life-threatening for humans, but represent a massive health and economic burden. Type 2 immune responses are essential to efficiently expel the worm and protect from re-infections and ILC2 were shown to participate in and amplify these immune responses.

Nippostrongylus brasiliensis

Nippostrongylus brasiliensis, a natural rodent helminth, is often used as a model of helminth infections. Upon subcutaneous injection of mice with worm larvae (L3), the larvae migrate into the lungs, molt, get coughed up, swallowed and then reach the intestine (146). ILC2 were initially identified in studies using *N. brasiliensis* as a helminth infection model (1–3). Early on during the infection, ILC2 are induced and responsible for the induction of type 2 signature cytokines, especially IL-13 (1–3). IL-13 release by ILC2 is essential for clearance of the infection since lack of IL-13 results in inefficient worm expulsion and transfer of wild-type ILC2 into IL-13-deficient mice can restore worm clearance (2). In addition, eosinophil counts significantly increase in the lungs upon *N. brasiliensis* infection (2, 3, 147). At the early stage of infection, the lung tissue is extremely fragile, as demonstrated by the increase of red blood cells in

the bronchiolar alveolar lavage (147, 148). Using reporter mice, pulmonary ILC2 were identified as the early source for IL-13 and IL-9 during *N. brasiliensis* infection (3, 71, 116). As IL-9 is an important autocrine cytokine that regulates fitness of ILC2, ILC2 and eosinophil levels were found to be reduced in IL-9 receptor-deficient mice (116) (see also paragraph about IL-9). Moreover, the recently identified nILC2 and iILC2 in the lungs are both induced upon *N. brasiliensis* infection albeit with different kinetics: iILC2 are induced early on and nILC2 are dominant in the lungs at late timepoints post infection, suggesting that iILC2 may act as transient progenitors of nILC2 in this setting (43).

Bacterial Infections

Bacterial infections and their link to ILC2 have not yet been addressed in many settings. Two models of bacterial infections, *Haemophilus influenzae* and *Staphylococcus aureus* have been reported to induce a decrease in GATA3 expression in pulmonary ILC2 similar to what has been reported in influenza infection (85). However, role(s) of pulmonary ILC2 in bacterial infections and associated immunopathologies still need to be further elucidated.

Allergen-Driven Disease Models

Since the description that ILC2 are able to rapidly secrete large amounts of type 2 signatures cytokines upon activation, their role in pulmonary allergic reactions and asthma models has been studied extensively.

House Dust Mite

Allergic reactions to HDM are a very common clinic manifestation in allergic patients (149). HDM can also induce allergic reactions in mice when administered intranasally and is therefore used as a model system to study cellular and molecular mechanism of allergic asthma (AA) (150). HDM extract is a mixture of different components including Der p1 (endopeptidase 1/mite) and Der p2 (TLR4 agonist) as well as serine and cysteine proteases, exo- and endochitinases and endotoxin (LPS) (151). Collectively, these mixtures have been shown to induce both innate and adaptive immune responses including ILC2, Th1, Th2, and Th17 in combination with eosinophils and neutrophils (152, 153). Dose, kinetics and number of administrations as well as the route of administration were shown to influence the outcome of the pulmonary immune response (152, 153). However, ILC2 and T cells follow similar kinetics upon HDM administration and T cells are required for complete ILC2 function in this model (153).

Papain Model

Papain is a cysteine protease present in papaya (and other fruit such as pineapple) and has been used in food and drug industries as meat tenderizer but also as tooth whitener in toothpaste and mints. Upon long-term exposure to papain dust, workers in these industries have been reported to develop papain induced asthma in combination with papain specific IgE antibody responses (154). Papain is able to induce an IL-33- and TSLP-dependent type 2 immune response including eosinophilia independent of adaptive immunity in mice (66, 155). Early upon intranasal papain administration, pulmonary ILC2 are the dominant source of IL-5 and IL-13 (66). Dependent on the amount of papain and the number of administrations, increasing levels of NKT and T cells can be detected in the lungs but these seem to be independent of the fitness of pulmonary ILC2 (156). If early (day 0 and 1) and late (day 13 and 20) administration of papain is combined, the support for dendritic cell-dependent priming of Th2 cells by ILC2-derived IL-13 has been reported (127). Thus, papain is an important model to study IL-33-dependent ILC2 function in the pulmonary environment (66).

Cytokine Administration Models to Study ILC2 Biology

Cytokine administration models (IL-33 or IL-25) are commonly used to study ILC2 biology as they mimic acute type 2 immune responses. These models are less physiological than allergen-dependent or infection models but their advantage is that they use the controlled activity of specific cytokines or cytokine combinations. Interestingly, IL-33 has been reported to be a more potent trigger of type 2 immune responses than IL-25 upon intranasal administration (42). However, intranasal administration of NMU in combination with IL-25 was shown to greatly enhance type 2 immune responses (41).

HUMAN ILC2 AND ASTHMA

As soon as adverse effects of ILC2 were reported in mouse models of pulmonary disease, the concept emerged of establishing ILC2 prevalence in peripheral blood as a biomarker for diagnostic purposes in lung diseases. Increased levels of IL-33 and ILC2 numbers ($Lin^-IL\text{-}7Ra^+FceRI^-IL\text{-}33R^+$) could be detected in bronchioalveolar lavage of asthma patients when compared with disease controls (patients with a disease history) (157). A different study analyzed ILC2 prevalence (gated as $Lin^-CD127^+CRTH2^+$ or $Lin^-CD127^+CD44^{high}$) in the peripheral blood of allergic asthma (AA) and allergic rhinitis (AR) patients compared to those of healthy donors (158). In general, ILC2 sorted from PBMCs released large amounts of IL-5 and IL-13 when stimulated with IL-2 and IL-33 but only negligible amounts of IL-4. Type 2 cytokine release of PBMCs from AA was increased compared with AR patients upon stimulation. However, PBMCs from healthy donors also secreted a robust level of type 2 signature cytokines but ILC2 prevalence per mL blood was slightly increased in AA patients compared with allergic and healthy donors. Another study compared ILC2 levels (gated on $Lin^-CD45^+CD127^+ST2^+$) in blood and sputum of severe asthma, steroid-naïve atopic asthma with healthy controls (159). ILC2 levels as well as type 2 signature positive ILC2 were increased in severe asthma compared with mild asthma patients and eosinophilia was positively correlated. Interestingly, high-dose corticosteroid did not completely reduce ILC2 numbers and signature cytokines, which might be due to TSLP-induced STAT5 phosphorylation in ILC2 which is non-steroid reversible (160). Another report investigated ILC2 ($Lin^-CRTH2^+CD127^+$) in eosinophilic and non-eosinophilic asthmatic patients, observing a correlation of high ILC2 levels with eosinophilic asthma (161). Asthma is a chronic, heterogeneous disease characterized by airway inflammation and hyper-responsiveness and is more

a syndrome than a disease with one specific pattern. Moreover, asthmatic patients have been reported with higher neuropeptide levels, adding once more the neuro-immune axis (162). Thus, ILC2 characterization in different asthmatic subgroups and their comparison is needed and the use of ILC2 levels as a biomarker needs to be carefully evaluated for each subgroup.

COUNTERACTING ILC2 TO AMELIORATE PULMONARY DISEASES

Group 2 innate lymphoid cells are critical initiators and amplifiers of type 2 immune responses. Therefore, understanding the regulation of ILC2 will help to understand how type 2 immune responses can be regulated in general. Type I and II interferons have been recently shown to be important in restricting ILC2 effector functions (8, 12, 135). Importantly, also IL-27, a member of the IL-12 cytokine family, is able to suppress ILC2 function (12, 135, 163). These reports showed that both interferons and IL-27 restrain ILC2 by STAT1 activation and thereby act as negative regulators of ILC2-mediated responses (12, 135). Interestingly, IL-27 can also restrict ILC2 *via* activation of STAT3 (163). However, STAT1 activation has been recently reported to not only restrain ILC2 but also exhibit negative regulatory potential on ILC3 (143). Interestingly, the IL-10 receptor is expressed on ILC2 from the mesentery, but no inhibitory effect of IL-10 was observed *ex vivo* (1, 12). However, pulmonary ILC2 upon challenge with papain or IL-33 are restrained when cultured *ex vivo* with IL-10 (164). This is of special interest as the recently described regulatory ILC execute their suppressive anti-inflammatory functions independent of Foxp3 but *via* the release of IL-10 (122). As such, regulatory ILC might constitute important negative regulators of ILC2. Importantly, negative regulation of ILC2 by IL-10 was shown to be dependent on their stimulation by IL-33 (164), further highlighting the importance of the cytokine milieu for ILC2 function. ILC2 sense and express the respective receptors for IL-33, namely ST2 and IL-1 receptor accessory protein and TSLP, TSLP receptor chain together with the IL-7Rα chain (107). IL-33 signal transduction results in MyD88-dependent activation of NF-κB and MAPK pathways, whereas TSLP mainly signals *via* STAT5. In human ILC2, TSLP induces cytokine expression by triggering GATA3 expression through STAT5 phosphorylation (17). Polymorphisms in genetic loci of IL-33, its receptor *ST2* (*IL1RL1*), and *TSLP* have been identified among other genes in genome-wide association studies in asthmatic patients (165) and monoclonal antibodies specific for TSLP have been positively evaluated in asthmatic patients (166, 167). Thus, this might be a therapeutical strategy to restrain ILC2 by capturing ILC2 eliciting effectors such as TSLP; however, plasticity of ILC2 might hinder this approach.

SUMMARY AND OUTLOOK

Since their detailed description in 2010, ILC2 have been studied and followed with huge interest and excitement. In this review, we focused on ILC2 biology and function within their pulmonary environment, giving an overview about lung physiology as well as different disease and allergy models in the lungs in which ILC2 are known to play a role. The pulmonary environment is especially fascinating to study ILC2 biology as they are the predominant ILC population within the lung. Thus, ILC2 play an important role in the initiation but also orchestration of the pulmonary immune response. However, we are just beginning to understand their exact cellular and molecular interactions, their dynamics, and especially their ability of surveillance within the pulmonary tissue at steady state and upon challenge. ILC2 have been mainly reported to play a role in deregulated type 2 immune responses. However, the positive impact of type 2 immune responses has also been proposed (168) and the contribution and the role of ILC2 in this regard has only been started to be elucidated.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Cytokine Networks between Innate Lymphoid Cells and Myeloid Cells

Arthur Mortha and Kyle Burrows*

Department of Immunology, University of Toronto, Toronto, ON, Canada

Innate lymphoid cells (ILCs) are an essential component of the innate immune system in vertebrates. They are developmentally rooted in the lymphoid lineage and can diverge into at least three transcriptionally distinct lineages. ILCs seed both lymphoid and non-lymphoid tissues and are locally self-maintained in tissue-resident pools. Tissue-resident ILCs execute important effector functions making them key regulator in tissue homeostasis, repair, remodeling, microbial defense, and anti-tumor immunity. Similar to T lymphocytes, ILCs possess only few sensory elements for the recognition of non-self and thus depend on extrinsic cellular sensory elements residing within the tissue. Myeloid cells, including mononuclear phagocytes (MNPs), are key sentinels of the tissue and are able to translate environmental cues into an effector profile that instructs lymphocyte responses. The adaptation of myeloid cells to the tissue state thus influences the effector program of ILCs and serves as an example of how environmental signals are integrated into the function of ILCs via a tissue-resident immune cell cross talks. This review summarizes our current knowledge on the role of myeloid cells in regulating ILC functions and discusses how feedback communication between ILCs and myeloid cells contribute to stabilize immune homeostasis in order to maintain the healthy state of an organ.

Keywords: innate lymphoid cells, Group 1 ILCs, ILC2, ILC3, myeloid cells, macrophages, dendritic cells, cytokines

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Avinash Bhandoola, National Institutes of Health (NIH), United States

****Correspondence:***
Arthur Mortha
arthur.mortha@utoronto.ca

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INTRODUCTION

Maintaining a physical barrier to the external environment is vital to proper physiology and function of the body's organs. However, environmental signals including nutrients, xenobiotic chemicals, microbial metabolites, together with cell intrinsic triggers like proliferation, cell death, damage, and metabolism play an essential role in influencing tissue homeostasis within organs. The immune system is a fundamental sensory system that contributes to physiology far beyond its classical role as modulator of immunity (1). Transient and long-lasting changes in the composition of these signals require appropriate interpretation by tissue-resident immune sensors, to ensure appropriate adjustments to environmental stimulation. Failure to achieve a balanced response through adequate instruction of non-sensory tissue-resident immune cells and incoming recruited immune cells resets the local tissue immune tone and ultimately affects the homeostatic physiology of an organ (2). While this description is generalized, it reflects many of the recent findings on tissue-resident elements of the immune system across lymphoid and non-lymphoid organs (3). The contribution of tissue-resident cells to organ homeostasis through sensory mechanisms and specialized effector functions sets the homeostatic tissue tone and is thus an essential process to preserve proper physiology of our body and its organs.

Innate lymphoid cells (ILCs) are a new family of innate immune cells composed of at least three independent lineages with distinct transcriptional profiles and effector functions. These cells predominantly reside in non-lymphoid tissues like the skin, lung, liver, intestine, and adipose tissue with organ-specific enrichment of different ILC subsets (4). Even though ILCs constitutively secrete cytokines and effector proteins, required to locally sustain tissue and immune homeostasis, their capacity to sense perturbations in the surrounding environment is limited (5, 6) (www.immgen.org). Interestingly, ILCs sparsely replenish their tissue-resident pool from circulating precursors but rather, self-maintain locally (7). ILCs thus resemble a tissue-resident immune cell type that executes local effector functions depending on the interpretation of the environmental state by an instructive sensor. However, there are two exceptions, the composition of the nutrient and metabolite pool in the immediate environment and secreted neuropeptides seams to be directly recognized by ILCs and influences ILC development and function during steady state and inflammation (8–12). The neuron–immune interaction through neuropeptides represents a new aspect of tissue-ILC crosstalk and environmental sensing. Even though this area of research is still in its infancy, it greatly advances our view on innate immune control by the neuronal network (13). Environmental metabolic components (i.e., amino acids, polyaromatic carbohydrates, vitamins, and lipids) control the ligand-activated transcription factors Aryl Hydrocarbon Receptor (Ahr) and members of the Retinoic-acid Receptor (Rar) family (14, 15). These mechanisms, although utilized by many cells, decorate ILCs with relevant direct sensory capacity impacting tissue development, protection, and homeostasis (16, 17). Although nutrient sensors like Ahr or Rar are widely expressed across the immune compartments and serve as sensor for metabolites, these receptors affect the development of several immune cells including ILCs (18). In addition, several non-dietary ligands are able to bind Ahr and result in its transcriptional activity or inhibition. These observations renders the actions of Ahr on its target cell highly dependent on environmental signals (19). Most strikingly, pathogen-associated Ahr ligands have been shown to enable the direct recognition of pathogens by Ahr-expressing ILCs and may thus be seen as ILC-intrinsic sensor of microbial ligands (17).

Myeloid cells are the earliest immune cells arising in our body and reflect a heterogeneous family of cells containing phagocytic and granulocytic cells. Members of these family either rapidly enter the tissue out of the blood circulation or self-maintain locally as tissue-resident patrolling sensors of the environment. Their migratory properties and excellent sensory machineries enable myeloid cells to recognize perturbations (i.e., cell death or infection) and immediately initiate a specific, local immune response (20, 21). Detailed genomic analysis of myeloid cells on high dimensional levels revealed tissue-specific epigenetic marks coupled to characteristic gene expression profiles depending on the local tissue environment (22–25). Interestingly, transfer of myeloid cells from one tissue to another will reprogram the myeloid gene expression profile, supporting the idea that myeloid cells are superior in recognizing and adapting to their local tissue environment (23). With ILCs being a tissue-resident cell type with limited capacity to recognize microbial signals, myeloid cells, as superior sensors of microbial and other tissue-derived signals, play a crucial role in controlling ILC homeostasis and function (26, 27). Acute and chronic activation of tissues is accompanied by changes in the local myeloid immune cell pool followed by adaptation of the tissue-resident ILC compartment (28–31). Our recent progress in understanding the tissue-specific distribution of myeloid cells and ILCs coupled to a detailed picture of their gene expression profile sheds light on the potential local dialog of these cells during health and disease. Current research is uncovering new pathways and cross talks that may be suitable to target these interactions with potential therapeutics, to reset diseased tissues to a healthy homeostatic state. Within this review, we highlight our current knowledge on the interactions of ILCs and myeloid cells, focusing on the cytokine-mediated cross talk of ILCs and myeloid cells.

DIVERSITY OF INNATE LYMPHOCYTES

Unlike T and B cells, ILC-poiesis is independent of Recombination-activated gene (*Rag*) and *Rag*-dependent rearrangement of antigen receptors for their development but require signals through the common gamma chain (γ_c). The cytokines thymic stromal lymphopoietin (TSLP), stromal cell-derived interleukin (IL)-7, and IL-15 also play dominant roles in their survival and homeostasis (32, 33). Concerted actions of these cytokines and others lead to the activation, priming and execution of effector functions of ILCs (34). The family of ILCs contains three lineages of ILCs (group 1 ILC, group 2 ILC, and group 3 ILC). These groups comprised natural killer (NK) cells (included in group 1 ILC), ILC1, ILC2, ILC3, and lymphoid tissue inducer (LTi) cells (included in group 3 ILC) that collectively mirror the effector profiles of most T helper (h) cells and cytotoxic T cell lineages (35) (summarized in **Figure 1**).

The development of all ILCs is tightly controlled by transcriptional programs that are shared with T cells, but additionally requires a unique composition of transcription factors to determine ILC commitment (e.g., Tox, Id2, Plzf, Nfil3, and Gata3) (38). In general, common innate lymphocyte precursors (CILPs) are lymphoid precursor cells in the adult bone marrow that are identified as Lin^- $CD127^+$ $Id2^+$ $Nfil3^+$ Tox^+ $Plzf^{high}$ cells and have unrestricted potential to differentiate into all groups of ILCs (39). Common helper innate lymphocyte precursor (CHILP) is an immediate descendent of CILPs and characterized as $Id2^{high}$ and $Plzf^-$ cells, which retain the potential to exclusively give rise to ILC1, ILC2, and ILC3, while lacking the potential to differentiate into NK cells and LTi cells (36). NK cells arise from precursor NK cells that are generated from CILPs, while LTi cells arise early during ontogeny from a fetal liver lymphoid precursor sharing transcriptional homologies with ILC3 (36, 39, 40). Being unique in their developmental origin, CHILP-derived ILCs are separated into three lineages (ILC1, ILC2, and ILC3) characterized by lineage-specific transcription factors and effector functions that mirror Th1, Th2, and Th17 cells (6, 41–44). Observations in *Rag2*$^{-/-}$ mice identified lymphoid cells secreting cytokines commonly associated with Th1, Th2, or Th17 lineage commitment supporting the functional and developmental homology of ILCs

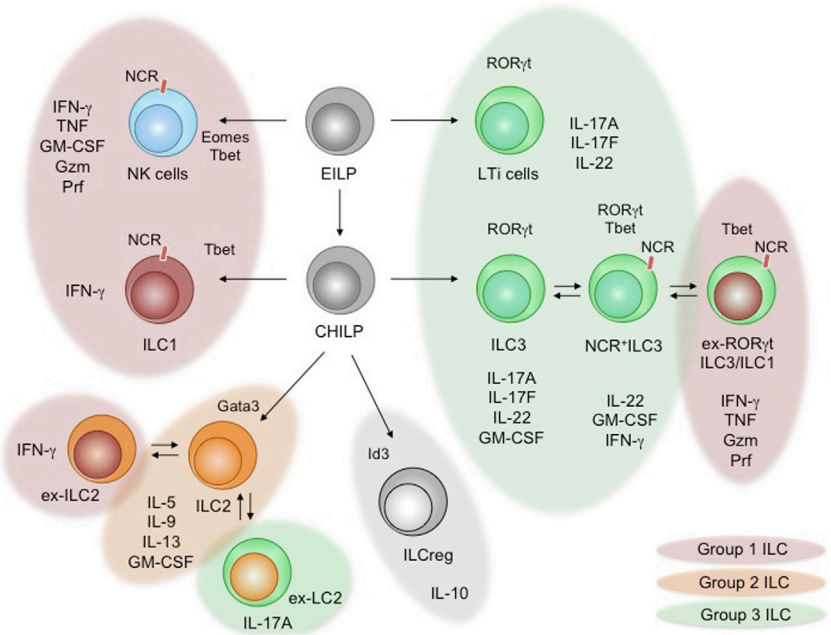

FIGURE 1 | Development and functional specialization of innate lymphoid cells (ILCs). The diagram shows the developmental pathways of ILCs and branching into their different lineages. The *early innate lymphoid precursor* (EILP) and the *common helper innate lymphoid precursor* (CHILP) are progenitors to all ILCs (36, 37). Critical lineage-determining transcription factors are shown. Arising ILCs [natural killer (NK) cells, lymphoid tissue inducer (LT) cells, ILC1–3, and ILCreg] are displayed, including arising subsets. Individual groups of ILCs are indicated through color schemes. The expression of natural cytotoxicity receptors (NCRs) on ILC subsets is indicated. Specific and shared effector cytokines secreted during inflammation and steady state are listed below the indicated subsets of ILCs. Documented plasticity within group 2 ILCs and group 3 ILCs is indicated using overlapping and colored bubbles.

and Th cells (45–48). During the steady state and even stronger during the onset of tissue inflammation, ILCs are a potent local source of cytokines that rapidly prime the "immunological tone" of a tissue (49). Given the limited capacity of ILCs to directly recognize tissue inflammation, the effector profile of these cells strikingly relies on cells interpreting the state of the tissue and communicating the presence of homeostasis, danger, or damage to ILCs.

Group 1 ILCs (ILC1) are identified as lin$^-$ NK1.1$^+$ CD49b$^-$ KLRG1$^-$ IL-7R$^+$ CD117$^-$ cells that secrete high levels of interferon (IFN)-γ and TNF-α but express little to no Granzyme (Gzm) or Perforin (Prf). Like Th1 cells, ILC1 are developmentally dependent on the T box transcription factor *Tbx21* (Tbet) and produce high amounts of their signature cytokine IFN-γ to protect from intracellular pathogens and contribute to chronic inflammatory pathologies (6, 36, 50, 51). Unlike NK cells that share an effector program with cytotoxic CD8$^+$ T cells, ILC1 are independent of *Eomesodermin* (Eomes), originate from CHILP and lack the capacity to lyse target cells (27, 36). Based on their phenotypic similarity to NK cells and other ILC subsets (discussed below), group 1 ILCs might thus represent a heterogeneous population of innate effectors (51). The increasing appreciation of plastic behavior within ILC subsets may thus simply reflect the activation of a developmentally different and heterogeneous pool of ILCs within the ILC1-like features (**Figure 1**) (52).

Group 2 ILCs (ILC2) are identified as lin$^-$ KLRG1$^+$ IL-7R$^+$ CD117$^-$ IL33R$^+$ IL1R2$^+$. ILC2 represent an innate Th2 counterpart, which is developmentally tied to high expression of the transcription factor Gata-binding protein 3 (Gata3) and essential in the anti-parasite/helminth defense through the production of the cytokines IL-4, -5, -9, -13, and GM-CSF (6, 43, 51, 53, 54). ILC2 have further been associated with allergic reactions and tissue repair (55–57). Interestingly, different ILC2 subsets and activation states have been identified, enhancing the interest in understanding the contribution of different ILC2 subsets to defense, autoimmunity, or tissue repair (51). Changes in ILC2-specific cytokine secretion and adaptation of mixed effector phenotypes within ILC2 have been uncovered, adding functional plasticity to the ILC2 lineage (58, 59). Under chronic inflammatory conditions of the airways, ILC2 start to show features of ILC1 and express Tbet and IFN-γ (59–62). This shift toward an ILC1-like phenotype depends on the cytokines IL-1, IL-12, and IL-27 secreted by macrophages and dendritic cells (DCs) (63). Interestingly, adaptation of IL-17 production by ILC2 has been reported during Candida infection, suggesting a highly plastic behavior in order to adapt to the inflammatory context (64).

The last and currently most diverse group of ILCs matching a Th cell lineage, are group 3 ILCs (ILC3), including LTi cells. LTi cells (likely including ILC3 by the time of discovery) were first identified as a population of lymphocytes accumulating in the developing lymph nodes of embryos to initiate lymph node and lymphoid cluster formation through surface Lymphotoxin ($sLT\alpha_1\beta_2$ and $LT\alpha_3$)–LTβ receptor interaction with stroma cells. *In vitro* cultures of purified LTi-like cells revealed that LTi-like cells are capable of expressing NK cell receptors and major histocompatibility complex (MHC) II on their surface,

resembling features of NK cells and antigen-presenting cells (40). The NK cell receptor-expressing population was later identified as an independent subset of the ILC3 lineage (45, 46, 65–67). Recent reports confirmed the expression of MHCII$^+$ ILCs, particularly on subsets of the ILC3 and ILC2 lineages, granting these ILCs with the ability to regulate adaptive T cell responses directly (68–70). LTi cells and ILC3 most closely mirror retinoic acid-induced orphan receptor (ROR)γt-dependent Th17 cells through their production of the cytokines IL-17A, IL-17F, IL-22, and GM-CSF (34). Importantly, ILC3s contain a population of RORγt$^+$ cells, expressing natural cytotoxicity receptors (NCRs; i.e., NKp46, NKG2D, NK1.1), sharing transcriptional and functional features with NK cells and ILC1 (i.e., expression of Tbet, Gzm, Prf, IFN-γ and IL-12R) (71). These cells were termed NCR$^+$ILC3 and led to the subdivision of ILC3s into NCR$^-$ILC3s and NCR$^+$ILC3s (72). The expression of the transcription factor RORγt in NCR$^+$ILC3 does not define a stable end state and is repressed by the cytokines IL-12 and IL-15 (71, 73, 74). NCR$^+$ILC3 that lost their expression of RORγt were identified using genetic fate mapping and conclusively termed ex-RORγt ILC3 (71). Like ILC1 these cells express Tbet, high levels of IFN-γ and are attributed with the capacity to contribute to colitis-like pathologies through the secretion of IFN-γ and GM-CSF (31, 50, 71, 75). The stage of ex-ILC3s, as a final/stable differentiation state, was challenged by a recent report demonstrating that ex-ILC3 are able to induce RORγt in the presence of IL-23 and retinoic acid (RA) potentially adapting their effector function to changes in the environmental cytokine milieu from inflammatory to homeostatic (75). Fate mapping of ILCs using NKp46-Cre mice reveals that NCR$^+$ILC3 might actually downregulate the expression of NCRs to adopt an NCR$^-$ILC3 phenotype (76). These routes of plasticity among ILC3s appear to be shifted from RORγt$^+$ILC3 toward ex-RORγt ILC3 under chronic inflammatory conditions, suggesting environmental signals as regulator of ILC3 plasticity.

Natural killer cells are the oldest and most studied population of ILCs. They were identified as lymphocytes capable of lysing target cells in the absence of antigen-presentation, associated by the secretion of high amounts of the inflammatory cytokines IFN-γ, TNF-α and IL-2. NK cells are able to recognize classical self-MHCI and non-classical MHCI-like molecules, as well as NCR-ligands through activating or inhibiting NCRs during steady state and during stress (77). While the expression of activating and inhibitory NCRs allows the direct sensing of environmental states, the integration of these signals into NK cell functions heavily depends on the signaling module associated with the recognizing NCR (78). Activating NCRs allow NK cells to lyse and kill ligand expressing target cells and thus, unlike ILC1, share functional similarities with cytotoxic CD8$^+$ T cells (79). The transcriptional regulators Tbet and Eomes are essential for NK cell development and function, including the production of IFN-γ and the execution of cytotoxicity, emphasizing their importance in anti-viral defense and anti-tumor immunity through direct cytotoxicity (80, 81). Interestingly, the exclusive role of NK cells in anti-tumor immunity was challenged by reports demonstrating a role for ILC3 and ex-ILC3 in contributing to potent anti-tumor immunity (82, 83).

Making up a highly diverse family of innate effector cells, ILCs execute a diverse range of homeostatic and inflammatory functions that are important for repair and defense of the body. Being of tissue-resident nature, ILCs permanently execute their effector functions locally driven by tissue-derived signals (26). Even though ILCs retain features of direct environmental sensing, their capability to recognize foreign microbial patterns remains poor and requires extrinsic sensory immune elements. Myeloid cells are perfectly equipped to undertake this task and play a dominant role in tissue homeostasis and inflammation by triggering repair and defense (84, 85). Communication between myeloid cells as sensors of the environment and ILCs as effector at the local tissue site is an attractive model accounting for early adaptations of organs to infections and resolution.

MYELOID CELL DEVELOPMENT

Myeloid cells across our body arise from clonogenic myeloid-primed precursors (MPPs) branching up into granulocytic or monocytic-primed progenitors giving either rise to polymorphonuclear leukocytes (PMNL) like basophils, mast cells, eosinophils and ganulocytes, as well as mononuclear phagocytes (MNPs) like monocytes, macrophages, DC, and plasmacytoid DC. The transcriptional circuits responsible for this diverse branching of the hematopoietic tree have been extensively reviewed elsewhere and will not be discussed in this review. However, myelopoiesis is one of the earliest processes during embryonic hematopoiesis, it continuously occurs throughout adulthood. While the yolk sac and the fetal liver are early sources for myelopoiesis, its primary source during adulthood is the bone marrow-resident precursor pool (85). The strongly inflamed tissue poses as an exception of this pattern, as undifferentiated myeloid precursors locally differentiating into mature myeloid cells have been reported in the acute and chronically inflamed spleen, lung. and intestine of mice (20, 86–88). Tissue-resident MNPs arise from early erythroid-myeloid precursors (EMP) in waves of myelopoiesis during ontogeny. These cells seed the developing organs and locally self-renew in the steady state, while chronic inflammation fosters the replacement of tissue-resident myeloid cells by progeny arising from circulating precursors (89, 90). MNPs including monocytes, macrophages, and DCs are generated from a common monocyte DC precursor (MDP) that requires cytokine-mediated signaling for its differentiation (84). A clonogenic progenitor with restricted potential to the monocyte/macrophage lineage has been described as Common Monocyte Precursor (cMoP) giving rise to both Ly6C^{low} and Ly6C^{high} monocytes, the latter being the immediate precursor to macrophages of the adult tissue. A recent report suggested a linear lineage positioning of cMoP upstream of Ly6C^{hi} monocytes that differentiate into Ly6C^{lo} monocytes following the fate to develop into a macrophage (91). The ontogeny of macrophages during different stages of development, including their transcriptional regulation has been reviewed excessively, is the subject of ongoing research and will not be the focus of the review (3). However, monocytes leaving the bone marrow into the circulation enter tissues for their terminal differentiation into macrophages. Recent epigenetic and transcriptomic characterizations of macrophages across almost the entire body demonstrated

conserved commonalities within macrophages across all tissue but more interestingly identified substantial tissue-dependent heterogeneity within the enhancer landscape and the gene expression profile in a tissue-dependent fashion (22, 23). Monocytes as immediate precursors of macrophages do not display any of these tissue-dependent traits prior to local differentiation into macrophages, pointing at tissue-derived signal as key driver of terminal macrophage heterogeneity. On the contrary, single cell profiling of precursor DCs (preDCs) uncovered pre-committed subpopulations giving rise to the two major DC lineages termed cDC1 and cDC2 previously called $CD103^+$ DC or $CD11b^+$DC, before leaving the bone marrow to migrate into the circulation (24, 92). cDC1-primed and cDC2-primed preDCs then enter this tissues and fully differentiate into their mature progeny to adopt tissue-specific activation marker expression. Even though diverse activation stages within the tissue have been identified for terminally differentiated granulocytes, basophils, eosinophils, or mast cells, it is currently not known if tissue specification within PMNLs exists across different organs or within their early precursor population (93). Multiple-primed precursors (MMPs) with PMNL potential have been identified; their capacity to differentiate into multiple myeloid cell types renders this population rather uncommitted and heterogeneous (94). While eosinophils develop from an IL-5 receptor expressing population within the granulocyte-macrophage precursor (GMP) pool, basophils and mast cells develop from a common basophil-mast cell precursor, giving rise to further committed basophil or mast cell precursor cells (95, 96). Surprisingly, common routes of myeloid cell development can be short cut and located to non-bone marrow niches to generate MNPs and PMNLs under conditions of acute infection or chronic inflammation. This process, called "emergency granulopoiesis" or "extramedullary myelopoiesis", rapidly generates large quantities of granulocytes, mast cells, basophils eosinophils, and monocytes depending on the type of inflammation. Reports identified the spleen and the intestine as prominent sources of extramedullary myelopoiesis (86, 88). Even though very few myeloid precursors are found in the steady state intestine or spleen, chronic autoimmune-driven intestinal inflammation or parasitic worm infections drive a substantial increase in these populations. Under these conditions, GMPs and MPPs give rise to granulocytes and macrophages, or basophils, eosinophils, and mast cells, respectively (86, 88). However, the contribution of bone marrow versus extramedullary myelopoiesis during steady state is currently unknown.

INDUCTION OF FEEDBACK COMMUNICATION BETWEEN TISSUE-RESIDENT ILCs AND MYELOID CELLS

Within diverse microenvironments and through selective precursor populations, myeloid cell development displays a highly complex and heterogeneous process adapted to the physiology of the hosting organ. With functions far beyond immunity, myeloid cells execute processes that are tailored to serve the physiology and homeostasis of the organ. Thus, functional diversification of myeloid cells, adapting to the local milieu adds another layer of complexity to the processes of myeloid specialization. ILCs are a potential tissue determinant of myeloid diversification as their tissue-resident production of cytokines, upon stimulation, can act on myeloid cells to reprogram their effector profile. However, with myeloid cells being the prominent tissue-resident sensors of the tissue environment, their activation precedes the activation of ILCs. Thus, it is important to first understand myeloid-derived signals as potent drivers of ILC function in order to ultimately determine the effects of cytokine-mediated feedback responses by ILCs on myeloid cells. These tissue-specific innate immune cell cross talks reprogram the local cytokine milieu and set the immune tone for tissue infiltrating immune cells prior to the initiation of an adaptive immune responses.

MNP-DERIVED STIMULI

Monocytes, macrophages, and DCs express highly conserved sensory machineries enabling them to recognize danger signals, pathogens, and foreign antigens (i.e., malignant cells, infected cells, bacteria, parasites, and viruses) to initiate protective immunity (97, 98). Toll-like receptors, NOD-like receptors, lectins, and Fc-receptors are intra- and extracellular sensors that trigger the activation of myeloid cells to alert the local cellular microenvironment. MNPs utilize the secretion of the cytokines IL-1α, IL-1β, IL-33, and IL-18 in order to achieve this goal (97, 98). As epithelial cells are also able to produce IL-1α, IL-18, and IL-33, tissue-resident non-myeloid sensors also contribute to the pool of cytokines to activate tissue-resident immune cells. However, as the sensory machinery of epithelial cells and MNPs differs, these different sensory compartments may be poised to react to distinct and defined stimuli. Monocytes, macrophages, and DCs secrete the cytokines IL-12, IL-15, TL1A, and IL-23 upon engagement of pattern recognition receptors and are able to synergistically amplify cytokine signaling in ILCs by IL-1α, IL-1β, IL-33, and IL-18 (26, 71, 99–101) (**Figure 2A**). Collectively, IL-1α, IL-1β, IL-12, IL-15, TL1A, IL-18, IL-23, and IL-33 are MNP-derived, locally produced cytokines that allow rapid stimulation of tissue-resident ILCs and thus determine the local immune tissue-tone upon danger recognition (**Figures 2A,B**). However, similar to the communication of DCs and T cells through MHC-peptide-antigen receptor complexes, MNPs including DCs are able to express MHC-like surface receptors that engage activating and inhibiting NCRs on ILC subsets (102, 103) (**Figure 2C**). These receptor-ligand interactions modulate the tissue environment and are an essential element in fine-tuning ILC-responsiveness in the steady state as well as during infection and cancer (104). Noteworthy, MNPs are metabolically active cells able to catabolize lipids, vitamins and amino acids, all of which are implicated in controlling the development and function of different ILC subsets (11, 105). With metabolites affecting ILC function and development, MNPs may modulate tissue-specific immunity by dampening and inhibiting the activation of ILC subsets through their contribution of specific metabolites (**Figure 2D**). MNPs further secrete the cytokines IL-10 and TGF-β, both immunologic inhibitors. Similar to inhibitory NCR-MHC-like receptor-ligand interactions, anti-inflammatory cytokines are potential negative regulators of ILCs arising from MNPs (106).

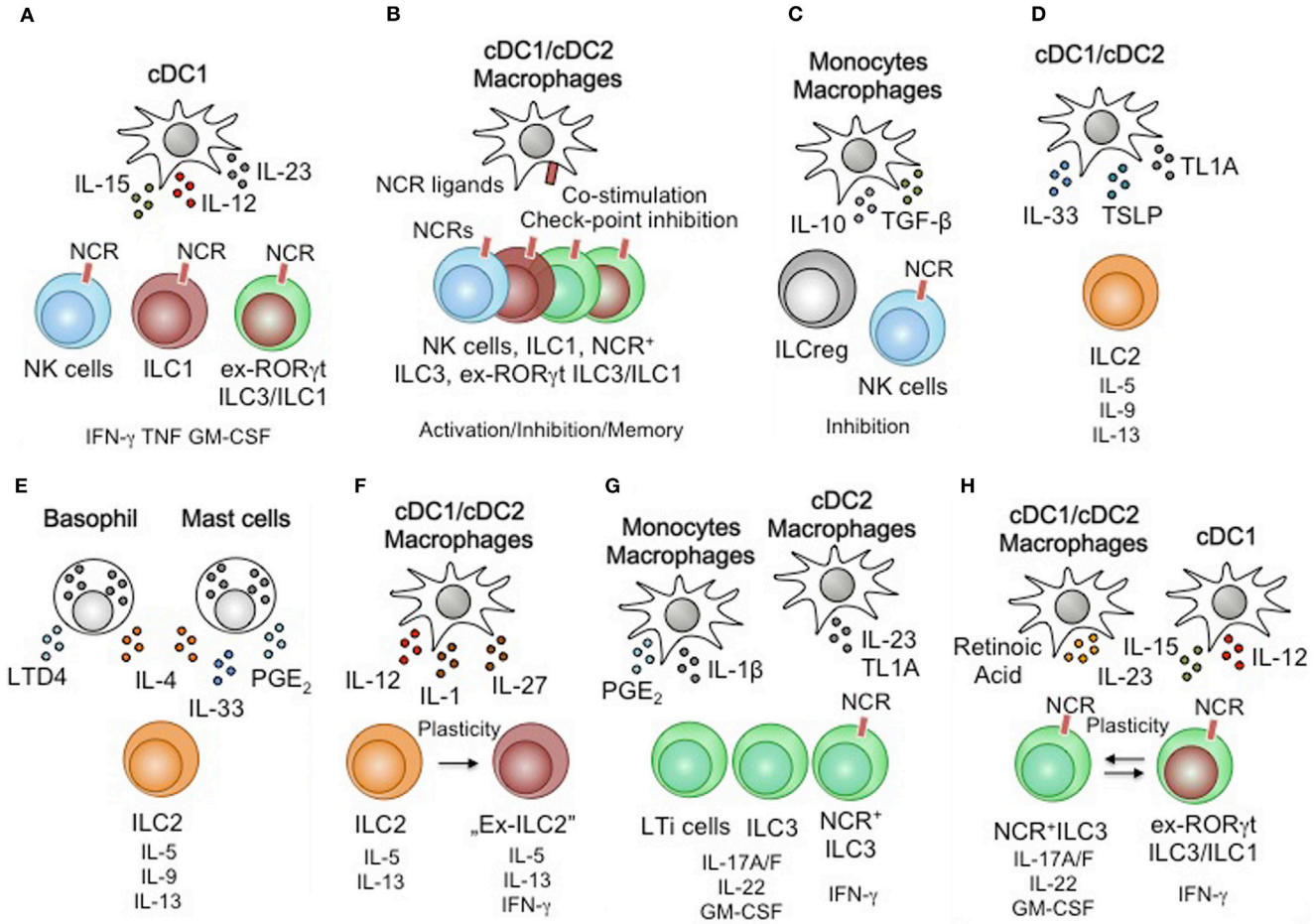

FIGURE 2 | Myeloid regulation of innate lymphoid cells (ILCs) function and plasticity. **(A)** Dendritic cells (DCs) activate natural killer (NK) cells, group 1 ILCs (ILC1), ex-ILC3/ILC1 through interleukin (IL)-12, IL-15, and IL-23. **(B)** Natural cytotoxicity receptor (NCR)-expressing ILC subsets are subject to receptor-ligand mediated activation or inhibition. Improved immune responses through re-occurring engagement of activating receptors have been associated with memory-like behavior in NK cells. Co-stimulatory receptors and checkpoint inhibitory ligands could control the interactions of macrophages/DCs and ILCs. **(C)** Monocyte/macrophages inhibit NK cells via IL-10 and TGF-β secretion. **(D)** DC subsets are capable of activating ILC2 through the production of IL-33, thymic stromal lymphopoietin (TSLP), and TL1A. **(E)** PMN leukocytes (basophils and mast cells) activate ILC2 through IL-33, IL-4, and lipid mediators. **(F)** DC subsets and macrophages influence the stability of ILC2 by inducing type 1 immune features through IL-12, IL-1, and IL-27 leading to plastic behavior of ILC2. **(G)** ILC3 are activated by monocyte/macrophages and DCs. Lipid mediators and the cytokines IL-1β, IL-23, and TL1A drive ILC3 effector functions. **(H)** Retinoic acid, IL-23, IL-15, and IL-12 are myeloid-derived cytokines that control the plasticity and transition of NCR+ILC3 and ex-RORγtILC3/ILC1.

MNPs thus collectively provide activating and inhibiting signals through cytokines, NCR-ligands and metabolites to control the activation of tissue-resident ILCs. With recent findings in mice, identifying an Id3-dependent ILC subset that secrets IL-10 and TGF-β, such inhibitory signals could also arise internally from within the ILC pool (107).

PMNL-DERIVED STIMULI

Basophils, eosinophils, mast cells, and neutrophils are PMNL that either reside locally within tissues or rapidly enter tissues from the circulation. PMNL are equipped to recognize tissue damage, parasitic worm infections and antigen-antibody complexes of the IgE isotype thus differing significantly from MNP (108, 109). PMNL contain cytosolic granules loaded with mediators of inflammation (e.g., eicosanoids and prostaglandins). Within minutes after activation, PMNL release these mediators, histamine, serotonin, proteases, reactive oxygen species, and anti-microbial peptides from their preloaded cytosolic granules followed by a delayed wave of secreted cytokines and chemokines to recruit additional myeloid cells. The immediate response of PMNLs in the tissue, similar to the response of MNPs, changes the local cytokine milieu and activates surrounding tissue-resident innate immune cells. PMNL therefore sets the local immune tone through activating signals that differ from MNPs (95, 110). Much like their tissue-resident MNP counterpart, basophils and mast cells are locally residing cells that adapt a tissue-specific transcriptional profile in the steady state (93, 111). IL-4 and IL-33 are prominent cytokines released by activated basophils and mast cells that are potent activators of ILCs (112) (**Figure 2E**). Eosinophils and neutrophils infiltrate injured tissues out of the circulation, initiate a local antimicrobial immune response, and serve as an amplifier of

mast cell and basophil-induced tissue inflammation. Eosinophils and neutrophils do not respond to mast cell and basophil-derived cytokines but require stimulation by additional cytokines. PMNL are producers of prostaglandine E2 and leukotrine D4 both lipid mediators and potent drives of ILC activation (113–115) (**Figure 2E**). PMNLs are thus tissue-resident or infiltrating myeloid cells controlling the local cytokines production by other tissue-resident immune cells like ILCs. This activation results in a feedback crosstalk of infiltrating myeloid cells and locally activated ILCs.

MYELOID ACTIVATION OF ILCs

Tissue-resident and infiltrating myeloid cells utilize diverse sensory machineries to integrate disturbances of the tissue into the activation of local immune cells. This is achieved through the secretion of cytokines, the release of lipid mediators and activating NCR-ligands. While other non-hematopoietic activators of tissue-resident immune cells have been identified (e.g., neuron, glial cells, fibroblasts, and epithelial cells) (10, 12, 34, 116), their sensory machinery and location may differ from myeloid cells and thus allow for the integration of more complex stimuli. In addition to activation, myeloid cells are able to inhibit tissue-resident immunity through cytokines and inhibitory NCR ligands. With ILCs being tissue-resident cells, these mechanisms control their local, tissue-specific activation, development, and function. Myeloid cells and other sensory non-hematopoietic cells are thus potent regulators of ILCs depending on their sensory receptors and downstream effector mechanisms.

Innate lymphoid cells are a diverse group of cells with characteristic transcriptional profiles determining their lineage commitment, differentiation, and function (35). Their effector machinery is primed to rapidly secrete large amounts of cytokines following antigen-independent stimulation by tissue-resident sensors. With myeloid cells being elementary in interpreting and integrating the local tissue state, MNP-derived or PMNL-derived stimulation selectively activates subsets of ILCs to determine the appropriate amplification of the tissue immune response. Type 1, 2 and 3 immune responses are tailored to defend an organ from specific pathogens. Type 1 combats viruses, intracellular bacteria, intracellular parasites and malignant cells. Type 2 protects against parasitic worm infections, while type 3 defends against fungal and extracellular bacterial attacks (117). The transcriptional programs of ILC1, ILC2 and ILC3s are tailored to selectively support these antimicrobial immune responses, respectively. The activation of either of these ILC subsets, however, is determined by signals arising from specific myeloid cells based on their cytokine receptor profiles (118).

TYPE 1 IMMUNE RESPONSES AND MYELOID ACTIVATION OF ILC1, NK CELLS, AND *ex*-ILC3

Natural killer cells, ILC1, and ex-ILC3 are capable of receiving activating signals by myeloid cells through cytokine receptors. These ILCs express IL-12Rb1, IL-12Rb2, IL-2Rb, and IL-18R and transduce signals upon engagement by IL-12, IL-15, and IL-18 (6) (**Figure 2A**). Stimulation via these cytokines initiates the production of INF-γ, TNF-α, and GM-CSF and triggers a Type 1 immune response for the defense against intracellular bacteria, intracellular parasites, viruses, and tumors, but can also be attributed to the development of autoimmune pathologies like inflammatory bowel disease (34). Myeloid cells are capable of activating group 1 ILCs beyond secreted cytokines. The expression activating NCRs (e.g. NKp46, NKG2D, NKG2A and Ly49s) on NK cells, ILC1 and ex-ILC3 offer a receptor-ligand-mediated mechanism for ILC activation (41, 50, 71) (**Figure 2B**). Engagement of activating NCRs can drive cytotoxicity in NK cells and trigger the release of cytokines by ILC1 and ex-ILC3. Direct cell–cell contact of ILCs and myeloid cells thus contributes to an increase in effector function. Astonishingly, "memory-like" NK cells expressing the receptor Ly49H have been found to sustain their activation state and exhibit accelerated responses upon reengagement of this receptor (119). With recent observations describing the expression of PD1 on ILC3 subsets and additional inhibitory receptors being expressed by NK cells and ILC2 subsets (e.g., Ly49A, KLRG1, or CTLA4), a critical role of check point inhibitors and their ligands on myeloid cells requires future investigations (120–122). Noteworthy, two recent reports identified regulatory functions in ILC subsets suggest ILC-mediated negative regulations, particularly during inflammation (107, 123). Comparable to observations made in NK cells, ILC subsets can be inhibited by the suppressive cytokines IL-10 and TGF-β secreted by macrophages and a recently identified set of regulatory ILCs (106, 107) (**Figure 2C**). These suppressive activities are exclusively observed under conditions of strong or chronic inflammation. It thus remains to be shown if suppression of ILCs by ILCs or macrophages takes place during the steady state in order to maintain homeostasis.

TYPE 2 IMMUNE RESPONSES AND MYELOID ACTIVATION OF ILC2

Group 2 ILCs express the cytokine receptors IL-2Ra, IL-4Ra, IL-9R, IL-17Rb, IL-1Rl1, and TSLPR and low levels of death receptor (DR)3, IL-1R2, and IL-12Rb1. These receptors render ILC2 susceptible to signaling through the cytokines IL-2, IL-4, IL-9, IL-25, TL1A, IL-33, and IL-12 (6, 34). These cytokines are produced by activated basophils, mast cells, cDCs, and macrophages and induced the release of GM-CSF, IL-4, IL-5, IL-9, and IL-13 by ILC2 (**Figures 2D,E**). Triggering the expression of IL-5, IL-9, and IL-13 is critical for the defense against parasitic worms and respiratory flu infections (47, 88). Noteworthy, exacerbated secretion of IL-5, IL-9, and IL-13 has been reported to be a driver of allergic reactions and chronic inflammatory conditions of the lung and skin (56, 124). More strikingly, mast cell and eosinophil-derived lipid mediators are strong non-cytokine-related drivers of ILC2-specific cytokine production (112, 113, 115) (**Figure 2E**). Conversely, the cytokines IFN-α, IFN-β, and IL-27 have been reported to inhibit ILC2 activity. Although the exact source of these cytokines within the hematopoietic compartment remains to be identified, it is anticipated to stem from myeloid cells (58). Concerted actions of inhibitory and activating cytokines, atypical to type 2 immune responses, revealed plastic adaptions of the

secreted cytokine profile by ILC2 (59–61) (**Figure 2F**). Nonetheless, tissue-derived inhibition of ILC2 through the engagement of KLRG1 by E-cadherin further suggests ligand receptor-mediated suppression to dampen and control ILC2 activity (125).

TYPE 3 IMMUNE RESPONSES AND MYELOID ACTIVATION OF ILC3

Group 3 ILCs express the cytokine receptors IL-1R1, IL-2Ra, IL-2Rb, IL-12Rb1, IL-12Rb2, IL-18R, IL-23R, DR3, and IFN-γR. ILC3 are thus responsive to IL-1, IL-2, IL-12, IL-15, IL-23, IL-18, TL1A, and IFN-γ. Stimulation through these receptors drives the production of IL-22, IL-17A, IL-17F GM-CSF, TNF-α, and IFN-γ (6, 34) (**Figure 2G**). These ILC3-derived cytokines play an important role in the defense against extracellular bacteria and fungi (26, 34, 126). Expression of ILC3-produced cytokines also supports tissue and immune homeostasis during the steady state through induction of immunosuppressive functions of DC and macrophages as well as the modification of local and systemic antibody responses (26, 116, 127). More strikingly, exacerbated activation of ILC3 has been implicated in chronic inflammatory diseases like inflammatory bowel disease (86, 128, 129). The activation of ILC3 thus requires contextual interpretation to fine tune their secreted signature cytokines to ensure homeostasis and prevent chronic inflammation. A mechanism negatively regulating ILC3 functions includes the epithelial cell-derived cytokine IL-25 (130). IL-25 is highly produced by Tuft cells, a crucial stimulus to activate ILC2 and essential to control parasitic worm infections (131). Understanding the exact molecular pathways to facilitate ILC3 inhibition requires more detailed future investigations. Interestingly, cytokine-independent myeloid activation of ILC3s represents a way of communication between NCR^+ILC3 and their tissue-resident myeloid partners. NCR^+ILC3 express the activating NCR NKp44. Crosslinking this receptor increases the capacity of NCR^+ILC3 to secrete cytokines and induces a functional switch from homeostatic toward pro-inflammatory phenotype with an increase in INF-γ and TNF-α production (102) (**Figures 2B,H**). In line with these observations, enrichment of NKG2D-expressing ILC3s has been observed in psoriatric lesions of patients, suggesting that the expression of NKG2D ligands in the skin leads to the activation of skin-resident ILC3 (132). While these findings suggest receptor-ligand signaling through activating NCRs to stimulate ILC3s, the role of inhibitor NCRs on these cells remains enigmatic. In light of growing efforts to block inhibitory receptors on immune cells, to revitalize their effector program, receptor-ligand interactions controlling ILC3s are of great interest in order to therapeutically target ILC3s during chronic inflammatory diseases.

TYPE 1 IMMUNE CONTROL THROUGH THE ILC1/NK CELL/ex-ILC3–MYELOID CELL AXIS

Group 1 ILCs, NK cells, and ex-ILC3 produce high levels of INF-γ, TNF-α, and GM-CSF. Neutrophils, monocytes, macrophages, and DC express INF-γR1, IFN-γRII, TNFR and the GM-CSF-specific receptor α and common β chain to integrate stimulation of these cytokines into their differentiation and maturation (97, 98) (**Figure 3A**). Each of these cytokines is able to individually activate monocytes, macrophages and DC through their corresponding receptor, resulting in specific effector functions like phagocytosis, antigen-presentation, lysosome degradation and the production of reactive oxygen species (133). Interestingly, stimulation by GM-CSF is used to drive the differentiation of monocytes into macrophages and DC, while synergistic activation through concerted actions of GM-CSF, IFN-γ, and TNF-α results in strong activation and inflammatory phenotypes (134). Recent findings demonstrated that GM-CSF and TNF-α play important role during the steady state by controlling myeloid cell homeostasis (135, 136). The steady state production of these cytokines thus controls the local myeloid cell population. The absence of GM-CSF, IFN-γ, or TNF-α renders mice highly susceptible to infections by viruses, intracellular bacteria, and intracellular parasites particularly at mucosal surfaces. While steady state expression of GM-CSF and TNF-α controls the local myeloid homeostasis, concerted actions of additional inflammatory cytokines (i.e., IFN-γ) or changes in the locally produced concentrations of GM-CSF and TNF-α could contribute to the development of tissue inflammation. The myeloid cell-induced production of IFN-γ, TNF-α and GM-CSF by ILC1, NK cells, and ex-ILC3 are thus essential feedback signals required to initiate antimicrobial immunity and inflammation, while steady state sources of these cytokines might control homeostatic functions of myeloid cells (42). ILC1 and ex-ILC3 have been found to accumulate and exacerbate chronic intestinal inflammation- driven by IL-12, IL-23, and IL-15 (41, 50, 71, 75, 86). In these studies, RORγt-expressing ILC3 lost their RORγt-controlled effector functions and adapted an ILC1-like phenotype associate with increased levels of secreted IFN-γ, which promote a pro-inflammatory phenotype in neutrophils and monocytes resulting in tissue damage and destruction. Furthermore, ILC-derived inflammatory cytokines promoted the secretion of matrix metalloproteases ultimately destabilizing the epithelial barrier (137). High levels of IFN-γ and GM-CSF influence the local myeloid cell population, but additionally induce changes in infiltrating myeloid cells and the local epithelial barrier. Changes in the location of myeloid cells within the inflamed tissue are associated with adaptations in myeloid cell recruitment and differentiation of myeloid precursor (86, 138). The effector functions of ILC1/NK cells and ex-ILC3 during inflammation therefore control the local and peripheral development of myeloid cells leading to a reprogramming of the local tissue-resident myeloid effector pool followed by an increased myeloid cell output by bone-marrow myelopoiesis and extramedullary granulopoiesis. Collectively, the activation of ILC1, NK cells, and ex-ILC3 by myeloid cells drives strong tissue inflammation and potentiates the pro-inflammatory type 1 immune response.

TYPE 2 IMMUNE CONTROL THROUGH THE ILC2-BASOPHIL/EOSINOPHIL/MAST CELL AXIS

Group 2 ILCs are potent producer of IL-4, IL-5, IL-13, IL-9, and GM-CSF. ILC2 secreted cytokines are able to activate myeloid cells through the common β chain, IL-5Rα chain, IL-9Rα chain,

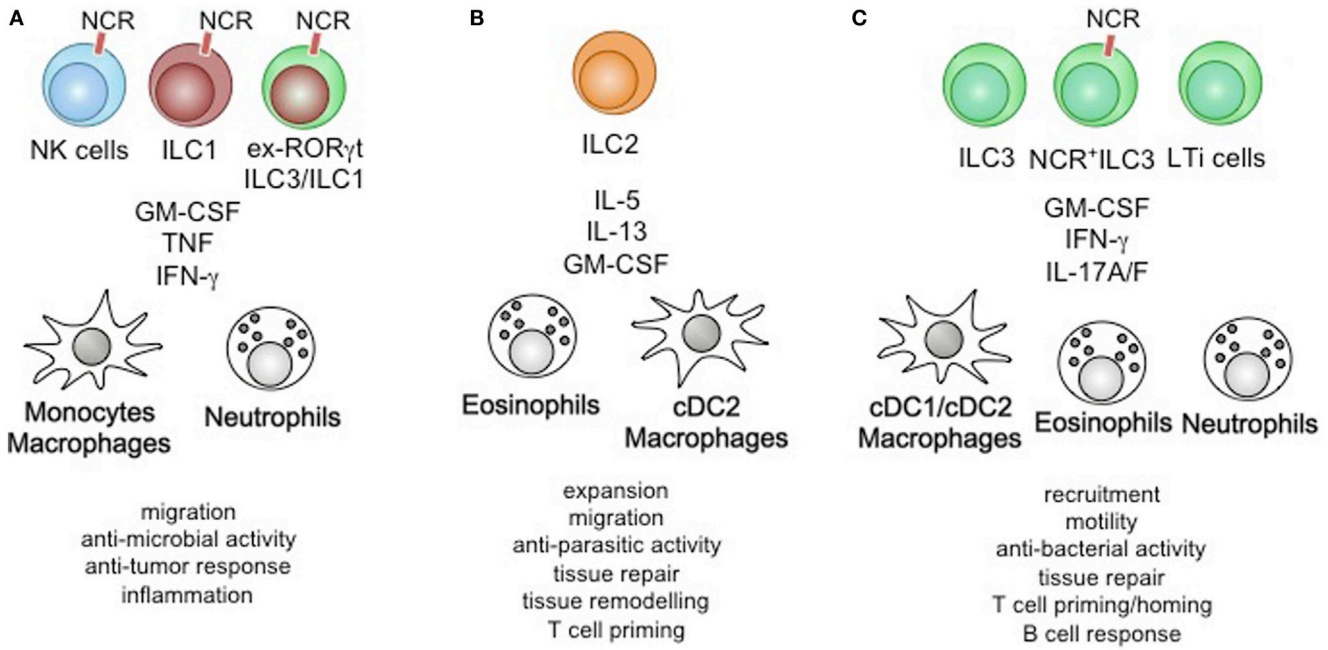

FIGURE 3 | Summary of ILC-mediated myeloid cell activation on immunity and physiology. **(A)** Natural killer (NK) cells, Group 1 ILCs (ILC1), and ex-RORγt ILC3/ILC1 control the migration of monocyte/macrophages and neutrophils, modulate their anti-microbial activity and anti-tumor response leading to sustained inflammation. **(B)** ILC2-derived cytokines act on eosinophils, dendritic cells (DCs), and macrophages to control their proliferation, migratory behavior, role in tissue remodeling and repair. Furthermore, ILC2-mediated activation of myeloid cells is essential in anti-parasitic immunity and optimization of Th2 priming. **(C)** ILC3s control the recruitment, motility, and tissue repair through the activation of DC/macrophages, eosinophils, and neutrophils. The anti-bacterial immunity driven by ILC3-produced cytokines contributes to host-defense, while homeostatic execution of ILC3-specific effector functions improves T cell priming and innate B cell responses.

IL-13R1, IL-13R2, common γ chain, and GM-CSFR α chain. Mast cells, basophils, eosinophils, macrophages, and DC express these receptors and are potential target cells for ILC2-derived cytokines (93, 97, 98) (**Figure 3B**). While IL-4, IL-9, IL-33, IL-25, and TSLP act on ILC2 to drive the secretion of IL-4, IL-5, IL-13, IL-9, and GM-CSF, a few of these cytokines act in an autocrine fashion on ILC2 to sustain the secretion of cytokines—being an important example of a positive feedback loop (34). IL-5 and GM-CSF are essential differentiation and activation factors for MPPs in the bone marrow and mucosal tissue. ILC2-produced cytokines were found to be key drivers of MPP-derived progeny cell differentiation (i.e., basophils, mast cells, eosinophils, and macrophages) upon stimulation by IL-25 and IL-33 and contributed to the local anti-parasitic host defense by enlarging the pool of tissue-resident myeloid cells through extramedullary myelopoiesis (87, 88). Interestingly, synergistic activity of IL-5 and IL-13 or IL-5 and IFN-γ was shown to modulate the phenotype of eosinophils, suggesting plastic behavior of eosinophils in the context of ILC activation (139). Concerted actions of INF-γ and IL-33 on ILC2-specific cytokine production revealed a similar pattern of adaptation to environmental cytokines by ILC2 (140). ILC2-derived IL-13 has been shown to acts on mast cells, macrophages, and DCs (34). In conjunction with other myeloid growth and differentiation factors like GM-CSF, IL-4 and IL-13 promote an alternatively activated macrophage phenotype that is essential in controlling adipocyte differentiation, lung tissue fibrosis, and anti-helmith immunity (141–143). DC exposed to the ILC2-produced cytokines IL-4 and IL-13 are more prone to licensing a type 2 Th cell response and support the recruitment of memory T helper cells to the inflamed airways during allergy (144). ILC2-derived cytokines further play a significant role in maintaining tissue macrophages through the recruitment of monocyte and the initiation of the differentiation into alternatively activated macrophages. More strikingly, ILC2-produced IL-13 is a critical factor driving allergic reactions and could be an interesting target for future therapeutic manipulation. Collectively, ILC2-derived cytokines control extramedullary myelopoiesis and specific adaptations of tissue-resident myeloid cells in the context of type 2 immune responses.

TYPE 3 IMMUNE CONTROL THROUGH THE ILC3–MONOCYTE/DC/GRANULOCYTE AXIS

Group 3 ILCs express the Th17-associated cytokines IL-17A, IL-17F IL-22, and GM-CSF in the steady state and IFN-γ during inflammation (6, 34). These cytokines engage the IL-17R, IFN-γR, and the GM-CSFR on myeloid cells. Macrophages, neutrophils, eosinophils, and DC are particularly responsive to GM-CSF even though the expression level of the GM-CSF-specific GM-CSFR alpha chain varies among these cells (23, 97) (**Figure 3C**). Much like other cytokines expressed by ILC3 (i.e., IL-22 and IL-17A), GM-CSF production requires instructive signal from the intestinal microbiota commonly sensed by myeloid cells and epithelial cells (26). ILC3-derived GM-CSF has been implicated

in connecting the innate and adaptive immune response through its actions on myeloid cells. It imprints macrophages, DC and neutrophils with the capacity to produce RA, IL-10, and BAFF, cytokines that contribute to the generation of regulatory T cells, gut homing of T cell, and the production of innate antibody responses in the steady state (26, 71, 126, 127). Under inflammatory conditions, GM-CSF acts in concert with IFN-γ, TNF-α, and pathogen associated molecules to induce a switch in effector functions and polarization (134). It controls the anti-microbial activity of neutrophils and eosinophils and contributes to exacerbated inflammation at mucosal tissues (128, 137). Most striking, both the absence of and the chronic production of GM-CSF result in high susceptibility to intestinal pathology, demonstrating the importance of its balanced expression and activity to maintain homeostasis (145, 146). Additionally, promiscuous expression of GM-CSF at tissue sites that commonly show little to no expression of GM-CSF has been associated with an increased pathology (147). These findings suggest that increased expression of ILC3-derived GM-CSF or ectopic expression in concert with other cytokines support inflammation. It will be important to dissect the role high versus low level of GM-CSF expression in the context of tissue inflammation, as well as the localized actions on the myeloid effector pool. With some reports demonstrating IFN-γ production in ILC3, the concerted expression of GM-CSF and IFN-γ by ILC3 could help to identify unique inflammatory ILC3 subsets that would be an interesting target for treatment of chronic inflammatory conditions.

DISCUSSION

Research over the last decade extended our understanding of ILCs as a new and exciting cell type within the innate immune system. ILCs are a potent source of homeostatic cytokines in the steady state and essential in tuning inflammatory innate and adaptive immune responses (4). Being members of the tissue-resident immune compartment, ILCs respond to cytokines released by activated myeloid cells in order to sustain their own cytokine profile. Several ILC-derived cytokines signal back to myeloid cells and in turn control their survival, recruitment, functional profile or developmental origin. The communications of ILC subsets and their matching myeloid counterpart within the tissue are elementary to early host defense and significantly contribute to chronic inflammatory processes like IBD, psoriasis, multiple sclerosis, obesity, or asthma. Deciphering the essential homeostatic and inflammatory cytokines, their modes of orchestrated action within the network of ILC-regulating cytokines will greatly advance our understanding of how the myeloid cell–ILC axis drives defense or triggers chronic inflammation. New *in vitro* cell culture systems will be instrumental in characterizing the responsive signaling pathways within ILCs to identify selective drug targets (148–150). With ILC-derived cytokines acting on myeloid cells and their precursors, functional and developmentally distinct myeloid cells arise at sites of tissue-resident ILC activation. Identifying the contribution of individual cytokines to the onset of this myeloid reprogramming will uncover the role specific ILC-derived cytokines during defense or chronic inflammation and should be another priority to target ILCs for tissue-specific modulation. While ILCs can secrete several cytokines at the same time, subsets of ILCs at the site of activation could collaborate to confer more then two cytokine-signals to myeloid cells. The analysis of ILC subsets and their cytokine profiles during health and disease will help identify inflammatory cytokine-producing ILC subsets that either initiate defense, or chronic inflammation. A systematic approach is required to understand myeloid-derived cytokine networks that control the crosstalk of myeloid cells and ILCs in order to tailor interventions of their communication. Understanding the changes of ILC and myeloid cell-derived cytokines at the local tissue site during, steady state, infection or chronic inflammation will allow the design of strategies to revert tissue-specific changes in innate immune activation associated with inflammation. Several clinical trials targeting inflammatory cytokines are in pre-clinical or clinical phase, aiming to block tissue-specific inflammation by systemic neutralization of cytokines. While successful therapies are based on this approach of cytokine neutralization (e.g., Infliximab or Anakindra), other targeting strategies resulted in severe side effects that exacerbated the pathology (151). The cytokine-mediated cross talks described in this review offer several targets that could be utilized to intervene in the myeloid cell–ILC communication. Targeting approaches however, should take into account that fine-tuning the myeloid cell-ILC axis, rather then blocking it completely might be desirable. Sustained, homeostatic activation of ILCs should be maintained during targeting approaches. With strong developmental and functional homology between mouse and human ILCs across tissues, animal models are an attractive system to screen for myeloid cell–ILC interactions that could be translated into the human setting. In summary, understanding the cytokine-mediated cross talks between myeloid cells and tissue-resident ILCs could lead to a better understanding of tissue homeostasis and chronic tissue inflammation.

AUTHOR CONTRIBUTIONS

AM and KB wrote the manuscript and designed figures.

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REVIEW
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Regulation of Innate Lymphoid Cells by Aryl Hydrocarbon Receptor

*Shiyang Li[1], John W. Bostick[2] and Liang Zhou[1]**

[1] Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL, United States, [2] Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, United States

With striking similarity to their adaptive T helper cell counterparts, innate lymphoid cells (ILCs) represent an emerging family of cell types that express signature transcription factors, including T-bet$^+$ Eomes$^+$ natural killer cells, T-bet$^+$ Eomes$^-$ group 1 ILCs, GATA3$^+$ group 2 ILCs, RORγt$^+$ group 3 ILCs, and newly identified Id3$^+$ regulatory ILC. ILCs are abundantly present in barrier tissues of the host (e.g., the lung, gut, and skin) at the interface of host–environment interactions. Active research has been conducted to elucidate molecular mechanisms underlying the development and function of ILCs. The aryl hydrocarbon receptor (Ahr) is a ligand-dependent transcription factor, best known to mediate the effects of xenobiotic environmental toxins and endogenous microbial and dietary metabolites. Here, we review recent progresses regarding Ahr function in ILCs. We focus on the Ahr-mediated cross talk between ILCs and other immune/non-immune cells in host tissues especially in the gut. We discuss the molecular mechanisms of the action of Ahr expression and activity in regulation of ILCs in immunity and inflammation, and the interaction between Ahr and other pathways/transcription factors in ILC development and function with their implication in disease.

Keywords: aryl hydrocarbon receptor, innate lymphoid cell, mucosal immunity, gut, microbiota and immunity

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****Correspondence:***
Liang Zhou
liangzhou497@ufl.edu

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INTRODUCTION

Innate lymphoid cells (ILCs) are newly identified cell populations, which mirror helper T cells, such as Th1, Th2, and Th17 cells, by expressing similar transcription factors and cytokines (1–3). ILCs are divided into group 1 ILCs (ILC1) (T-bet$^+$), group 2 ILCs (ILC2) (GATA3$^+$), and group 3 ILCs (ILC3) (RORγt$^+$) (1). To join the group, a new type of ILC that express the transcription factor Id3 and exhibit regulatory function [known as regulatory ILC (ILCreg)] have also recently been identified (4). Notably, natural killer (NK) cells have been defined as distinct population from ILC1, based on eomesdermin (Eomes) expression and a distinct progenitor from other ILCs (5). ILCs are predominantly locate at the mucosal barriers and participate in various biological processes, such as control of pathogenic infection, progression of autoimmune disease, as well as development of cancer (2, 6, 7). Different from adaptive immune cells, ILCs lack the antigen stimulation step and respond quickly under certain contexts of disease (8). The aryl hydrocarbon receptor (Ahr) is a ligand-dependent transcriptional factor, which can sense environmental and endogenous compounds generated by commensal, dietary, or cellular metabolism (9–11). Ahr has been studied in the development and/or function of various immune/non-immune cells (11) and recently found to be key regulator of ILC3 (12–14). There are many extensive reviews on Ahr in other immune cells. In this review, we focus our efforts on summarizing the recent progresses on decoding Ahr physiological functions in the development and function of ILCs, as well as Ahr-mediated cross talk between ILCs and other immune/non-immune cells in host tissues, especially in the gut. We discuss the

molecular regulation of Ahr expression and activity in ILCs, and the interaction between Ahr and other pathways/transcription factors in ILC development and function. We also identify areas that need further study, especially the role of Ahr in group 1 and group 2 ILCs.

DESCRIPTION AND FUNCTION OF ILCs

Innate lymphoid cells share the same progenitor, common lymphoid progenitors (CLPs), as adaptive immune cells, including T and B cells (15). CLPs differentiate toward unique direction to α-lymphoid precursor, and then common helper innate lymphoid progenitor (CHILP), to become ILCs, including NK cells, ILC1, ILC2, ILC3, and ILCreg (5, 16). The lineage-defining transcription factors, key regulators, stimuli, and effector molecules are summarized in **Table 1**.

Group 1 ILC

While NK cells are predominantly circulating in the blood and secondary lymphoid organs such as the lymph nodes and spleen, NK cells are also found in some non-lymphoid tissues such as the liver, uterus, and lung (17). Closely related in function to NK cells, ILC1 are present in various non-lymphoid tissues, including intestine, liver, salivary glands, and the female reproductive tract (18). The development and function of ILC1 depend on T-bet, while the requirement of T-bet by NK cells appears to be complicated since deletion of T-bet reduces the numbers of NK cells in liver, spleen, and peripheral blood (19, 20), but not in bone marrow and intestine (5, 19, 20). The transcription factor, Eomes, distinguishes NK cells from ILC1 and is indispensable for the development of NK cells (5). Recent studies indicate that NK cells and ILC1 derive from different progenitors, which further separate NK cells from ILC1 (5). Although developmentally identified as two distinct populations, NK cells and ILC1 can be stimulated by IL-12, IL-15, or IL-18 to produce interferon γ (IFNγ) and tumor necrosis factor (TNF) (18), which are critical for the immune response to control intracellular pathogens, viruses, and tumors (5, 21, 22). NK cells have the ability to secrete granzyme and perforin to promote cytotoxic function, which imparts NK cells tumor suppression activity, distinct from ILC1 (23). Different from intestinal lamina proprial ILC1 that express T-bet but not Eomes, intraepithelial ILC1 have been shown to express both T-bet and Eomes, and produce granzyme and perforin; however, lack of the requirement of IL-15 signals for their maintenance distinguishes intraepithelial ILC1 from NK cells (24).

Group 2 ILC

Group 2 ILCs have been identified to localize in various lymphoid/non-lymphoid tissues, including intestine, lung, adipose tissue, spleen, nasal tissue, and skin, while immature ILC2 are also reported in bone marrow (25–27). The development and function of ILC2 require GATA3, RORα, Gfi1, TCF1, Bcl11b, and Notch signaling, of which GATA3 acts as the defining marker of ILC2 (26, 28–34). Upon stimulation with IL-25, IL-33, or thymic stromal lymphopoietin (TSLP), ILC2 can produce IL-5, IL-13, and IL-4, similar to Th2 cells, which contribute to the control of helminth infection and pathology of allergic inflammation (25, 35–39). ILC2 can also express IL-9 to promote the epithelial cell maintenance in the lung (40, 41). Amphiregulin is an effector molecule produced by ILC2 to participate in the tissue repair in the gut (42). Additionally, ILC2 have been shown to promote the beiging of white adipose tissue to control obesity through the production of methionine-enkephalin peptides (43, 44).

Group 3 ILC

Group 3 ILCs are mainly found in gastrointestinal tract, while few ILC3 are present in other tissues (45, 46). ILC3 are

TABLE 1 | Features of innate lymphoid cell (ILC) subsets.

Nomenclature		Lineage-defining transcription factors	Key transcription factors	Stimuli	Effector molecules
Group 1 ILCs (ILC1)	Natural killer cells	T-bet, Eomes	ETS1, Blimp1, KLF4, Helios, TOX, Nfil3, Id2, aryl hydrocarbon receptor (Ahr)	IL-12 IL-15	IFNγ, TNFα, perforin, granzymes
	ILC1	T-bet	GATA3, Nfil3, Id2, Ahr	IL-18	IFNγ, TNFα
Group 2 ILCs (ILC2)	ILC2	GATA3	Gfi1, RORα, Bcl11b, TCF1, G9A, ETS1, Nfil3, Id2, Notch	IL-25 IL-33 Thymic stromal lymphopoietin TNF-like ligand 1A IL-15	IL-4, IL-5, IL-9, IL-13, Areg
Group 3 ILCs (ILC3)	NCR$^+$CCR6$^-$ ILC3	RORγt, T-bet	Ahr, WASH, GATA3, Nfil3, Id2, Ikaros, Notch	IL-23 IL-1β	IL-22, IFNγ, GM-CSF
	NCR$^-$CCR6$^+$ ILC3	RORγt	Ahr, GATA3, Nfil3, Id2, Ikaros	IL-15	IL-22, IL-17
	NCR$^-$CCR6$^-$ ILC3	RORγt, T-bet	Ahr, GATA3, Nfil3, Id2, Ikaros	IL-18	IL-22, IL-17, IFNγ
	Fetal lymphoid tissue inducer	RORγt	Id2, Ikaros, GATA3, Nfil3	NA	Lymphotoxin
Regulatory ILC (ILCreg)	ILCreg	Id3	Id3	TGFβ	IL-10

heterogeneous, and can be divided, based on the expression of the natural cytotoxicity receptor (NCR or NKp46/NKp44) and chemokine receptor 6 (CCR6), into three major groups: NCR^+CCR6^- ILC3, NCR^-CCR6^+ ILC3, and NCR^-CCR6^- ILC3 (47). It should be noted that the above discussion is on ILC3 after birth. Fetal ILC3, also known as lymphoid tissue inducer (LTi) cells, which express RORγt, function in the formation of secondary lymphoid organs, such as lymph nodes and gut-associated lymphoid tissue (48–50). Postnatal $CCR6^+$ ILC3 found in the gut and other lymphoid organs are known as LTi-like cells (51). While RORγt is the common transcription factor that is required for the development, maintenance, and function of all ILC3 (52), NCR^+ ILC3 also appear to depend on T-bet for development and function (53). When stimulated, all three subsets of ILC3 produce IL-22, while NCR^+ ILC3, relying on T-bet, can express IFNγ (53). In addition, ILC3 can also secret IL-17A and GM-CSF (51, 54). GATA3 is required for development of all IL-7Rα-expressing ILCs (55). Although GATA3 expression is high in ILC2, it is also expressed at a lower level in ILC1 and ILC3 and required for their maintenance (5, 56). It has been shown that GATA3 is important for ILC3 function to produce IL-22 (47). ILC3 are involved in clearance of bacterial and fungal infection, control of enteric virus infection, and maintenance of microbiota (57–62), while recent studies suggest that GM-CSF, as well as IL-22, expressed by ILC3 participate in ILC-driven colitis (63–65). After birth, ILC3 are also required for the development of cryptopatches and isolated lymphoid follicles (ILFs) in the gut through expression of lymphotoxin and CCR6 (66–69).

Regulatory ILC

In addition to ILCs discussed above, a new ILC subset, with the ability to suppress ILC1 and ILC3 to promote the resolution of intestinal inflammation, has been identified recently in mice (4). Although further work is needed to confirm the existence and function of this cell type, ILCreg, mainly populate in the gut, develop from CHILP in bone marrow, and require transcription factor Id3 for their development. The regulatory function of ILCreg is mediated by IL-10. TGFβ1 is required for the expansion of ILCreg during inflammation (4). In human, the regulatory ILC (ILCreg) are also reported in the context of cancer recently (70), to suppress the expansion of tumor-associated T cells. Different from the mouse ILCreg that do not express other ILC signature transcription factors, the human ILCreg, present in the tumor tissue, express high levels of Eomes, T-bet, GATA3, RORα, and Ahr, suggesting an overlapping transcriptional profile of the human ILCreg and other ILC subsets.

Ahr STRUCTURE AND ACTIVATION

Aryl hydrocarbon receptor is a ligand-dependent transcription factor and belongs to Per-Arnt-Sim (PAS) superfamily (71, 72). Various Ahr ligands have been identified, including environmental pollutants such as dioxins, and multiple physiologic ligands generated by microbiota, diet, and host metabolism (73–76). Without ligand binding, Ahr localizes in the cytoplasm, and this inactive status is maintained by interacting with 90-kDa heat shock protein (HSP90) (77). Ahr also interacts with Ahr-interacting protein (AIP) which protects Ahr from degradation (78), as well as p23 (79). Upon ligand activation, the conformation of Ahr is changed, leading to the release of Ahr from the protein complex and the translocation of Ahr into the nucleus, where Ahr interacts with Ahr nuclear translocator (ARNT) through PAS-A domain and bHLH domain (80) and acts as a transcription factor targeting dioxin response element (DRE)-containing genes, which are prototypically cytochrome P450 family, like Cyp1a1, but also include genes involved in other important biological processes (13, 81). Several partners of Ahr have been identified, such as RORγt, sterol regulatory element binding transcription factor 1, LXR, NF-κB (13, 82, 83). The involvement of ARNT in these reported interactions remains to be determined.

Aryl hydrocarbon receptor was initially identified as the sensor for 2,3,7,8-tetracholrodibenzo-p-dioxin (TCDD) (84). Later, a variety of Ahr ligands were identified from different physiological sources, such as tryptophan (Trp) metabolism and microbiota. The metabolism of Trp generates Ahr ligands through catalysis by indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) to kynurenine (Kyn), which acts as an Ahr ligand (76, 85, 86). Independent of IDO/TDO, Trp can also be metabolized by the tryptamine and serotonin pathway, of which the metabolites can act as Ahr agonist (87, 88). Notably, Trp can be photo-oxidized by ultraviolet light or metabolized by other pathways to 6-formylindolo[3,2-b]carbazole (FICZ), which has been proven as a physiologically relevant Ahr agonist (89, 90). Of note, a higher concentration of Kyn, at micromolar concentration, compared to nanomolar of TCDD or FICZ, is required for Ahr activation.

In addition to cellular metabolism, commensal bacteria can catalyze Trp into Ahr ligands as well (74, 91). Lactobacilli expand when the energy source switches from sugar to Trp, and produce indole-3-aldehyde which acts as Ahr ligand to promote IL-22 production by ILC3 (74). Consequently, the Ahr-IL-22 axis provides resistance to the fungus *Candida albicans* and protection from dextran sulfate sodium (DSS)-induced colitis. In accordance with the importance of Trp in mice, recent research suggests that dysregulation of commensal bacteria that use Trp to generate Ahr ligands may correlate with the pathogenesis of human inflammatory bowel disease (IBD) (92). Besides the Ahr ligands generated by cellular metabolism or commensal bacteria, bacterial pigment factors such as the phenazines from *Pseudomonas aeruginosa* and the naphthoquinone phthiocol from *Mycobacterium tuberculosis* can also act as ligands for Ahr, and contribute to the antibacterial response through activation of the Ahr pathway (93).

Ahr EXPRESSION IN ILCs

Aryl hydrocarbon receptor is thought to be expressed ubiquitously in various organs and cell types, including immune cells, such as Th17 cells, IL-17-producing γδ T cells, Treg cells, CD8αα IEL lymphocytes, B cells, Langerhans cells, monocytes, and splenic dendritic cells (DCs) (94–100). However, the expression of Ahr in ILCs, at both mRNA and protein level, remains to be clarified. Genome-wide transcription analysis

of different ILC populations, which is available at IMMGEN.ORG, has shown that *Ahr* mRNA is detectable among ILCs (101). It has been reported that cytokine stimulation, including IL-2, IL-12, or IL-15, can enhance Ahr expression in splenic NK cells (102, 103). In addition, the transcription factor, Distal-Less Homeobox 3 is found to enhance Ahr transcription in NK cells, while its function remains to be determined (104).

We and other groups have reported the expression of Ahr in ILC3. Differential levels of Ahr were observed in different subsets of ILC3 (13, 37, 41). NCR^+ ILC3 express higher Ahr than the other two subsets of ILC3, which lack NCR on the surface (13). How Ahr expression is regulated in ILCs has been a subject of active research. Recent study has shown that in NCR^+ ILC3, Wiskott-Aldrich syndrome protein and SCAR homolog (WASH) activates Ahr expression by recruiting AT-Rich Interaction Domain 1A (Arid1a) to the *Ahr* promoter, and thus maintains NCR^+ ILC3 in the gut (105).

Although further investigation on Ahr expression, especially at the protein level, needs to be conducted, the public data at IMMGEN.ORG appears to show that the special microenvironment of the gut correlates with the high Ahr transcriptional expression, since lower Ahr expression is observed in spleen or liver NK cells or ILC1. In a *Cyp1a1* (a target gene of Ahr) reporter mouse, Ahr was shown mainly active in the gut in homeostatic conditions (106). A recent paper using a mouse model in which GFP was knocked into the endogenous locus of Ahr showed that among Tregs in various tissues, gut Treg cells express the highest amounts of Ahr, suggesting a tissue adaptation of Ahr expression (107). Identification of the gut specific factors, such as cytokines/metabolites and transcription factors that facilitate Ahr expression will provide insights into the regulation of Ahr expression in ILCs, and potentially be translated into clinical manipulation of the Ahr pathway. To get a molecular understanding on the regulation of Ahr expression, it is of importance to analyze chromatin status of the Ahr locus and Ahr interactions with key transcription factors in different ILC populations.

INVOLVEMENT OF Ahr IN ILC FUNCTION AND REGULATION

Ahr and NK Cells/ILC1

In tumor, Ahr promotes NK cell cytotoxicity and its production of IFNγ (103). During *T. gondii* infection, Ahr is also required for maximal IL-10 production by NK cells (102). It has also been shown that Ahr maintains liver-resident $CD49a^+$ cells by regulating cytokine-induced cell death (108). Notably, CD49a is considered as a marker for ILC1 in the liver, instead of NK cells (18). Therefore, these data may suggest that Ahr is required for liver ILC1 maintenance (108).

So far, the studies on Ahr in NK cells or ILC1 have been predominantly focused in the liver or spleen. The function of Ahr in the gut ILC1 and NK cells still remains to be elucidated, given that the gastrointestinal tract is another site for these two cell populations, especially for ILC1 (5).

Ahr and ILC2

Currently, limited knowledge is available on the function of Ahr in ILC2. IFNγ has recently been shown to inhibit ILC2 activation (109, 110). In addition, IFNγ can induce *Ido1* mRNA and Ido protein expression in some cell types (111, 112). Given that Ido1 is able to catalyze Trp to Kyn, which acts as a ligand for Ahr, it is tempting to speculate that Ahr ligands, such as Kyn, might suppress ILC2 function but additional works are needed to test this hypothesis. TNF-like ligand 1A (TL1A) has been shown to promote expansion and function of ILC2 in the gut (113). RNA-seq data reveal that TL1A enhances Ahr expression in the presence of IL-33 and IL-25 in human ILC2 (114). Thus, the function of Ahr in ILC2 and in *in vivo* models of ILC2-driven pathology remains to be investigated.

Ahr and ILC3

Aryl hydrocarbon receptor has been relatively well studied in gut ILC3. Although it is dispensable for fetal LTi development, Ahr is essential for the maintenance and IL-22 production of ILC3 (12, 13, 45). Although the precise mechanisms by which Ahr regulates the homeostasis of ILC3 still remain to be determined, it has been described that Ahr can regulate survival and/or proliferation of ILC3 (**Figure 1**). First, it is reported that Ahr is important for the survival of ILC3 by promoting the expression of anti-apoptotic proteins, such as Bcl-2. Ahr upregulates IL-7 receptor (IL-7R) in ILC3, in line with the role of IL-7/IL-7R signaling pathway in the supporting the survival of ILC3 (13). Second, it has been shown that Ahr-deficient ILC3 have reduced Ki67 expression, indicating that decreased proliferation may lead to the defective expansion of ILC3. Furthermore, Ahr can regulate the expression of Kit through binding to DRE at the promoter of *Kit* locus, suggesting direct regulation of Kit expression by Ahr at the transcriptional level (12). Finally, Ahr supports the development of ILC3 presumably through promoting the transcription of Notch 1 and Notch 2, although defects in Notch signaling have more effect on NCR-expressing ILC3 than NCR-negative ILC3 (45). By regulating the maintenance and function of ILC3, Ahr is critical for the clearance of *Citrobacter rodentium*, a murine pathogen that models human enterohemorrhagic *Escherichia coli* and enteropathogenic *E. coli* infections in the gut (12, 13, 64), as well as for the pathology of anti-CD40-incuced colitis (64).

Ahr in ILC Plasticity

The plasticity of ILCs has been observed in both human and mouse systems under steady state or certain disease models, while the mechanism that drives the plasticity of ILCs is still not well understood. The conversion of ILC3 to ILC1 is characterized by the loss of RORγt and gain of T-bet expression to become exILC3 (115, 116). These exILC3 stop the production of IL-22, and begin to secrete IFNγ. IL-15 and IL-12 can lead to downregulation of RORγt, and enhance IFNγ expression (116). In support, IL-12 has been shown to participate in the transition of ILC3 to ILC1 in humans (115, 117). There is an increase of ILC1 and decrease in ILC3 in the intestines of patients with Crohn's disease, suggesting the ILC3-derived ILC1 might

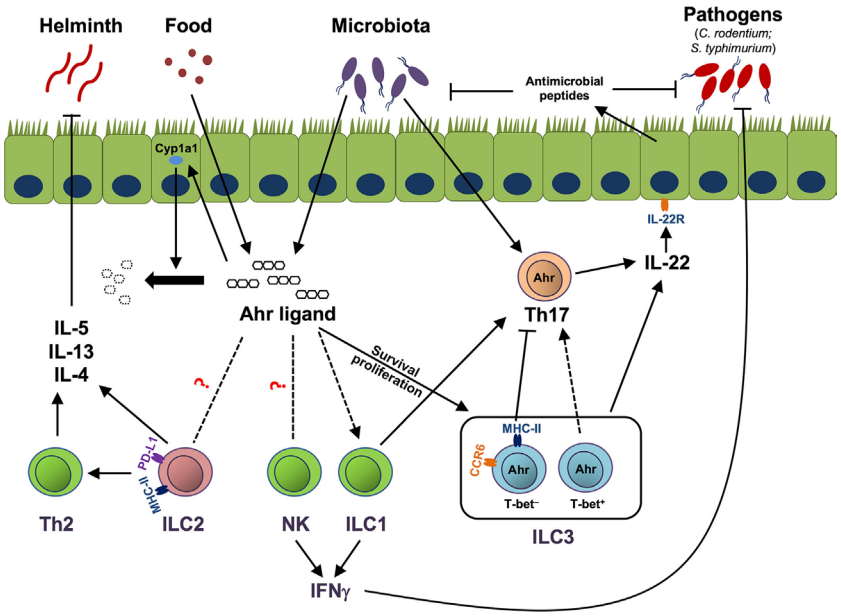

FIGURE 1 | Aryl hydrocarbon receptor (Ahr)-mediated cross talk between innate lymphoid cells (ILCs) and immune/non-immune cells. Ahr ligands derived from the diet or microbiota activate Ahr to promote group 3 ILCs (ILC3) homeostasis by enhancing the survival or proliferation of ILC3. MHC-II$^+$ ILC3, which are mainly CCR6$^+$, suppress pathogenic Th17 response to commensal bacteria, while T-bet$^+$ ILC3, together with group 1 ILC (ILC1), promote Th17 cells. Both Th17 cells and ILC3 can produce IL-22 to control commensal/pathogenic bacteria through facilitating the production of antimicrobial peptides by epithelial cells. Ahr ligand could potentially regulate natural killer (NK) cells, ILC1, and group 2 ILCs (ILC2) through the Ahr pathway in the gut. NK cells and ILC1 can help the host to clear pathogens, like *Salmonella typhimurium*, by production of effector cytokine IFNγ. ILC2, through expression of MHC class II (MHC-II) and programmed death ligand 1 (PD-L1), enhance Th2 cells. ILC2 and Th2 cells protect the host from helminth infection by secreting type 2 cytokines, including IL-5, IL-13, and IL-4. Ahr ligand enhances Cyp1a1 expression in gut epithelial cells, and as a feedback negative control loop, Cyp1a1 degrades Ahr ligand to prevent overt Ahr-mediated immune responses. Solid lines and arrows depict known regulation. Dotted lines and arrows depict to-be-determined regulation in the gut.

contribute to the pathology of human IBD (115, 117). Human ILC1 can also convert to ILC3 in the presence of IL-2, IL-23, and IL-1β, and retinoic acid can accelerate this process which may depend on the receptors for retinoic acid (117). Although Ahr has been shown to prevent the differentiation of human ILC3 to NK cells (118), it is of interest to determine whether Ahr participates in the transition of ILC3 to ILC1. Of note, microbiota has been shown to maintain the RORγt expression by ILC3 through IL-7 signaling in the gut (116). Since commensal bacteria have the ability to produce Ahr ligands, Ahr might receive the signals from microbiota to maintain ILC3 through upregulating IL-7R.

The plasticity of ILC2 has recently been reported (31, 119–121). Gfi1, a key transcription factor for ILC2 development and function, appears to sustain ILC2, as deletion of Gfi1 in ILC2 leads to upregulation of RORγt and IL-17 production by these ILC2 (29). Similarly, Bcl11b, a recently defined transcription factor for ILC2, maintains the stability of ILC2 by suppressing RORγt and Ahr expression (31). ILC2 were found to convert to IFNγ-producing ILC1 in the lung by IL-12 and IL-18 (119–121). The conversion is dependent on T-bet expression, and enhanced by IL-1β through induction of the IL-12 receptor alpha (Il12rα). As the frequency of ILC1 shows positive correlation with disease severity in patients with chronic obstructive pulmonary disease or chronic rhinosinusitis with nasal polyps (119, 121), the plasticity of ILC2 could be a therapeutic target for these respiratory diseases. Whether Ahr plays any role in ILC2 conversion to ILC1 needs to be established.

Ahr-MEDIATED MODULATION OF THE CROSS TALK BETWEEN ILCs AND OTHER CELLS

Cross Talk with Innate Immune Cells

Innate immune cells, such as dendritic cells (DCs) and mononuclear phagocytes (MNPs), have been shown to interact with ILCs. Both CX3CR1$^+$ MNPs and CD103$^+$ DCs can induce IL-22 production by ILC3, while CX3CR1$^+$ MNPs can also recruit ILC3 to the gut through CXCL16–CXCR6 pathway (122–124). Ahr controls the differentiation and function of DCs by arresting the differentiation of progenitors, as well as regulating antigen presentation in DCs (125–128). In addition, recent work has shown that Ahr controls differentiation of monocyte to monocyte-derived macrophages in the human system (129). Thus, it is possible that Ahr controls ILC3 through regulating these innate immune cells. On the other hand, innate immune cells can be attracted by ILC-secreted cytokines. IL-5 and IL-13, produced by ILC2, can recruit eosinophils which are essential for the clearance of helminth infections (25). ILC3 can secrete IL-17A, which is proved to attract neutrophils into the intestine (130, 131). Thus, lack of ILC3 in Ahr-deficient mice may account for the resistance of anti-CD40 colitis (64).

Cross Talk with Adaptive Immune Cells

The absence of Ahr in ILC3 leads to defects in the IL-22-producing ability of ILC3. The impaired IL-22 production in the gut of Ahr-deficient mice causes a decrease in antimicrobial peptide production by gut epithelial cells (62, 132), leading to increased segmented filamentous bacteria (SFB) which has been established to induce Th17 cells in the gut (133, 134). However, recent papers also show that SFB induced IL-22 production by ILC3 can induce epithelial production of Serum Amyloid A, which in turn promotes Th17 cells (135, 136). Thus, the role of ILC3-derived IL-22 in regulating Th17 cells will require further investigation into the underlying molecular mechanisms, which are most likely indirect given the lack of expression IL-22R by immune cells. By supporting ILC3 homeostasis, Ahr controls cryptopatches formation, and consequently the genesis of ILFs in the gut (12, 137). As ILFs have been recognized as a site for the production of intestinal IgA responses (138), it is possible that Ahr contributes to B cell responses *via* the regulation of ILC3, in addition to its B cell-intrinsic roles (96, 97, 139).

Recent research showed that ILC2 are critical for memory Th2 cell responses, as impaired Th2 cells are found in sensitized mice, which lack ILC2 (140). During helminth infection, ILC2 have been shown to express the checkpoint molecule Programmed Death Ligand 1, through which ILC2 support Th2 polarization, and effective Th2 dependent-anti-helminth response (141). Additionally, ILC2, through producing IL-9, can sustain the proliferation of ILC2 and activation of Treg cells in arthritis, by which promote the resolution of inflammation (142). It is of interest to note that, with the resistance to IL-7-induced down-regulation of IL-7R, ILCs limit the availability of IL-7 for T cells, thus controlling the homeostasis of T cells (143). Given that Ahr deficiency leads to reduction of ILC3, it remains to be determined whether enhanced T cell proliferation and Th17 cell differentiation observed in Ahr knockout mice are caused by increased IL-7 that is made available to T cells.

In addition to the cross talk between ILCs and adaptive immune cells through cytokines, ILCs interact with T cells through the expression of MHC class II (MHC-II) molecules on the surface. The MHC-II-mediated interaction between ILCs and T cells controls the activation or anergy of T cells (**Figure 1**). For example, ILC2, *via* MHC-II and co-stimulatory molecules, CD80 and CD86, interact with and activate T cells (144). Different from ILC2, ILC3 expressing MHC-II but not the co-stimulatory molecules CD80 and CD86, induce T cell apoptosis and tolerance in the gut (145, 146). However, ILC3 express CD30 ligand and OX40 ligand, which may contribute to the maintenance of $CD4^+$ T cell memory (147). Although there is no direct evidence indicating whether Ahr regulates MHC-II or co-stimulatory molecule expression by ILC2 and ILC3, Ahr may mediate the cross talk between ILCs and T cells, at least through regulating ILC numbers (**Figure 1**). A recent study reveals that NCR-expressing ILCs, including ILC1 and NCR^+ ILC3, support Th17 cells in inflamed central nervous system (148), which raises intriguing questions that whether similar event is evident in the gut, and how the host keeps the balance between the induction of Th17 cells by NCR^+ ILCs, and the inhibition of Th17 cells by $CCR6^+$ ILC3 through MHC-II expression (**Figure 1**).

Cross Talk with Epithelial Cells

The cross talk between gut epithelial cells and ILC3 has been recently investigated. Over-expression of Cyp1a1, a target gene of Ahr, in epithelial cells consumes Ahr ligands in the gut, which consequently leads to the decrease of gut ILC3 (106) (**Figure 1**). These findings raise the possibility that activation of Ahr may not only promote gut ILC3 in a cell-intrinsic manner, but also maintain the ILC3 at a physiological level through controlling the availability of Ahr ligands in the gut. On the other hand, ILC3, *via* expression of IL-22 and lymphotoxin, regulate the fucosylation of epithelial cells which is critical for the host to control *Salmonella typhimurium* infection (149). In addition, ILC3, *via* producing IL-22, promote the expansion of intestinal stem cell, and consequently promote the regeneration of intestinal epithelium after gut injury (150, 151).

Cross Talk with Commensals

Aryl hydrocarbon receptor appears to mediate the interaction of ILC3 and microbiota. The absence of caspase recruitment domain family member 9 (CARD9) results in alteration of microbiota, and the altered microbiota fail to metabolize Trp into Ahr ligands, leading to decreased ILC3 and IL-22 production, and increased susceptibility of the host to colitis (92). Accordingly, Ahr ligands are found decreased in the microbiota of IBD patients, especially in the individuals with IBD-associated single-nucleotide polymorphism within *CARD9* (rs10781499), suggesting microbiota–Ahr ligand axis may be a therapeutic target of colitis in humans (92). Although the cross talk between ILCs and microbiota remains to be further explored, genome-wide analysis at the transcriptional level of ILCs has been conducted using RNA-seq by comparing specific pathogen-free mice to those with microbiota depletion (152). Marked numbers of transcripts change significantly in all ILCs upon antibiotics treatment, but the expression profile is generally maintained. Intriguingly, depletion of microbiota shows more effects on the gene expression of ILC1 and ILC2 than that of ILC3. Given the important role of Ahr in ILC3 and Ahr could sense ligands generated by commensals, for example, *Lactobacillus reuteri* (74, 91), these findings may suggest ligands from other sources (e.g., diet) could activate the Ahr pathway in the absence of microbiota.

Regulation of ILCs by ILCreg

With the minimal Ahr expression in mouse ILCreg at least under the steady state (4), it remains to be determined whether Ahr plays a role in ILCreg. In contrast to the mouse ILCreg, human ILCreg in cancer that suppress T cell expansion appear to express high level of Ahr, indicating potential role of Ahr in this population (70). The mouse ILCreg have been shown to regulate ILC1 and ILC3 (4), it is unclear whether ILCreg can suppress ILC2.

ILC-Nervous System Interaction

The nervous system has been shown to affect ILCs. Glial cells in the gut, through secreting neurotrophic factors that bind to the neuroregulatory receptor rearranged during transfection (RET) on ILC3, promote the expression of IL-22, and consequently decrease the susceptibility to intestinal inflammation and infection (153). Recent studies demonstrate that among various

hematopoietic cells, ILC2 uniquely express the neuropeptide neuromedin U (NMU) receptor 1 (NMUR1), which makes them respond to NMU (154–156). The activation of ILC2 by NMU leads to enhanced cell expansion and type 2 cytokine production, which promote the clearance of helminth in the gut. It remains to be determined that whether Ahr modulates ILC responses to neuromediators.

Cooperation of Ahr and Partners in Regulating ILCs

Aryl hydrocarbon receptor has been studied for decades, and some interacting proteins, like HSP90 and AIP, have been well documented. However, only a few partners of Ahr have been functionally implicated in ILCs. In Th17 and IL-17-producing γδ T cells, Ahr regulates IL-22 expression while the molecular mechanism of action of Ahr is unclear (94, 95). However, Ahr has been shown to interact with RORγt in an overexpression system to promote IL-22 expression (13). RORγt is required for the recruitment of Ahr to the *Il22* locus, as Ahr alone fails to bind to the *Il22* locus. In contrast to the *Il22* locus, Ahr is recruited to the *Cyp1a1* locus independent of RORγt. These data raise a question regarding how Ahr, by cooperating with other transcription factors (e.g., RORγt), regulates gene expression in ILC3 and other lymphocytes (e.g., Th17 and γδ T cells). In addition to RORγt, the C2H2 zinc finger transcription factor Ikaros, a key regulator of hematopoiesis, is a binding protein of Ahr in ILC3 (157). Ikaros negatively regulates ILC3 through zinc finger 4-dependent inhibition of transcriptional activity of the Ahr by disruption of the Ahr–ARNT complex. It will be of interest to investigate whether Ikaros participates in a complex of Ahr and RORγt to regulate RORγt activity in ILC3 development and/or function. Intriguingly, Ikaros but not Ahr is required for fetal LTi cell development, demonstrating the distinct transcriptional regulation of fetal and postnatal ILC3.

As ILC3 resemble Th17 cells in regards to key transcription factor and cytokines, knowledge of the function of Ahr in Th17 cells might be adopted into ILC3 potentially. Transcription factor Musculoaponeurotic Fibrosarcoma (MAF) has been shown to be induced by TGFβ in Th17 cells to promote IL-17 production and suppress IL-22 secretion (158). Although the interaction between Ahr and MAF has been only implicated in type 1 regulatory T cells (159), the cross talk of these two proteins may provide insight into the molecular regulation of IL-22 expression in ILC3.

Aryl hydrocarbon receptor has been shown to interact with RelB, a key component of NF-κB signaling, and synergize to induce the transcription of certain genes, such as IL-6 and IL-8 in DC or macrophage (160, 161). Additionally, another component of NF-κB, RelA, binds to Ahr, and the interaction consequently promotes IL-6 transcription (162). Therefore, the interplay between Ahr and NF-κB pathway might be important for ILCs since the critical function of NF-κB has been investigated throughout various cell types.

Not limited to transcriptional function, Ahr has been reported to participate in posttranslational regulation in non-immune cells. It is described that Ahr acts as a component of cullin 4B ubiquitin ligase complex, which targets sex steroid receptors for degradation (163, 164). More investigation directed to confirm and extend this non-genomic function of Ahr in ILC and other cell types will be necessary to understand how Ahr is linked to protein degradation in different contexts.

In non-immune cells, Ahr exhibits a rhythmic expression, and its sensitivity to Ahr ligands is time-dependent (165). Reciprocally, genes associated with circadian clock and the behavioral responses of mice to circadian clock are regulated by Ahr (165). Ahr has been shown to interact with Bmal1, which forms a complex with Clock to facilitate the transcription of circadian genes (166–168). ILC2 activation and consequent eosinophil recruitment is responsive to the circadian clock, suggesting a conserved circadian mechanism in ILCs (25). Understanding of the synergetic function of Ahr and circadian signaling could improve our understanding of the basic biology of ILCs, and provide new targets of interest for regulation of ILCs.

TRANSLATIONAL POTENTIAL OF Ahr IN ILCs

Changes in ILCs have been reported in the patients with IBD. IL-22-producing ILC3 decreased in the intestine of Crohn's patients (115, 169, 170), in line with the protective role of IL-22 on the integrity of gut barrier which has been implicated several mouse models (171). Other studies also reveal that IL-22 produced by ILC3 increased in inflammatory sites of the colons in both CD and UC patients (122, 172), which might be due to a compensatory response of the host to inflammation but also might reveal the pathological aspects of ILC3, especially NCR^+ ILC3 (63, 64). The MHC-II expression on ILC3 is critical to induce T cell tolerance to gut commensal bacteria and avoid overt inflammation. It has been shown that pediatric IBD patients have reduced MHC-II expression on colonic ILC3, consistent with the model that compromised ILC3 regulatory function can lead to T cell-mediated inflammation (146). It has been shown that the expression of Ahr is reduced in the gut tissues from IBD patients compared to healthy controls (173). Accordingly, treatment of Ahr ligand ameliorated the pathology of several mouse colitis models, including 2,4,6-trinitrobenzenesulfonic acid (TNBS)-, DSS-, and T cell transfer-induced colitis, in which IL-22 is required (173, 174). Considering the role of Ahr in the maintenance of gut ILC3 and IL-22 production by ILC3, Ahr pathway could be potentially manipulated to regulate gut inflammation by increasing ILC3 in the gut of IBD patients. However, given the different functions between NCR^+ ILC3 and NCR^- ILC3, special considerations are needed while targeting the Ahr pathway in IBD.

Type 2 immunity has been considered to mediate ulcerative colitis in human, which has been modeled by oxazolone-induced colitis in mice (175). A known Ahr ligand, 3,3'-Diindolylmethane, has been found to alleviate oxazolone-induced colitis, probably through inhibition of Th2/Th17 cells and induction of Treg cells (176). Since ILC2 express large amounts of type 2 cytokines, this population could potentially play a pathogenic role in ulcerative colitis (177). Despite the reduced expression of Ahr in IBD, the role of Ahr in ILC2 and disease pathogenesis remains to be determined. In addition, it will be of interest to investigate the balance between ILC2 (or type 2 immunity) and ILC3 in colitis. IL-33, a cytokine that acts on ILC2 and Th2 to promote the cytokine

production, increased in IBD patients and in experimental colitis models of mice, including TNBS and DSS model (178). Ablation of IL-33-ST2 pathway relieves experimental colitis in mice. Of note, IL-33 and soluble ST2 have been shown increased in the colons of IBD patients (179), in line with the proinflammatory role of type 2 immunity. Thus, the functions of ILC2 and ILC3 in colitis could be dissected into two phases as ILC2 initiate the pathology *via* IL-13 (177), while ILC3, probably through IL-22, facilitate the tissue repair in the later phase of disease. However, IL-22 could also participate in the gut inflammation, highlighting its "double-edged sword" nature (65, 180). A recent study reveals that IL-33 stimulates ILC2 to secrete amphiregulin to promote tissue repair in experimental colitis (42), suggesting ILC2 at different stage of the disease and/or some subset of ILC2 (i.e., amphiregulin$^+$ ILC2) may have protective function in the resolution of colitis as well.

Allergic asthma is a chronic inflammatory disease, in which type 2 cytokines, IL-4, IL-5, and IL-13 are associated with the pathology (181). These type 2 cytokines are required for IgE response, recruitment of eosinophils, and mucus production. ILC2 have been implicated in asthma, since ILC2 produce large amounts of IL-5 and IL-13, as well as IL-4 under certain context, in response to IL-33, IL-25, and TSLP (182). Additionally, recent study showed that ILC2 increase in the airways of severe asthma patients, suggesting ILC2 may contribute to airway inflammation in mouse and human (183). Although the function of Ahr in ILC2 remains to be determined, several Ahr ligands have been reported to suppress allergic airway inflammation in different mouse models, through suppressing type 2 cytokines, IL-4 and IL-5, production, eosinophilia, and specific IgE expression (184–186). Thus, study of the role of Ahr in ILC2 would provide another potential target for clinical intervention in airway inflammation, like asthma. Although type 2 cytokines have been well documented in asthma, elevated IL-17 has been noticed clinically (187). Given that IL-25 can induce a population of lung ILC2 with IL-17-producing ability, the potential role of this special ILC2 subset in the pathology of asthma in humans needs to be studied in the future.

Both pro- and antitumor action of Ahr has been implicated (188), and the potential function of Ahr in ILC-mediated tumor immunology remains largely unknown. Ahr has been demonstrated to promote the antitumor activity of NK cells (103). IL-22, mainly produced by ILC3 under the steady state, has been shown to associate with increased risk in colon cancer (189). Accordingly, IL-22-producing ILC3 are found to promote an experimental cancer model in mice (190). Therefore, understanding of the precise function of Ahr in ILCs in cancer needs to be carefully studied.

CONCLUDING REMARKS

The tissue microenvironment may be involved in regulating the differentiation, homeostasis, and function of ILCs. Thus, the expression and activity of Ahr in ILCs from different organs under the steady state need to be carefully considered when designing therapeutics to target Ahr. Furthermore, it will be of great interest to investigate whether the Ahr level/activity in ILCs can be changed under different contexts, like in infection, inflammation, and/or cancers.

Cell-intrinsic role of Ahr in ILCs has to be determined given the broad expression of Ahr in other cell types. The molecular mechanism by which Ahr regulates the development or homeostasis of ILCs remains to be explored. Mechanistic insights of Ahr expression and/or activity in various ILC subsets or any given ILCs in different tissues are important for designing targeted strategy to modulate the Ahr function pharmacologically. It is of interest to investigate whether various ILCs have different sensitivity to Ahr ligand, or unique machinery to uptake Ahr ligand. Furthermore, single cell-omics studies involving RNA-seq and ATAC-seq analyses, together with ChIP-seq analysis of Ahr, will delineate the functional pattern and role of Ahr in regulating transcriptional landscape of ILCs. Identification of Ahr-binding partners in ILCs will provide insights into the mechanism by which Ahr cooperates with other factors to differentially regulate gene expression. These molecular findings could uncover more specific and effective therapeutic targets on the Ahr pathway, in cell-type/tissue-specific manner, in disease treatment and prevention.

AUTHOR CONTRIBUTIONS

SL wrote the manuscript with JB's contribution. LZ supervised the research and edited the manuscript.

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Metabolic Regulation of Innate Lymphoid Cell-Mediated Tissue Protection—Linking the Nutritional State to Barrier Immunity

Christoph Wilhelm, Schekufe Kharabi Masouleh and Alexander Kazakov*

Unit for Immunopathology, Department of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, University of Bonn, Bonn, Germany

Innate lymphoid cells (ILC) are a recently described group of tissue-resident immune cells that play essential roles in maintaining and protecting the tissue barrier against invading pathogens. Extensive research has revealed that ILC-mediated immune responses are controlled by dietary components and metabolites. An additional role of ILC as important direct regulators of host metabolism and glucose tolerance is emerging. This suggests that ILC may act as key dietary sensors integrating nutritional and metabolic stress to facilitate both maintenance of barrier sites and a coordinated immune response protecting these tissues. In this respect, investigations have begun to determine how different ILC responses are metabolically fueled and the impact of nutrient availability on the regulation of ILC function. Here, we discuss the current literature concerning dietary and metabolic control of ILC. In particular, we address whether the dietary and metabolic control of ILC and their simultaneous influence on host metabolism may function as a coordinated program of barrier defense.

Keywords: innate lymphoid cells, metabolic syndrome, allergic inflammation, immunometabolism, aryl hydrocarbon receptor, vitamin A, lipid mediators

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****Correspondence:***
Christoph Wilhelm
christoph.wilhelm@uni-bonn.de

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INTRODUCTION

Innate lymphoid cells (ILC) have recently emerged as an integral component of tissue immunity with several subsets described both in mice and man (1, 2). Type 1 ILC (ILC1) including NK cells express the transcription factor T-bet, produce the cytokine interferon (IFN)-γ, and are implicated in protection against intracellular pathogens such as *Toxoplasma gondii* (3, 4). ILC2 express the transcription factor GATA-3 and produce the cytokines interleukin (IL)-5, IL-9, IL-13, and amphiregulin, promoting immunity against helminth infections and tissue repair but also allergic inflammation and asthma (1, 5). By contrast, ILC3 are characterized by the expression of the

Abbreviations: AA, arachidonic acid; AAM, alternatively activated macrophage; AhR, aryl hydrocarbon receptor; AHR, airway hyperreactivity; Arg1, Arginase 1; CAM, classically activated macrophage; CRTH2, chemokine receptor homologous molecule expressed on Th2 lymphocytes; CYP1, cytochrome P4501; DC, dendritic cells; FAO, fatty acid oxidation; IFN-γ, interferon-γ; IL, interleukin; ILC, innate lymphoid cells; HFD, high-fat diet; LTD_4, leukotriene D4; LTi, lymphoid tissue inducer cell; LTs, leukotriens; LXA_4, lipoxin A4; LXs, lipoxins; MetEnk, methionine-enkephalin; OXPHOS, oxidative phosphorylation; PGD_2, prostaglandin D2; PGE_2, prostaglandin E2; PGs, prostaglandins; PUFA, Omega-3 polyunsaturated fatty acid; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; Treg, cell T regulatory cell; UCP1, uncoupling protein 1; VAT, visceral adipose tissue.

transcription factor RORγt and the cytokines IL-22 and IL-17 (1). Both cytokines mediate anti-bacterial immune responses and prevent bacterial translocation across barriers. However, aberrant regulation of ILC3 and in particular the expression of IL-17 is a potential driver of chronic gastrointestinal inflammation (1, 6, 7). Exposure to cytokines results in the activation of ILC and both IL-12 and IL-18 stimulate ILC1, the epithelial cell-derived cytokines IL-25, IL-33, or TSLP lead to the activation of ILC2, while ILC3 readily respond to IL-1 and IL-23 with the production of IL-22 (1).

Current data suggest that ILC may play an additional role by maintaining metabolic regulation and glucose tolerance of the host (8–10). Simultaneously, specific ILC functions appear to be coupled to the availability of nutrients and diet-derived factors (11–18). Thus, the function of ILC may extend beyond the simple maintenance of barrier surfaces to the maintenance of body homeostasis and metabolic control of the organism. Here, we discuss the function of ILC in the context of these new findings.

CONTROL OF ILC BARRIER FUNCTION BY DIETARY-DERIVED PRODUCTS

The intestinal immune system is equipped to directly sense and react to dietary nutrients and substances (19). This sensing is largely dependent on the expression of specialized receptors many of which belong. This includes receptors for the recognition of vitamin A and tryptophan metabolites, the retinoic acid receptors (RAR) and aryl hydrocarbon receptor (AhR), respectively. Of note, both receptors are expressed in ILC suggesting a particular importance of these pathways for ILC-mediated barrier immunity.

Vitamin A

Vitamin A is a fat-soluble essential micronutrient obtained through the diet as carotenoids (in fruits and vegetables), or as vitamin A itself in the form of retinyl esters (in foods of animal origin) (20). RA is required for the growth and development of the organism but also has profound effects on the immune system controlling innate and adaptive immune responses (19, 21–23). Lately, the essential effects of RA in control of ILC3 responses were revealed (14–16, 24). Animals deficient in vitamin A, display reduced numbers of ILC3 in contrast to mice fed vitamin A. This reduction in ILC3 has functional consequences for intestinal immunity, as these mice are more susceptible to infection with the bacterial pathogen *Citrobacter rodentium* than vitamin A competent animals (15). This was primarily due to a lack of ILC3-mediated IL-22. Conversely, delivery of exogenous RA drives production of IL-22 from ILC3, which in turn protects against *C. rodentium* and ameliorates the pathology of DSS-induced colitis (14). RA appears to control ILC3 on multiple levels. Activated RAR binds directly to *il22* and *rorc* promoter regions in ILC3, as well as promoting transcriptional activity of genes important for ILC3 development and function (14, 16). Moreover, homing of ILC3, but not ILC2, to the gut depends on RA, which upregulates CCR9 and $\alpha_4\beta_7$ (25). Finally, this axis appears to be active in humans, since ILC3 differentiation, IL-22 production, and RORγt-mediated conversion of human ILC1 to ILC3 also relies on RA (26). The effects of vitamin A deficiency extends to development of the immune system, as inhibition of maternal uptake of retinoids early in ontogeny impairs the development of lymphoid tissue inducer cells and, thus, the development of secondary lymphoid organs of the offspring (16). In addition to its effects on ILC3, RA suppresses ILC2 through downregulation of IL-7Rα (15). Although, the molecular basis and why this mechanism is only affecting ILC2 is unknown, increased IL-7Rα expression in the absence of RA may enable ILC2 to compete more efficiently for limiting intestinal IL-7. This may be particular effective since the maintenance and differentiation of ILC3 is simultaneously decreased. Thus, vitamin A deficiency induces an adaptation of the intestinal immune response by adjusting the proportions of ILC2 and ILC3, resulting in expansion of IL-13 producing ILC2 and efficient expulsion of helminth (15) (**Figure 1A**). This enables maintenance of the intestinal barrier in the context of chronic parasite infections, but not bacterial infections.

Ligands Activating the AhR

Ahr, best known for its ability to metabolize environmental toxins, was recently shown to directly effect immune cells, including ILC3 through tryptophan-derived metabolites (11–13, 27). These metabolites are formed either exogenously directly from the diet (often found in *Brassicaceae* such as cabbage or broccoli) or produced endogenously (mostly tryptophan-derived metabolites). Disruption of AhR signaling resulted in a loss of ILC3 and a decrease in IL-22 production, most likely due to loss of AhR binding to the *il22* promoter region (11–13). Consequently, AhR-deficient mice or mice fed a AhR-ligand deficient diet failed to clear intestinal bacterial infections (11–13). The importance of endogenous AhR ligands is further emphasized by constitutive intestinal expression of cytochrome P4501 (CYP1) enzymes, which are necessary for detoxification and degradation of AhR ligands (28, 29). Constitutive expression of Cyp1a1 resulted in reduced ILC3 numbers, decreased Th17 differentiation, and increased susceptibility to intestinal infection (30). This was mediated by constant depletion of endogenous AhR ligands and could be counterbalanced by increasing the availability of external dietary ligands. Interestingly, internal ligands may be provided by commensal bacteria such as *Lactobacilli*, which may help to prevent infections with *Candida albicans* through increased expression of IL-22 (31). However, this effect was dependent on unrestricted access to dietary tryptophan. Thus, the dietary availability of both RAR and AhR ligands is crystalizing as a key feature controlling ILC3-mediated barrier protection against bacterial pathogens (**Figure 1A**).

Lipid Mediators

Lipids are a third class of dietary components in direct control of immune functions (32). The majority of immunologically active lipid mediators, including PGs, leukotrienes (LTs), and lipoxins (LXs), are derived from arachidonic acid (AA). AA is synthesized from the essential polyunsaturated fatty acids (PUFAs) α-linolenic acid (18:3) and linoleic acid (18:2), critically linking the dietary supply of lipids to the ability to generate lipid signaling molecules. The importance of these lipid mediators in ILC function is multifaceted. PGD_2 receptor [chemokine

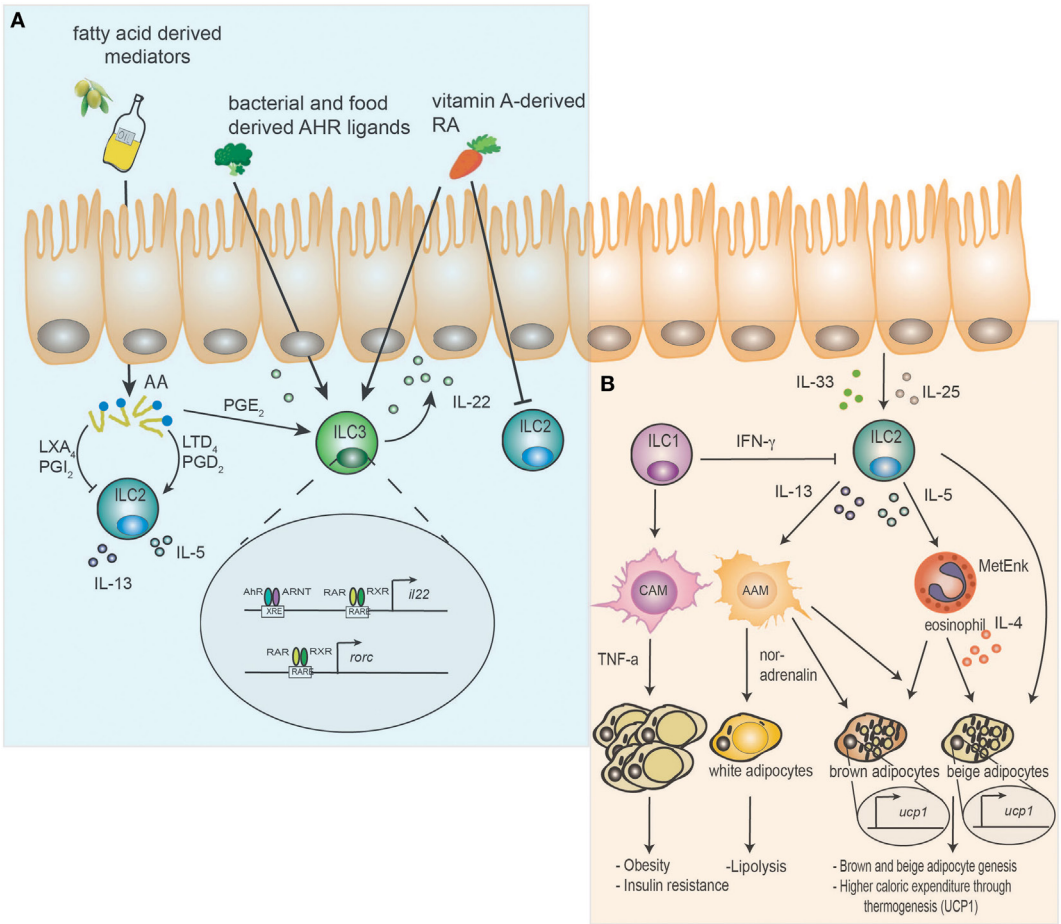

FIGURE 1 | Dual function of innate lymphoid cells (ILC) as relays of dietary components and host metabolism. **(A)** Dietary components such as aryl hydrocarbon receptor (AhR) ligands and vitamin A-derived retinoic acid (RA) are sensed by ILC. AhR/ARNT signaling promotes interleukin (IL)-22 release from ILC3 by directly binding to xenobiotic response elements in the *il22* locus. Vitamin A-derived RA additionally enhances IL-22 secretion by binding of retinoic acid receptor (RAR) and retinoic X receptor (RXR) to retinoic acid response elements on the DNA and induces rorc expression. Furthermore, retinoic acid (RA) can directly inhibit ILC2. Essential plant-derived fatty acids (FA) are converted into arachidonic acid (AA) in the host and further metabolized into prostaglandins (PGs), leukotrienes (LTs), and lipoxins (LXs). Prostaglandin D_2 (PGD_2) and leukotriene D_4 (LTD_4) activate ILC2 and support expression of the cytokines IL-5 and IL-13. In contrast prostaglandin I_2 (PGI_2) and lipoxin A_4 (LXA_4) inhibit the function of ILC2. Prostaglandin E_2 (PGE_2) supports the function of ILC3 and IL-22 expression. **(B)** Epithelial-derived cytokines IL-33 and IL-25 activate ILC2 to release methionine-enkephalin (MetEnk), IL-13 and IL-5, which promote the activation of alternatively activated macrophages (AAM) and eosinophils. IL-4 secreted by eosinophils and AAM enhance brown and beige adipocyte genesis and mediate higher caloric expenditure through induction of uncoupling protein 1 (UCP1). Additionally, AAM may promote lipolysis in white adipocytes *via* noradrenalin. ILC2 are inhibited by ILC1 derived interferon (IFN)-γ, which drives classically activated macrophages (CAM) and the development of obesity and insulin resistance in a TNF-α-dependent manner.

receptor homologous molecule expressed on Th2 lymphocytes or (CRTH2)] is used as a defining feature of human ILC2, pointing to a particular importance of PGs in the regulation of ILC2 responses (33). Indeed, PGD_2 acts as a strong chemoattractant and activator of ILC2 in the context of inflammation in mouse and man, but is negligible for their maintenance (34, 35). These features make CRTH2 a promising target in clinical trials for the treatment of asthma (36). Additional lipid signaling molecules of ILC2-mediated pathology include LT, which, in concert with IL-33, act to amplify ILC2-driven airway inflammation (37, 38). Leukotriene D_4 (LTD_4) also mediates barrier protection by promoting ILC2-derived IL-4 and induction of Th2 cell responses in helminth infections (39).

The inflammatory potential of some lipid mediators is counterbalanced by the pro-resolving function of others. LXA_4 is capable of suppressing PGD_2 driven IL-13 secretion by ILC2, while PGI_2 suppresses ILC2 functions in both mice and humans (40, 41). PGI_2-receptor (IP)-deficient mice display features of uncontrolled ILC2-mediated lung pathology upon intranasal challenge with the fungus *Alternaria alternata* (41). In this study Cicaprost, a PGI_2 analog, showed promising therapeutic potential as it limits the secretion of pro-inflammatory cytokines from ILC2. A more indirect effect was observed for maresin-1, a pro-resolving mediator derived from Omega-3 PUFAs, which suppresses ILC2-derived IL-5 and IL-13 through the induction of T regulatory cells (Tregs) and TGF-β secretion (42). Finally, by

binding to the receptor EP_4, PGE_2 can prevent systemic inflammation through increased ILC3-derived IL-22 (43). Consequently, lipid mediators are essential modulators of ILC-mediated barrier immunity in general, but in particular of ILC2-mediated immune functions (**Figure 1A**).

ILC in Control of Host Metabolism

ILC2 are a cell type considered to be dedicated to the defense and maintenance of the tissue barrier. However, the first description of ILC2 as a unique cell population was as resident cells of the mesenteric fat (44). This finding eventually led to the discovery that ILC2 are a major component of healthy but not obese adipose tissue, important for the maintenance of a lean state in mice and humans (8–10, 45). Non-obese adipose tissue-infiltrating immune cells mainly consist of alternatively activated macrophages (AAM), eosinophils, Tregs, and ILC2 (8, 45–47). This homeostasis is perturbed in high-fat diet (HFD) induced obesity, where a loss of ILC2 coincides with increases in ILC1, neutrophils, inflammatory macrophages, and activated T cells (8, 45, 46, 48, 49). The importance of ILC2 in regulation of host metabolism was further demonstrated by depletion of ILC2 in wild-type or in obese $Rag1^{-/-}$ mice, which increased weight gain and glucose intolerance (10). Additionally, gain- and loss-of-function studies have verified the importance of IL-33 or IL-25 stimulated ILC2 in white adipose tissue homeostasis. Treating obese mice with IL-33 or IL-25 results in weight loss and increased glucose tolerance, while the opposite effect is observed in mice lacking IL-33, which demonstrates the importance of activated ILC2 for the overall metabolic fitness of the organism (8–10).

Multiple mechanisms of action have been proposed for ILC2 to control host metabolism. One includes that type 2 cytokines, such as IL-5 and IL-13, induce AAM and the accumulation of eosinophils. Both cell types promote the beiging of white adipocytes (9, 50), AAM potentially through the release of noradrenalin and induction of thermogenesis in brown and lipolysis in white adipocytes (51). Lack of IL-13 is associated with weight gain, reduced eosinophils and AAM in adipose tissue (10). However, the direct involvement of AAM in adipocyte metabolism was lately challenged (52), hence more research is needed to identify the exact modes of action. Besides these effects, eosinophil and ILC2-derived IL-4 directly prompted the proliferation and differentiation of adipocyte precursors into beige adipocytes (9). Beige fat is characterized by large quantities of mitochondria and expression of uncoupling protein 1 (UCP1) (53, 54). Here, IL-33 mediated release of methionine-enkephalin from ILC2 increases UCP1 expression, thermogenesis and beiging of white adipose tissue. This results in accelerated energy expenditure preventing obesity and metabolic inflammation (8). However, IL-33 may be able to directly induce beiging of white adipose tissue by regulating the appropriate splicing of *ucp1* mRNA (55). In support of a direct mode of action, IL-33 can prevent adipose tissue inflammation through induction of lipolysis (56).

Although the majority of reports investigated the role of ILC2 in control of host metabolism, one study emphasized the importance of IL-22 expression from ILC3 and T cells for the prevention of diabetes and obesity. Mice displaying genetic ablation of the IL-22 receptor gene were prone to development HFD induced obesity and insulin resistance (57). IL-22 promoted the expression of genes involved in triglyceride lipolysis and fatty acid oxidation (FAO) in adipocytes. Treatment of obese mice with IL-22 suppressed TNF-α expression in visceral adipose tissue (VAT) and improved insulin resistance (57). Finally, a functional switch from cytotoxic to IFN-γ producing ILC1 may promote the inflammatory state in obesity. This may be mediated by simultaneous activation of classically activated macrophages and inhibition of IL-33-induced ILC2 in VAT by IFN-γ (48, 58, 59). Thus, the accumulation of these data suggests a role of ILC beyond the guarding functions at barrier sites, in the maintenance of host metabolism and prevention of obesity-associated inflammation (**Figure 1B**).

METABOLIC REGULATION OF ILC

A prerequisite to a better understanding of the regulation of the immune system by dietary components and metabolites is to reveal the metabolic constrains fueling immune cells. Recent advances were made to understand the metabolism of T cells. Naïve T cells are quiescent before activation and rely on FAO and oxidative phosphorylation (OXPHOS) involving the mitochondria (60). Upon antigen encounter and activation, the metabolic requirements of T cells rapidly adapt to match a high energy demand required for proliferation, growth, and cytokine production (61, 62). This is achieved by a switch to aerobic glycolysis, a metabolic process that generates less ATP molecules than OXPHOS but is performed at a faster rate (62). After pathogen clearance, remaining T cells become long-lived memory T cells, which in contrast to effector T cells mainly depend on mitochondrial FAO for persistence and function (62). Despite the relative abundance of data available describing the metabolic control of T cells little data exist revealing the metabolic regulation of ILC.

Metabolic Regulation of ILC1

The primary role of ILC1 including NK cells is the protection of the host against intracellular pathogens and tumors. In analogy to naive T cells, freshly isolated splenic NK cells preferentially use OXPHOS over glycolysis prior to activation (63) (**Figure 2A**). Activation of splenic NK cells *in vivo* with poly(I:C) (activating toll-like receptor 3 and Rig-I) resulted in increased uptake of the glucose analog 2-NBDG and expression of the L-amino acid transporter CD98, suggesting dependence on glutamine and glucose metabolism upon activation. Indeed, metabolic profiling of *in vitro* activated NK cells demonstrated induction of both glycolysis and OXPHOS, although extended stimulation with high dose IL-15 (100 ng/ml for 18–120 h) was required for significant induction of glycolysis (63, 64). These findings apply to human NK cells, suggesting a conserved mode of action in mammals (65). The bioenergetic adaptation of NK cells upon activation is regulated by the mammalian target of rapamycin (mTOR) and both *in vitro* and *in vivo* stimulation with IL-15 or poly:IC, respectively, increased mTOR activity (64, 66). Targeting mTOR with rapamycin decreased expression of both IFN-γ and granzyme B *in vivo* and *in vitro* but also in cultured human NK cells (64, 66). The functions of mTor are cell intrinsic, since acute genetic deletion of mTOR in NKp46 expressing cells and transfer

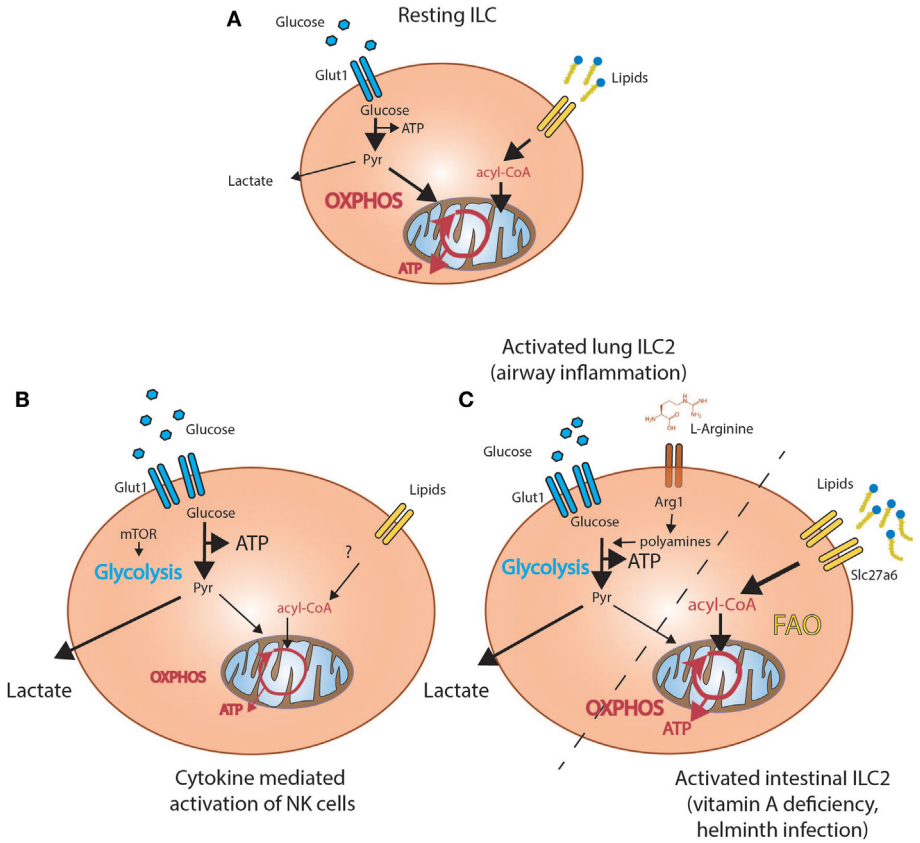

FIGURE 2 | Metabolic programs active in innate lymphoid cells (ILC). **(A)** Resting ILC predominately rely on oxidative phosphorylation (OXPHOS) and take up lipids from the environment. **(B)** Activation of lung ILC2 in the context of airway inflammation (left side) results in increased glycolysis driven by conversion of arginine by Arginase-1 (Arg1) into polyamines. Activation of intestinal ILC2 in the context of helminth infections or vitamin A deficiency (right side) increases uptake of lipids from the environment and results in increased fatty acid oxidation (FAO)-dependent oxidative phosphorylation (OXPHOS). **(C)** Cytokine-mediated activation of NK cells results in increased uptake of glucose and increased mammalian target of rapamycin (mTOR)-dependent glycolysis.

into wild-type mice, revealed a metabolic disadvantage in nutrient uptake and activation (64). Thus, in analogy to T cells, the activation of NK cells favors glycolysis, a switch recently shown to be controlled by the transcription factor Srebp (67) (**Figure 2B**). Interestingly, blocking mTOR signaling in the resolution phase of inflammation has the opposite effect and promotes the survival of memory NK cells through stimulation of autophagy as does the treatment of mice with the anti-diabetic drug metformin (68, 69).

Metabolic Regulation of ILC2

Nonetheless, whether induction of glycolysis upon activation is a defining feature of all ILC populations remains unclear. A recent study demonstrated that amino acid metabolism might play a central role for the regulation of ILC2. Arginase-1 (Arg1), essential for the metabolism of L-arginine into urea and ornithine, is expressed by progenitor and mature ILC2. In a model of allergen-induced airway inflammation, genetic deletion of Arg1 in all lymphocytes reduced the amount and proliferation of ILC2 and inflammation (17). The overall reduction of ILC2 was caused by decreased conversion of L-arginine into polyamines, which impaired aerobic glycolysis fueling ILC2 proliferation and accumulation (17). By contrast, deletion of Arg1 in macrophages and neutrophils had no effect on overall pathology. This supports the idea that ILC2-specific expression of Arg1 is a critical driver of airway inflammation and was the first report demonstrating a potential role of glycolysis for the pathogenicity of lung resident ILC2 (**Figure 2C**).

Yet, this finding appears to contradict other studies suggesting a gene expression pattern enriched for FA metabolism in intestinal ILC2 (70). In support of this assumption, intestinal ILC including ILC2 acquire high amounts of FA from the environment in comparison to other tissue-resident cell types such as Tregs (18). In particular, proliferation and accumulation of ILC2 in the context of malnutrition caused by vitamin A deficiency was fueled through increased uptake of extracellular lipids. Supporting the idea of a preferential dependence on FA metabolism, inhibition of systemic FAO by treatment with etomoxir impaired accumulation of ILC2, the production of IL-5 and IL-13 and ablated anti-helminth immune responses (**Figure 2C**) (18). Similar effects were observed by inhibition of FA uptake by the lipase inhibitor orlistat but not upon impairment of systemic glycolysis. Thus, proliferation and effector functions of ILC2 may be based on a unique FA-fueled metabolic program functioning in settings of low glucose availability and malnutrition.

CONCLUDING REMARKS: METABOLIC CONTROL OF ILC AS COORDINATED PROGRAM OF BARRIER DEFENSE?

Taken together, can we identify a common nominator to understand the specific function of each ILC subset by linking the dietary and metabolic control to the corresponding effector function? One obvious difference is the relative dependence of ILC3 on dietary components, such as AhR ligands or vitamin A metabolites. Thus, the functionality of ILC3 maintaining barrier integrity and fighting intestinal pathogens appears to be critically linked to the availability of food-derived metabolites. Although, few data are available on the metabolic control of ILC3, gene expression analysis identified carbohydrate metabolism and glycolysis as a potentially defining metabolic feature of ILC3 (71). Lack of RA results in downregulation of genes involved in glycolysis (18), which could explain the subsequent loss of ILC3 in vitamin A deficiency. In addition, AhR deficiency may increase FAO and protect against HFD-induced obesity (72). Accordingly, dietary-derived tryptophan and vitamin A metabolites may both fuel glycolysis in ILC3, linking the effector function and the metabolic program of ILC3 to dietary availability. Along the same lines, anti-viral immunity mediated by ILC1 likewise appears to depend on glycolysis. Thus, we propose that acute infection-induced ILC predominately use glycolysis to mediate barrier protection against invading pathogens and that this function is closely coupled to nutritional availability. By contrast, the maintenance and function of damaged-induced ILC2 appears to be controlled by host-derived metabolites, lipids in particular. Tissue damage caused by helminth infections requires ILC2 activation and repair. Assuming that such responses are critically dependent on FA availability, lipid mobilization may be crucial to fuel ILC2 functions, which as a side effect may prevent the development of metabolic syndrome. Furthermore, different metabolic constrains in obesity, such as high blood glucose may impose on the metabolic maintenance of adipose ILC2, which ultimately results in a loss of these cells and aggravation of metabolic inflammation. Finally, a different physiological context of ILC2 activation outside helminth-induced tissue repair could explain a divergent metabolic program of allergen-induced ILC2 (17). In conclusion, the metabolic control of ILC and the corresponding effector functions may be intimately intertwined, which offers a new approach to study ILC responses by unraveling their metabolic profile.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Shaping Innate Lymphoid Cell Diversity

Qiutong Huang[1,2], Cyril Seillet[1,2] and Gabrielle T. Belz[1,2]**

[1] Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia, [2] Department of Medical Biology, University of Melbourne, Melbourne, VIC, Australia

Innate lymphoid cells (ILCs) are a key cell type that are enriched at mucosal surfaces and within tissues. Our understanding of these cells is growing rapidly. Paradoxically, these cells play a role in maintaining tissue integrity but they also function as key drivers of allergy and inflammation. We present here the most recent understanding of how genomics has provided significant insight into how ILCs are generated and the enormous heterogeneity present within the canonical subsets. This has allowed the generation of a detailed blueprint for ILCs to become highly sensitive and adaptive sensors of environmental changes and therefore exquisitely equipped to protect immune surfaces.

Keywords: innate immunity, differentiation, gene expression, immune protection, innate lymphoid cell

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****Correspondence:***
Gabrielle T. Belz
belz@wehi.edu.au;
Cyril Seillet
seillet@wehi.edu.au

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INTRODUCTION

The importance of innate immunity has been recognized for many years. These cells play a key role in rapid responses to pathogens to protect the mucosal and external surfaces of the body. Natural killer (NK) cells and lymphoid tissue-inducer (LTi) cells are the founding members of the innate lymphoid family with the former identified more than 40 years ago. Over the past 10 years, a number of new family members have been discovered revealing an entire network of innate cells that complement the adaptive immune network. These cells went largely unrecognized for several decades which begs the question as to how they were overlooked. In this review, we summarize the current knowledge on innate lymphoid cell (ILC) differentiation and critically discuss the key challenges in the field in understanding ILC homeostatic regulation.

CANONICAL ILC SUBSETS

The ILC family is divided into three major groups: group 1 ILCs (ILC1s) which includes NK cells and ILC1s that produce interferon-γ (IFN-γ) and depend on the transcription factors Eomesodermin (Eomes) and T-bet; group 2 ILCs (ILC2s) that secrete IL-5 and IL-13 and are characterized by Gata3 expression; and group 3 ILCs (ILC3s) that express the RAR-related orphan receptor, Rorγt, and includes LTi cells and multiple subsets of ILC3s capable of producing IL-17 and/or IL-22 (**Figure 1A**). The ILCs are distinguished from adaptive immune cells by their lack of germline rearranged antigen-specific receptors and generalized lack of lineage-specific markers normally used to distinguish B and T cells. ILCs are not thought to traffic through tissues and are often referred to as "tissue-resident" (1) but their precursors can be isolated in humans from blood (2) indicating that these cells are not completely sessile throughout their life cycle. At the very least, they transit around the body to achieve their strategic positioning close to barrier surfaces to allow them to respond rapidly to local environmental changes.

Innate lymphoid cell subsets were initially categorized based on their phenotype, function, and the key transcriptional regulators that drive their development. In many aspects, these subsets mirror $CD4^+$ T cell subsets although some populations such as ILC1s have been quite difficult to position due to their lack of specific distinguishing markers (3, 4). The current classification model has served as an important framework to focus our thinking around canonical subset classifications. However, recent analyses of elegant reporters and genomic probing of individual cells has revealed that ILCs

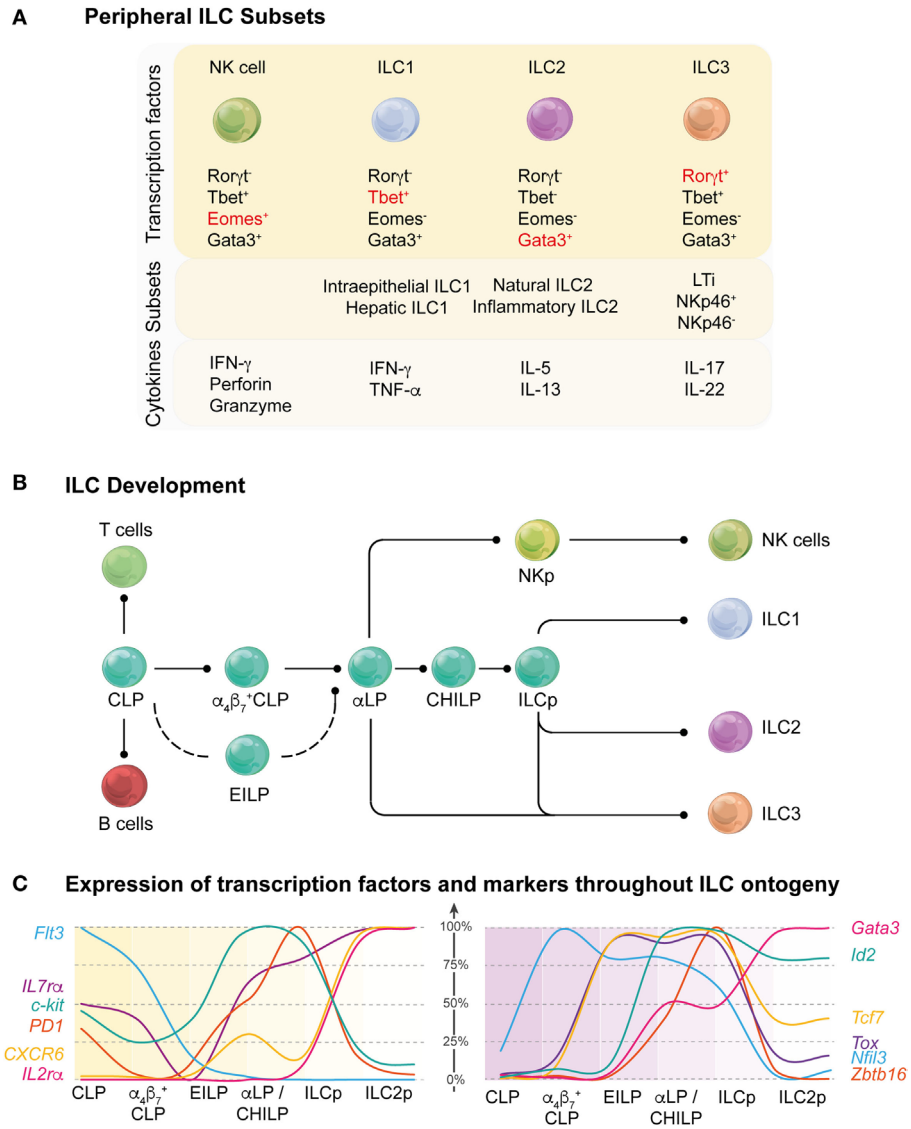

FIGURE 1 | Overview of stages of innate lymphoid cell (ILC) development. **(A)** Current understanding of the regulation of peripheral ILC subsets. A variety of different transcription factors are required for the development of peripheral ILC subsets. Key transcription factors (red) are responsible for the lineage determination of the canonical ILC subsets (top panel). ILC subsets can also be further differentiated and categorized based on the organ in which they reside, functional differences, or the expression of different receptors and surface markers (middle panel). In response to activation signals, ILCs are able to produce effector molecules and cytokines to mediate an appropriate immune response (bottom panel). **(B)** Schematic showing the current understanding of ILC development from the common lymphoid progenitor (CLP) through multiple intermediary stages on their way to becoming mature ILC subsets ILC1, 2, and 3. CLP has multi-lineage potential, including T and B cell fate, but this potential is gradually lost as the progenitors differentiate into the more lineage restricted αLP. This occurs through the intermediate α4β7+ CLP and αLP/common helper-like ILC precursor (CHILP) progenitors or through an alternative pathway *via* the early innate lymphoid progenitors (EILPs). Within the αLP population, the natural killer (NK) cell lineage diverges from the ILC lineage and the ILC precursor (ILCp) exclusively develops into the remaining mature ILCs in the periphery. **(C)** Dynamic regulation of the surface markers (left panel) and transcription factors (right panel) throughout the ILC ontogeny. The graphs show the relative RNA expression among the different ILC progenitor stages (100% represents the highest expression for each gene detected across the six different populations).

are dynamically tuned resulting in enormous heterogeneity (5–8). Potentially, this property would enable ILCs to respond to diverse stimuli in "real time." It is widely accepted that CD4+ T cell subsets display extraordinary plasticity allowing them adapt to a broad spectrum of inflammatory signals, but such a program among ILCs has not been appreciated until recently. Indeed, the capacity for ILCs to exhibit a highly flexible program may be an essential element for tuning ILCs to ensure responsiveness to continuous changes in signals encountered at mucosal barriers.

Recent findings in the field have identified conceptually new ideas about how the immune system is regulated and how the innate arm might contribute to this process. For example, the ILC network forms an extensive interface between the external environment and the adaptive immune system. Their regulation

is highly dynamic and relies on a highly integrated molecular signaling network, resulting in heterogeneity and plasticity. Finally, it appears to be highly complementary to the adaptive immune system providing a fail-safe mechanism for ensuring immune protection and repair processes. Excitingly, we are only just beginning to understand how this network of cells might work.

CORE TRANSCRIPTION FACTORS ESTABLISH THE ILC DIFFERENTIATION FRAMEWORK

Innate lymphoid cells arise from the common lymphoid progenitor (CLP) through multiple intermediary stages with changes in surface expression of key surface molecules and the temporal regulation of transcriptional regulators to become mature ILC1, 2, and 3 subsets (**Figure 1B**). Induction of the downstream molecular program involves the induction of $\alpha_4\beta_7$ which identify the $\alpha4\beta7^+$ CLP (9) [also called αLP1 (10)] follow by the downmodulation of Flt3 expression leading to the emergence of the α lymphoid progenitor (αLP, also called αLP2) (10, 11). While the αLP can generate all ILC subset, a subpopulation seems to have lost the ability to generate NK cells and named common helper-like ILC precursor (CHILP) (12). The distinction between the αLP and CHILP is not clear as they appear to be very highly similar in their surface marker or transcription factor expression. Finally, the induction of promyelocytic leukemia zinc finger (PLZF) in the ILC precursor (ILCp) mark the bifurcation between LTi and NK cells with the other ILC1, 2, and 3 subsets (13). Their fate is guided by lineage-determining transcription factors that are also involved in specifying different subsets of T cells. Transcription factors control multiple aspects of the development of immune cell lineages including proliferation, migration, metabolism, and effector function (**Table 1**). Some transcription factors play unique roles in defining the fate of early progenitors, but increasingly it is emerging that overlapping and synergistic contributions by transcription factors may be critical in setting the threshold for fate decisions and the function of an individual cell. A major challenge for the field now is to understand the combinatorial interactions between transcription factors and how they define ILC developmental choices.

The emergence of the innate cells from the CLP and the divergence of this pathway away from the adaptive lineages is

TABLE 1 | Requirement for different transcription factors during innate lymphoid cell (ILC) development.

Gene	Progenitors	Mature cells				Mouse phenotype
		Natural killer (NK) cells	ILC1	ILC2	ILC3	
Nuclear factor, interleukin 3 *(E4bp4)*	✓	–	–	–	–	Loss of αLP, small and fewer Peyer's patch; normal lymph nodes, significantly reduced NK cells (14, 15)
Inhibitor of DNA binding 2 *(Id2)*	–	✓	n.d.	n.d.	n.d.	Complete loss of lymph node and Peyer's patch formation, significantly reduced NK cells in KO and reduced IL-15 responsiveness in cKO (16, 17)
RAR-related orphan receptor gamma, RORγt (*Rorc*)	✓	–	–	–	✓	Complete loss of lymph node and Peyer's patch formation, loss of all ILC3s (18)
B-cell lymphoma/leukemia 11B BCL11B (*Bcl11b*)	✓	–	–	✓	–	Impaired function of ILC2 *via* dysregulation of Gfi1 and IL-33 receptor (ST2) (19–21)
Thymocyte selection-associated high mobility group protein *(Tox)*	✓	n.d.	n.d.	n.d.	n.d.	Normal NKp, loss of lymph node, and Peyer's patch formation, reduced NK cells, loss of mature NK cells (22–24)
ETS proto-oncogene1, ETS1 *(Ets1)*	✓	✓	n.d.	✓	n.d.	Reduced NK cells, hyporesponsive to IL-15 and impaired killing and degranulation, impaired ILC2 development.
T cell-specific transcriptions factor 1 *(Tcf7)*	✓	–	n.d.	n.d.	n.d.	Small Peyer's patches, reduced NK cells in bone marrow but normal peripheral compartment (25, 26)
Promyelocytic leukemia zinc finger *(Zbtb16)*	✓	–	✓	✓	✓ [not lymphoid tissue-inducer (LTi)]	Not required in peripheral NK cells of LTi cells (13)
GATA-binding protein 3, GATA3 *(Gata3)*	✓	✓/–	✓	✓	–	Loss of GATA3 impairs NK cell maturation (27–29)
Growth factor independent 1 transcriptional repressor, GFI1 *(Gfi1)*	n.d.	n.d.	n.d.	✓	n.d.	Regulates GATA3 expression together with responsiveness *via* IL-33 receptor (ST2) (30)
Tbx21	–	✓	✓	n.d.	✓	Reduced NK cells, ILC1 and NCR^+ ILC3; reduced mNK cells
Eomesodermin	–	✓	n.d.	n.d.	n.d.	Reduced NK cells and loss of mNK
Pdcd1	–	✓	n.d.	✓	n.d.	Normal secondary lymphoid tissue formation (5, 9)

✓, required for development and/or maintenance; –, not required for development and/or maintenance; mNK cells, mature NK cells; KO, germline deletion; cKO, conditional deletion; n.d., not determined.

an extremely controlled process that involves the coordinated actions of several transcription factors. Detailed analysis of the transcriptional landscape of the ILC development from the earliest precursor to the committed cells has revealed that regulation of the different developmental stages is highly dynamic. The sequential expression of nuclear factor interleukin 3 (NFIL3), inhibitor of DNA binding 2 (ID2), thymocyte selection-associated high mobility group box protein (TOX), and GATA-binding protein 3 (GATA3) establishes the framework for ILC differentiation (7, 9) (**Figure 1C**). ID2 counterbalances the effects of E proteins to direct cell choices away from T and B cell outcomes (31). Other transcription factors such as EOMES, PLZF, transcriptions factor 1 (TCF-1), and RUNX influence subset divergence. We now have new insight to the key factors that determine the fate outcome of progenitor cells under steady-state conditions. However, it still remains unclear how higher order genomic architecture establishes and maintains the differentiation program.

THE EARLY REGULATORS: A QUARTET

Two major transcription factors, NFIL3 and TOX, have emerged as critical initiators of development of early αLP. Induction of NFIL3 appears to be the critical initiating step in driving the αLP toward the ILC lineage (7, 9). NFIL3 is induced in $\alpha4\beta7^+$ CLP, but the factors responsible for this induction have yet to be elucidated (**Figures 1B,C**). Concomitantly, TOX and ID2 are only expressed at low levels in the CLP but TOX expression rapidly increases in the early innate lymphoid progenitors (EILPs) while ID2 levels remains low until the late αLP and common helper ILC (CHILP) stages (12, 32–34). Nevertheless, the expression of ID2 has two patterns in ILCps; the first phase in which ID2 is expressed at low levels (and the E protein E2A is concomitantly high) and does not appear to be required for ILC development, and the second in which ID2 is strongly upregulated with concurrent downregulation of E2A and is essential for ILC lineage progression (9, 31). Indeed, while the deletion of NFIL3 blocks the development of the $\alpha4\beta7^+$CLP, ID2 deletion has does not appear to affect the development of the αLP. However, all cells derived from the ILC progenitor are absent in $ID2^{-/-}$ mice suggesting that ID2 is more important in late differentiation and in maintaining the long-term identity of ILCs (16, 35). Loss of ID2 in ILCs has also been shown to repress genes belonging to the stem cell program such as *Gfi1b*, *Tal1*, *Lmo2*, *Gata2*, and *Hhex* (9).

Precisely how NFIL3 and TOX regulate the development of the ILC progenitors remains unclear. TOX-deficient progenitors appear to lack the expression of key factors thought to be essential for ILC development including *Gata3*, *Rora*, *Rorc*, *Tcf7*, and *Zbtb16* (22). Despite this, *Nfil3* expression, which could be regulated by TOX, was not found to be different from that of wild-type cells. Thus, more work will be necessary to ascertain whether NFIL3 is a direct target of TOX or not. NFIL3 has been shown to directly bind to ID2; however, it is not clear that this binding is actually responsible for the induction of ID2 (36). In ILC progenitors, ID2 expression has been found to be reduced when NFIL3 is deleted in the hematopoietic compartment. However, in mature cells, ablation of NFIL3 did not alter ID2 expression suggesting that the developmental stage of the cell influenced the interactions (37, 38). Furthermore, overexpression of either ID2 or TOX in NFIL3-deficient CLPs revealed that both transcription factors could at least partially rescue ILC development independent of NFIL3. Therefore, it is likely that the key role of NFIL3 is to promote the emergence of ILCs by induction of the expression of these two key transcription factors.

EXPRESSION PATTERNING OF NFIL3 AND ID2 ESTABLISHES THE LANDSCAPE FOR ILCs

Although several details of the fine tuning of NFIL3 remain unanswered, the timing and action of NFIL3 is very interesting. $Nfil3^{flox/flox}$ mice crossed to the $Id2^{ERT2Cre}$ strain generated a model in which deletion of NFIL3 could be timed relative to ID2 (9). This approach demonstrated that NFIL3 expression preceded that of ID2 but that surprisingly, deletion of NFIL3 in $ID2^+$ cells either *in vivo* or *in vitro* did not affect the subsequent development of any of the ILC subsets. These findings are consistent with earlier work showing that NFIL3 was not required for the maintenance of mature NK cells (14). What was particularly unexpected, however, in the study, was the very transient nature of the NFIL3 expression which was both necessary and sufficient to promote ILC development. Indeed, only a short pulse of NFIL3 expression in the progenitors was required and it subsequently rapidly decreased as ILC progressed through each developmental stage (7, 9) and is repressed in mature cells in the periphery (15).

This pattern of short-lived expression found in NFIL3 may represent a more generalized pattern for orchestrating the complex integration of different transcriptional signals. This expression pattern has also been reported for *Zbtb16* (that encodes PLZF), another transcription factor important for ILC development but which was originally implicated in NKT cell development (39). Mature ILCs do not express PLZF; however, lineage tracing experiments revealed that 60–75% of ILCs exhibited a fluorescent imprint marking their previous expression of PLZF during development (13). In this setting, only ~5% of LTi cells and ~20% of NK cells were labeled indicating that bifurcation of these lineages from other ILC subsets occurred before the induction of PLZF. The role of the transient expression of PLZF in ILCp is not known. Using the PLZF-reporter mice, Constantinides et al. (13) also showed that PLZF is expressed only transiently. This allowed the identification of the ILCp, but PLZF was subsequently downregulated after this stage. PLZF does not appear to be absolutely required for ILCs as deletion of PLZF results in ~4-fold reduction in the number of ILCs that develop in contrast to ablation of NFIL3 which results in more than a 10-fold reduction. PLZF is expressed after NFIL3 coordinately with E2A and ID2 resulting in a stepwise progression through the early progenitor stage to generate $E2A^{hi}PLZF^{-}ID2^{lo}$, $E2A^{hi}PLZF^{+}ID2^{lo}$, $E2A^{lo}PLZF^{hi}ID2^{hi}$, and $E2A^{lo}PLZF^{-}ID2^{hi}$ expressing cells (31). In competitive situation, PLZF-deficiency appears selective, mainly affected ILC2s in the lamina propria and ILC1s in the liver (13). It was notable that ~40% of the ILC2s in this study were not labeled in these tracing experiments, so it

remains possible that they could be derived from an alternative pathway that is independent of PLZF.

LOSS OF IL-7R EXPRESSION: A NEW PROGENITOR OR AN ALTERNATIVE PATHWAY?

The identification of the early ILC progenitor in the bone marrow, or EILP, noted for its high expression of TCF-1, has raised some questions around the linear model of ILC development (25). This EILP lacks B or T cell potential but can generate all ILC subsets including the NK cells similar to the αLP progenitor capacity, but it differs from the other precursors described as it lacks IL7Rα expression (25). One possibility is that IL-7Rα expression is lost between the αLP and the ILCp stages. Such changes could be regulated *via* posttranslational modifications though the biological relevance for such downregulation is unclear. A second possibility is that the EILP is a precursor for an alternative pathway for ILC development. EILPs express high levels of NFIL3, TOX, and TCF-1, low levels of ID2 and PLZF is almost undetectable. Interestingly, the EILPs are not affected by ID2 deletion, thus the EILPs appear to be very similar to the IL-7R-expressing αLP. A comparison between these two cell types may help to better understand the relationship between the IL7Rα$^+$ and IL7Rα$^-$ ILCps and define the factors that regulate IL-7R signaling in ILCs that is essential for their development. To date, TCF-1 and ID2 are known to be upregulated in EILP but additional transcriptional requirements of this progenitor have not been investigated. Therefore, whether EILP represents an intermediate stage of the ILCp, or an alternative precursor that does not fit in the current linear model of ILC development, remains an open question.

HETEROGENEITY AND PLASTICITY OF ILCs ARE KEY TO MAINTAIN HOMEOSTASIS

Initial categorization of ILC subsets relied on cytokines and effector molecules they produced combined with signature transcription factors that appeared to be central regulators of the different subsets. The patterns found in ILCs were aligned to the categorization of CD4$^+$ T cell subsets. Unlike CD4$^+$ T cells, however, it quickly emerged that several ILC phenotypes did not necessarily neatly fit into the unified nomenclature coined in 2013 with subsets described as being "like" other subsets (40). The full spectrum of ILC heterogeneity has recently been unveiled using comprehensive single-cell sequencing combined with mass cytometry approaches which has allowed the field to bridge between our understanding of ILCs in mouse and man (2, 6, 41, 42) (**Figure 2A**). This approach revealed that potentially as many as 15 transcriptionally distinct identities could be delineated in the small intestine and that crosstalk orchestrated *via* cytokines such as IL-12 and IL-23 and microbial signals were substantially responsible for mediating this subset plasticity (6). It is undoubtable that these approaches are transforming our understanding of diversity in immune cell subsets but these data also throw up new technical and intellectual challenges in understanding how diversity arises. Historically, various surface markers have been considered to represent "lineage specificity" but we now recognize that this is seldom the case, challenging that how we interpret complex data and underlying that subsets cannot be defined exclusively by particular markers or transcription factors (43). Nevertheless, it provides a rich landscape for understanding how different stimuli affect the homeostatic balance of ILC subsets. It has been revealed that ILCs exhibit tissue-specific characteristics and this in part reflects alterations in phenotype that can be significantly regulated by responses to inflammation and infection.

The first example of ILC ability to adopt a different phenotype was the identification of ILC3s that downregulated RORγt but expressed T-bet, NK1.1, and produced IFN-γ (also known as ex-ILC3s) (12, 44, 45) under chronic stimulation and RORγt$^+$ ILC3 that produced IL-17 which were found in the large intestine (46) (**Figure 2B**). IL-12 was found to be a key driver of this pathway. Similarly, IL-1β, together with combinations of IL-4, IL-12, and IL-33, can drive ILC2s to adopt an IFN-γ-producing phenotype called ex-ILC2 (47–49). ILC2s can be divided into two subsets: homeostatic or natural ILC2s which typically reside in barrier tissues and respond to IL-33; and inflammatory ILC2s which are generally not found in peripheral tissues at steady-state but respond to IL-25 which induces multipotency (50–53). In this setting, T-bet (encoded by *Tbx21*) was induced, whereas GATA3 was diminished. These studies highlight the variability that can occur in ILCs, particularly *in vitro* as demonstrated for ILC2s (47–49). Indeed, even NK cells can undergo this sort of transformation (54). Signaling through the TGF-β pathway can convert NK cells (CD49a$^-$CD49b$^+$Eomes$^+$) into intermediate ILC1 (CD49a$^+$CD49b$^+$Eomes$^+$) and *bone fide* ILC1 (CD49a$^+$CD49b$^+$Eomes$^{+/-}$) within a tumor microenvironment. Strikingly, these latter ILC1 were disabled in their capacity to control local tumor growth and to prevent metastasis, while NK cells retained their ability to undertake immune surveillance (54). It is striking that once activated through these different pathways, each subset converges on an "IFN-γ-producing ILC1-like" phenotype. This suggests that this fate may represent a common outcome for multiple ILC subsets, even perhaps an essential adaptive program for all ILCs. It remains a challenge, however, to understand how these IFN-γ-producing cells arise and whether it represents a protective response or the first steps to loss of immune control (**Figure 2B**). Teasing this apart will require much more extensive study particularly in the context of pathogen infection, inflammation, and tumor development.

ARE MORE SUBSETS POSSIBLE?

How ILCs populate tissues after birth or maintain their presence in peripheral tissue is not clear. Our current understanding indicates that ILCs are almost exclusively tissue resident and that they do not routinely circulate throughout the body. This perception is predicated on the findings that during engraftment following irradiation, donor ILCs largely fail to replace ILCs originally found in the host (1). Instead, they depend on local proliferation to expand and replace ILCs, and it is only late in an infection or physiological disturbance that replenishment from blood-borne precursors

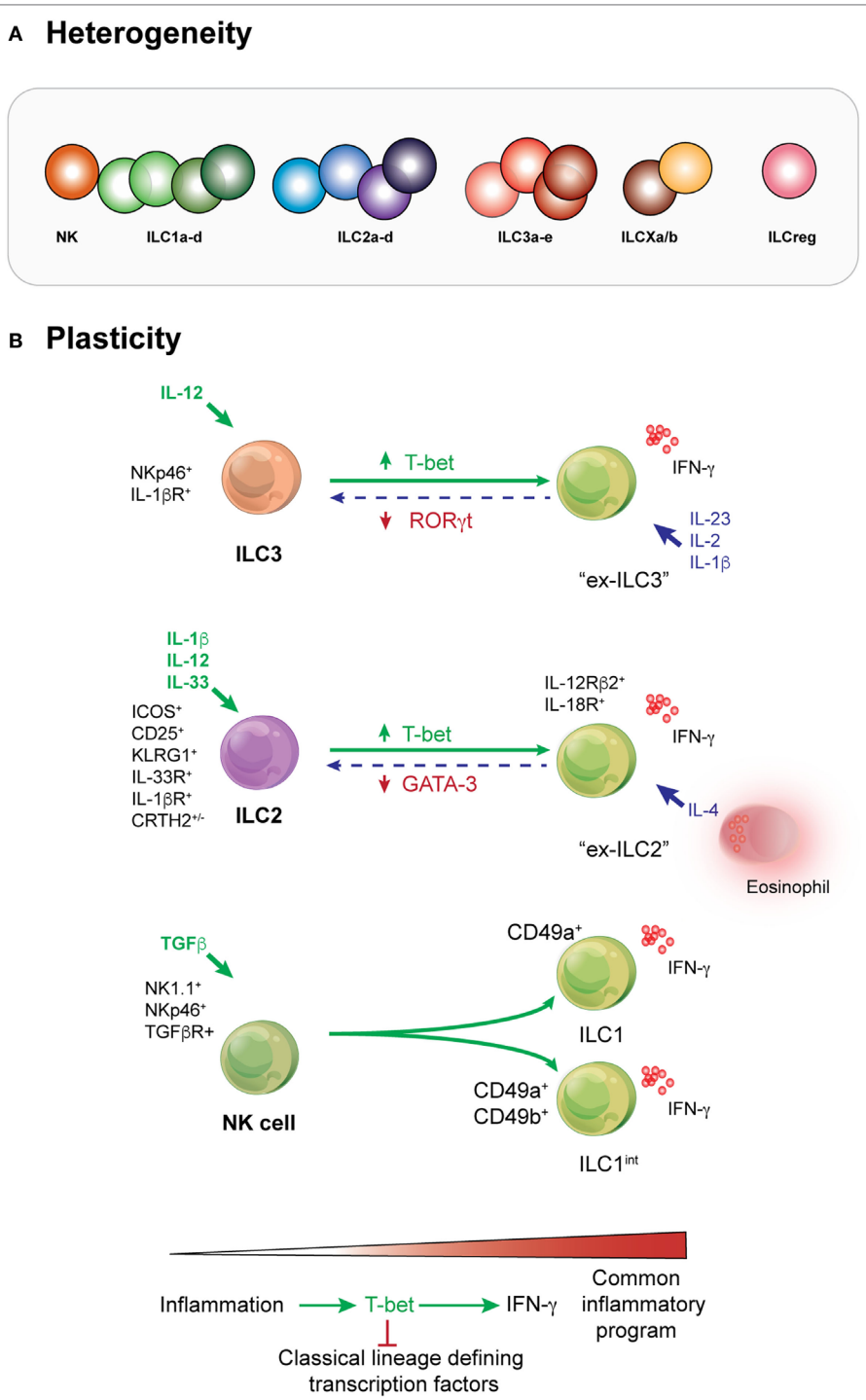

FIGURE 2 | Heterogeneity and plasticity are prominent features of innate lymphoid cell (ILC) behavior across all subsets. **(A)** Deep analysis into the transcriptome of ILCs demonstrates that heterogeneity occurs within every subset. Depending on the type or intensity of the stimuli received by these cells, different molecular pathways may be activated by cells of the same subset. This results in phenotypic or functional variation and a subsequent spectrum of ILCs within each compartment. Whether additional subsets such as the proposed regulatory subset exist is yet to be fully determined. **(B)** ILC3s can adopt an ILC1-like phenotype when activated by IL-12. They are known as "ex-ILC3s." In human cells, this pathway can be reversed by the action of IL-23, IL-2, and IL-1β. ILC2s are activated by IL-2 and IL-33. Stimulation with IL-1β primes the responsiveness of ILC2 by enhancing the expression of cytokine receptors such as IL-25R, IL-33R, and TSLP to potentiate ILC2 responsiveness and induce a significant increase in the population. Critically, however, IL-12 is essential to effect remodeling of the chromatin landscape in ILC2 allowing them to induce phenotypic changes and become more like ILC1s (ILC1-like or "ex-ILC2s") that produce IFN-γ. Natural killer (NK) cells respond to TGF-β to form "intermediate ILC1" reflecting their acquisition of CD49a and *bone fide* ILC1. In many situations, it appears that the transcription factor T-bet is key to augmentation of the inflammatory program and concurrently represses signature transcription factors that typically define individual lineages.

restores the integrity of the ILC compartment. But have we got this right? These findings depend on a number of assumptions. For example, it is presumed that the transcriptional regulators, surface molecules, maturity, and frequency of relatively "mature" ILCs are also the most useful for pinpointing circulating ILCs. In addition, the high similarity of markers in murine ILCs that are comparable in man may have obscured our ability to identify circulating progenitors, or more mature cells, that are critical to maintain tissue homeostasis.

Circulating Precursors

The first clue that programming might potentially differ between mouse and man came from Scoville et al. (55) who observed that RORγt was expressed in all human ILCs. This was in striking contrast to murine ILCs where RORγt expression was highly restricted to the ILC3 subset (18). Interestingly, $CD34^+$ progenitors expressing c-kit and RORγt could generate all ILC subsets, including NK cells. These progenitors selectively resided in secondary lymphoid tissues. This helped to identify the pathway of ILC development in man; however, the involvement of these precursors remained unclear. Later, an extensive analysis of the $c\text{-}kit^+$ ILCs from the blood and tissues revealed that circulating ILCp in humans exist and do not typically express many of the markers associated with mature cells but do express low levels of RORγt (2). Such cells are maintained in RORC-deficient patients and retain the potential to produce different populations of ILC except ILC3. Thus, it appears that high expression of RORγt is necessary to generate an ILC3 in both man and mouse, but these observations raise the question of whether in mice similar progenitors have been simply discounted due to their low expression of this transcription factor. However, while the role of RORγt in the development of human ILCps is unclear, transcriptomic and epigenomic analysis of circulating human ILCps revealed the upregulation of many transcription factors known to be critical for ILC development in mice such as *NFIL3*, *ID2, TOX, TCF7*, *ZBTB16*, and *GATA*3 (2). This suggests that transcriptional regulation of ILC development share common factors in human and mouse.

Regulatory ILCs

Innate lymphoid cell subsets appear to largely mimic those defined for $CD4^+$ T cells. Recent evidence suggests that this extends to the presence of regulatory ILCs. Among the ILCs, NK cells have been described to produce IL-10 (56, 57) that acts to depress B cell (58) and dendritic cell immune responsiveness (59, 60) and suppress activation. The Ohashi group (61) strengthens the notion that a regulatory subset might exist. During their evaluation of a tumor-infiltrating cell-based adoptive immunotherapy for ovarian cancer, it was noticed that a high frequency of $CD56^+CD3^-$ cells was strongly correlated with suppression of tumor-infiltrating cell outgrowth and proposed that these cells played a regulatory role. While this effect has only been tested *in vitro*, they ascertained that regulatory and conventional $CD56^+CD3^-$ ILCs exhibited high levels of the transcription factors *ID2, ZBTB16 (PLZF), RUNX3*, and *TOX,* but similar amounts of *EOMES, TBX21, GATA3, RORA*, and *AHR*, factors also shared with NK cells, ILC2s, and ILC3s. Very recently, however, Wang et al. (62) provide the first description of regulatory ILCs in mice and humans which were shown to be important in response to gut inflammation such as *Citrobacter rodentium*. This subset arises from an ID2-expressing progenitor and depends on a second inhibitor of DNA-binding protein, ID3 but not PLZF or RORγt. Intriguingly, this subset lacks expression of any of the classical transcription factors required for the early steps in development by other ILCs including NFIL3, TOX, TCF-1, GATA3, or PLZF. Thus, it remains unclear whether the early progenitor is the ILCp, or alternatively a distinct progenitor gives rise to this subset. However, like other ILC subsets they lack typical lineage markers but do express CD25 and CD90 while autocrine expression of TGF-β1 drives expansion of this $IL\text{-}10^+$ subset during inflammation and results in suppression of activation of ILC1 and ILC3.

Combined, these important studies point toward the existence of a regulatory subset, but important questions still remain. For example, how and when do regulatory ILCs emerge, what other transcription factors drive this process, and do they express receptors that can be targeted to restore immune homeostasis during chronic and autoimmune diseases. If they do, this proves another avenue to unleash the protective power of ILCs either within tumors or during inflammation and in maintaining normal homeostasis to prevent autoimmunity.

CONCLUDING REMARKS

With increasing understanding of the regulation of the ILC, we realize how extensive is their ability to adapt their microenvironment. While ILC subsets are often seen as innate counterpart of T helper cells, it may be interesting to imagine the ILCp as an innate counterpart of naive T cells. ILC subsets also to appear to be an extremely plastic population that can profoundly change their predicted responses in reaction of extracellular mediators. One important challenge will be to identify the large variety of environmental and host-derived signals they can integrate to understand the role of ILCs in the homeostasis of the tissue and during inflammation.

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript and read, edited, and approved the final manuscript.

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MINI REVIEW
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GATA3 Regulates the Development and Functions of Innate Lymphoid Cell Subsets at Multiple Stages

*Jinfang Zhu**

Molecular and Cellular Immunoregulation Section, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States

Innate lymphoid cells (ILCs) are regarded as the innate counterpart of effector CD4 T helper (Th) cells. Just as Th cells, ILCs are classified into distinct subsets based on their functions that are delivered mainly through effector cytokine production. Both ILCs and Th cells play critical roles in various protective immune responses and inflammatory diseases. Similar to Th cell differentiation, the development of ILC subsets depends on several master transcription factors, among which GATA3 is critical for the development and maintenance of type 2 ILCs (ILC2s). However, GATA3 is expressed by all ILC subsets and ILC progenitors, albeit at different levels. In a striking parallel with GATA3 function in T cell development and differentiation, GATA3 also has multiple functions in different ILCs at various stages. In this review, I will discuss how quantitative and dynamic expression of GATA3 regulates the development and functions of ILC subsets.

Keywords: GATA3 transcription factor, innate lymphoid cells, development, cytokines, transcriptional activation

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Jennifer Walker, MRC Laboratory of Molecular Biology (MRC), United Kingdom

***Correspondence:**
Jinfang Zhu
jfzhu@niaid.nih.gov

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INTRODUCTION

Innate lymphoid cells (ILCs) are innate counterparts of CD4 T helper (Th) cells, which are considered professional cytokine-producing cells during adaptive immune responses. Based on their cytokine production and functions, just as Th cells, ILCs are divided into at least three major groups—group 1 ILC (ILC1), group 2 ILC (ILC2), and group 3 ILC (ILC3) subsets (1–4). ILC1s mainly produce IFN-γ; ILC2s produce IL-5 and IL-13; whereas ILC3s primarily produce IL-22. T-bet, GATA3, and RORγt are the master transcription factors for the development and functions of Th1, Th2, and Th17 (IL-17-producing Th) cells, respectively (5). Similarly, these master regulators are also critical for the development of ILC1s, ILC2s, and ILC3s, respectively (**Figure 1**). Natural killer (NK) cells are considered as the innate counterpart of CD8 T cells since both have cytolytic activity and depend on the transcription factor Eomes for their development.

Genome-wide analyses indicate that, in addition to the similarity in their transcriptomes, the epigenome of a given ILC subset is very similar to that of the Th counterpart especially at the cytokine loci (6, 7). Because of their similar capacity in producing effector cytokines (6–8), same class of ILC and Th subset may participate in specific type of immune responses (1, 6–11). Like Th1 cells, ILC1s may be involved in protective immunity against intracellular pathogens (12). Similar to Th2 cells, ILC2s are involved in type 2 immune responses to helminth infections (13–16). ILC3s and Th17 cells are important in dealing with infections of extracellular bacteria and fungi (17, 18). While ILC2s contribute to allergic inflammation (19–23), activation and expansion of ILC1s and ILC3s may induce certain types of autoimmunity (24). Interestingly, ILC activation may be sufficient to induce inflammation or to mount an effective immune response to infections in the absence of T cells under certain conditions (16, 25). Among the ILC3s, there is a distinct subset that expresses

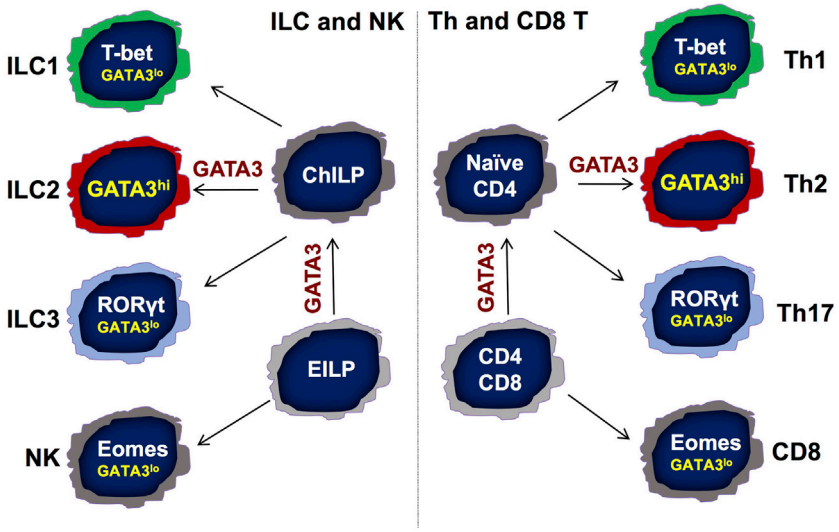

FIGURE 1 | Symmetry between the development of adaptive and innate lymphocytes. There are following three major T helper (Th) subsets: Th1, Th2, and Th17 cells. Transcription factors T-bet, GATA3, and RORγt are the master regulators for the differentiation of Th1, Th2, and Th17 cells, respectively, from naïve CD4 T cells. Th2 cells express high levels of GATA3 that is critical for the development and functional maintenance of Th2 cells. The importance of low GATA3 expression in Th1 and Th17 cells is not clear. During T cell development, GATA3 is critical for the development of CD4 but not CD8 T cells in the thymus. Similarly, GATA3 is indispensable for the development of all IL-7Rα-expressing helper-like innate lymphoid cells (ILCs) but not natural killer (NK) cells. While GATA3 is critical for the functional maintenance of ILC2s, it is also required for the homeostasis, function, and further maturation of other ILCs, including ILC1s and ILC3s. Although GATA3 is not necessary for the development of NK and CD8 T cells, GATA3 expression at low levels contributes to the homeostasis and maturation of these cells. EILP, early ILC progenitor that expresses TCF7; ChILP, common helper-like ILC progenitor that expresses Id2.

chemokine receptor CCR6 in mice (26). These mouse CCR6$^+$ ILC3s represent lymphoid tissue inducer (LTi) or LTi-like cells that play a critical role in the development of lymphoid tissues including lymph nodes and Peyer's patches (27, 28).

T helper and ILC subsets also differ from each other in many aspects. Because ILCs lack antigen receptors, they mainly respond to inflammatory cytokines to produce their own effector cytokines; IL-1 and IL-12 family cytokines including IL-1, IL-18, IL-33, and IL-23, as well as IL-25, are the major players in activating ILC subsets (1, 29, 30). Although Th cells mainly respond to antigens through activation of their T cell receptors, they can also respond to inflammatory cytokines in an antigen-independent manner, and these cytokines may play an important role in the maturation of Th cells (31, 32). Since antigen-specific Th cells need to be activated and expanded during an immune response while ILCs preexist in the tissue (33, 34), ILCs provide first line of host defense.

Another striking difference between Th cells and ILCs in their development is their dependence on cytokine signaling. While Th cell differentiation critically depends on cytokine-mediated activation of signal transducer and activator of transcription (STAT) family proteins (35), many STATs including STAT3, STAT4, and STAT6 are not required for ILC development (36–38). However, the response of mature ILCs to cytokines may still require the activation of STAT proteins (36–38). Nevertheless, STAT proteins that are important for cell proliferation and survival, especially STAT5 proteins (STAT5A and STAT5B), are important for the homeostasis of both Th cells and ILCs (39). In fact, ILCs are largely absent in common gamma chain (γc)-deficient animals and STAT5 is a major downstream molecule activated by cytokines that utilize γc (40).

Id2 plays an important role in ILC development but has a minimal effect on T cell development (12, 26, 41, 42). Some Id2-expressing progenitors in the bone marrow represent common helper-like ILC progenitors (12). On the other hand, the essential factor for T cell development Bcl11b only controls the development of ILC2s but not of other ILCs (26, 41, 43–47). Nevertheless, the development of ILC and Th cell subsets depends on many shared transcription factors such as TCF7, Tox, and GATA3 (8, 41, 48–50). Some TCF7-expressing cells in the bone marrow represent early ILC progenitor with a potential to become either NK cells or helper-like ILCs (48). Possibly because LEF1 (a TCF7 family member) (51) and Tox2 (52–54) are also expressed by ILCs and T cells, the blockage of ILC and T cell development in TCF7- or Tox-deficient animals is incomplete. On the other hand, since GATA3 is the only GATA protein expressed in T cells and ILCs, GATA3 is absolutely required for the development of T cells and ILCs (8, 55). In mature ILCs, while ILC2s express high levels of GATA3, all other ILCs express low levels of GATA3. In this review, I will discuss the functions of quantitative GATA3 expression in different ILC subsets and ILC progenitors in parallel with its important functions in the development and functions of T cell subsets.

CRITICAL FUNCTIONS OF GATA3 IN ILC2s

ILC2s are enriched in the gut, lung, skin, and adipose tissues with few of them found in lymphoid tissues and in blood, and

all the ILC2s express high levels of GATA3 (1, 13, 20). Like Th2 cells, ILC2s depend on GATA3 for their development. Even after ILC2s are fully mature, GATA3 remains important for their maintenance and functions, just as the essential role of GATA3 in differentiated Th2 cells (8, 56–64). In fact, GATA3 regulates a common set of important genes in both cell types, including *Il5*, *Il13*, *Areg*, *Il1rl1*, and *Ccr8*, which are well-known genes important for type 2 immune responses (8). This may explain identical functions of Th2 cells and ILC2s. Interestingly, the transcriptomes of ILC2s and Th2 cells harvested during helminth infection are remarkably similar (6).

Genome-wide analysis of GATA3 binding through ChIP-Seq shows that GATA3 binds to the *Il4/Il13* gene locus at multiple sites in both Th2 cells and ILC2s (65, 66). GATA3 also binds to the *Il5* and *Il13* promoters to induce their transcription (67, 68). Although the function of GATA3 in regulating epigenetic modifications at cytokine gene loci in ILC2s is not clear, GATA3 plays an important role in chromatin remodeling at the *Il4/Il13* locus in Th2 cells (65). GATA3 also directly binds to many genes that are involved in type 2 immune responses including *Il1rl1*, which encodes the IL-33 receptor subunit T1/ST2 (65, 66) and *Il17rb*, which encodes IL-25R (8). By regulating IL-33R and IL-25R expression, GATA3 is necessary for mature ILC2s to respond to inflammatory cytokines IL-33 and IL-25. ILC2s die quickly after GATA3 removal (8). It is possible that GATA3-deficient ILC2s fail to respond to cytokines. Alternatively, GATA3 may directly regulate cell proliferation and/or survival related genes in ILC2s (8).

GATA3 FUNCTIONS IN ILC PROGENITORS

The functions of GATA3 in lymphocyte development and functional regulation are far beyond its critical role in Th2 cells and ILC2s, because all T cells and ILCs express different levels of GATA3 (66, 69, 70). We have previously reported that the development of IL-7Rα-expressing ILCs (or Th-like ILCs) but not of NK cells depends on GATA3 (8). Another report also indicates that ILC3 development requires GATA3 expression (50). This is consistent with the critical function of GATA3 during CD4 but not CD8 cell development (69, 71–73). It also supports the notion that ILC1s are developmentally distinct from conventional NK cells. Therefore, the development of innate (ILC) and adaptive (T cells) lymphocytes is highly symmetrical (**Figure 1**).

GATA3 promotes IL-7Rα expression in all T cells and ILCs, indicating that this may be a common mechanism through which GATA3 regulates lymphocyte homeostasis (66, 74). GATA3 expressed at low levels is sufficient to bind to the *Il7r* locus and regulates IL-7Rα expression in all ILCs and T lymphocytes (66, 74); the fact that the GATA3 binding pattern to the *Il7r* gene in ILC3s is identical to that in ILC2s and Th2 cells suggesting the existence of a high-affinity GATA3 binding site at the *Il7r* gene (66). However, GATA3-mediated IL-7Rα expression does not explain its critical role in the development of IL-7Rα-expressing ILCs because we have found that IL-7Rα transgene fails to rescue the ILC developmental defect in the absence of GATA3.

It has been reported that the ILC1s, ILC2s, and non-LTi ILC3s are derived from ILC progenitors that express both PLZF (75) and PD-1 (76). These PLZF-expressing progenitors are known as common precursors to ILCs (ILCPs). However, CCR6$^+$ LTi or LTi-like cells do not have a history of PLZF expression according to PLZF-fate-mapping experiments (75). We have previously reported that ILC numbers are dramatically reduced but not absent in the *Gata3*$^{fl/fl}$-Vav-Cre conditional knockout mice (Vav-Cre is active at the hematopoietic stem cell stage) (8). Our recent results further demonstrate that LTi or LTi-like cells are the only ILCs that still remain in the GATA3 conditional knockout mice. By contrast, ILC1s, ILC2s, and CCR6$^-$ ILC3s are undetectable in these mice. Interestingly, the PLZF-expressing ILCPs express high levels of GATA3 expression whereas the LTi or LTi-like progenitors express low levels of GATA3 in the bone marrow. Strikingly, the PLZF-expressing progenitors are completely lost in the bone marrow of *Gata3*$^{fl/fl}$-Vav-Cre mice. The critical function of GATA3 for the generation of PLZF-expressing ILC progenitors is independent of cytokine signaling and/or IL-7Rα regulation since we have observed that these progenitors are present in normal numbers in γc-deficient animals. Thus, GATA3 is a critical regulator for the generation of PLZF-expressing ILC progenitors (**Figure 2**).

GATA3 FUNCTIONS IN ILC3 SUBSETS

ILC3s are the major ILC population highly enriched in the gut (8, 18). RORγt, the master regulator of Th17 cells (77), is critical for the development and functions of ILC3s including both NKp46-expressing ILC3s and CCR6-expressing LTi or LTi-like cells (27). Although NKp46$^+$ ILC3s may have some specialized functions in inflammation and gut homeostasis, by producing IL-22, NKp46$^+$ ILC3s and CCR6$^+$ ILC3s are functionally redundant in host defense (54, 78). NKp46-expressing ILC3s co-express T-bet and RORγt, whereas CCR6$^-$ ILC3s in mice only express RORγt (66, 79, 80). Interestingly, both types of ILC3s express low levels of GATA3 (66).

T-bet and RORγt are critical for the development of NKp46$^+$ ILC3s. During Th cell differentiation, transcription factors induced in one lineage can repress the expression and/or functions of transcription factors that are expressed by other lineages. For example, T-bet suppresses GATA3 expression as well as its function (81–83). In addition, T-bet and GATA3 bind to Th1- or Th2-specific genes at the same regions (83–85). T-bet also inhibits RORγt during T cell differentiation (86). NKp46$^+$ ILC3s express slightly lower levels of RORγt than CCR6$^+$ ILC3s, suggesting that T-bet may inhibit RORγt expression in these cells (80). Such inhibition may explain how "ILC1-like" "ex-ILC3s" can be generated through turning off RORγt expression (80). Interestingly, these "ex-ILC3s" may re-express RORγt and become ILC3s by IL-1/IL-23 stimulation (87).

Since the master transcription factors cross-regulate each other and they can be co-expressed at the single cell level, quantitative expression of different master regulators may alter the balance among these factors resulting in phenotypical changes. Indeed, haplo-insufficiency of the master regulators, including T-bet, GATA3, and RORγt has been reported (58, 66, 88). Interestingly, all three transcription factors, T-bet, GATA3, and RORγt, are expressed by NKp46$^+$ ILC3s. More strikingly, low levels of GATA3 are also required for the development of NKp46$^+$ ILC3s,

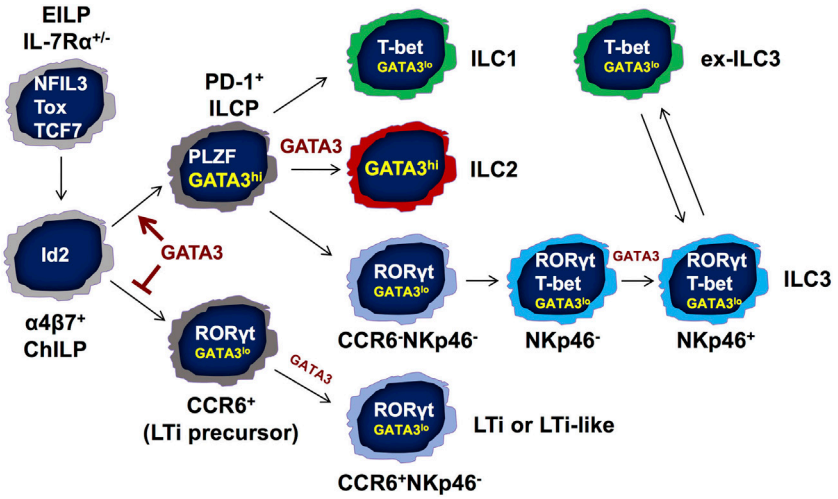

FIGURE 2 | GATA3 regulates innate lymphoid cell (ILC) development and functions at multiple stages. GATA3 is expressed by all ILCs including lymphoid tissue inducer (LTi) cells and natural killer (NK) cells, albeit at different levels. GATA3 is required for the development of IL-7Rα-expressing ILCs but not NK cells as shown in **Figure 1**. At a possible developmental branch point for the bifurcation of PD-1$^+$ PLZF-expressing ILC progenitors (ILCPs) and CCR6$^+$ LTi progenitors, GATA3 is highly expressed in and absolutely required for the generation of "non-LTi" PLZF-expressing ILCPs. High level of GATA3 expression may suppress LTi developmental program. Nevertheless, GATA3 expression at low levels during the early stage of LTi cell development regulates LTi function. In mature ILCs, GATA3 is required for the functions and maintenance of ILC2s. GATA3 is also important for the homeostasis of ILC1s. Furthermore, the development of NKp46$^+$ ILC3s that co-express T-bet and RORγt depends on low levels of GATA3 expression. Finally, GATA3 promotes IL-22 production in RORγt-expressing ILCs and regulates IL-7Rα expression in all ILC subsets. EILP, early ILC progenitor that expresses TCF7, NFIL3, and Tox; ChILP, common helper-like ILC progenitor that expresses Id2; ILCP, common ILC progenitor that expresses PLZF and GATA3.

and GATA3 regulates the balance between RORγt and T-bet (66). GATA3-deficient ILC3s express ~2-fold higher RORγt indicating that GATA3 inhibits RORγt expression in ILC3s. Correcting RORγt expression levels by breeding GATA3-deficient mice onto the *Rorc* heterozygous background restores the development of NKp46$^+$ ILC3s, indicating that GATA3 regulates the balance between RORγt and T-bet during NKp46$^+$ ILC3 development (**Figure 2**).

As mentioned earlier, GATA3 is not required for the development of LTi or LTi-like cells. However, these LTi cells are nonfunctional, since *Gata3*$^{fl/fl}$-VavCre mice do not have lymph node structures (8). Single cell analysis of gene expression may address whether GATA3-deficient LTi cells fail to express some LTi-specific genes that determine LTi function. On the other hand, high levels of GATA3 may be inhibitory for the development of LTi progenitors since we have found that the expression levels of GATA3 are negatively associated with many LTi-specific genes at the single cell level. Furthermore, deletion of *Gata3* in NKp46$^+$ ILC3s results in upregulation of CCR6$^+$ ILC3-specific genes (66). Therefore, high levels of GATA3 expression at the PLZF-expressing progenitor stage are important for suppressing LTi lineage fate, and low expression of GATA3 in NKp46$^+$ ILC3s is continuously required to maintain NKp46$^+$ ILC3 cell identity by repressing the expression of LTi lineage-related genes.

GATA3 is also important for the optimal expression of *Il22* (66). Interestingly, GATA3 binds to the *Il22* promoter only in ILC3s but not ILC2s. Since GATA3 promotes IL-22 expression in both CCR6$^+$ ILC3s and NKp46$^+$ ILC3s, mice with ILC3-specific *Gata3* deletion mediated by RORγt-Cre are susceptible to *Citrobacter rodentium* infection. However, these mice develop normal lymph node structures. These results suggest that while GATA3 regulates LTi function at an early stage of their development, maintenance of LTi functions does not need continuous expression of GATA3 in LTi cells (66).

GATA3 FUNCTIONS IN ILC1s AND NK CELLS

ILC1s including tissue-resident NK cells are enriched in the liver and T-bet is the master regulator for the development of ILC1s (12, 34). Similar to ILC3s, ILC1s also express low levels of GATA3 (12, 66). It has been reported that GATA3 is important for the maintenance of ILC1s (12). However, it is not known whether such GATA3 function is related to its effect on IL-7Rα expression in ILC1s. As discussed earlier, GATA3 is not necessary for the development of conventional NK cells (8, 89, 90). However, GATA3 is also expressed by NK cells, and they need GATA3 for their maturation and cytokine production (89).

REGULATION OF GATA3 IN ILCs AND THEIR PROGENITORS

Since GATA3 plays important roles in different ILC subsets and progenitors, and its function is associated with its dynamic and quantitative expression, it is critical to understand signals that regulate GATA3 expression. During Th2 differentiation, IL-4-mediated STAT6 activation is the major driving force responsible for the upregulation of GATA3 expression. TCR-mediated signaling especially triggered by low dose of antigens

can also upregulate GATA3 expression (91). However, ILCs do not express antigen receptors, and ILC2 development seems to be IL-4-STAT6 independent (37).

Notch signaling induces whereas TGFβ downregulates GATA3 expression (92, 93). These signaling pathways may be important in regulating GATA3 expression in different ILC subsets at different stages. Indeed, it has been reported that TCF7, which can be induced by Notch signaling, positively regulates GATA3 expression during early stages of ILC development (48, 59). Besides transcriptional regulation of *Gata3* gene expression, posttranscriptional and posttranslational regulations of GATA3 expression and functions should be also considered. For example, GATA3 activity is regulated by tyrosine kinase p38 in both T cells and ILCs (60, 94). Furthermore, GATA3 protein quickly disappears in ILC2s upon *Gata3* deletion indicating that there is an active mechanism for GATA3 protein degradation in these cells.

As another critical transcription factor involved in ILC development, Id2 may regulate GATA3 expression or *vice versa*. In the future, it is essential to understand the relationship between Id2 and GATA3. Because gene regulation mediated by GATA3 is highly cell type and stage specific, its induction may also depend on cell context. Identification of the cofactors for GATA3 and the gene regulatory networks in different cell types at various developmental stages is essential to understand the biology of GATA3 in ILCs.

CONCLUDING REMARKS

T-bet, GATA3, and RORγt are the lineage-defining factors that are critical for the development and functions of ILC1s, ILC2s, and ILC3s, respectively. Besides its critical role in the development and maintenance of ILC2s, GATA3 is also indispensable for the generation of PLZF-expressing ILC progenitors that give rise to non-LTi ILCs. Furthermore, GATA3 regulates the acquisition of LTi cell function, ILC3 effector function, and ILC homeostasis (**Figure 2**).

T-bet, GATA3, and RORγt can be co-expressed at the single cell level resulting in heterogeneity and possible plasticity of ILC subsets. In particular, NKp46$^+$ ILC3s express all three transcription factors. GATA3 expression in NKp46$^+$ ILC3s cells, albeit at low levels, is important for regulating the balance between T-bet and RORγt. Because quantitative changes in GATA3 expression may result in qualitative developmental effects, a model with titratable GATA3 expression may be needed to study its dose effects on the development and functions of ILC subsets.

In addition to studying the dose effect of GATA3 in ILC development and functions, investigating cell type- and stage-specific GATA3 regulation and function will greatly enhance our knowledge on ILC development. It is important to identify cell type- or stage-specific enhancers within the 1Mb *Gata3* gene locus through epigenetic analyses. Future studies should also identify the similarities and differences between the actions of GATA3 in different cell types at various developmental stages, including both T cell and ILC lineages. Revealing cell type-specific gene regulation and epigenetic modifications mediated by GATA3 as well as cell type-specific binding patterns of GATA3 at a genome level will help us understand detail mechanisms. Finding GATA3 partners in different cell types, either by using co-immunoprecipitation followed by mass spectrometry or by genome-wide screening through bimolecular fluorescence complementation methods, may explain cell type-specific gene regulation mediated by GATA3.

Besides GATA3, many other transcription factors such as Id2, TCF7, NFIL3, Tox, and Runx proteins are also involved in ILC development (8, 41, 42, 48, 49, 95, 96). Further investigation on the relationship between GATA3 and other important transcription factors that are involved in ILC development and functions, including studies at a single cell level, may yield a deeper understanding of the ILC biology.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Epigenomic Views of Innate Lymphoid Cells

Giuseppe Sciumè[1], Han-Yu Shih[2], Yohei Mikami[2] and John J. O'Shea[2]*

[1] Department of Molecular Medicine, Sapienza University of Rome, Laboratory Affiliated to Istituto Pasteur Italia – Fondazione Cenci Bolognetti, Rome, Italy, [2] Lymphocyte and Cell Biology Section, Molecular Immunology and Inflammation Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH, Bethesda, MD, United States

The discovery of innate lymphoid cells (ILCs) with selective production of cytokines typically attributed to subsets of T helper cells forces immunologists to reassess the mechanisms by which selective effector functions arise. The parallelism between ILCs and T cells extends beyond these two cell types and comprises other innate-like T lymphocytes. Beyond the recognition of specialized effector functionalities in diverse lymphocytes, features typical of T cells, such as plasticity and memory, are also relevant for innate lymphocytes. Herein, we review what we have learned in terms of the molecular mechanisms underlying these shared functions, focusing on insights provided by next generation sequencing technologies. We review data on the role of lineage-defining- and signal-dependent transcription factors (TFs). ILC regulomes emerge developmentally whereas the much of the open chromatin regions of T cells are generated acutely, in an activation-dependent manner. And yet, these regions of open chromatin in T cells and ILCs have remarkable overlaps, suggesting that though accessibility is acquired by distinct modes, the end result is that convergent signaling pathways may be involved. Although much is left to be learned, substantial progress has been made in understanding how TFs and epigenomic status contribute to ILC biology in terms of differentiation, specification, and plasticity.

Keywords: innate lymphoid cells, NK cells, epigenetic, regulomes, DNA accessibility, transcriptomes, transcription factors

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****Correspondence:***
Giuseppe Sciumè
giuseppe.sciume@uniroma1.it

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INTRODUCTION

The immune system employs a variety of effector cells that ensure protection against diverse types of infections. An important strategy for host defense is that distinct immune responses are evoked by different pathogens, one aspect being the production of selective cytokines (1, 2). Intracellular bacteria and viruses are usually eliminated through the so-called type 1 response, which is dominated by the release of interferon (IFN)-γ as a signature cytokine. Moreover, cells infected by these pathogens are recognized by immune cells with the ability to directly kill their targets by releasing perforin and granzymes and inducing programmed cell death (3–5). In contrast, parasites and worms evoke a type 2 response, characterized by the production of interleukin (IL)-4, IL-5, and IL-13, necessary to drive their elimination/expulsion (6, 7). Finally, extracellular pathogens and fungi are associated with production of the signature cytokines IL-17 and IL-22, which provide host defense at mucosal surfaces (8).

In many respects, the effector functions described above have been mainly associated with the adaptive arm of the immune system, with a largely T cell-centric point of view (9). Although NK cells were recognized 40 years ago, other innate lymphocytes with helper features were unknown until recently (10). Based on the analogy with the effector functions of T cell subsets, innate lymphoid

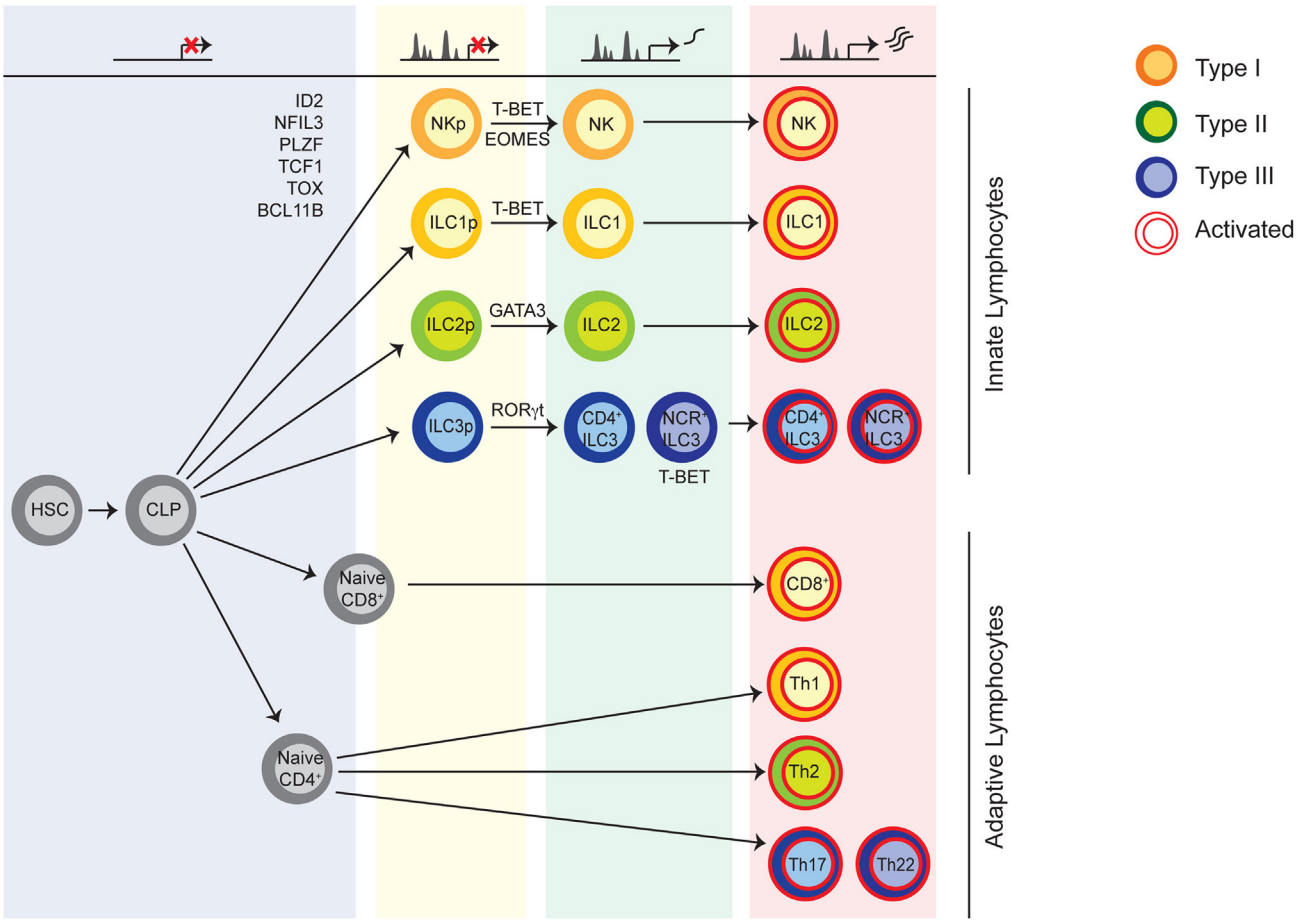

FIGURE 1 | Innate lymphoid cells (ILCs) are divided into three groups analogously to T cell subsets. NK cells and other interferon-γ producing cells, namely ILC1, belong to the group of type 1 ILCs. Helper ILCs producing T helper (Th)2- and Th17/22-cytokines are termed ILC2 and ILC3, respectively. Type 3 ILCs comprise lymphoid tissue inducer-like cells and NCR$^+$ ILC3. The different ILC subsets acquire their specific epigenomic features in a step-wise manner during development. In the course of specification, ILC precursors acquire regulatory elements for signature genes expressed by mature cells, while opposing fates are antagonized. During infection, ILCs and effector T cells undergo convergent epigenetic regulation. Transcription factors required during development and lineage-defining transcription factors for prototypical ILC subsets are shown.

cells (ILCs) are currently divided into three groups (**Figure 1**) (11). Type 1 ILCs include both NK cells and ILC1 which may be viewed as the innate counterpart of CD8$^+$ cytotoxic T lymphocytes, and T helper (Th) 1 cells, respectively. Similarly, ILC2 and ILC3 comprise helper lymphocytes that promote Th2- and Th17/22-related responses (12–16). Lymphoid tissue inducer (LTi)-like cells are included in the ILC3 group, as well as, ILCs expressing natural cytotoxicity receptors (NCRs), found in the mucosal tissues (17–22). More recently, the identification of an ILC subset that produces IL-10 and transforming growth factor (TGF)-β, and suppresses effector functions of other ILCs has broadened this view, adding yet another ILC subset that shares functional properties with regulatory T cells (23). Beyond these functions, several other concepts of T cell biology have been applied to ILCs, including memory-like responses of NK cells following viral infection (24–26) and the plasticity of ILCs that occurs in response to environmental changes (27, 28). Despite the similarities, an important distinction between innate and adaptive lymphocytes is the characteristic poised activation state of ILCs, as well as, their lack of antigen receptor engagement for acquisition of effector functions (29–31).

The field of ILC biology is an area of intense investigation and it is impossible to do justice to all the rapid advances. Not only are ILCs being considered in terms of host defense and immune mediated disease, but are emerging as players in the regulation of metabolic homeostasis, deposition of adipose tissue and, obesity, in both physiological and pathological conditions (32–35). However, in this short essay, we will focus on how the progress of next generation sequencing (NGS) technologies has provided new insights into some of the aspects of ILC biology from a genome-wide perspective. Clues to the nature of ILC identity and function are revealed by their global epigenomic features and transcriptomic programs, providing insights into heterogeneity and plasticity, and new paradigms for their relationships to T cells. These concepts can be integrated in the context of transcription factor (TF) networks, differentiation, microenvironment, and infections.

TRANSCRIPTIONAL PROGRAMS DEFINING ILCs

The transcriptional programs defining distinct ILC lineages have been investigated by using both genomic and genetic tools. These approaches have helped to elucidate shared and distinctive features of innate and adaptive lymphocytes, and the TFs required in regulation of ILC differentiation/functions. Although in homeostatic conditions ILCs share more genes with each other than they do with T cells (36, 37), the similarity between T cells and ILCs is exemplified by the common expression of a high number of genes and the clear reliance on many of the same lineage-defining transcription factors (LDTFs), also referred to as "master regulators" of T cell fate.

The expression of two LDTFs belonging to the T-box family, EOMES and T-BET (encoded by *TBX21*), is critical for NK cells. Deletion of *Eomes* in mouse leads to the loss of NK cells, which is not rescued by the expression of T-BET (38). On the other hand, *Tbx21*$^{-/-}$ NK cells show defects in cell turnover, trafficking, and functional properties (39). The constitutive expression of these two TFs helps to explain the poised features of NK cells and highlights functionalities shared with CD8$^+$ T cells, although the latter upregulate T-BET and EOMES expression after activation. Transcriptomic analyses have shown that the poised state of NK cells is not restricted to the expression of *Ifng* and genes required for the cytotoxic machinery, but comprises multiple effector molecules transcribed in resting mouse NK cells and also in activated/effector CD8$^+$ T cells (37).

In contrast to NK cells, ILC1 do not express EOMES and, instead, like Th1, require only T-BET for their development, as shown by *Tbx21*$^{-/-}$ mice (38, 40, 41). However, the ectopic expression of EOMES in ILC1 pushes their differentiation toward mature NK cells, suggesting that ILC1/NK conversion could involve induction of EOMES (42). Recently, cells with mixed ILC1/NK phenotype have been identified in mouse salivary gland, as well as, NK cells expressing EOMES and low levels of T-BET in human liver (43–45). Based on expression of cytokine/chemokine receptors and other surface markers, liver-resident ILC1 can be viewed as being related to NKT cells. More broadly though, the liver ILC1 program has greater global similarity to NK cells versus NKT cells (46). Although liver ILC1 are not considered prototypical cytotoxic ILCs, they do express high levels of the transcripts encoding for granzyme A and C (*Gzma*, *Gzmc*) and can kill through the expression of tumor necrosis factor-related apoptosis-inducing ligand (36, 46, 47). Together, these findings blur the boundaries among the different type 1 ILCs; although, strong evidence for functional sub-specialization of this group of cells is still limited.

Type 2 ILCs are dependent upon GATA-3 for their development, as this LDTF is for Th2 cells. Likewise, ILC3 require RORγt (encoded by *RORC*), which also promotes the fate of Th17/22 cells (12). Further complexity in the ILC3 group is provided by the combinatorial expression and requirement of T-BET, and GATA-3 in NCR$^+$ ILC3 (40, 48–53). The evidence for expression of more than one LDTF in ILCs highlights the functional importance of a coordinated network of TFs in regulation of ILC transcriptional programs. However, ILCs are not alone in this regard—both adaptive and innate T cells are now appreciated to rely on a network of TFs working in conjunction, rather than on a single TF as previously proposed in the monolithic view of T cell development (9, 31, 54).

Together with the functional options acquired by "default" during development, the phenotype of mature ILCs can be skewed toward different fates through distinct environmental stimuli (51, 55–60). Cytokines previously linked to the processes of differentiation and plasticity of Th cells are now considered major drivers of the functional plasticity of ILCs. For instance, type 1 features in ILC2 can be induced by IL-12 (57, 58, 60), whereas ILC3/ILC1 transitions can be driven by IL-12 and IL-23 (51, 55, 56). In addition, both canonical and non-canonical pathways downstream of TGF-β signaling regulate NK/ILC1 conversion, along with imprinting of the ILC1 features, NK cell activity, and differentiation (43, 61–65). Thus, the environment—including tissue-specific signals—becomes a fundamental element to consider in the regulation of global gene expression in ILCs, which can modify the effects of lineage identity driven by LDTFs (36, 66–68). In this regard, the role for signal-dependent TFs, such as AHRs, RORs, NOTCHs (including the induction of downstream TFs, BCL11B, and GFI1), and STATs has become evident in several settings (29, 69–73).

Our understanding of the functions of key TFs involved in ILC differentiation is often limited by the total loss of cells observed in knockout mice, which limits the ability to identify direct targets. To overcome this issue, deletion of a single TF allele may still permit cell development and thus, permit evaluation of the consequence of reduced TF expression. This genetic approach, combined with transcriptomic analysis, has been useful to identify a discrete number of genes directly regulated by T-BET and, more recently, STAT5 in ILCs (53, 74). In the case of STAT5, the presence of two distinct genes encoding *Stat5a* and *Stat5b*, provides multiple genotypes, and ILC phenotypes, to explore. Deletion of the entire *Stat5a/b* locus has profound effects on lymphoid development and NK cells were absent in the few mice that survived this genetic lesion (75). More recently, lack of NK cells has been observed in mice carrying a deletion of the *Stat5a/b* locus specifically in cells expressing NKp46 (76). The selective ablation of only one gene (using *Stat5a*$^{-/-}$ or *Stat5b*$^{-/-}$ mice) highlighted a major role for STAT5B upon STAT5A, in the maintenance and *in vitro* proliferation of NK cells (77). Thus, the employment of mice carrying only one allele for *Stat5a* (*Stat5a*$^{+/-}$*Stat5b*$^{-/-}$) or *Stat5b* (*Stat5a*$^{-/-}$*Stat5b*$^{+/-}$) represented a suitable compromise allowing to interfere with the amount of STAT5 without reaching a total ablation (74). As well, RNA-seq and ChIP-seq analyses helped to reveal a direct and constitutive role for STAT5 in maintaining the expression of genes defining the identity of NK cells, beyond its known role in regulation of survival and proliferation. Moreover, reduction in STAT5 signaling perturbed other NCR$^+$ ILCs, including ILC1 and NCR$^+$ ILC3, while having less of an impact on ILC2 and LTi-like ILC3. This preferential, or hierarchical, requirement for STAT5 is explained in part by a direct role in regulation of T-BET expression on NCR$^+$ ILCs.

Amidst this complexity, what is clear is that there seems to be no single "master regulator" for ILCs or ILC subsets (akin to *MyoD* in muscle or *Pax5* in B cells); instead, as with T cells, there

appears to be complex orchestration of TFs that presumably exert their effect in a combinatorial manner and LDTFs act in concert with SDTFs (78).

REGULOMES

The hard-wired effector functions of ILCs have been appreciated since the observation of the constitutive transcription of the *Ifng* gene in resting NK cells, favored by an accessible chromatin conformation of its promoter (79, 80). Thousands of accessible regions have been defined, which spread throughout the chromatin allowing/restraining access to TFs and other transcriptional regulators and determining the final outcome of gene expression. These sites include not only promoters, but also non-coding regulatory elements (REs), such as enhancers, silencers, repressors, and insulators, and are called, overall, regulomes (81). The different types of REs can be discriminated by the presence of selective histone modifications or histone modifiers. For instance, trimethylation of histone H3 at lysine 4 (H3K4me3) is a histone mark enriched at the promoter of active genes; while H3K4me1, H3K4me2, acetylation of H3K27 (H3K27ac), and the presence of the acetyltransferase p300 are found at enhancer sites (82). Below, we will discuss how the ILC epigenomic programs contribute to ontogeny and function.

Ontogeny of ILCs

In sharp contrast to T cells, signals from antigen receptors are not required for ILC effector function nor development (29–31). Instead, multipotent ILC precursors, including the α-lymphoid progenitor, early innate lymphoid progenitor, common helper innate lymphoid progenitor, and ILC progenitor, are regulated in mouse by the programmed expression of several TFs, including ID2 (inhibitor of DNA binding-2), TCF1 (encoded by *TCF7*), PLZF (encoded by *ZBTB16*), TOX, and NFIL3 (27, 83, 84). The above-mentioned ILC precursors progressively lose their multipotentiality, becoming unipotent ILC precursors, and a new set of TFs is required before lineage diversification, such as BCL11B for ILC2 or RUNX3 for ILC1 and ILC3 (85–88). However, expression of these TFs is not limited to the early stages of differentiation, as in the case of the basic helix-loop-helix TF, ID2, which is both required for the commitment of the entire ILC lineage and homeostasis of mature cells. One of ID2's primary roles is to inhibit the functions of E proteins, blocking T and B cell development in favor of the ILC fate (89–94). Unlike PLZF which is required for both invariant NKT (iNKT) cell and ILC development, deficiency of *Id2* does not alter the development of iNKT cells, indicating both specific and overlapping requirements for innate and innate-like T cell ontogeny (41, 94, 95). The early steps of ILC differentiation are also characterized by the requirement of the basic leucine zipper TF, NFIL3 (96–98). In *Nfil3*$^{-/-}$ mice, generation of B, T, and NKT cells is not affected, while development of NK cells (99–102) and other ILC subsets (35, 61, 98, 103) is highly impacted. However, in the context of mouse cytomegalovirus infection, the signals provided by the triggering of the activating NK cell receptor Ly49H and by the proinflammatory cytokine IL-12 can overcome the requirement for this TF, leading to generation of NK cells with intact functional properties and ability to mediate memory responses (104). The source of these NK cells remains unknown, but their origin could be explained by the presence of NFIL3-alternative pathways of NK cell generation, as shown for other type 1 ILCs, such as salivary gland NK cells (105, 106) and liver ILC1 (107), which can develop in the absence of NFIL3. Although during lymphoid development, the range of NFIL3 action is restricted to ILCs, this TF, as well as ID2, can have a broader role, being required for optimal production of IL-13 and IL-10 by adaptive and innate T cells and for terminal differentiation of Th17 (108–110).

How the expression of these and the other TFs expressed during ILC development impacts the acquisition of lineage-specific REs has not been investigated; although at present, the small numbers of such cells represent a technical challenge. However, when ILC precursors become unipotent, progressively acquire distinctive lineage-specific REs (66). Indeed, after lineage specification committed NK and ILC2 precursors show features of chromatin accessibility typically found in fully developed NK cells and ILC2, including not only loci related to signature cytokines but also genes encoding for molecules acquired only at late stages of differentiation, as is the case of KLRG1 (66). Another important aspect is that chromatin accessibility of lineage-specific genes starts to diverge at the precursor stage. Consequently, the loci encoding type 2 cytokines are not accessible in NK precursors, whereas the *Ifng* locus is not accessible in ILC2s, indicating that formation of the signature features of chromatin accessibility occurs during development, defining their effector function as well as restraining their alternative fates. In this regard, the lysine methyltransferase G9a, that catalyzes the repressive histone mark H3K9me2 (dimethylation of histone H3 at lysine 9), plays a key role in preserving the ILC2 fate (111). When its deletion is applied to the entire mouse hematopoietic system, both development in the bone marrow and homeostasis of tissue-resident ILC2 are highly impacted. Evidence of the repression of alternative fates mediated by G9a is the increased expression of genes associated to the type 3 response occurring after its deletion (111). Recently, a human ILC precursor able to give rise to all known ILC subsets has been defined in the peripheral blood (112). This precursor expresses several TFs related to murine ILC development, such as ID2, GATA-3, TOX, and TCF7, and its identity has been defined through the analysis of the genome-wide distribution of the histone modification H3K4me2 (dimethylation of Histone H3 at lysine 4) (112). This histone modification is present on both active and poised loci (113). Although LDTFs, as EOMES, TBX21, RORC, and other molecules present on mature ILCs are not expressed (including IL23R, CCR6, and signature cytokines), these genes show the presence of H3K4me2, indicating that they are epigenetically poised (112). Thus, both in human and mouse, ILC signature genes can acquire a complete set of REs at the precursor level, while the expression of these genes is induced/upregulated only after terminal differentiation.

Lineage Relationships and Identity

The elucidation of both mouse and human ILC regulomes has provided global molecular evidence for their poised state and also

for the current functional classification. Since ILC nomenclature is based on the common features with T cells, a compelling question is the extent to which ILCs and T cells are appropriately viewed as two distinct "lineages" or whether their epigenomic features converge reflecting their effector features. Interestingly, in homeostatic conditions, the chromatin accessibility of distinct ILC and T cell subsets seems to be divergent, as observed in both human and mouse (66, 67). Similar conclusions have been obtained through analyses of a class of enhancers highly linked with cell identity, called stretched- or super-enhancers (SE) (67). In contrast, the patterns of chromatin accessibility of ILCs and T cells converge in the infection settings, as a part of the shared transcriptional/epigenetic programs. Indeed, upon *Nippostrongilus brasiliensis* infection in mouse, the transition from naïve to Th2 cells requires a wholesale remodeling of the chromatin but nonetheless, Th2 regulomes converge with those of ILC2 (66). In the same way, after human cytomegalovirus infection, the DNA-methylation patterns of adaptive NK cells parallel those of cytotoxic/effector $CD8^+$ T cells (114). These NK cells show downregulation of the TF PLZF and peculiar functional properties which occur thanks to the regulation of the DNA-methylation state of key NK cell genes, including TFs and signaling proteins (114, 115).

When compared with transcriptomes, ILC regulomes are reportedly less dependent on acute environmental effects, and more sensitive to discriminate lineage identity (66); however, the extent to which ILC regulomes are malleable is clearly worthy of further investigation. Of note, after microbiota depletion, both intestinal ILC1 and ILC2 lose a portion of their signature REs and acquire type 3 features; while their global identity is preserved (68). Definition of ILC identity relies on the presence of very well-defined clusters of enhancers, as shown by the genome-wide distribution of p300 and histone marks (66–68). In contrast, our understanding of the specific role for single/specific REs in regulation of gene expression in ILCs is largely unknown. Another area that is in its infancy is the understanding of the function of cis-REs associated with expression of long non-coding RNAs (lncRNAs). One lncRNA termed RNA-demarcated regulatory region of ID2 (*Roid*) is essential for the expression of ID2 selectively on mature type 1 ILCs, in mouse (116). Beyond the initial role in driving differentiation of the ILC lineage, ID2 is required for homeostatic regulation of differentiated cells, such as type 1 and type 3 ILCs (116, 117), while the ectopic expression of ID1 in the thymus induces a general increase of the numbers of ILC2 in mice (118). Through regulation of the accessibility of the *Id2* locus, this cis-RE and its related lncRNA, *Roid*, control identity, differentiation, and functions of both NK and ILC1. These data point out that, along with TFs and REs, lncRNAs can represent another layer of epigenetic regulation in ILCs (119). In this context, another lncRNA, lncKdm2b, contributes to the maintenance of murine ILC3 by promoting the expression of ZFP292, recruiting SATB1, and the nuclear remodeling factor complex to the *Zfp292* promoter (120).

RECENT ADVANCES FROM SINGLE-CELL ANALYSES

The development of new strategies for single-cell analysis has made possible the characterization of the expression profiles of transcripts and proteins (121). Gene expression at the single-cell level can now be profiled by several single-cell RNA-sequencing (scRNA-seq) protocols, with different sensitivity, accuracy, cost-efficiency, and drawbacks (122, 123). Technical challenges notwithstanding, single-cell resolution has revealed substantial heterogeneity among ILCs. In mouse, 15 transcriptional states have been identified for intestinal $CD127^+$ ILCs, including two transcriptional profiles that did not fall in any previously recognized ILC category (68). Similarly, the features of $CD127^+$ ILCs and NK cells isolated from tonsils have been dissected in humans (124). The scRNA-seq approach has been also a key to clarify the heterogeneity of ILC precursors in mouse, which has led to establish the relevance of BCL11B in the differentiation process and to the identification of PD-1 as an early checkpoint in ILC2 development (125). Notably, treatment with PD-1 antibodies acts on ILCs in mouse models of infection (influenza and *N. brasiliensis*) and papain-induced acute lung inflammation (125, 126). In the context of fetal development, the requirement of NOTCH in lymphoid progenitors has been also dissected, such as the lineage relationship between LTi cells and other ILC lineages. These findings suggest a clear bifurcation in fetal lymphoid progenitors which involves an LTi precursor expressing TCF1 but not PLZF (127, 128). Despite evidence for such astonishing multiplicity of states of ILCs, what is less clear is whether these data are indicative of stable features of the cells or simply snapshots of transient states that emerge in many or all of the cells in a given population.

Recent methods allow for parallel measurement of single-cell transcriptomes and protein expression in addition to flow cytometry-based scRNA-seq techniques (129, 130). In the context of protein expression, time of flight mass cytometry followed by computational techniques has been used to dissect human ILCs in several tissues through evaluation of protein expression, based on a cytometry panel consisting of 38 transition element isotopes-labeled antibodies (131). Although this study contributes to unravel the complexity of ILCs between individuals and tissues, it also questions the existence of the prototypical ILC1 subset in human [as defined previously in Ref. (132)]. Beyond the technical details underlying the failure to reveal $T\text{-}BET^+$ ILC1 in human tissues and the limitations of this approach [explained in Ref. (133)], the definition of human type 1 ILC populations remains a subject of debate, due to the absence of specific markers and the expression of molecules associated to T cell biology, including intracellular expression of CD3 and surface expression of CD5 (134, 135). Despite the increasing awareness of ILC heterogeneity, it will be important to clarify to what extent the degree of complexity defines truly distinct ILC subpopulations, with different functions, or whether reflects a range of activation states within a single population.

CONCLUSION

Given that ILC regulomes are acquired independently of antigen receptor signals, a key unanswered issue is to understand how endogenous and exogenous factors function as drivers of chromatin organization. To what extent do endogenous TFs expressed during development contribute and how to exogenous

and environmental factors including the microbiome, diet, and cytokines contribute to the development of the epigenomic landscape? What factors are common to all lymphocytes and which are subset or situationally specific? One prevailing view is that lineage-specifying TFs bind to thousands of places in the genome and have "pervasive" effects on high-order chromatin structure and accessibility of critical loci. We are beginning to understand these processes in B cells and T cells (136–138). Even for differentiated ILCs, it will be important to understand how acute activation does or does not continue to modify regulomes to drive gene expression. How do epigenomic modifications, chromatin looping, and transcription relate? Technical limitations related to the small numbers of ILCs limit our understanding of global three-dimensional chromatin structure at present; however, given the rate of advances in this field it seems unlikely that this will remain a barrier for long. Additionally, unraveling the signals present within the different bone marrow niches underlying acquisition of regulomes and functional specification will help to discriminate the signaling pathways involved.

The development of mouse models with conditional deletion of genes or cell types has helped to understand how similar ILCs and T cells are in terms of differentiation and function, and in which contexts they can play distinct roles. In human, primary immunodeficiency syndromes have revealed specific functions for NK cells in controlling viral infections (139, 140), while evidence for ILC redundancy has been recently provided (141). The appearance of distinct ILC subsets can precede that of B and T cells during vertebrate evolution (142), suggesting that prototypical effector programs have evolved along with the repertoire of effector lymphocytes. Of consequence, the redundant transcriptional/epigenetic regulation and, probably, functions of ILCs, innate-like, and adaptive T cells can be certainly seen as a key factor to improve the overall fitness of a species. Interestingly, the different effector programs appear to follow different routes of evolution. Indeed, type 2 cytokines seem to emerge after type 1 and type 3 cytokines, despite the early appearance of TFs belonging to the GATA family, implying an initial role in lymphoid development for GATA-3, above regulation of type 2 response (143, 144). Thus, one aspect to evaluate will be the degree of conservation relative to the SEs, spreading along cytokine loci, and defining cell identity, and the TFs underlying these important switches. The increased resolution and sensitivity of NGS technology has been providing a pivotal contribution to elucidate the mechanisms of epigenomic regulation underlying ILC development/effector functions and to identify REs distinctive of each ILC subset. The possibility of combining scRNA-seq and clustered regularly interspaced short palindromic repeats (CRISPR)-pooled screening will allow analysis of genomic perturbation on transcriptomes within the same cell (145, 146). Thus, this combination of genome-editing and single-cell techniques will enable elucidation of gene function and its loss-of-function phenotype at single-cell level, but also the functions of REs that contribute to identity, development and functions of lymphoid cells.

AUTHOR CONTRIBUTIONS

The final manuscript was a result of the joint efforts of all the authors.

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REVIEW
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Interactions between Innate Lymphoid Cells and Cells of the Innate and Adaptive Immune System

*Cornelia Symowski and David Voehringer**

Department of Infection Biology, University Hospital Erlangen and Friedrich-Alexander University Erlangen-Nuremberg (FAU), Erlangen, Germany

Type 2 innate lymphoid cells (ILC2s) are a major source of cytokines, which are also produced by Th2 cells and several cell types of the innate immune system. Work over the past few years indicates that ILC2s play a central role in regulating type 2 immune responses against allergens and helminths. ILC2s can interact with a variety of cells types of the innate and adaptive immune system by cell–cell contacts or by communication *via* soluble factors. In this review, we provide an overview about recent advances in our understanding how ILC2s orchestrate type 2 immune responses with focus on direct interactions between ILC2s and other cells of the immune system.

Keywords: allergic inflammation, helminths, mast cells, innate lymphoid cells, eosinophils, basophils, Th2 cells, alternatively activated macrophages

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***Correspondence:**
David Voehringer
david.voehringer@uk-erlangen.de

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INTRODUCTION

Innate lymphoid cells (ILCs) are characterized by their lack of expression of rearranged antigen-receptors and absence of cell surface markers present on other common hematopoietic cell lineages. They arise from a common lymphoid progenitor, requiring expression of the transcriptional repressor Id2 and relying on cytokine signaling through the common gamma-chain (γ_c chain) of the IL-2 receptor family. ILCs are classified into three distinct populations termed group 1, 2, and 3 ILCs based on their ability to produce effector cytokines associated with Th1, Th2, or Th17 cells, respectively (1–4).

Group 2 ILCs (ILC2s) share the expression of the transcription factor GATA-3 and characteristic cytokines with Th2 cells indicating a similar role for both cell types in type 2 immune responses (5, 6). ILC2s respond extremely rapidly to epithelial cell-derived cytokines, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), associated with barrier disruption (7) and act as "early sentinel" cells of the innate immune system orchestrating type 2 immune responses at mucosal surfaces and adipose tissue (8–10).

The canonical type 2 immunity-associated cytokines IL-4, IL-5, and IL-13 are pivotal in immunity toward gastrointestinal helminths inducing eosinophilia, elevated IgE levels, goblet cell metaplasia with enhanced mucus production, and smooth muscle hyperreactivity (11, 12). It is evident that cells of the adaptive and innate immune system, including Th2 cells, ILC2s, eosinophils, basophils, and mast cells, produce significant levels of these cytokines inducing and sustaining ongoing type

Abbreviations: ILC, innate lymphoid cells; PGD2, prostaglandin 2; CRTH2, cysteine-three-histidine protein 2; NK cell, natural killer cell; VAT, visceral adipose tissue; CLP, common lymphoid progenitor; ICOS, inducible T cell co-stimulator; VPAC2, vasoactive intestinal peptide receptor 2; FALC, fat-associated lymphoid cluster.

2 responses (13, 14). Although the prominent role of Th2 cells in type 2 immune responses is well established, the secretion of IL-13 from ILC2s rather than Th2 cells is of particular importance for controlling the intestinal immune response and worm expulsion during infection of mice with the helminth *Nippostrongylus brasiliensis* (9, 10, 15). On the other hand, T cell-derived IL-4/IL-13 are needed for effector cell recruitment, germinal center formation, IgE switching, and paracrine Th2 differentiation (16, 17).

In contrast to the protective function of ILC2s, dysregulated ILC2 responses contribute to inflammatory processes, such as airway hyperreactivity (18), allergen-induced lung inflammation (19, 20), and atopic dermatitis (21). Despite the substantial gain of knowledge about ILC2s development and mediators that positively or negatively modulate ILC2 homeostasis, activation, and functions (22, 23), the regulation of ILC2 functions is becoming more complex, and it is of high importance to understand the immunoregulatory mechanisms to improve therapeutic treatments of pathological type 2 immune responses. Besides producing cytokines, ILC2s may interact with other effector immune cells and coordinate immune responses as part of the complex immune system network important for immune defense and allergic reactions. Recent data indicate that ILC2s can influence T cell responses in a reciprocal manner, either through cytokines, indirect effects on accessory cells, or direct cell–cell contact relaying signals to the adaptive immune system. Additionally, ILC2s also contribute to the maintenance of eosinophils (24) and affect the functions of cells such as basophils (25), macrophages (26), dendritic cells (DCs) (27, 28), and mast cells (29), which on the other hand can also activate ILC2s (30) or suppress their activity (31).

Defining the complex network of interactions and mutual communications of ILC2s with immune cells from the innate and adaptive immune system and understanding the specific contributions of ILC2s leading to protective immunity against helminths or development of pathologic responses may reveal critical checkpoints that can be manipulated for controlling type 2 immunity-mediated responses and will be important to investigate new possible therapeutic interventions.

INTERACTIONS OF ILC2s WITH CELLS OF THE ADAPTIVE IMMUNE SYSTEM

ILC2s and T Cells

Th2 cells are a major source of IL-4 and IL-13 and they play an important role in type 2 immune responses. Recently, our group revealed that specific depletion of IL-4/IL-13 in $CD4^+$ T cells results in reduced accumulation of innate effector cells (eosinophils, basophils, ILC2s) in the lung of *N. brasiliensis*-infected mice as compared to wild-type mice and that $CD4^+$ T cell-derived IL-4/IL-13 cytokines promote Th2 polarization in a paracrine manner (16, 17). Beside eosinophils, basophils, and mast cells, mouse ILC2s are also known to transcribe and produce low amounts of IL-4 induced by the lipid mediator leukotriene D4 (15, 32, 33). Leukotrienes can act in a synergistic manner together with IL-33 to activate ILC2s (34, 35). Human ILC2s appear to secrete larger quantities of IL-4 as compared to mouse ILC2s especially upon IL-33 stimulation and in the presence of TSLP (36). During infection with the helminth *Heligmosomoides polygyrus*, ILC2-derived IL-4 has been shown to contribute to ILC2 expansion and to drive early Th2 differentiation without direct cell–cell contact (33). Furthermore, mice lacking ILC2s showed impaired Th2-skewed inflammatory responses following helminth infection or local exposure to the protease-allergen papain, or house dust mite antigens (27, 37, 38). This suggests that ILC2s can promote Th2 responses under certain experimental conditions. In addition, IL-13-producing ILC2s were shown to cooperate with $CD4^+$ T cells during *N. brasiliensis* infection to mediate larval killing in the small intestine during primary infection (38) and in the lung following secondary infection (26). Furthermore, *N. brasiliensis* could be expelled by transfer of ILC2s into IL-13-deficient mice, but not into Rag2-deficient mice (9). This indicates that IL-13 from ILC2s is sufficient for clearance of primary *N. brasiliensis* infection, but $CD4^+$ T cells are still required for effective worm expulsion Interestingly, T cell-derived IL-2 can induce ILC2 proliferation and IL-13 secretion (39). In addition, it was shown that in mice exposed to the pro-allergic protease papain ILC2-derived IL-13 rather than IL-4 promotes migration of DCs into lung-draining lymph nodes, where activated DCs support Th2 cell differentiation (27).

Innate lymphoid cells not only contribute to Th2 cell differentiation by cytokine secretion but can also directly interact with $CD4^+$ T cells. Using an *in vitro* culture system, it was reported that ILC2s promote Th2 polarization in a cell–cell contact-dependent manner (39). In addition, both costimulation by OX40/OX40-L interaction and ILC2-derived IL-4 was shown to enhance Th2 cell proliferation and Th2 cytokine production when the isolated cell populations were cultured together (40). Beside expressing costimulatory molecules, ILC2s have also been shown to express MHC class II (9, 39, 41). Recent data identified ILC2s as antigen-presenting cells (APC) able to process and present peptide antigens and modulate naive $CD4^+$ T cell activation in a cell contact-dependent manner (38, 39, 42). Expression of MHC-II on ILC2s was required to receive activating signals by T cell-derived IL-2 causing efficient secretion of IL-13 (38). This suggests that ILC2s and T cells can communicate in an antigen-dependent manner. However, whether ILC2s play a significant role as APC during priming of the Th2 response remains to be investigated.

ILC2s and Treg Cells

Subsequent studies demonstrated that Treg cells and ILC2s engage in reciprocal regulation. Treg cells are regulators of adaptive immune responses through direct cell–cell contact, as well as through the suppressive activities of IL-10 and TGF-β. The importance of Treg cells on control of ILC2 activity and homeostasis has recently been shown by inhibition of the transcription factors Id2 and Id3 in Treg cells, which lead to a spontaneous increase in ILC2 counts, as well as accumulation of eosinophils in the lungs and resulted in the development of fatal inflammatory disease (43). While Treg cells regulate ILC2 expansion and suppress their pro-inflammatory cytokine secretion *in vivo* and

in vitro, ILC2-derived IL-4 plays a requisite role in mediating sensitization to food allergens by compromising allergen-specific regulatory T cell function and thereby promoting food allergy (44). In contrast, ILC2-derived IL-9 was required for Treg activation and resolution of inflammation in an arthritis model (45). In addition, ILC2s may enhance the suppressive activity of Treg cells by secretion of amphiregulin as it has been described for mast cell-derived amphiregulin (46).

IL-33 can either directly activate ST2$^+$ Treg cells in the intestine (47) or promote their expansion indirectly by inducing IL-2 secretion from DCs (48). Others have shown that accumulation of Treg cells in adipose tissue by IL-33 was directly dependent on ILC2s by a process requiring ILC2-intrinsic MyD88 signaling and cognate interactions between inducible T cell co-stimulator (ICOS) on Treg and ICOS ligand (ICOS-L) on ILC2s (49). As IL-33 alone can activate significant Treg cell proliferation independently of ILC2s, one can assume that ILC2s act mainly to promote Treg survival. Coculture experiments revealed that ICOS-L/ICOS interaction is important for the ILC2-mediated survival of Treg cells (49). Furthermore, ILC2-derived IL-4 may promote the conversion of Treg cells to Th2 cells after *H. polygyrus* infection (50). A recent study demonstrated that induced Treg cells (iTreg), but not natural Treg cells, effectively suppressed human and mouse ILC2 function and this effect was dependent on ICOS/ICOS-L interactions (51). Autocrine ICOS/ICOS-L interactions on ILC2s were also reported to play an important role to enhance survival of ILC2s (42, 52). IFN-γ was found to counter-regulate the effects of IL-33 mediated ILC2 activation and Treg cell expansion (49), indicating that Th1-dominated immune responses can actively suppress the IL-33-ILC2-Treg axis and thereby cause loss of barrier tissue integrity and shifts in fat metabolism. Understanding the contributions of ILC2s and Tregs to optimal immune regulation might help preventing excessive tissue damage and the development of chronic inflammations.

ILC2s and B Cells

In addition to their mutual interactions with T cells, ILC2s may also interact with B cells. It is known that fat-associated lymphoid clusters (FALCs), found in human and in mouse mesentery, contain large proportion of B1 cells and contribute to innate B cell activation and germinal center differentiation (53). So far, it is described that ILC2s from FALCs maintain homeostasis of the self-renewing B1 cells in an IL-5-dependent manner and support production of parasite-specific antibodies by B cells (8, 54). Interestingly, it was described that lung ILC2s not only enhance the proliferation of B1 but also follicular B2 type B cells and promote the secretion of IgM, IgG1, IgA, and IgE by these cells in the absence of T cells (55). It is also assumed that ILC2s have the ability to promote T cell-dependent antibody responses as serum IgE levels are reduced in ILC2-deficient mice challenged with papain (27). Human ILC2s were found to directly activate B cells isolated from tonsils (56).

Furthermore, it has been described that ILC2s can enhance IgE production from B cells *in vitro*, raising the possibility that ILC2s orchestrate IgE-dependent allergic sensitization (57). Whether ILC2s can interact with ICOS-L-expressing B cells is still an open question and additional studies are required to identify B cell-derived mediators that feedback on ILC2s. However, the addition of anti-ICOS antibodies showed no effect on the ability of ILC2s to enhance B cell IgM and IgG1 production indicating that cytokines like IL-5 and other soluble factors that are secreted by ILC2s may play a key role (55).

Still, many questions remain to be answered regarding the interactions between ILC2s and cells of the adaptive immune system. Taken together, ILC2s regulate and dictate the nature of downstream T cell- and B cell-mediated immune responses, while T cells also influence the survival, proliferation, and function of ILC2s (**Figure 1**).

INTERACTIONS OF ILC2s WITH CELLS OF THE INNATE IMMUNE SYSTEM

The alarmins IL-25, IL-33, and TSLP initiate a local inflammatory response through the recruitment and activation of ILC2s and other innate effector cells. Investigating the role of ILC2s during coordination of this early innate immune response is critical to uncover their contribution for type 2 immunity during infections, allergic responses, or autoimmune diseases.

ILC2s and Macrophages

Innate lymphoid cells can promote the differentiation of so-called "alternatively activated" or M2 macrophages, which have protective functions in some helminth infection models and contribute to tissue repair responses (58). Using a mouse model for cerebral malaria, IL-33-elicited ILC2s were found to promote M2 polarization and Treg cell expansion resulting in protective immunity

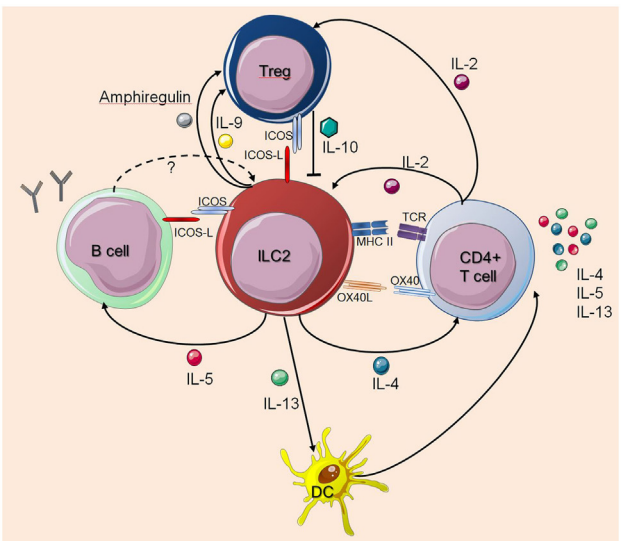

FIGURE 1 | Interactions between innate lymphoid cells (ILC2s) and T cells or B cells. The figure illustrates how ILC2s interact with T cells or B cells as described in the main text. The dashed arrow indicates a potential feedback regulation for which there is currently no experimental evidence. Solid arrows indicate published evidence for activating mechanisms whereas Treg-derived IL-10 was shown to suppress ILC2s. Cell symbols where taken from http://smart.servier.com website.

(59). It was further described that IL-33- or IL-2-elicited ILC2s are sufficient to induce M2 macrophage-mediated larval killing of *N. brasiliensis* helminths in the lung (26). Antifungal type 2 immune responses are also regulated by ILC2-mediated M2 polarization as demonstrated with a *Cryptococcus neoformans* infection model (60). In addition, IL-25-elicited ILC2-derived IL-13 was shown to promote activation of lung-resident macrophages to a profibrotic phenotype, driving collagen deposition from fibroblasts (61).

M2 macrophages in visceral adipose tissue (VAT) play an important role for glucose and fat metabolism and ILC2s as well as eosinophils have been described to promote M2 macrophage accumulation in VAT during helminth infection (62, 63). Furthermore, it has been reported that IL-33-activated ILC2s elicit the differentiation of alternatively activated macrophages through IL-4 receptor signaling and regulate directly beige fat biogenesis (64). However, recent studies indicate that IL-4-mediated macrophages have no relevant effect on white and brown adipocyte function and do not likely modulate adipocyte metabolism by catecholamine production (65, 66).

The other way round, alveolar macrophages are able to secrete IL-33, which is likely important for direct activation of ILC2s to produce substantial amounts of IL-13, as mice lacking the IL-33 receptor failed to develop AHR or airway inflammation independently of adaptive immunity (67). Thus, cross talk between macrophages and ILC2s might be critical to promote an early feed forward process during type 2 immune responses.

ILC2s and DCs

Type 2 innate lymphoid cell (ILC2)-derived IL-13 was reported to induce migration of DCs into the draining lymph node, where DCs drive naive T cells to become Th2 cells (27). However, how IL-13 controls the migratory function of DCs still remains to be defined. Furthermore, ILC2-derived IL-13 promotes the secretion of the chemokine CCL17 from DCs for the recruitment of $CCR4^+$ memory Th2 cells to the site of allergen exposure (28). Eosinophils recruited into tissues and lymph nodes can also control DC activation and migration and promote Th2-cell-mediated immunity *via* degranulation of eosinophil peroxidase (68), demonstrating how innate cells work in a complex network to drive type 2 inflammatory responses.

The tumor necrosis factor (TNF) family cytokine TL1A is known to be produced by DCs and macrophages in response to toll-like-receptor and Fc receptor cross-linking and regulates the adaptive immune response by co-stimulating T cells (69). It was demonstrated that TL1A synergizes with IL-25 *in vivo* to directly promote ILC2 expansion, survival, and function (70, 71). The activation of ILC2s by TL1A could provide new insight into interaction of ILC2s and activated myeloid cells.

Recent observations revealed that type I and type II IFNs as well as IL-27 play a critical role as negative regulators of ILC2s to restrict type 2 immunity and its associated pathologies (49, 72–74). It has been reported that plasmacytoid dendritic cells (pDC) play a critical role in dampening the function and survival of ILC2s in the context of allergic pulmonary inflammation. IFN-α production by activated pDCs can inhibit proliferation and increases the apoptosis rate of ILC2s (31). Similarly, polyinosinic–polycytidylic acid (pI:C) activated NK cells inhibit the proliferation and cytokine production of ILC2s *via* IFN-γ during the early stage of lung inflammation, reminescent of the classic antagonism between Th1 and Th2 differentiation (75). As interferons are produced by many different cell types including Th1 cells, NKT cells, and others, one can assume that ILC2s may be suppressed by various cellular sources of these mediators.

ILC2s and Eosinophils

Innate lymphoid cells are a major source of IL-5 and thereby enhance proliferation, survival, and recruitment of eosinophils (24, 49). By comparing $Rag2^{-/-}$ and $Rag2^{-/-}\ \gamma_c{}^{-/-}$ mice, lung ILC2s were shown to promote eosinophilia independently of signals from the adaptive immune system (76). Naive lung ILC2s secrete IL-5 constitutively, as indicated by elevated *Il5* mRNA levels and fluorescent signals in *Il5*-reporter mice (24, 77). ILC2-derived IL-5 is required for systemic maintenance of eosinophils and during type 2 inflammation ILC2s are induced to co-express IL-13, resulting in localized eotaxin production for recruitment and activation of eosinophils during allergic inflammation and helminth infection (24, 62, 78). It was further shown that vasoactive intestinal peptide, a hormone regulated by caloric intake, stimulates ILC2s *via* the vasoactive intestinal peptide receptor 2 receptor to release IL-5, linking eosinophil levels with metabolic cycling (24). IL-33 can directly stimulate eosinophil survival (79) and activate the production of IL-4 (80), which can stimulate ILC2s and thereby mediate the cross talk between eosinophils and IL-5-producing ILC2s (81).

ILC2s and Basophils

Basophils were shown to promote ILC2 proliferation by secretion of IL-4 in lung and skin inflammation models (25, 82). Basophils are relatively short-lived circulating cells, which have specified effector functions in type 2 immunity such as protective functions against helminths and ticks as well as pro-inflammatory functions in response to allergens. Lung ILC2 activation and numbers are reduced in basophil-specific IL-4-deficient mice, indicating that the ILC2s respond to basophil-produced IL-4 (25, 83). Furthermore, clusters of basophils and ILC2s where shown to accumulate in a mouse model for atopic dermatitis where basophil-derived IL-4 was required for ILC2 accumulation and proliferation in inflamed skin (82). Whether basophil activity, survival, or tissue recruitment is regulated by ILC2-derived factors remains to be analyzed.

ILC2s and Mast Cells

Innate lymphoid cells have been found in proximity to tissue mast cells in human lung (84). Furthermore, dermal ILC2s and skin-resident mast cells have been reported to physically interact in contact dermatitis models in mice using intravital multiphoton microscopy, supporting the idea that ILC2s can directly communicate with mast cells (85). It was shown that ILC2s have the potential to dampen pro-inflammatory mast cell response through the production of IL-13, thereby reducing IL-6 and TNF-α production by mast cells (85).

The close proximity of ILC2s and mast cells could be caused by production of inflammatory mediators such as PGD2 by mast

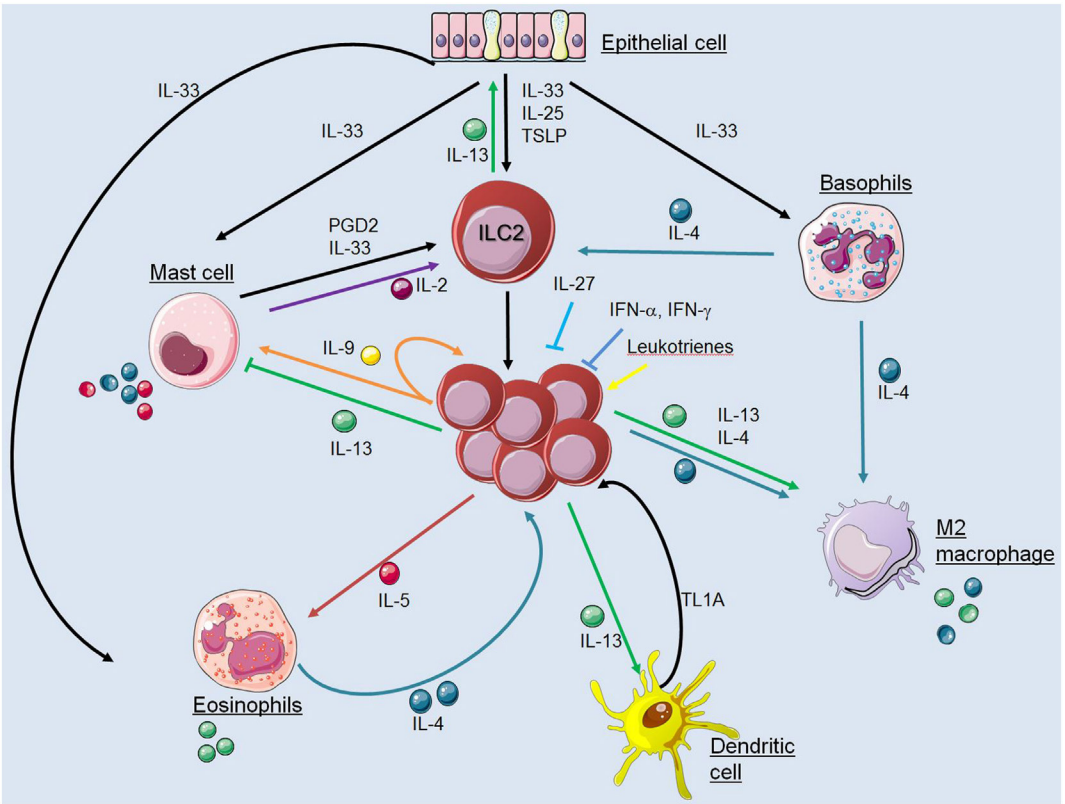

FIGURE 2 | Interactions between innate lymphoid cells (ILC2s) and cells of the innate immune system. The figure illustrates communication pathways between ILC2s and other cells of the innate immune system by secretion and recognition of soluble factors as described in the main text. Solid arrows indicate published evidence for activating mechanisms whereas ILC2-derived IL-13 was shown to suppress mast cells and IL-27 or interferons inhibit ILC2 functions. Cell symbols where taken from http://smart.servier.com website.

cells, which induces the chemotaxis of ILC2s through activation of cysteine-three-histidine protein 2 and promotes IL-13 production in a synergistic manner with airway epithelial cytokines IL-25 and IL-33 leading to tissue eosinophilia (84, 86–88). Moreover, mast cells can influence ILC2 activity indirectly by releasing non-caspase proteases chymase and tryptase that cleave IL-33 into a more bioactive isoform (89).

Mast cells can also release IL-33 upon antigen-specific IgE-mediated activation (90) and in response to extracellular ATP, which in turn activates ILC2s to produce IL-13 resulting in clearance of helminth infection (30). Conversely, recombinant IL-33 can directly activate mast cells to produce several cytokines including IL-4 and IL-5. IL-33-activated mast cells are known to produce IL-2, which was shown to indirectly limit ILC2 proliferation by the expansion of Treg cells and their production of IL-10 (91). On the other hand, mast cells also produce IL-2 when activated by IL-9 from IL-33-elicited ILC2s. This mast cell-derived IL-2 leads to expansion of pro-inflammatory CD25$^+$ ILC2s, which in turn activate Th9 cells leading to an amplified allergic inflammation (92). However, comparing *N. brasiliensis*-infected wild-type mice with IL-9 receptor-deficient mice showed similar mast cell accumulation in the lung arguing against a major role of ILC2-derived IL-9 for mastocytosis in this model (93).

CONCLUSION

Innate lymphoid cells are important "early sentinel" cells, which bridge the gap between the innate and adaptive type 2 immune response by sensing environmental changes and releasing immune-regulatory cytokines. A large variety of pathways that regulate the functions of ILC2s have been identified in the recent past and key interactions between ILC2s and cells of the innate and adaptive immune system were characterized (**Figure 2**). Apart from cross talk with various immune cell types, ILC2s may also have effects on structural cells including epithelial cells, smooth muscle cells, and fibroblasts. These interactions in mice and humans include communications by direct cell–cell contact and by secretion and recognition of soluble factors like cytokines, chemokines, hormones, and lipid mediators. However, regulation of ILC2 functions in various tissues during steady state conditions and upon infection with different pathogens becomes more and more complex and many pathways are still unknown. Further investigations will help to improve our understanding of how interactions between ILC2s and other immune cells are regulated. This information is essential to dissect the complexity of type 2 immune responses with the hope to identify critical checkpoints that are accessible for therapeutic interventions in allergic inflammation and immunity against helminths.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Group 3 Innate Lymphoid Cells: Communications Hubs of the Intestinal Immune System

David R. Withers[1] and Matthew R. Hepworth[2]**

[1] College of Medical and Dental Sciences, Institute of Immunology and Immunotherapy (III), University of Birmingham, Birmingham, United Kingdom, [2] Manchester Collaborative Centre for Inflammation Research (MCCIR), Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, School of Biological Sciences, Manchester Academic Health Science Centre, University of Manchester, Manchester, United Kingdom

The maintenance of mammalian health requires the generation of appropriate immune responses against a broad range of environmental and microbial challenges, which are continually encountered at barrier tissue sites including the skin, lung, and gastrointestinal tract. Dysregulated barrier immune responses result in inflammation, both locally and systemically in peripheral organs. Group 3 innate lymphoid cells (ILC3) are constitutively present at barrier sites and appear to be highly specialized in their ability to sense a range of environmental and host-derived signals. Under homeostatic conditions, ILC3 respond to local cues to maintain tissue homeostasis and restrict inflammatory responses. In contrast, perturbations in the tissue microenvironment resulting from disease, infection, or tissue damage can drive dysregulated pro-inflammatory ILC3 responses and contribute to immunopathology. The tone of the ILC3 response is dictated by a balance of "exogenous" signals, such as dietary metabolites and commensal microbes, and "endogenous" host-derived signals from stromal cells, immune cells, and the nervous system. ILC3 must therefore have the capacity to simultaneously integrate a wide array of complex and dynamic inputs in order to regulate barrier function and tissue health. In this review, we discuss the concept of ILC3 as a "communications hub" in the intestinal tract and associated lymphoid tissues and address the variety of signals, derived from multiple biological systems, which are interpreted by ILC3 to modulate the release of downstream effector molecules and regulate cell–cell crosstalk. Successful integration of environmental cues by ILC3 and downstream propagation to the broader immune system is required to maintain a tolerogenic and anti-inflammatory tone and reinforce barrier function, whereas dysregulation of ILC3 responses can contribute to the onset or progression of clinically relevant chronic inflammatory diseases.

Keywords: innate lymphoid cells, group 3 innate lymphoid cell, intestinal inflammation, interleukin-22, inflammatory bowel disease

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Cyril Seillet, Walter and Eliza Hall Institute of Medical Research, Australia
Arthur Mortha, University of Toronto, Canada

****Correspondence:***
David R. Withers d.withers@bham.ac.uk; Matthew R. Hepworth matthew.hepworth@manchester.ac.uk

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BACKGROUND

Innate lymphoid cells (ILCs) constitute a family of tissue-resident innate lymphocytes with increasingly appreciated roles in tissue homeostasis, immunity, and inflammation (1–5). A rapidly developing body of evidence derived from mouse and human studies has begun to demonstrate how ILCs play critical, non-redundant roles in maintaining tissue health or in driving disease pathology.

ILCs possess several characteristics that make them particularly suited to rapidly respond to perturbations in tissue homeostasis, infection, or tissue damage including (i) constitutive presence in barrier tissues and lymphoid organs, (ii) a "poised" transcriptional and epigenetic landscape, and (iii) the ability to respond rapidly and robustly to signals in the tissue microenvironment.

As reviewed extensively elsewhere (1–5), ILCs can be subdivided into groups on the basis of transcription factor expression and cytokine secretion profile. In this review, we will focus on group 3 innate lymphoid cells (ILC3), which are characterized by the expression of the transcription factor retinoic acid (RA)-related orphan receptor γ isoform t (RORγt) and the capacity to produce the cytokines interleukin (IL)-17A, IL-17F, IL-22, and GM-CSF. ILC3 differ from other ILC groups in that they constitute at least two bona fide subsets that are transcriptionally, developmentally, and functionally distinct and inhabit distinct tissue microenvironments [reviewed in Ref. (6)]. In mice, these subsets are distinguished by surface expression of natural cytotoxity receptors (NCR), particularly NKp46 (termed NCR$^+$ ILC3), and molecules and receptors historically associated with fetal lymphoid tissue inducer (LTi) cells, such as lymphotoxin and the chemokine receptor CCR6 (termed LTi-like ILC3) (5, 6). ILC3 are found in a range of tissues and organs, most notably the gastrointestinal tract and associated lymphoid tissues, although the relative distribution of ILC3 subsets differs dependent upon tissue location. Indeed, while NCR$^+$ ILC3 are the most prevalent ILC3 subset in the small intestine, LTi-like ILC3 appear to dominate in the colon and lymphoid tissues. Importantly, emerging evidence suggests that these two subsets also play distinct functional roles that relate to tissue-specific biological challenges.

Tissue-resident ILC3 sense and respond to a wide range of environmental and host-derived signals within the local microenvironment and integrate these cues to modulate cell-intrinsic transcription and to relay information to other cells, either through the production of cytokines or through cell–cell interactions. Indeed, as discussed below, ILC3 concurrently sense a multitude of soluble signals and environmental cues, which may change dynamically following infection or tissue damage, thus posing the question as to how ILC3 integrate and interpret these signals to respond appropriately. The balance of ILC3 effector functions may set the immunological tone of the tissue and help orchestrate the wider immune response. Although other ILC subsets also share the capacity to respond to a wide range of cues within the tissue microenvironment [reviewed in detail elsewhere—(4, 5, 7)], here, we will discuss the concept of ILC3 as tissue-resident sentinels and key "communications hubs" of the intestinal immune response.

COMMUNICATING WITH THE OUTSIDE WORLD: ILC3 AS EARLY COLONIZERS AND SENTINELS OF BARRIER TISSUES

ILC3 are derived from fetal liver progenitors during embryogenesis and are among the first lymphocytes to seed barrier tissues, in particular the intestinal tract, prior to birth (8, 9). In this context, ILC3 are among the first-responders to colonization by commensal microbes, as well as diet-derived antigens introduced following weaning. Furthermore, these cells are central organizers of secondary lymphoid tissue organogenesis (10). ILC3 are therefore in prime position to shape the emerging mucosal immune system. Developmentally both ILC3 subsets derive from a common lymphoid progenitor ancestor; however, recent evidence has highlighted a divergent developmental relationship between ILC3 subsets and other ILC family members. The development of the wider ILC family is mediated through a series of transcriptional decisions that generate distinct progenitor populations with increasing commitment toward the ILC lineage, as reviewed extensively elsewhere (3, 11, 12). A key stage in this development is the bifurcation of the "cytotoxic" ILC lineage (i.e., classical NK cells), from the remaining "helper" ILC lineage (ILC1, ILC2, and ILC3). This is characterized by the development of a common helper ILC progenitor (CHILP) that expresses high levels of the transcription factor ID2 and IL-7R and which is able to generate ILC1, ILC2, and both ILC3 subsets but has lost the potential to generate progeny from other lymphocyte lineages (13). Additionally, a subset of ID2$^+$ progenitors develops downstream of the CHILP, characterized by the expression of the transcription factor PLZF and surface PD-1—termed the common ILC precursor (ILCp) (14, 15). However, while ILCp can give rise to ILC1, ILC2, and NCR$^+$ ILC3, they are unable to generate LTi-like ILC3 progeny (14), suggesting that a bifurcation of ILC3 subset development occurs during the progression from CHILP to ILCp. In line with these findings, LTi-like ILC3 have been demonstrated to derive from an ID2$^+$ $\alpha_4\beta_7^+$ CXCR5$^+$ progenitor cell that diverges upstream of ILCp, prior to the acquisition of PLZF (16–18). As such LTi-like ILC3 develop *via* a distinct route from the remaining ILC family members, including NCR$^+$ ILC3, which may underlie key transcriptional and functional differences recently highlighted between ILC3 subsets (6, 19–21). Moreover, recent studies have uncovered plasticity among ILC3 and other ILC populations that is dictated by changes in the cytokine milieu within the tissue microenvironment (22–24). Thus, in addition to transcriptional decisions made during development, plasticity of mature ILC3 may shape the composition of these cells in tissues.

Seeding of intestinal tissues and associated lymphoid structures by ILC3 occurs during embryogenesis and is further regulated by environmental signals encountered following birth. Seminal studies demonstrated LTi-like ILC3 are present in the fetal gut, whereas NKp46$^+$ ILC3 are largely absent but proliferate rapidly following birth to become the dominant ILC3 subset in the small intestine (8). ILC3 subset maturation and migration to the gut is in part dictated by maternal, microbial and dietary signals (**Figure 1**; Inputs). The extent to which ILC3 can directly sense microbial-derived cues remains poorly understood. Indeed, unlike many myeloid cell populations, ILC3 do not appear to express toll-like receptors or other canonical pattern recognition receptors. Rather ILC3 responses to microbial cues are dependent upon other key intermediaries, such as resident mononuclear phagocyte (MNP) populations that convey information to ILC3 (detailed below). ILC3 are particularly sensitive to alterations in the microbiota and their numbers are modulated following neonatal colonization by commensal microbes, in part

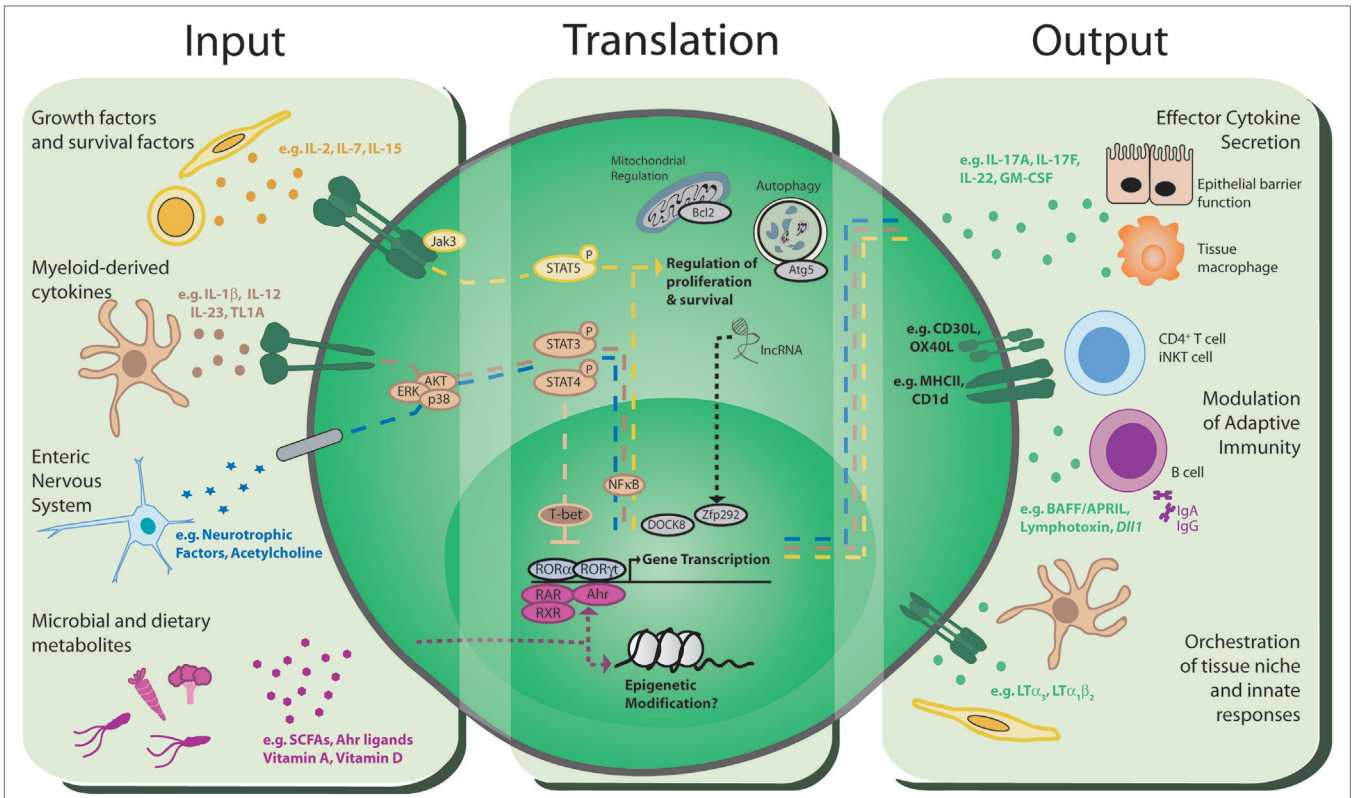

FIGURE 1 | Group 3 innate lymphoid cells (ILC3): communications hubs of the intestinal immune system. *Input*: ILC3 have the capacity to receive environmental cues and host-derived signals from a range of sources including the immune system, stroma, the enteric nervous system, the microbiota, and the diet. *Translation*: these signals are integrated through a range of intracellular signaling mechanisms, including phosphorylation of STAT proteins, ligand-binding transcription factors, epigenetic modification, and multiple intracellular processes with the potential to support long-term survival of ILC3. Integration of multiple signals likely happens simultaneously, indicating a complicated and dynamic process that determines the threshold, magnitude, and type of ILC3 responses that are propagated to communicate with other cells in the intestinal environment. *Output*: translated signals are conveyed to other cells within the local environment through a variety of distinct mechanisms, most notably *via* the production of effector cytokines and growth factors that modulate and orchestrate the wider immune response and intestinal barrier function. The nature and balance of the signals perceived by ILC3 ultimately determine the downstream effector response, and dysregulation of homeostatic signals in disease may dramatically alter ILC3 responses and drive pro-inflammatory phenotypes. Finally, direct cell–cell communication through antigen presentation and co-stimulatory molecules can directly regulate the adaptive immune response.

through an IL-25-dependent negative feedback mechanism that restricts expansion of ILC3 in a microbiota-dependent manner (9). Recent studies suggest that ILC3 are directly regulated by bacterial metabolites, such as short-chain fatty acids (SCFAs) produced through microbial metabolism of dietary fiber. Consistent with this, NCR$^+$ ILC3 present in the Peyer's patches (PP) of the ileum were found to express *Gpr109a*—the receptor for butyrate—and SCFA signaling acted to restrict NCR$^+$ ILC3 numbers and cytokine production (25). Additionally, NCR$^+$ ILC3 are dependent upon the aryl hydrocarbon receptor (Ahr) for their development and persistence in the small intestine (26–29). Ahr ligands can be derived from intestinal bacteria, as well as dietary and endogenous sources and further regulate cytokine production of mature adult ILC3, while immunoglobulin-bound Ahr ligands are also transferred from the mother to promote the migration of NCR$^+$ ILC3 to the neonatal small intestine (30).

In addition to microbial metabolites, ILC3 are subjected to further direct modulation by dietary cues. Maternal retinoids transferred during embryogenesis favor the development and maturation of LTi-like ILC3 by promoting and stabilizing RORγt expression (31), while dietary vitamin A-derived RA is required for the maintenance of adult small intestinal ILC3 subsets (32–34). In support of a role for dietary vitamins in the regulation of ILC3 responses, human ILC3 were found to up-regulate vitamin D receptor expression following cytokine stimulation and vitamin D treatment subsequently resulted in suppression of the IL-23R, implicating vitamin D as a regulator of ILC3 function (35). In line with this vitamin D receptor-deficient mice exhibit elevated ILC3 numbers and IL-22 production (36). Taken together, these studies implicate dietary vitamins as key modulators of ILC3 numbers and function and demonstrate the capacity of ILC3 to directly sense dietary signals.

SETTING THE TONE: HOMEOSTATIC INPUTS MAINTAIN ADULT ILC3 SUBSETS

In addition to environmental cues derived from the microbiota and diet, the numbers and relative balance of ILC3 subsets at steady state are additionally regulated by host-derived inputs

(**Figure 1**; Inputs). In contrast to the dynamic changes observed within the neonatal intestine, adult ILC3 populations appear to be stable and long-lived with little to no replenishment from the bone marrow under homeostatic conditions (37). Current understanding suggests that ILC3 are maintained through self-renewal and/or replenished from tissue-resident precursors and several groups have reported the presence of tissue-resident RORγt$^+$ progenitors that can give rise to mature ILC3 subsets in both mice and humans (38, 39). In particular, intestinal resident NKp46$^-$CCR6$^-$RORγt$^+$ ILC3 precursors give rise to NCR$^+$ ILC3, and to a lesser extent the LTi-like ILC3 compartment, a process that is regulated by local tissue cytokines and Notch signaling (40–44). Adult ILC3 subsets differ in their tissue localization; CCR6$^+$ ILC3 are largely restricted to lymphoid structures such as the mesenteric lymph node and cryptopatches and ILFs within the small intestine and colon (45, 46), while NCR$^+$ ILC3 are largely excluded from lymph nodes and colon but reside within both the lamina propria and ILFs of the small intestine, with localization of NCR$^+$ ILC3 dictated in part through CXCR6 expression (47–51). Thus, local signals perceived by ILC3 subsets likely dictate their maintenance and survival within specific tissue microenvironments.

While the cell-extrinsic and cell-intrinsic signals that support ILC3 under homeostatic conditions remain incompletely defined, recent evidence suggests that ILC3 subsets are differentially dependent on constitutive signals received *via* pro-survival cytokines for their maintenance. ILC3 constitutively express high levels of common γ chain cytokine family receptors including IL-2R, IL-7R, and IL-15R. Of these, the IL-7:IL-7R interaction has been most extensively studied and IL-7 deficient mice have reduced numbers of lymph nodes, consistent with a loss of LTi cells in the embryo, and decreased ILC3 numbers in the adult (40, 52–54). Furthermore, enhanced signaling through IL-7Rα increases LTi-like cell numbers in the adult (53), arguing that IL-7 has the capacity to directly regulate ILC3 numbers. Notably overexpression of TSLP, which signals through a heterodimer of TSLPR and IL-7R, could overcome the effect of IL-7 deficiency, further arguing that other signals can compensate (55). A more recent study demonstrated that residual numbers of all ILC groups persist in the absence of IL-7, with IL-15 necessary to support the survival of the remaining NCR$^+$ ILC3, but not LTi-like ILC3, in the intestinal tract (56). Provision of survival signals such as IL-7, IL-15, and TSLP is mediated largely through critical interactions with non-hematopoietic, stromal cell populations (**Figure 1**; Inputs). Studies of IL-7 reporter mice identified marginal reticular cells as a key source of this cytokine in lymph nodes (57), although analysis of IL-7 mRNA indicates that fibroblastic reticular cells (FRCs) also produce IL-7 (58). Strikingly, production of IL-7 by stromal cells requires signaling through the lymphotoxin β-receptor (59), indicating that continued interactions between stroma and lymphotoxin expressing immune cells support normal FRC function, which in turn maintains ILC3. Similarly, FRCs in lymph nodes and PP are a key source of IL-15, which supports NCR$^+$ ILC3 as well as NK cells and ILC1 (56, 60). Thus, the stromal infrastructure of tissue microenvironments provides much of the survival requirements of ILC3. However, there is evidence that some LTi-like ILC3 persist in the absence of both of IL-7 and IL-15 (56), arguing for a role for other cytokine signals or indicating that alternative mechanisms of survival exist. Given that LTi-like ILC3 also highly express CD25 (IL-2R) and are enriched in lymphoid structures in close proximity to proliferating T cells (61, 62), it is possible that IL-2 may play an additional role in the long-term maintenance of LTi-like ILC3. Together these findings suggest that stromal cells form critical niches within secondary and tertiary lymphoid tissues that provide signals required to support ILC3 survival.

TALKING TO YOURSELF: DIVERSE HOST-DERIVED SIGNALS REGULATE ILC3 EFFECTOR RESPONSES

As addressed earlier, ILC3 have the capacity to receive input from cells and soluble molecules within their local tissue microenvironment (**Figure 1**; Inputs). In addition to stromal cells, other innate immune cells, such as MNPs, play key roles in both homeostatic and effector ILC3 responses (63, 64). Within the intestine it is clear that IL-23, produced by CX$_3$CR1$^+$ MNPs, is a key regulator of ILC3 function (65–67). CX$_3$CR1$^+$ MNPs were observed to cluster with ILC3 in distinct intestinal lymphoid tissues, such as cryptopatches. Following weaning sensing of the microbiota by intestinal lymphoid tissue, MNPs result in local production of IL-23 and the induction of IL-22 from lymphoid tissue-resident LTi-like ILC3 (46). Moreover, depletion of CX$_3$CR1$^+$ MNPs results in a failure to control *Citrobacter rodentium* infection due to impaired IL-22 production by ILC3. In addition to IL-23, IL-1β drives IL-22 production by ILC3 and this is further augmented by TL1A, again produced by CX$_3$CR1$^+$ MNPs (67). The balance of cytokine signals perceived by ILC3 is also critical in determining phenotype and function. Indeed, intestinal ILC3 exhibit significant plasticity and signals including IL-12, IL-15, and IL-18 released in the context of infection and inflammation promote the progressive up-regulation of T-bet and production of pro-inflammatory cytokines such as IFN-γ and TNF-α by NCR$^+$ ILC3 and a subsequent loss of RORγt expression by this subset, resulting in these cells being labeled "ex-ILC3" (40, 41). Conversely, upon resolution of infection or inflammation, restoration of homeostatic levels of MNP-derived signals including IL-23 and IL-1β favors the reconversion of inflammatory "ex-ILC3" back to an RORγt$^+$ NCR$^+$ ILC3 phenotype (43, 68).

ILC3 may also respond to other fundamental innate immune factors, such as the complement system. A subset of ILC3 appear to be sensitive to the complement cascade *via* expression of the C3aR (69), while Complement Factor P—a positive regulator of the alternative complement pathway—was found to directly bind NKp46 (70), suggesting NCR$^+$ ILC3 in particular may sense pathogen infection *via* interactions with the complement cascade. Beyond interactions with the immune system, an increasing body of evidence indicates intestinal resident ILC3 can directly sense cues from the enteric nervous system. In a series of studies, LTi-like ILC3 were demonstrated to express RET—a receptor for neurotrophic factors (71, 72). RET expression is

required for PP formation and accumulation of lymphotoxin-producing LTi cells, although this occurs indirectly *via* ligation of RET on lymphoid tissue-initiator cells and induction of chemokine production to sequester immune cells, including LTi cells, to drive PP formation. Nonetheless, adult CCR6$^+$ LTi-like ILC3 express RET and can directly respond to glial-derived neurotrophic factor (GDNF) family ligands. Production of GDNF by intestinal glial cells in response to stimulation by microbial ligands acts to reinforce intestinal barrier function *via* regulation of IL-22 transcription and secretion by LTi-like ILC3 (72). Interestingly, enteric neurons also have the capacity to provide RA signals, which are critical for the maturation of LTi cells (73), thus suggesting that alternative, non-dietary sources of RA may also play roles in ILC function and immune homeostasis. In addition to local neuronal signals, systemic nervous signals may modulate the tone and magnitude of ILC3 responses. Vagal nerve innervation of the colon is required for the formation of tertiary lymphoid structures, *via* regulation of local chemokine production (74). Although a direct effect of vagal denervation on ILC3 was not demonstrated in this study, more recent evidence suggests that the vagal nerve acts to regulate ILC3 responses to bacterial pathogens in the peritoneal cavity (75). This effect was in part dependent upon the ability of NCR$^+$ ILC3 to enzymatically generate lipid precursors that were in turn metabolized by resident macrophages to promote resolution of inflammation (75). Together these findings suggest signals from both tissue-resident and systemic neurons may directly regulate ILC3 numbers and function during homeostasis or following infection. Interestingly, ILC3 may also play important roles in the central nervous system and a recent study demonstrated that NCR$^+$ ILC3 are present in the meninges and promote neuroinflammation in a model of multple sclerosis by licensing entry of inflammatory Th17 cells into the brain (76). It is highly likely that other signals from the nervous system and beyond may impact upon ILC3 function. For example, host-derived lipid mediators—such as prostaglandin E_2—have been demonstrated to directly promote homeostatic ILC3 cytokine production (77). Similarly, endocrine signals, particularly sex hormones, are long appreciated to regulate innate immunity. In this regard testosterone directly inhibits ILC2 responses (78); however, the capacity of androgens, estrogens, or other hormones to modulate ILC3 is yet to be investigated.

TRANSLATING THE MESSAGE: INTEGRATION AND TRANSCRIPTIONAL DYNAMICS

The ability of tissue ILC3 to sense a breadth of exogenous and endogenous cues of significantly varying natures provokes the question as to how these various inputs are simultaneously integrated, and prioritized, in order to control ILC3 function. While significant advances have been made in understanding the effector functions of ILC3, as well as the transcriptional decisions that determine ILC development, relatively little is known about the signaling pathways and transcriptional dynamics that act to integrate external cues and determine the nature and magnitude of downstream ILC3 responses. Advances in RNA sequencing and epigenetic profiling have begun to redress this balance and allowed for enhanced resolution of individual cellular states among ILC populations (18–21, 79, 80). Furthermore, comparison of the transcriptional networks of ILC3 subsets and their Th17 counterparts will likely prove useful in identifying common and distinct signaling pathways utilized by RORγt-expressing cells (81). While significant overlap may be expected in this case, it is critical to note that ILC3 and Th17 cells also demonstrate key difference in their transcriptional regulation. For example, while Th17 is acutely dependent on RORγt for the maintenance of a mature functional phenotype, ILC3 were able to maintain core effector functions and phenotype following deletion of RORγt (82). Surprisingly, human IL-22-producing ILC3 can be generated from circulating ILCps even when derived from *Rorc*-deficient donors (83). Thus, it is likely that ILC3 also exhibit unique and differing signaling transduction pathways and transcriptional regulation that underlie their innate functions (**Figure 1**; Translation). As detailed earlier, ILC3 transcription dynamics are acutely modified by dietary and bacterial metabolites *via* Ahr and it is likely epigenetic changes may be imprinted *via* histone deacetylases downstream of SCFA-sensing, as in other lymphocytes (84).

ILC3 additionally integrate multiple soluble signals including common gamma chain cytokines and growth factors—many of which induce signal transduction *via* phosphorylation of STAT5 (85). Thus, pSTAT5 and downstream signal activation (ERK/AKT) are likely to play key roles in orchestrating intracellular responses in ILC3. Similarly, both IL-23 and RET signals are transduced in part by pSTAT3 to regulate IL-22 production (72, 86). Thus, it is likely that a threshold of intracellular signaling downstream of multiple receptors, sensing cues from multiple biological systems, acts to establish the tone and magnitude of the ILC3 response (**Figure 1**; Translation). The signaling pathway engaged following stimulation by cytokines or other cues is likely to determine the biological processes that are modulated. For example, ILC3 from mice with mutations in *Jak* signaling exhibited an impaired ability to phosphorylate STAT5 in response to IL-7, while inhibiting *Jak3* signaling in mature human ILC3 suppressed proliferation of these cells but not cytokine production (87).

Maintenance of ILC3 subsets is likely to be regulated transcriptionally *via* multiple mechanisms (**Figure 1**; Translation). Mice lacking the scaffolding protein dedicator of cytokinesis 8 (DOCK8) demonstrate reduced ILC3 numbers and defective immunity to *C. rodentium*, in part due to a reduced ability of DOCK8-deficient ILC3 to respond to IL-7 and IL-23 and an increased rate of apoptosis (88). Similarly, survival of ILC3 is modulated *via* long non-coding RNAs that orchestrate downstream gene accessibility (89). In particular, *lncKdm2b* expression by ILC3 controls activation of transcription factors, including *Zfp292*, and recruitment of chromatin organizational machinery that promote ILC3 maintenance in vivo (89). ILC3 survival may also be dependent upon expression of anti-apoptotic machinery. Indeed, LTi-like ILC3 highly express the anti-apoptotic molecule Bcl-2, expression of which fluctuates following perturbation of local cytokine signals such as IL-7 (56). In addition, regulation of intracellular organelle degradation through autophagy has

been implicated in ILC survival (90). The autophagy related factor *Atg5* was found to be required for ILC family development, suggesting that autophagy may play a role in ILC3 persistence and maintenance (90). Nonetheless, and despite these advances, many of the precise mechanisms through which ILC3 maintenance is instructed *via* changes in gene expression or epigenetics and the environmental signals that are required to promote ILC3 survival are yet to be defined.

PASS IT ON: ILC3 ORCHESTRATION OF INTESTINAL TISSUE IMMUNE RESPONSES

ILC3 possess multiple mechanisms through which they relay host and environmental signals to orchestrate the intestinal immune response and to maintain tissue function (**Figure 1**; Outputs). ILC3 were initially identified as potent sources of effector cytokines, most notably IL-22. ILC3-derived IL-22 plays critical roles in regulating host-commensal bacteria interactions in the healthy intestine in addition to mediating responses to enteric pathogen infections (27, 86, 91–98). IL-22 mediates its effects on IL-22R-expressing non-hematopoietic cells, including intestinal epithelial cells, epithelia-associated stem cells, and Paneth cells (99–101), and promotes barrier function and segregation of commensal bacteria from the underlying immune system *via* induction of epithelial tight junction proteins (97, 102), fucosylation of epithelial cell-associated glycans (103–105), secretion of mucin, and production of anti-microbial peptides (e.g., S100A8/A9 and RegIIIβ/γ), which together induce bacterial killing and prevent translocation of commensal organisms into the circulation and peripheral organs (102).

Although the importance of ILC3 in regulating intestinal health and inflammation has largely been ascribed to the production of IL-22, ILC3 produce several other cytokines that contribute to intestinal immune responses. In particular, ILC3 subsets have the capacity to produce IL-17A and IL-17F (2). Although both embryonic LTi and adult ILC3 isolated from the small intestine can be induced to secrete IL-17A upon ex vivo stimulation (9), fate mapping of IL-17A producing cells revealed that only a small proportion of ILC3 in the intestine exhibit a history of IL-17A expression (106). Nonetheless, ILC3-derived IL-17A may contribute to the formation of pulmonary tertiary lymphoid structures following infection and inflammation (107), can contribute to host immunity to fungal and bacterial pathogens (63, 108, 109), and has been implicated in the pathogenesis of obesity-associated airway hyper reactivity (110). Additionally, dysregulated IL-17A production by ILC3 may act to exacerbate inflammation and disease pathology in a range of inflammatory diseases, including psoriasis and IBD (65, 111, 112). IL-17F has high homology to IL-17A and can be secreted as either a homodimer or heterodimer with IL-17A (113). ILC3 are a dominant source of IL-17F after induction of colitis in *Rag*1$^{-/-}$ mice (65, 114), upon oral infection with *Candida albicans* and following skin wounding (108, 115), suggesting that IL-17F production by ILC3 may play critical roles in inflammation and immunity or resolving immune responses. Although IL-17F has largely been ascribed pro-inflammatory roles and may play pathogenic roles in colitis models (116, 117), it can also synergize with IL-22 to enhance production of anti-microbial peptides (118). Despite this evidence, the exact role of ILC3-derived IL-17F and its mechanisms of action in infection and disease, and how its effects differ from those of IL-17A, remain incompletely understood.

Recent studies have additionally highlighted ILC3 as a potent source of the cytokine and growth factor GM-CSF (67, 119–121). GM-CSF modulates myelopoiesis in the bone marrow, as well as extramedullary hematopoiesis in tissues, and acts on mature peripheral myeloid cells including monocytes, macrophages and neutrophils by regulating their activation, maturation, and migration into tissues (122). ILC3 are the predominant source of GM-CSF at steady state in the intestinal tract, with both NCR^{+} and LTi-like ILC3 capable of GM-CSF secretion. Under homeostatic conditions ILC3-derived GM-CSF acts to maintain immune tolerance by regulating DC subsets that further promote regulatory T-cell populations (120). Thus, constitutive homeostatic ILC3-derived GM-CSF secretion acts to maintain a tolerogenic environment. The interplay between MNPs and ILC3 in the intestine is bidirectional. Indeed, microbiota-dependent signals, including IL-1β, IL-23, and TL1A, derived from intestinal MNPs act to potentiate GM-CSF production by ILC3 (67, 120), suggesting the potential for a regulatory feedback loop in the intestine regulated by ILC3-derived GM-CSF-dependent crosstalk with myeloid cells. This crosstalk may also be important in the context of intestinal inflammation and following perturbation of intestinal barrier function as MNP-derived cytokines were found to regulate ILC3 production of IL-22, in addition to GM-CSF, in mouse models of colitis and human IBD patients (67). In contrast to a tissue-protective role for ILC3-derived GM-CSF two independent studies demonstrated that ILC3-derived GM-CSF acted to exacerbate intestinal pathology in an innate cell driven model of colitis (anti-CD40 treatment of *Rag1*$^{-/-}$ mice), in part through recruitment of inflammatory monocytes (121, 123). Furthermore, onset of colitis results in migration of ILC3 out of intestinal cryptopatches and into the lamina propria in a GM-CSF-dependent manner (121), further demonstrating that GM-CSF-dependent ILC3-MNP crosstalk may dictate the migration and localization of immune populations within the intestinal tissue.

REGULATION OF ADAPTIVE IMMUNE RESPONSES

Embryonic LTi cells are required to generate secondary lymphoid tissues, and this role has been expertly reviewed before (10, 124) Further to this role in establishing the microenvironments that foster B- and T-cell responses, more recent studies have revealed that CCR6^{+} LTi-like ILC3 contribute to the regulation of adaptive immune responses *via* both indirect and direct interactions with the adaptive immune system (**Figure 1**; Outputs). CCR6^{+} ILC3 reside within the spleen, mucosal-associated lymphoid tissues, and lymph nodes—particularly those draining mucosal sites such as the mesenteric and mediastinal

lymph nodes (62). Phenotypically, adult LTi-like CCR6$^+$ ILC3 are very similar to the embryonic LTi population but additionally express molecules such as OX40L and CD30L that may foster interactions with lymphocytes (125–128). Interestingly, expression of OX40L can be induced in embryonic LTi cells through ex vivo culture with inflammatory cytokines, such as TL1A (129). Whether embryonic-derived LTi persist in the neonate and adult, and for how long, is unclear—but given the presence of long-lived LTi-like cells in the adult and the functional heterogeneity of ILC3 subsets, the potential persistence of bona fide embryonic LTi cells after birth needs to be addressed. It is striking that CCR6$^+$ ILC3 in adult PP, LNs, and spleen associate with stromal populations that closely resemble the embryonic "organizer" cells through which fetal LTi orchestrate lymphorganogenesis (57, 130). Thus, tissue microenvironments fostered early in the life in secondary lymphoid tissues by ILC3 may be maintained in the adult. In support of this, restoration of the splenic white pulp architecture after viral infection was delayed in the absence of ILC3 (131).

Direct regulation of CD4$^+$ T cells by ILC3 can be mediated through MHCII-dependent antigen presentation. ILC3-conditional deletion of MHCII resulted in moderate colitis due to a failure to control T-cell responses to commensal bacteria (61, 132). Thus, within the gastrointestinal tract, ILC3 appear to play a crucial suppressive role in regulating CD4 T-cell responses to commensal organisms to maintain tissue homeostasis. Mechanistically, ILC3 were found to control commensal bacteria-specific CD4$^+$ T-cell responses in part by outcompeting T cells for IL-2, thus starving that T cells of growth factors needed for proliferation and resulting in apoptosis (61). Similarly, ILC3 may regulate the T-cell pool by controlling the availability of other critical growth cytokines in lymphoid tissues, including IL-7 (133). Further investigations have indicated that the outcome of ILC3:T-cell interactions may be determined by the specific tissue environment, with splenic ILC3 found to support, rather than suppress, CD4 T-cell responses in an MHCII-dependent manner (134). This discrepancy could be partially explained by the ability of cytokines to modulate ILC3—for example, IL-1β can induce ILC3 expression of co-stimulatory molecules (e.g., CD80 and CD86) and alter the nature of ILC3 antigen presentation (134). In addition to the presentation of protein-derived antigenic peptides *via* MHCII, LTi-like ILC3 have the capacity to present lipid antigens to iNKT cells *via* surface CD1d (135). Furthermore, ILC3 are required to suppress homeostatic CD8$^+$ T-cell expansion in neonatal mice (136).

ILC3 also have the capacity to modulate humoral immunity (**Figure 1**; Outputs). ILC3 present in the spleen and PP support innate T-cell-independent IgA production through production of secreted and membrane bound lymphotoxin, which supports local DC populations and aids IgA class switching (137–139). Similarly, splenic ILC3 provide B-cell growth factors, including BAFF/APRIL and *Dll*1, to enhance local Ab production by marginal zone innate B cells (119). Despite these advances, the full extent and nature of the crosstalk between ILC3 and other lymphocyte populations, and how these signals are integrated alongside those provided by traditional antigen-presenting cell populations such as DC and B cells, remain to be fully elucidated.

LOST IN TRANSLATION: DYSREGULATED ILC3 COMMUNICATION AND DISEASE

Here, we have highlighted the roles of ILC3 in integrating signals from the environment and relaying information to surrounding immune and non-immune cells, thus functioning as a critical communications hub within intestinal tissue. Through being able to respond to both epithelial and myeloid-derived cytokines, vitamins, metabolites, and also neuropeptides, ILC3 integrate a wealth of regional cues to maintain the appropriate balance of key effector molecules and ensure local tissue homeostasis. Thus, while ILC3 have a clear protective role in the tissue, dysregulation or dramatic changes in environmental cues can result in disrupted ILC3 communication and may contribute to disease pathology, in part *via* altered ILC3 effector functions. For example, dysregulated cytokine production in the context of mouse models of colitis can promote ILC3 production of disease driving pro-inflammatory cytokines such as IFN-γ and IL-17A (40, 68, 111). Similarly, while ILC3-derived IL-22 is critical for supporting homeostatic intestinal barrier function, epithelial cell repair and regeneration, chronic overproduction of IL-22 by ILC3 may promote colorectal cancers (140, 141). Interestingly, genetic polymorphisms associated with chronic inflammatory disease or cancer may also alter the inflammatory milieu and have the potential to drive dysregulated ILC3 communication. For example, Card 9 deficiency results in disrupted IL-1β production and impacts upon epithelial cell proliferation and colitis-associated cancer due to perturbed ILC3-associated IL-22 production (142). It is likely that many other polymorphisms seen in patients with intestinal inflammatory disorders, including IL-23R, IL-10/IL-10R, *Atg16l1*, and *Nod2,* also impact upon ILC3 function either directly or by altering the integration of tissue-specific signals that are sensed, interpreted or propagated by ILC3. Dysregulation of protective ILC3 functions in intestinal disease is also not limited to effector cytokine production. Indeed, ILC3-intrinsic expression of MHCII has been observed to be reduced in two separate cohorts of Crohn's patients and found to correlate with enhanced Th17 responses in disease (61, 143), suggesting that the altered intestinal tissue environment may impact upon antigen presentation by these cells.

Infections may additionally disrupt ILC3 populations in the gut, resulting in a loss of their normal sentinel activity, the breakdown of gut barrier integrity, and intestinal pathology. In particular, infection with human or simian immunodeficiency viruses (HIV/SIV) results in depletion of ILC3 from the intestinal mucosa and lymph nodes (144–147). Thus, further investigation is needed to understand how alterations in ILC-related signals result in loss of these cells and how this balance can be redressed to restore homeostatic numbers and functions of ILC3. Finally, as the next generation of anti-inflammatory therapeutics enter the clinics, their relative impact on beneficial intestinal ILC3 need to be thoroughly addressed. In particular, monoclonal antibodies and small molecule inhibitors targeting common pathways shared by Th17 and ILC3 (e.g., anti-IL-23, anti-IL-12, anti-IL-17A, and small molecule antagonists of RORγt) have the potential to suppress inflammation but may have long-term consequences for patients by disrupting protective ILC3 pathways. In this

regard, an increased understanding of the differences and similarities between Th17 and ILC3 regulation is required to guide therapeutic interventions and treatment regimen. Promisingly, recent studies suggest that acute targeting of RORγt effectively reduces Th17-driven inflammation while leaving protective ILC3 responses intact (82), although chronic inhibition of this master transcription factor may eventually negatively impact upon ILC3 responses. Furthermore, ILC3-derived cytokines may have beneficial roles in maintaining epithelial barrier function and healthy host–microbe interactions, thus targeting that these cytokines and their receptors in the context of Th17-driven inflammation may result in undesirable consequences. Indeed, treatment with neutralizing monoclonal antibodies against IL-17A or its receptor has been reported to worsen disease and increase incidence of adverse effects in several IBD patient cohorts (148, 149), further highlighting the need to understand the potential repercussions of emerging therapeutics on ILC3. Future studies will lead to a further understanding of how these critical innate immune sentinels are regulated in order to harness their protective functions to maintain tissue health, while suppressing dysregulated responses that exacerbate disease.

AUTHOR CONTRIBUTIONS

This review was jointly written by both authors, who contributed equally.

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REVIEW
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Innate Lymphoid Cells in Intestinal Inflammation

Alessandra Geremia and Carolina V. Arancibia-Cárcamo*

Translational Gastroenterology Unit, Nuffield Department of Experimental Medicine, University of Oxford, Oxford, United Kingdom

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the intestine that encompasses Crohn's disease (CD) and ulcerative colitis. The cause of IBD is unknown, but the evidence suggests that an aberrant immune response toward the commensal bacterial flora is responsible for disease in genetically susceptible individuals. Results from animal models of colitis and human studies indicate a role for innate lymphoid cells (ILC) in the pathogenesis of chronic intestinal inflammation in IBD. ILC are a population of lymphocytes that are enriched at mucosal sites, where they play a protective role against pathogens including extracellular bacteria, helminthes, and viruses. ILC lack an antigen-specific receptor, but can respond to environmental stress signals contributing to the rapid orchestration of an early immune response. Several subsets of ILC reflecting functional characteristics of T helper subsets have been described. ILC1 express the transcription factor T-bet and are characterized by secretion of IFNγ, ILC2 are GATA3$^+$ and secrete IL5 and IL13 and ILC3 depend on expression of RORγt and secrete IL17 and IL22. However, ILC retain a degree of plasticity depending on exposure to cytokines and environmental factors. IL23 responsive ILC have been implicated in the pathogenesis of colitis in several innate murine models through the production of IL17, IFNγ, and GM-CSF. We have previously identified IL23 responsive ILC in the human intestine and found that they accumulate in the inflamed colon and small bowel of patients with CD. Other studies have confirmed accumulation of ILC in CD with increased frequencies of IFNγ-secreting ILC1 in both the intestinal lamina propria and the epithelium. Moreover, IL23 driven IL22 producing ILC have been shown to drive bacteria-induced colitis-associated cancer in mice. Interestingly, our data show increased ILC accumulation in patients with IBD and primary sclerosing cholangitis, who carry an increased risk of developing colorectal cancer. ILC may play an important amplifying role in IBD and IBD-associated cancer, through secretion of inflammatory cytokines and interaction with other immune and non-immune cells. Here, we will review the evidence indicating a role for ILC in the pathogenesis of chronic intestinal inflammation.

Keywords: innate lymphocytes, epithelial barrier integrity, mucosal immune response, microbial flora, cytokines, inflammatory bowel disease, cancer

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****Correspondence:***
Alessandra Geremia
alessandra.geremia@ndm.ox.ac.uk

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INTRODUCTION

Innate lymphoid cells (ILC) belong to a family of innate immune cells that share similarities with the phenotype and functions of T lymphocytes. They present classical lymphocyte morphology, but lack a rearranged antigen-specific receptor. ILC do not express T, B, or myeloid cell markers and, therefore, they are lineage negative (Lin$^-$), but express the IL2 receptor (CD25) and the IL7 receptor

(IL7R or CD127) (1). ILC also include cytotoxic NK cells and lymphoid tissue inducer (LTi) cells that contribute to lymph node organogenesis during embryonic development (2).

Innate lymphoid cells are crucial players mediating immunity against pathogens and maintenance of tissue homeostasis. They bridge the innate and adaptive immune systems, sense environmental changes, and are able to mediate these processes mainly by secreting and responding to a finely tuned cytokine crosstalk between various cell types. ILC are able to swiftly react to microbial and inflammatory challenges with cytokine production, limiting pathogen spread, and tissue injury. They are strategically located at sites where there is the highest exposure to the outside world and infections are more likely to first arise, such as the intestinal mucosa, the skin, and the lungs. They respond to environmental stress signals contributing to the rapid orchestration of an early immune response to pathogens, such as extracellular bacteria, helminthes, and viruses (**Figure 1**). However, recent evidence suggests that ILC are also involved in the pathogenesis of chronic inflammatory disorders, including allergic reactions, inflammatory bowel disease (IBD) and cancer (1).

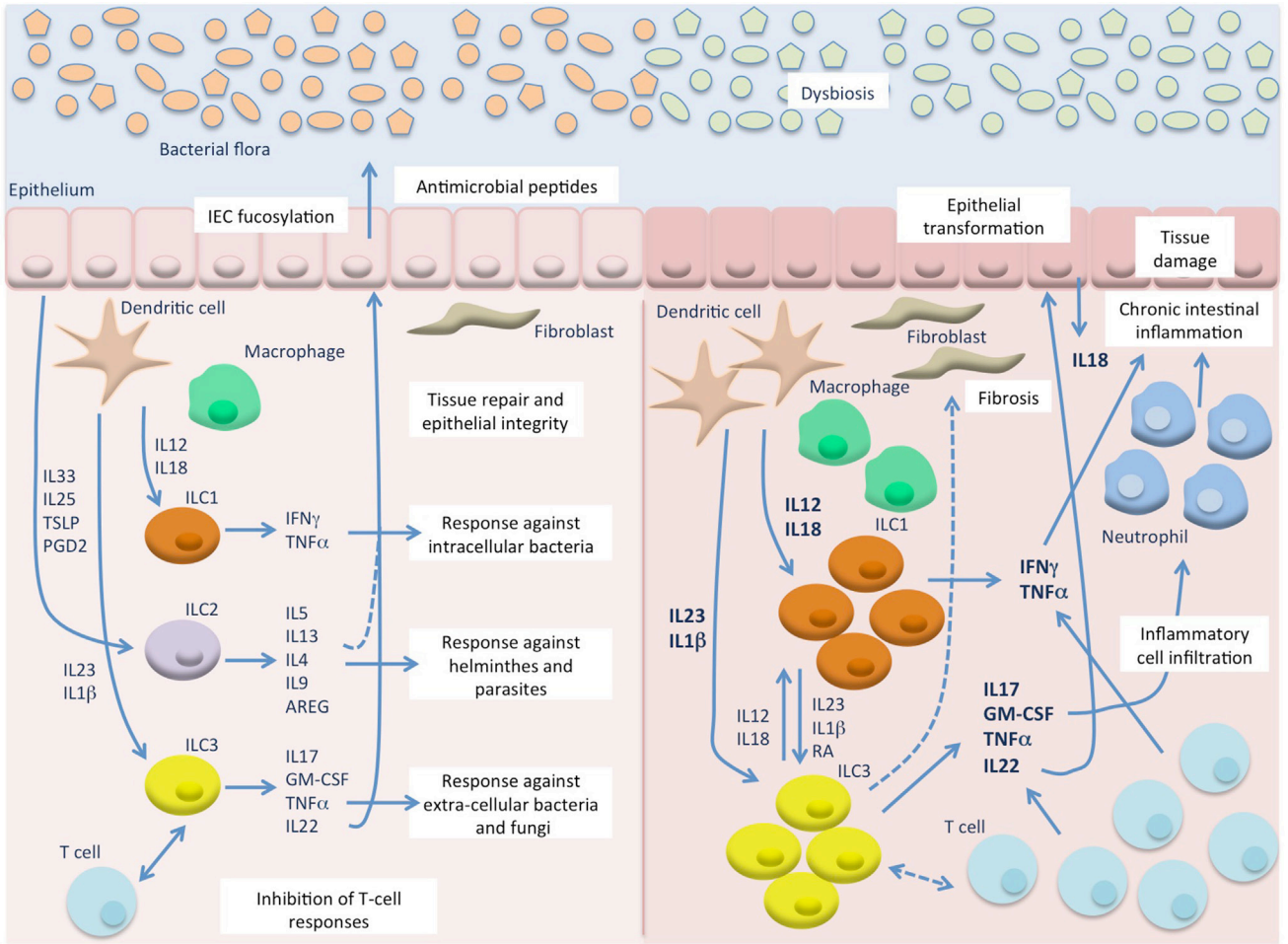

FIGURE 1 | Innate lymphoid cells (ILC) in intestinal homeostasis and chronic inflammation. In the healthy mucosa (**left**) ILC contribute to the maintenance of intestinal homeostasis through induction of protective immune responses against pathogens and promotion of tissue integrity. In response to microbial stimuli, innate cells, such as dendritic cells (DC) and macrophages, secrete IL12 and IL18 that stimulate ILC1 responses against intracellular pathogens, and IL23 and IL1β that induce ILC3 responses against extracellular bacteria and fungi. On the other hand, epithelial derived factors, such as IL33, IL25, TSLP, and prostaglandin D2 (PGD2), induce secretion of type 2 cytokines from ILC2 that contribute to the expulsion of helminthes. ILC3 and possibly ILC2 also promote epithelial integrity and tissue repair, particularly through IL22 production, and can inhibit T cell activation and proliferation. During chronic intestinal inflammation, such as Crohn's disease (CD) (**right**), in the presence of an altered bacterial flora, excessive levels of IL12, IL18, IL23, and IL1β are secreted by inflammatory DC and macrophages and lead to accumulation and activation of ILC1 and ILC3 that secrete high amount of type 1 and type 17 cytokines. These, also produced by accumulating T cells, induce chemotaxis of more inflammatory cells, such as neutrophils, and result in chronic inflammation and tissue damage. Besides the production of cytokines, ILC may also interact with immune (T cells) and non-immune cells (epithelial cells and fibroblasts) leading to secretion of more inflammatory mediators, such as IL18 from the epithelium, and to tissue reorganization (epithelial proliferation and fibrosis). A possible role for IL22 in epithelial transformation in colitis-associated cancer has also emerged from murine studies.

Although it has been reported that human ILC express toll-like receptors (3), they mostly respond to cytokines by making more cytokines. Unlike innate cells that respond directly to the pathogens and adaptive cells that respond through more specific antigen recognition, ILC act like messengers that in response to innate cell produced cytokines secrete more cytokines to orchestrate the local immune response. A small population, through an impressive per cell ability to release cytokines, can have an important amplifying role and the combination of different cytokines can lead to a dramatic shift in responses. ILC also go further by interacting with other cells, such as non-immune cells like epithelial and stromal cells, and may induce tissue reorganization. Moreover, they can influence T cell responses by promoting T helper (Th) cell differentiation and effector functions, have been shown to express MHCII and are able to present antigen to Th cells and interact with regulatory T cells (Tregs) (4–7) (**Figure 1**).

Innate lymphoid cells arise from the common lymphoid progenitor and their development is dependent on the transcription factors Id2, the common γ-chain, GATA3, and PLZF (8–10). Three main subsets of ILC have been described reflecting functional characteristics of the Th subsets (2). Hence, like Th1, ILC1 express the transcription factor T-bet and are characterized by secretion of IFNγ and TNFα and are involved in the immune response against intracellular pathogens. ILC2, mirroring Th2 cells, express GATA3, secrete IL4, IL5, IL9, and IL13 and are involved in protection against helminthes and in allergic reactions. Finally, ILC3 depend on expression of RORγt and secrete IL17, IL22, GM-CSF, and TNFα, resembling their Th17 counterpart, and participate in the response against extracellular pathogens at mucosal sites (**Figure 1**).

However, it is becoming apparent that, similarly to what is observed for Th subsets, ILC retain a degree of plasticity depending on exposure to environmental stimuli, such as cytokines or metabolites. It has been shown that ILC1 can differentiate into ILC3 in presence of IL1β and IL23, an effect that is enhanced by the vitamin A metabolite retinoic acid (RA) (11), and to ILC2 in the presence of IL4 (12). On the other hand, ILC3 when exposed to IL12, IL18, and/or IL1β up-regulate T-bet and can therefore express IFNγ, while repressing RORγt and the secretion of IL17 and IL22 (13). In mice, a gradient of expression of RORγt and T-bet by ILC3 is associated with functional plasticity (14). In particular, NKp46$^-$ ILC3 can develop into NKp46$^+$ "ex-ILC3" through induction of T-bet and repression of RORγt. The "ex-ILC3" down-regulate the IL23 receptor, up-regulate the IL12 receptor, and mainly produce IFNγ in response to IL12. Similarly ILC2 can also trans-differentiate into ILC1 after exposure to IL12 and IL18 (12, 15).

Recent studies have particularly challenged the existence of a defined ILC1 subset, distinct from conventional NK cells, and other ILC subsets. Simoni *et al.* did not find human ILC1 in various healthy and disease-derived tissues using CyTOF mass-cytometry to assess the expression of an extensive range of cell surface and intracellular parameters. The t-SNE analysis showed clear clustering of ILC2, ILC3, and conventional NK cells, but was unable to identify a distinct subset of ILC1 that appeared instead to be scattered throughout other clusters (16). Furthermore, transcriptomic analysis of murine ILC and NK cell populations has shown overlapping profiles for ILC1 cells, NKp46$^+$ "ex-ILC3" cells, and NK cells (17). On the other hand, in a human study using single cell RNA sequencing of tonsil derived CD127$^+$ ILC and NK cells, unbiased clustering of cellular transcriptomes revealed four distinct cell populations, corresponding to ILC1, ILC2, ILC3, and NK cells (18). These observations suggest that a dogmatic classification of ILC in rigidly defined subtypes is likely to represent an over-simplification of the real scenario. It appears more plausible to conceive that ILC, like their T cell counterpart, can exert flexible and adaptable functional properties in response to the evolving environment at mucosal sites.

ILC are key contributors to the already complex immune system playing a critical role in health and disease. In this review, we focus on the role of non-cytotoxic or helper-like ILC (called ILC from now on for simplicity) in intestinal homeostasis and inflammation.

ILC IN INTESTINAL HOMEOSTASIS

The maintenance and regulation of the intestinal microenvironment to keep a stable and functional healthy gut are not a small task and involve constant tweaking of physiological, biochemical, and immune pathways. The intestinal environment has been described as the Wild West of the immune system and represents the epitome of the complexity of immune organization. In this environment, several factors, such as commensal microbes, foods, and metabolites, can modify the immunological balance of the region and the gut needs to distinguish unharmful from pernicious agents. The process of homeostasis in the intestine is not a passive process, but it actually involves several active mechanisms on the part of local cells especially from ILC.

The distribution of ILC varies across the different parts of the human gastrointestinal (GI) tract with a progressive increase of total ILC from the proximal to the distal intestine (19). Moreover, distinct subsets are represented at different sites. Indeed, ILC1 are enriched in the upper GI tract, while ILC2 are only present at very low frequencies in the entire intestine, and ILC3 increase toward the colon and this correlates with higher distal expression of IL7. Interestingly, the functional properties of these cells were also found to differ across different parts of the intestine, with highest secretion of IL22 being observed in the ileum and in the esophagus (19).

ILC play a role in the maintenance of intestinal homeostasis by contributing to the protective immune response toward intestinal pathogens, as it has emerged by murine studies. In particular, T-bet expressing ILC participate in the defense against *Helicobacter typhlonius*, a commensal in the murine microbiota that closely resembles *Helicobacter pylori,* the frequent colonizer of the human stomach associated with gastritis, peptic ulcer, and gastric cancer. Mice lacking T-bet in the innate immune compartment develop colitis spontaneously triggered by *H. typhlonius* through induction of TNFα secretion by dendritic cells (DC). In turn, TNFα synergizes with IL23 to induce IL17 secretion by ILC in this animal model (20). These findings are particularly interesting in light of the high representation of ILC1 in the human upper GI tract where they may be involved in the inflammatory and

pro-carcinogenic responses induced by *H. pylori*. ILC1 were also shown to contribute to protection against intracellular pathogens, such as *Salmonella enterica*. During *Salmonella* infection T-bet expressing ILC (including ILC1 and "ex-ILC3") are the main source of IFNγ, which drives the secretion of mucus-forming glycoproteins required to protect the epithelial barrier (14). Similarly, ILC1 are the main producers of both IFNγ and TNFα during *Toxoplasma gondii* infection and T-bet deficient mice fail to control parasite replication (21).

On the other hand, ILC2 were found to play an important role in mounting protective innate responses against parasites and helminthes through induction of eosinophilia and goblet cell hyperplasia. In particular, IL25 and IL33 responsive ILC2 are critical for *Nippostrongylus brasiliensis* expulsion in mice that lack adaptive immune cells (6, 22, 23). As for all other ILC, ILC2 development depends on the common γ-chain cytokine receptor, IL7 and the transcription factor Id2 and GATA3 (24, 25), and they also require RORα (26, 27) and TCF-1 (28). ILC2 respond to stimulation with epithelial derived cytokines, such as IL33, IL25, and TSLP, but also eicosanoids, such as prostaglandin D2 (PGD2) (29, 30) and leukotriene D4 (31), and secrete type 2 cytokines, mainly IL5 and IL13, but also IL4, IL9 (31–34), and the epidermal growth family member amphiregulin that is responsible for lung epithelial repair after murine infection with the H1N1 influenza virus (35).

Human ILC2 have been identified in the peripheral blood and in fetal and adult tissues, including the gut, even if at low frequencies (19, 36). These cells express the IL33R (ST2) and the IL25R, and are positive for the chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes (CRTH2) (36). CRTH2 is a G-protein coupled receptor for PGD2, which is released by activated mast cells during allergic reactions. PGD2 binding to CRTH2 has been shown to induce ILC2 migration, production of type 2 cytokines, and up-regulation of IL33R and IL25R possibly creating a positive feedback loop that amplifies type 2 responses during allergy (30).

Three independent groups initially identified ILC2 in mice and applied different denominations to the groups, "natural helper cells," "nuocytes," and "innate helper 2 cells" (6, 22, 23). "Natural helper cells" were identified in lymphoid clusters associated with the adipose tissue in the peritoneal cavity. Interestingly, similar fat-associate lymphoid clusters (FALC) were also found in the human mesentery. These cells are Lin^-, but express c-Kit, Sca-1, the IL7R and the IL33R, produce large amounts of Th2 cytokines, such as IL5 and IL13 and also induce B cell proliferation. After helminthic infection and in response to IL33, FALC Lin^-c-$Kit^+Sca\text{-}1^+$ cells produce large amounts of IL13, which leads to goblet cell hyperplasia and contribute to the expulsion of helminthes. The location of these cells in the visceral fat, and not at the classical barrier surface, is interesting. Recent studies indicate that ILC2 play a role in adipose tissue deposition and metabolism. In fact, they were shown to promote weight loss and glucose tolerance, through induction of caloric expenditure and browning of the adipose tissue (37–40). "Nuocytes" were identified in the IL13 reporter mice as non-B, non-T lymphocytes that expand *in vivo* in response to IL25 and IL33, and mediate the early protective immune response to helminthes through secretion of IL13. Similarly, "innate helper 2 cells" were identified as Lin^-, IL25, and IL33 responsive cells in IL4 and IL13 reporter mice. These cells are widely distributed and expand after helminthic infection and secrete IL13 resulting in worm clearance. More recently, it has been shown that IL33-mediated induction of IL13 by ILC2 is necessary to mediate protection against helminthes (41).

Around the same time, an IL25 responsive Lin^- multipotent progenitor precursor called MPP^{type2} was also identified in the gut-associated lymphoid tissue (GALT). These cells are also able to elicit protective type 2 responses during parasitic infection (42). Compared with the other three populations, however, MPP^{type2} are Id2 independent, show a distinct transcriptional profile and are able to differentiate into multiple monocyte/macrophage and granulocyte populations and are therefore classically not included in the ILC family (43).

ILC2 were shown to be critical for epithelial repair in the lung following influenza virus infection through secretion of amphiregulin (35). Murine gut-associated ILC2 can also secrete amphiregulin in response to IL33 and were shown to have a protective role in the dextran sodium sulfate model of intestinal damage and inflammation (44). These data suggest that intestinal ILC2 may play a role in the maintenance of epithelial integrity in the gut (**Figure 1**).

Together with fetal LTi cells, ILC3 belong to the Group 3 of ILC. Both types of cells depend on RORγt and IL7 for their development and function (2). The classical Group 3 LTi, in conjunction with stromal cells, are responsible for the induction of secondary lymphoid tissues during embryonic development (45). On the other hand, ILC3 expand postnatally in response to microbiota derived signals (46–48). Phenotypically, fetal LTi cells and adult ILC3 populations can be distinguished on the basis of CCR6 expression, with LTi cells being of $CCR6^{hi}$ phenotype and adult ILC3 populations of $CCR6^{low/-}$ phenotype (14).

ILC3 are (arguably) the most interesting ILC in the intestine and are the most abundant ILC subset at mucosal tissues, particularly in the intestinal tract. In fact, ILC3 constitute the majority of ILC in the ileum (13, 49) and in the colon (19). Interestingly, it has been shown that the composition of the different ILC subsets correlates with localized IL7 expression. IL7 stimulation increases RORγt expression and this is a striking result, as the implication would be that IL7 does not just control ILC survival but can modulate which ILC subsets you have, i.e., the more IL7, the more ILC3.

In the healthy gut, ILC3 exhibit a range of cytokine dependent and cell surface receptor mediated mechanisms to apply homeostatic control of intestinal immunity. Indeed, one of the major functions of ILC3 is to maintain barrier integrity in an environment where a tug of war is constant between the enhancement and inhibition of immune responses against the microbiota (50).

Once they sense bacterial antigens, $CX3CR1^+$ mononuclear cells and DC are major sources of IL23 or IL1β in the gut (**Figure 1**). In turn, these cytokines stimulate ILC3 to produce the effector cytokines IL22, GM-CSF, and IL17. The IL22 cytokine has a key role in homeostatic control and is secreted by Th17, Th22, and ILC3 (51–54). IL22 expression by ILC3 is also controlled by the aryl hydrocarbon receptor (Ahr), a ligand

dependent transcription factor that is activated by xenobiotic and endogenous ligands (55). Ahr controls the development of adult, but not fetal RORγt$^+$ ILC in mice, and RORγt$^+$ ILC that lack Ahr were shown to produce less IL22 through both direct inhibition of *Il22* gene transcription and reduced responsiveness to IL23 (47, 48).

In the intestine, the IL22 dependent crosstalk between ILC3 and epithelial cells has been shown to influence the composition of the intestinal microbiota in mice, resulting in augmented host resistance to colonization with pathogenic organisms. The production of IL22 by ILC3 is critical for innate responses against intestinal bacterial pathogens such as *Citrobacter rodentium* (56), while their production of IL17 is central for antifungal responses against the opportunistic pathogen *Candida albicans* in the oral mucosa (57).

IL22 derived from ILC3 can also prevent bacterial translocation and keep opportunistic pathogens in check. Indeed, mice lacking IL22 or ILC3 show disrupted barrier integrity and hence cannot contain commensal bacteria resulting in translocation. ILC3 derived IL22 has been shown to protect mice against systemic dissemination of pathogenic bacteria following *Clostridium difficile* infection and infection-induced damage to the intestinal epithelium. ILC3 derived IL22 was required for the induction of complement factor C3 in the liver and intestine, which enhanced bacterial phagocytosis and allowed effective control of antigen load in peripheral organs (58). Additionally, IL22–IL22R interactions on intestinal epithelial cells (IEC) induce STAT3 phosphorylation and stimulate production and secretion of anti-microbial peptides and proteins such as β-defensins, RegIIIβ and RegIIIγ, calgranulins S100A8 and S100A9, and lipocalin-2 and mucins like Muc1, Muc3, Muc10, and Muc13 (59–63). In mice, it has been shown that suboptimal expression of anti-microbial factors by IEC results in systemic dissemination of commensal bacteria of the genus *Alcaligenes spp*, which is normally contained in GALT and Peyer's patches. Peripheral *Alcaligenes spp*. dissemination was associated with low-grade systemic inflammation, which could be reversed by treating mice with recombinant IL22 (64).

IL22 binding to IL22R in IEC triggers a signaling cascade necessary for the production and maintenance of IL18 mRNA and pro-IL18 induction in epithelial cells (65). In addition, activation of the inflammasome in these cells leads to the downregulation of IL22 binding protein and hence increases the effects of IL22 (65, 66). Therefore, IL18 and IL22 production is tightly and mutually controlled contributing to the maintenance of gut homeostasis. When this delicate balance is altered under inflammatory conditions, intestinal pathology ensues. In mice, it has been shown that ILC3 responses are indirectly downregulated *via* IL25, which is secreted by microbiota-stimulated epithelial cells and likely acts *via* DC, which in turn limit ILC3 activation through a, so far, unknown mechanism (67).

In addition to being regulated by immune cell subsets such as DC (68), ILC3 have the capacity to regulate the activity of other immune cells *via* direct cell–cell interactions with important implications for gut homeostasis. Moreover, ILC3 respond to IL1β with the secretion of GM-CSF, a crucial regulator of mononuclear phagocytes, which induce intestinal Tregs. In fact, IL1β stimulation elicits production of pro-inflammatory cytokines by ILC3, including TNFα, IL6, and GM-CSF as well as regulating the expression of MHCII and co-stimulatory ligand expression in both *ex vivo* and *in vitro* generated mouse ILC3 (4). Hepworth et al. have demonstrated a requirement for class II transactivator, a master transcriptional regulator of MHCII, in driving MHCII expression by ILC3 (69). ILC3 are indeed capable of processing ingested protein antigens and load them onto MHCII for presentation to CD4 T cells, although the efficiency at which ILC were shown to do this was expectedly much lower than that of professional antigen presenting cells (5, 7).

ILC3 inhibit commensal-specific T cells *via* the MHCII receptor together with a withdrawal of IL2. Under steady-state conditions, IL2 and MHCII dependent interactions between intestinal ILC3 and effector CD4 T cells can inhibit CD4 T cell responses (5, 69). In humans, HLA-DR has been shown to be expressed on intestinal ILC3 as well as in ILC2. ILC3 have also been reported to limit commensal-specific CD4 T cell responses, through direct inhibitory interactions with CD4 T cells (5, 69) and through induction of tolerogenic RA and IL10 secreting intestinal DC and macrophages (70).

Data from our unit (Anna-Lena Schaupp et al.) have shown that ILC populations in human blood and the intestinal lamina propria express HLA-DR and co-stimulatory ligands, such as CD80, CD86, OX40, and PD-L1. In the colon, ILC3 were found to contain the highest proportion of HLA-DR expressing cells, with intermediate expression by ILC1 and lowest expression by ILC2. A similar distribution of HLA-DR expression among mouse ILC subsets in the small intestine has been reported with ILC3 populations containing a significantly higher percentage of HLA-DR$^+$ cells compared with ILC2 or ILC1 (5).

Furthermore, ILC3 can also constrain commensal dissemination through effects on B cell responses. Thanks to their expression of B cell activating factors, such as BAFF, APRIL, and CD40L, as well as through lymphotoxin-dependent pathways, ILC3 promote the production of T cell-dependent and -independent IgA and IgG antibodies by mucosal and splenic B cell populations (71, 72). In turn, mucosal IgA and low-affinity systemic IgG antibodies contribute to the maintenance of mucosal homeostasis by regulating the composition, size, and anatomical containment of the intestinal commensal microbiota (73).

ILC3 express high levels of CD1d on their surface, therefore, they can acquire and present lipids to iNKT cells and this engagement stimulates them to produce IL22 (74). IL22 plays a central role in epithelial barrier function and tissue repair (75), and thus, ILC3 integration of signals obtained through CD1d, along with those received from cytokines in the local inflammatory milieu, such as IL23, may contribute to maintenance of homeostasis and to regulation of immune responses. By sensing the environment *via* the interaction microbial lipids-CD1d, ILC3 make an important contribution to the maintenance of barrier function.

The function of TLR on ILC3 is still an open question. TLR1, 2, 5, 6, 7, and 9 transcripts in human ILC3 have been shown to be expressed, albeit at low levels, however, only TLR2 engagement on ILC3 induces cytokine production in the presence of IL2, IL15, and IL23 (3). These results imply that ILC3 may have a role during *C. albicans* infection.

Another mechanism through which intestinal ILC3 have been shown to control commensal homeostasis is the regulation of IEC fucosylation (**Figure 1**). Commensal bacteria induce IEC fucosylation and epithelial fucose is used as a dietary carbohydrate. Fucosylated carbohydrates on IEC are thought to be important for the generation of an intestinal niche for commensal microbes (76). IL22 engagement with its receptor and secretion of lymphotoxin by ILC3 triggers fucosylation of epithelial cells (77). Indeed, IEC fucosylation is mediated by the fucosyltransferase enzymes Fut1 and Fut2 (78). ILC3 have been shown to induce intestinal epithelial Fut2 expression and fucosylation in mice. Abrogation of IEC fucosylation in mice lacking Fut2 has been linked to intestinal commensal dysbiosis and led to increased susceptibility to infection with the pathogens *Salmonella typhimurium* and *C. rodentium* (76). Thus, by regulating IEC anti-microbial functions, IL22 producing ILC3 plays an important role in preventing the systemic dissemination of microbial commensals.

ILC IN INTESTINAL PATHOLOGY

Recent evidence from murine and human studies indicates that ILC participate in the pathogenesis of chronic intestinal inflammation and colitis-associated cancer in patients with IBD. On the other hand, a protective role for ILC has emerged in murine models of graft versus host disease (GVHD), a major complication of allogeneic hematopoietic stem cell transplant (A-HSCT). These findings identify ILC as potential therapeutic target for patients' treatment and suggest that modulation of ILC function and activity may be beneficial in pathological conditions.

Inflammatory Bowel Disease

Inflammatory bowel disease is a chronic inflammatory disorder of the GI tract that encompasses two main forms, such as Crohn's disease (CD) and ulcerative colitis (UC). In UC, inflammation is confined to the colonic mucosa while in CD it involves all layers of the gut wall and can affect any part of the GI tract, often leading to stricturing or fistulisation. Extra-intestinal manifestations of the disease are frequent with possible involvement of joints, skin, eyes, and kidneys. Moreover, the risk of developing other chronic immune disorders, such as psoriasis, ankylosing spondylitis, or primary sclerosing cholangitis (PSC), is also increased. Patients with long standing IBD colitis also have increased risk of colon cancer, and this risk is five times higher in patients with PSC-associated IBD (79). Patients are typically treated with immune suppressive treatment (corticosteroids, immunosuppressant agents, and biologic therapies, such as anti-TNFα or anti α4β7 antibodies), but surgery is often required during the course of the disease (80). The etiology of IBD remains unknown, but the available evidence suggests that IBD is caused by an abnormal immune response against the microorganisms of the intestinal flora in genetically susceptible individuals (81). Both dysregulated innate and adaptive immune pathways have been implicated in the pathogenesis of intestinal inflammation in these patients (82).

Early IBD research mainly focused on understanding the contribution of abnormal adaptive T cell responses to disease pathogenesis. CD has long been considered to be driven by an IL12 dependent Th1 response, while UC has been rather associated with a non-conventional Th2 response. However, an important role for IL23 driven Th17 responses in the pathogenesis of IBD has emerged by GWAS studies, and was confirmed in animal models and in translational studies in patients (83). Furthermore, advances arisen from genetic studies contributed to moving the focus of IBD pathogenesis on to mucosal innate immune responses. It became apparent that effector cytokines traditionally attributed to CD4 or CD8 T cells could also be secreted by unconventional innate lymphocytes, including ILC. This prompted an extensive research into the role of ILC in intestinal inflammation.

Buonocore et al. have shown that IL23 responsive ILC3 are required for induction of innate bacterial driven colitis in mice lacking B or T cells following infection with the pathogenic bacterium *Helicobacter hepaticus*. Colitis development was dependent on IL23 induced IL17 and IFNγ production by ILC3 that accumulated in the gut in response to IL1β. RORγt$^+$ ILC3 were critical for colitis development, since intestinal inflammation was abrogated in mice that lack ILC3 (84). As previously mentioned, pathogenic IL17 production by intestinal ILC3 has also been shown to underlie the development of spontaneous colitis in mice lacking the type 1 transcription factor T-bet. In this model, ILC3 accumulate in the intestinal lamina propria and are activated in response to TNFα, IL23, and IL6 produced by activated DC (20).

Pearson et al. set out to investigate whether the movement of ILC within tissues contributed to immune and inflammatory responses (85). Using the anti-CD40 animal model of colitis, they observed ILC movement within cryptopatches. The authors concluded that by accumulating, clustering, and moving within the cryptopatches, ILC might initiate an inflammatory immune cascade resulting in intestinal inflammation. Interestingly, ILC appeared to wriggle their way directly through the tissue, possibly following a chemokine gradient.

Commensal dysbiosis secondary to ILC3 dysfunction can promote systemic inflammatory responses in mice. Outgrowth of commensal segmented filamentous bacteria, which are implicated in the development of pro-inflammatory Th17 cells (86), has been observed in mice lacking Ahr with lower levels of intestinal ILC3 derived IL22. Further reduction of ILC3 numbers and IL22 production in Ahr$^{-/-}$Rorcγt$^{GFP/-}$ mice promoted development of spontaneous, Th17-driven colitis, indicating that ILC3/IL22 mediated regulation of the commensal flora plays a role in mitigating the induction of potentially pathogenic Th17 responses to commensal antigens (48).

Furthermore, as already discussed, IL22 induces production of IL18 by IEC in mice and uncontrolled IL18 can amplify intestinal inflammation hence disrupting the mucosal barrier and creating a feedback loop of injury (65). Moreover, IL18 is up-regulated in intestinal lesions and peripheral blood in patients with CD (87–89), suggesting the presence of an altered IL22-IL18 regulation (**Figure 1**).

Interestingly, in both mice and humans, ILC3 were also found to secrete GM-CSF, which can recruit myeloid cells and further promote intestinal inflammation (85). In the same study, it was shown that ILC3 move within the intestinal tissue when activated. These two mechanisms can contribute to the induction and progression of inflammation throughout the gut.

In intestinal samples from IBD patients, we observed accumulation of $Lin^-CD56^-CD127^+$ ILC in the inflamed ileum and colon of patients with CD, but not UC. These cells were able to respond to IL23 *in vitro* and they expressed IL17 and IFNγ (90). More, recently, we also found increased ILC frequencies in patients with PSC-associated IBD, but again no accumulation was observed in UC patients (91). Follow-up studies have confirmed that ILC contribute to inflammation in CD and suggested that IFNγ secreting ILC1 are especially increased in the intestinal lesions in these patients. Two populations of ILC1 have been described in the human intestine. Bernink et al. identified a population of $CD127^+NKp44^-c\text{-}Kit^-$ cells that express high levels of T-bet and IFNγ compared to $c\text{-}Kit^+$ ILC3 (13). Similarly to NK cells, these cells respond to IL12 and IL18 stimulation *in vitro* with induction of IFNγ, however, they do not express granzyme B or perforin, which are classically associated to NK cell cytotoxic ability. Another population of IFNγ secreting intraepithelial ILC1, which are $CD127^{-/low}$ and express the epithelial homing integrin CD103, was identified in the human gut (92). Like $CD127^+$ ILC1 and $CD127^{-/low}CD103^+$ ILC1 are $T\text{-}bet^+$, but they express the transcription factor Eomes and perforin and granzyme B granules, similarly to conventional cytotoxic NK cells. IFNγ secreting $CD127^+NKp44^-C\text{-}Kit^-$ ILC1 were found to accumulate in the inflamed intestine of patients with CD (13). The frequency of intraepithelial $CD127^{-/low}CD103^+$ ILC1 was also found to be increased in the inflamed tissue of patients with CD (92). These data are in agreement with our first observation of increased frequencies of ILC in the inflamed lamina propria of patients with CD and may reflect ILC functional flexibility and their exposure to high levels of IL12 in the mucosa (90, 93) (**Figure 1**).

A role for ILC2 in pathologic intestinal inflammation has also been suggested by results in the oxazolone induced model of colitis, where ILC2 participate in intestinal inflammation in an IL25-dependent manner (94). Interestingly, increased frequencies of ILC2, which are able to secrete IL13 and IFNγ, have been detected in intestinal samples from CD patients (15). In the same study, stimulation of ILC2 with IL12 *in vitro* induced the expression of T-bet and IFNγ. IL12 is classically increased in the mucosa of patients with CD (93) and the finding of increased frequency of IFNγ secreting ILC2 may, therefore, reflect the functional plasticity of ILC depending on the mucosal microenvironment. $CRTH2^+$ ILC2 were found to accumulate in esophageal tissues of patients with the allergic disease eosinophilic esophagitis (95), in nasal polyps of patients with chronic rhinosinusitis (36), an allergic condition characterized by high levels of IL5 and IL13, and in the inflamed skin of patients with atopic dermatitis (96), suggesting they can contribute to type 2 cell-mediated disease. In these allergic conditions, $CRTH2^+$ ILC2 can be activated by mast cell released PGD2, up-regulate IL33R and IL25R and secrete IL4, IL5, and IL13 leading to the amplification of the type 2 response (30). On the other hand, while IL13 induction has been associated to mucosal inflammation in patients with UC (97, 98), there are no reports of increased ILC2 frequency in UC to date. Very low frequencies of ILC2 are found in the human colon and they may not represent major players in the colonic immune response, at least in the absence of parasitic infections (19). Interestingly, frequencies of $CD127^+$ ILC were similar between UC patients and controls in our studies (90, 91), suggesting these cells may not be involved in intestinal inflammation in these patients.

Colorectal Cancer

Inflammation is one of the 10 hallmarks of cancer (99). The immune system can profoundly influence carcinogenesis with both favorable anti-tumorigenic effects and detrimental pro-tumorigenic activities. Despite the protective role of mucosal inflammation to limit damage and promote tissue repair, a dysregulated, chronic and extensive intestinal inflammation is regarded as a promoter of carcinogenesis. The exact mechanisms remain unknown, but inflammatory cytokines can act on IEC to promote many processes involved in neoplastic transformation, including proliferation, inhibition of apoptosis, invasion, angiogenesis, epithelial to mesenchymal transition, and metastasis (100).

Evidence from animal models suggests that ILC and especially IL23 responsive ILC3 may contribute to intestinal carcinogenesis. In particular, Kirchberger et al. identified a role for IL23 driven IL22 producing ILC in bacteria-induced colitis-associated cancer in T and B cell deficient mice infected with *H. hepaticus* long-term or co-treated with 2-azoxymethane (a cancer inducing agent) (101). In this setting, IL22 producing $CD4^-Nkp46^-Thy\text{-}1^+$ ILC accumulate at the expense of $Nkp46^+$ and $CD4^+$ ILC populations. Depletion of $Thy\text{-}1^+$ ILC or IL22 blockade can reverse established invasive carcinoma and inflammation. Importantly, $IL22^+$ T cells and non-T cells are present in human colorectal cancer, and *IL22* gene expression is increased in cancer tissue relative to matched adjacent normal tissue in these patients. In another model, transgenic expression of IL23 was shown to drive the *de novo* development of duodenal adenomatous tumors that was dependent on IL17 secreting $Thy\text{-}1^+$ $Sca\text{-}1^+$ ILC (102). However, one must be careful to translate results from T cell depleted animals into clinical settings, especially in light of the beneficial effects of IL22 in promoting barrier protection and epithelial regeneration. It is evident that IL22 can act as a "double edged sword" with both protective and pathogenic activities depending on the context.

Patients with IBD are at increased risk of developing intestinal cancer. This risk is further increased in patients co-diagnosed with PSC, a chronic progressive disorder of the hepato-biliary system, and strongly associated to IBD. PSC is characterized by inflammation, fibrosis, and stricturing of intrahepatic and extrahepatic bile ducts, leading to liver cirrhosis (103). Interestingly, patients with UC and PSC have a fivefold increased risk to develop colon cancer than those with UC alone (104). Patients with PSC are also at increased risk of other malignancies, including cholangiocarcinoma, gall-bladder adenoma, dysplasia, and carcinoma (105).

Our data show that secretion of Th17-cytokines from colonic T cells did not differ between patients with PSC–IBD, patients with UC and healthy controls, while we observed enhanced Th1 responses in PSC–IBD. On the other hand, ILC accumulate in the colon of PSC–IBD patients suggesting that ILC may play a role in intestinal inflammation and increased cancer risk in PSC and IBD (91). However, further studies are needed to evaluate the functional activity of ILC in these patients, and whether they contribute to pro-carcinogenic responses, including cytokine secretion and/or cell–cell interaction, that can promote epithelial transformation in PSC–IBD.

Graft Versus Host Disease

Allogeneic hematopoietic stem cell transplant is a potentially curative treatment for patients with leukemia and lymphoid malignancies (106). However, A-HSCT is frequently complicated by potentially fatal GVHD (107), caused by allo-reactive donor T cells that are activated against recipient antigens and lead to severe tissue damage, most frequently involving the GI tract, liver, and skin, where ILC are particularly represented.

Murine studies showed that IL23 responsive ILC3 secrete IL22 after bone marrow transplant, but are decreased in mice with acute GVHD. The reduction of ILC3 is associated with decreased secretion of IL22, loss of intestinal stem cells, impaired epithelial barrier function, and increased intestinal inflammation (108). A recent study showed that ILC2 are highly reduced after radiation and chemotherapy before A-HSCT in mice, and only limited repopulation with donor bone marrow derived ILC2 is observed in the GI tract. Interestingly, infusion of IL33–activated donor ILC2 significantly reduced the severity of GI GVHD in two separate murine models and this effect was dependent on the recruitment of donor myeloid-derived suppressor cells. Furthermore, ILC2 treatment of recipients with established GVHD reduced disease severity and increased survival (109).

Accordingly, ILC are depleted by pre-conditioning therapy in patients with acute leukemia and the reconstitution of ILC1, ILC2, and NKp44$^-$ ILC3 is slow compared with neutrophils and monocytes. On the other hand, NKp44$^+$ ILC3 that are not present in the peripheral blood of healthy individuals, appear after conditioning and A-HSCT. A significant proportion of NKp44$^+$ ILC3 expresses the gut-homing and skin-homing receptors, cutaneous lymphocyte-associated antigen and CCR10. The frequency of activated NKp44$^+$ ILC3 expressing skin- and gut-homing receptors were significantly higher in patients who did not develop therapy induced mucositis and acute or chronic GVHD. These data suggest that NKp44$^+$ ILC3, similarly to their murine counterpart, are involved in intestinal homeostasis and tissue repair contributing to GVHD prevention possibly through the secretion of IL22 (110).

On the other hand, ILC were shown to be dispensable in patients with severe combined immune deficiency that received A-HSCT. In these patients, while T cell and B cell reconstitution was obtained after A-HSCT, ILC were still deficient after transplant. However, no increased risk of infections or other disease was observed in the medium-long term in these subjects (111).

Besides their protective role in maintaining intestinal homeostasis, ILC have been shown to participate in chronic intestinal inflammation in both animal models and human diseases. In the presence of pro-inflammatory mediators in the microenvironment a shift from protective ILC populations toward pro-inflammatory and possibly pathogenic ILC subsets may occur. While IL22 mediated induction of epithelial proliferation and tissue repair may play a protective role in intestinal inflammation in GVHD, chronic exposure to IL22 may lead to acquisition of epithelial stemness and neoplastic transformation contributing to the increased risk of colon cancer that is associated to longstanding colitis.

FUTURE PERSPECTIVES AND THERAPEUTIC APPLICATIONS

The identification of ILC as a heterogeneous group of innate lymphocytes particularly abundant at mucosal sites has prompted both animal and human studies aimed to evaluate their role in intestinal pathophysiology. They are present at low frequencies, compared to other lymphocytic or myeloid populations, however, results from murine studies have highlighted their important contribution to maintaining intestinal homeostasis and to promoting chronic intestinal inflammation. As discussed, ILC can exert protective activities in the gut, through regulation of the microbial niche and contribution to epithelial and tissue repair. On the other hand, they can induce inflammation through the production of inflammatory cytokines and the regulation of innate and adaptive immune responses. It is becoming apparent that ILC can exert their function, not only through the secretion of soluble mediators, but also through their interaction with other cells, such as immune cells, including myeloid and T cell populations, and non-immune cells, such as epithelial cells and stromal cells, as suggested by the role of LTi-stromal cell crosstalk during lymphoid tissue embryogenesis. Targeting ILC activities, through cytokine blocking or modulation of ILC activation and their interaction with other cells, may result in beneficial effects in order to maintain homeostasis and prevent or treat inflammation. However, due to the lack of ILC specific markers, mediators or functions, at present any therapeutic approach will also have effects on other immune cells, and particularly on T cell mediated responses.

IL22 is emerging as a central mediator in intestinal homeostasis and tissue repair (75), while IL17 and IFNγ preferentially secreted by ILC3 and ILC1 have been associated to more pathogenic effects. A differential accumulation of IL22 secreting and IFNγ− and IL17A-secreting ILC has been found in patients with IBD and particularly in CD. Our own data suggest that ILC populations that respond to IL23 stimulation with secretion of IL17A and IFNγ accumulate in the inflamed intestine of patients with CD, but not in UC. Further studies have confirmed accumulation of IFNγ secreting ILC populations in both the lamina propria and the epithelium in CD (13, 90). Moreover, a reduction in the frequency of IL22 secreting ILC3, associated to IFNγ secreting ILC accumulation, has been described in CD (112, 113). Interestingly, no study to date has described accumulation of ILC in the gut of patients with UC and our studies may suggest they are not implicated in UC inflammation, even if larger studies are needed. On the contrary accumulation of ILC is present in patients with PSC–IBD, who classically exhibit mild colitis but significantly increased risk of colon cancer compared to other patients with IBD (91).

ILC1 secreted IFNγ may contribute to propagation of inflammation and tissue damage in CD. Both Th1 and ILC1 responses are increased in CD with higher levels of IFNγ expressed in the intestinal mucosa of these patients. However, Fontolizumab, a humanized murine anti-IFNγ antibody, did not show efficacy in clinical trials, even if some concerns about the design of these studies have been raised and Fontolizumab may induce some partial benefit in CD (114).

ILC3 derived IL17 promotes the recruitment of inflammatory neutrophils by inducing the release of chemokines from epithelial and endothelial cells. In this context, IL17 promotes epithelial barrier function (115). However, IL17 also presents cytokine functional dichotomy, and in the presence of IL23, IL17 and pathogenic Th17 cells have been implicated in intestinal inflammation and tissue damage (116). Hence, IL23 dependent IL17 production might be counter-indicative. Based on the results of genetic studies and immunological studies models and human tissue, blocking Th17- and unconventional lymphocyte-secreted IL17 seemed a reasonable therapeutic approach for patient with IBD. However, specific targeting of IL17 with Secukinumab, a human anti-IL17A monoclonal antibody, led to disappointing results in a multicentre phase IIa study (117). Secukinumab was not only ineffective in CD patients, but also actually resulted in disease exacerbation, likely interfering with the more protective IL17 functions or resulting in increased Th1 and IFNγ-mediated responses. The results of this study highlighted the complexity of the IL23/IL17 axis, with IL17 being only one of the many IL23 induced mediators with often redundant and dichotomic effects (118).

It is conceivable that insufficient levels of intestinal ILC derived IL22 in CD patients, resulting in impaired epithelial regeneration, innate anti-microbial defense and containment of commensal microbes, may contribute to disease pathogenesis. Selective enhancement of IL22 production by ILC may have protective effects in these patients, although this strategy would have to be carefully balanced against a potentially increased risk of colorectal cancer. In fact, chronic IL22 exposure promotes intestinal epithelial proliferation and may result in increased risk of colitis-associated cancer as suggested by murine studies (101). Targeting of IL22 or more specifically IL22 secreting ILC may be beneficial in the prevention or treatment of longstanding IBD-associated increase risk of colon cancer.

Studies in murine models also suggested that blocking ILC3 secreted GM-CSF or altering ILC3 movement or activity might help reduce intestinal inflammation (85). However, blocking GM-CSF activity could also result in the inhibition of important protective mechanisms (119, 120). Furthermore, anti-GM-CSF autoantibodies were found to be increased in IBD (121) and recombinant GM-CSF (Sargramostim) showed some efficacy in patients with CD (122). However, these results were not confirmed in further studies (123). In any case, clinical trials are underway to investigate the use of anti-GM-CSF in other inflammatory conditions, such as rheumatoid arthritis or multiple sclerosis and these studies could offer insight into whether this approach could have beneficial effect on intestinal inflammation in patients with concomitant IBD.

ILC3 might well be divided into several subsets and an understanding of the different functions of each one might help us in the design of specific therapies according to the specific roles of ILC3 in intestinal inflammation and homeostasis. A crucial open question of ILC biology is how these rare cells impact homeostatic or, in the case of IBD, pathogenic immune responses in the presence of a functional adaptive immune system. This is evidenced by the existence of functionally discrete ILC3 subsets, including those that present antigen and inhibit microbiota-directed T cell immunity and those that promote anti-microbial T cell responses *via* IL22 production.

Elevated levels of pro-inflammatory cytokines, such as IL12, IL23, or IL1β, in the intestine of patients with IBD may be responsible for the acquisition of pathogenic ILC phenotypes. IL12 and IL23, can be targeted by Ustekinumab and Briakinumab, monoclonal antibodies against their shared p40 subunit. This strategy has proven to be effective for patients with CD (124–126) and it remains to be elucidated how ILC and T cell populations are affected by IL23 and IL12 blockade. Interestingly, the efficacy of anti-IL12/IL23 treatment in CD is not comparable to the striking results obtained in patients with psoriasis (127, 128) highlighting the complexity of the immune mechanisms involved in intestinal inflammation in these patients (118). More recently Risankizumab, a monoclonal antibody against the IL23 specific subunit p19, showed higher efficacy than placebo at inducing clinical remission in CD patients in a phase 2 study (129).

It is conceivable that future therapeutic strategies could include interfering with ILC interaction with immune and non-immune cells. As discussed, ILC3 inhibit commensal-specific T cells *via* the MHCII receptor together with a withdrawal of IL2 (5, 69). However, in IBD an increase in the availability of IL2 (130) due to widespread immune activation could mean that IL2 uptake by ILC may no longer be a limiting factor for effector CD4 T cells. In this context, T cell-derived IL2 in conjunction with other pro-inflammatory cytokines (IL1β, IL6, IL12, and IL23) could furthermore drive inflammatory ILC3 activation. ILC3 derived effector cytokines (IL17A, IFNγ, TNFα, and GM-CSF) could then feedback to potentiate pathogenic effector CD4 T cell responses, either directly or indirectly *via* antigen presenting cells.

Taken together, a better understanding of the regulation of cytokine expression by ILC and their interaction with other immune and non-immune cells will help to develop new strategies to treat inflammatory diseases in humans.

AUTHOR CONTRIBUTIONS

AG and CVA-C contributed equally to this work.

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Human CD5$^+$ Innate Lymphoid Cells Are Functionally Immature and Their Development from CD34$^+$ Progenitor Cells Is Regulated by Id2

*Maho Nagasawa[1,2], Kristine Germar[3†], Bianca Blom[1,2] and Hergen Spits[1,2]**

[1] Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands, [2] Amsterdam Infection and Immunity Institute, Amsterdam, Netherlands, [3] Department of Clinical Immunology and Rheumatology Center, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands

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Chiara Romagnani, Deutsches Rheuma Forschungszentrum, Germany

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***Correspondence:**
Hergen Spits
hergen.spits@amc.uva.nl

†Present address:
Kristine Germar, Inovio Pharmaceuticals Inc., San Diego, CA, United States

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Innate lymphoid cells (ILCs) have emerged as a key cell type involved in surveillance and maintenance of mucosal tissues. Mouse ILCs rely on the transcriptional regulator Inhibitor of DNA-binding protein 2 (Id2) for their development. Here, we show that Id2 also drives development of human ILC because forced expression of Id2 in human thymic progenitors blocked T cell commitment, upregulated CD161 and promyelocytic leukemia zinc finger (PLZF), and maintained CD127 expression, markers that are characteristic for human ILCs. Surprisingly CD5 was also expressed on these *in vitro* generated ILCs. This was not an *in vitro* artifact because CD5 was also found on *ex vivo* isolated ILCs from thymus and from umbilical cord blood. CD5 was also expressed on small proportions of ILC2 and ILC3. CD5$^+$ ILCs were functionally immature, but could further differentiate into mature CD5$^-$ cytokine-secreting ILCs. Our data show that Id2 governs human ILC development from thymic progenitor cells toward immature CD5$^+$ ILCs.

Keywords: innate lymphoid cells, human, development, Id2, CD5, CD5$^+$ ILC

INTRODUCTION

Innate lymphoid cells (ILCs) belong to a novel lymphoid cell subfamily. ILCs can be found throughout the body, particularly enriched in mucosal tissues and secondary lymphoid structures where they have been implicated as important regulators of mucosal homeostasis, host–microbiota interactions, initiation of protective immunity, inflammation, and tissue repair (1–3). ILCs have been categorized into three groups; ILC1 and NK cells, ILC2, and ILC3, based on their expression of typical transcription factors, cell surface markers and their cytokine production profile (4).

Recent papers have provided insight into the mechanisms of ILC development and the intermediate stages in mice and also in humans although our understanding of human ILC development lags that of mice. It has been suggested that human ILC development can occur not only in bone marrow, but also in secondary lymphoid tissues and the intestinal lamina propria (5, 6). ILC have also been found in the thymus which might imply that ILC also develop in the thymus. This notion is supported by the observation in mouse and human that the thymus contains bipotential T/NK progenitors

Abbreviations: ILC, innate lymphoid cells; Lin, lineage; PNT, postnatal thymus; UCB, umbilical cord blood; SCF, stem cell factor; PLZF, promyelocytic leukemia zinc finger; Id2, inhibitor of DNA-binding protein 2.

(7–9), and it is plausible that such precursors may also develop into ILCs. Previously, we documented that lineage commitment of the bipotential thymic T/NK progenitors is dependent on the balanced expression levels of E-proteins, which are members of the basic helix-loop-helix (bHLH) transcription factor family, and their antagonist Id proteins (10, 11). Id2 is a member of a family of Helix-loop-helix factors consisting of four members, Id1, Id2, Id3, and Id4, which share the capacity of sequestering bHLH transcription factors, including E2A and HEB, which are needed for T and B cell development, and E2-2, required for development of plasmacytoid dendritic cells (pDC) (12–15). As a consequence, Id proteins can inhibit T and B cell and pDC development (10, 11, 16, 17). High levels of Id2 results in NK cell development by suppressing E2A and HEB activity, whereas a high ratio of E2A and HEB to Id2 favors T cell development (15). Id proteins inhibit T and B cell development at an early stage by blocking upregulation of RAG1 and RAG2 genes thereby prohibiting T cell receptor and B cell receptor rearrangements (10, 11, 16). This may set the stage for development of the precursors to ILCs. Indeed ILCs require expression of Id2 for their development, as its deficiency resulted in the complete absence of all ILCs in mice (18).

In addition to Id2, several other transcription factors have been identified that are required for mouse ILC development. Nuclear factor interleukin 3 (Nfil3) and thymocyte selection associated high mobility group box protein (TOX) are required for differentiation of the common lymphoid progenitor (CLP) into the integrin α4β7-expressing ILC progenitor α lymphoid precursor (αLP) (19–23). αLPs further differentiate into common helper ILC progenitors, which are progenitors of ILC1, ILC2, ILC3, and LTi cells, and promyelocytic leukemia zinc finger (PLZF) expressing ILC precursors (ILCp), which have lost the ability to develop into LTi cells, but retain potential to develop into the remaining ILC subsets (24, 25). The intermediate stages between stem cells and mature ILC in humans are less well defined. Recent studies in humans identified a potential ILCp displaying a RORγt$^+$ CD34$^+$ phenotype in tonsils and intestinal lamina propria that differentiated into IL-22 producing ILC3 (5). Another group identified a population of progenitor cells expressing RORγt and IL-1R1, the receptor for IL-1β, in secondary lymphoid tissues that differentiated into all ILCs, including NK cells, but not into T cells or DCs (6). More recently, a c-kit + ILCp was identified in peripheral blood of humans that *in vitro* could develop into all mature ILC subsets (26). As these cells were also found in various organs it was proposed that these circulating c-kit + ILC are able to home in the tissues and to develop into mature ILC in those tissues.

In the present study, we examined the capacity of Id2 to promote development of human ILC. We demonstrate that ectopic expression of Id2 blocked T cell differentiation, resulting in ILCs that expressed CD5 and intracellular (ic) CD3. *In vitro* generated ILCs expressing CD5 and icCD3 phenocopied ILCs that can be found *in vivo* in thymus and cord blood. *Ex vivo* isolated CD5$^+$ non-T cells showed typical features of ILCs and displayed a functionally immature phenotype based on their inability to produce cytokines upon activation. CD5$^+$ immature ILCs could be induced to differentiate into cytokine-producing CD5$^-$ ILCs *in vitro*.

MATERIALS AND METHODS

Monoclonal Antibodies and Cytokines

The following antibodies to human proteins were used. From BioLegend: fluorescein isothiocyanate (FITC)-conjugated anti-CD1a (HI149), anti-CD3 (OKT3), anti-CD4 (RPA-T4) anti-CD14 (HCD14), anti-CD16 (3G8), anti-CD19 (HIB19), anti-CD34 (581), anti-CD94 (DX22), anti-CD123 (6H6), anti-FcER1α (AER-37); phycoerythrin (PE)-conjugated anti-CD161 (HP-3G10), anti-NKp44 (P44-8), anti-IL-5 (JES1-39D10); Alexa Fluor 647-conjugated anti-NKp44 (P44-8); Alexa Fluor (AF) 700-conjugated anti-CD3 (UCHT1), anti-IL-17A (BL168); Allophycocyanin (APC)-conjugated anti-CD3 (OKT3), anti-CD56 (HCD56), anti-CD94 (DX22), anti-IL-13 (JES10-5A2); APC Cy7-conjugated anti-CD4 (OKT4); APC/Fire 750-conjugated anti-CD161 (HP-3G10); brilliant violet (BV) 421-conjugated anti-CD161 (HP-3G10), anti-CD3 (OKT3), anti-CD5 (UCHT2); BV510-conjugated anti-IFN-γ (4S.B3); BV650 streptavidin. From Becton Dickinson: FITC-conjugated anti-CD34 (581), anti-TCRαβ (IP26), ant-TCRγδ (B1), anti-CD8 (SK1); PE-CF594-conjugated anti-CD3 (UCHT1), anti-CRTH2 (BM16); AF647-conjugated anti-CRTH2 (CD294; BM16). From Beckman Coulter: PE-Cy7-conjugated anti-CD127 (R34.34); PE Cy5.5-conjugated anti-CD117 (104D2D1); PE-conjugated anti-CD1a (SFCI19Thy1A8). From Miltenyi: FITC-conjugated anti-BDCA2 (CD303; AC144); APC-Vio770-conjugated anti-CD5 (UCHT2). From eBiosciences: PE Cy7-conjugated anti-IL-22 (22URT1). From invitorogen: PE Cy5.5-conjugated anti-CD5 (CD5-5D7). From NIH AIDS research program: purified anti-α4β7. Human cytokines: IL-2 was obtained from Novartis, IL-15, stem cell factor (SCF), IL-1β, IL-6, and TNF-α were obtained from R&D systems (Abingdon, UK). IL-7 and Flt3L were obtained from Pepro Tech, Inc. (Rocky Hill, NJ, USA).

Cell Lines, Constructs, and Retrovirus Production

The naïve OP9 murine stromal cell line was kindly provided by Dr. T. Nakano (Osaka University, Osaka, Japan) OP9-Jag1, OP9-Jag2, and OP9–DL1 were generated as previously described (27). Id2 was isolated from the retroviral construct LZRS Id2 IRES GFP previously described (17) and subcloned into LZRS IRES mCherry by using restriction enzyme NotI (Roche, Germany). The empty construct was used in control transductions. Retroviral supernatant was obtained from transfected Phoenix-GALV packaging cells (28).

Isolation of CD34$^+$ Cells and ILCs from Postnatal Thymus (PNT) and Umbilical Cord Blood (UCB)

The use of PNT tissue and umbilical cord blood (UCB) was approved by the Medical Ethical Committee of the Academic Medical Center. Thymocytes were obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery and UCB was collected with informed consent of the patients in accordance with the Declaration of Helsinki. The tissue was disrupted by mechanical means and pressed

through a stainless steel mesh to obtain a single-cell suspension, which was left overnight at 4°C. The next day, thymocytes were isolated from a Ficoll–Hypaque density gradient (Lymphoprep; Axis-Shield). Subsequently, $CD34^+$ cells were enriched by immunomagnetic cell sorting, using a CD34 cell separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD34^+$ thymocytes were stained with Abs against CD34, CD1a, CD56, and BDCA2. $CD34^+CD1a^-CD56^-BDCA2^-$ cells, further referred to as $CD34^+CD1a^-$, were sorted to purity on a FACSAria (BD Biosciences). For the isolation of ILCs from PNT and UCB, mononuclear cells isolated from a Ficoll–Hypaque density gradient (Lymphoprep; Axis-Shield) were positively selected by labeling with PE-conjugated anti-CD161 (described above) plus anti-PE microbeads (Miltenyi). The $CD161^+$ cells were stained with Abs against Lineage (CD1a, CD3, CD4, CD8, CD14, CD19, CD16, CD34, CD94, CD123, TCRαβ, TCRγδ, FcER1α), CD161, CD127, CD117, CRTH2, NKp44, and CD5. Cells were sorted on a FACSAria, purity of the sorted cells in all experiments was >99%.

Retroviral Transduction and Differentiation Assay

For transduction experiments, $CD34^+CD1a^-$ postnatal thymocytes were cultured overnight in Yssel's medium (29) with 5% normal human serum, 20 ng/ml SCF and 10 ng/ml IL-7. The following day cells were incubated for 6–7 h with virus supernatant in retronectin-coated plates (30 μg/ml; Takara Biomedicals, Shiga, Japan). The development of ILCs and NK cells was assessed by coculturing the mixture of transduced and non-transduced $CD34^+CD1a^-$ progenitor cells with OP9 cells in MEMα medium (Life Technologies, Carlsbad, CA, USA) with 20% FCS (Hyclone Laboratories, Logan, UT, USA), 5 ng/ml SCF, 5 ng/ml IL-7, and 5 ng/ml FLT3L. 0.5 ng/ml IL-15 was added at the onset of the culture, the medium containing IL-15 was refreshed every week. Flow cytometric analyses were performed on a LSRII FACS analyzer (BD Biosciences); electronic gating was performed using FlowJo (Tree Star, Ashland, OR, USA). Numbers in each dot plot represent the percentage of cells in each quadrant. The fold expansion in absolute cell numbers was calculated using Microsoft Excel 2007 (Microsoft, Redmond, WA, USA) on the basis of total numbers of cells harvested from the cultures, percentages of transduced cells, and percentages of each population corrected for the number of input cells.

Quantitative Real-time PCR

Total RNA was extracted with a NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was synthesized with a High-Capacity cDNA Archive kit (Applied Biosystems). PCRs were performed in a Bio-Rad iCycler (Bio-Rad, France) with IQ SYBR Green Supermix (Bio-Rad, France) using the following primer sets or otherwise as previously described (30) Id2 forward 5′-TTGTCAGCCTGCATCACCAGAG-3′; Id2 reverse 5′-AGCCACACAGTGCTTTGCTGTC-3′; PLZF forward 5′-GAGCTTCCTGATAACGAGGCTG-3′; PLZF reverse 5′-AGCCGCAAACTATCCAGGAACC-3′; Nfil3 forward 5′-TGGAGAAGACGAGCAACAGGTC-3′; Nfil3 reverse 5′-CTTGTGTGGCAAGGCAGAGGAA-3′; TOX forward 5′-CGCTACCTTTGGCGAAGTCTCT-3′; TOX reverse 5′-CTGGCTCTGTATGCTGCGAGTT-3′. Bio-Rad CFX Manager 3.1 software was used for quantification of expression. All samples were normalized to the expression of GAPDH and results are presented in arbitrary units.

ILC Differentiation, Single Cell Cloning, and Expansion

For differentiation assays, isolated ILCs were cultured in Yssel's medium supplemented with 1% (v/v) human AB serum (Invitrogen) for 5–7 days. Cytokines used in these assays were IL-2 (10 U/ml), IL-7 (10 ng/ml), IL-1β (50 ng/ml), IL-6 (50 ng/ml), and TNF-α (50 ng/ml). Single cell or bulk ILCs were expanded *in vitro* by culturing with 2×10^6/ml irradiated (25 Gy) allogenic peripheral blood mononuclear cells, 2×10^5/ml irradiated (50 Gy) JY Epstein–Barr virus-transformed B cells, phytohemagglutinin (1 μg/ml; Oxoid), IL-2 (100 U/ml), and IL-7 (10 ng/ml) in Yssel's medium.

RESULTS

ILCs Are Present in Thymus and Express Id2

We and others have demonstrated that the thymus contains bispecific T/NK cell progenitors (7–9, 15). In humans, these cells are contained within $CD34^+CD1a^-CD5^+$ cells (9). We expected that thymic T/NK cell progenitors would also be able to develop into ILC within the thymus. Therefore, we first investigated the presence of ILC subsets in the human thymus. We observed that human thymus contained ILCs at a frequency of approximately 1 in 100,000 total thymocytes. All ILC subsets, ILC1, ILC2, and ILC3 (both $NKp44^+$ and $NKp44^-$) were present (**Figures 1A,B**) and that all subsets expressed higher levels of Id2 as compared to $CD34^+CD1a^-$ thymic progenitor cells (**Figure 1C**).

Id2 Induces Differentiation of CD161-Expressing ILCs

Previously, we have demonstrated that thymic progenitors that overexpress Id2 develop into NK cells in response to IL-15 (15). Here, we investigated the effect of forced expression of Id2 in thymic precursors on ILC development. Id2-transduced $CD34^+CD1a^-$ cells were cocultured with OP9 mouse stromal cells in the presence of IL-7, SCF, and FLT3L. We observed that Id2 increased the development of ILC-like cells that expressed CD127, CD161, and integrin α4β7 (**Figures 2A,B**), both in proportion as well as in absolute cell numbers. Consistent with our previous report (15), Id2 inhibited differentiation into T cells and pDC, showing that the balance between E-proteins and Id2 is important for the cell lineage decision between T cells, pDC, and NK/ILC cells (14, 15, 17). Like CD127, CD5 was expressed on virtually all thymic progenitor cells and no difference in expression of these antigens was observed between control and Id2 conditions (**Figure 2A**). Thus, Id2 transduction resulted in the expansion of a lineage (CD1a, CD3, CD4, CD8, CD94, BDCA2) negative $CD5^+CD161^+CD127^+$ cell population resembling ILCs (**Figure 2A**; Figure S1A in Supplementary Material). While

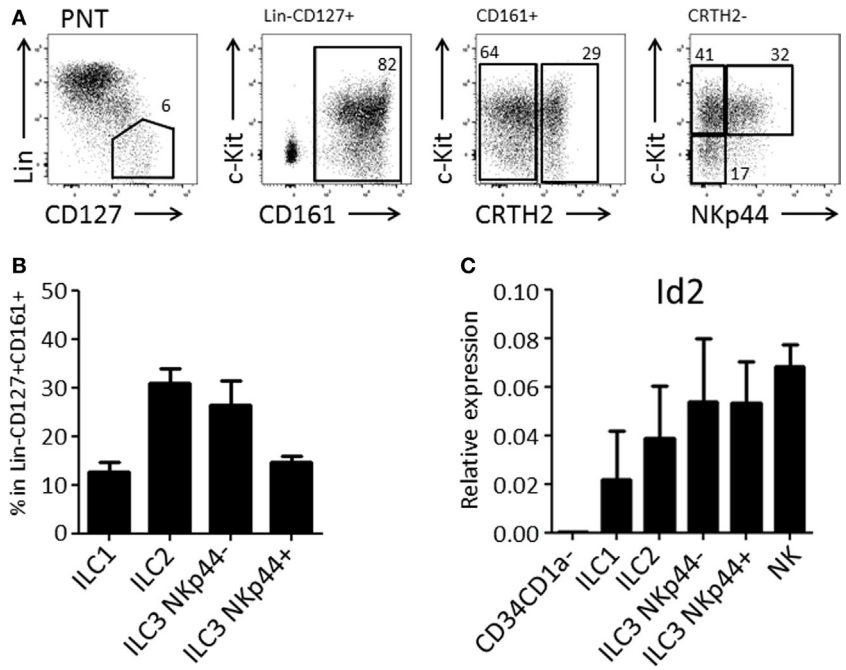

FIGURE 1 | Human postnatal thymus (PNT) contains all Innate lymphoid cell (ILC) subsets. **(A)** Gating strategy by flow cytometry of thymic ILC subsets. CD161 MACS-enriched thymocytes were stained with Lineage (CD1a, CD3, CD4, CD8, CD14, CD16, CD19, CD34, CD94, BDCA2, TCRαβ, TCRγδ, FcεRI), CD127, CD161, CRTH2, c-Kit, and NKp44. **(B)** Frequency (%) of ILC subsets in Lin$^-$CD127$^+$CD161$^+$ ILC population. The data shown are average of five donors. **(C)** qPCR analysis of Id2 mRNA expression in thymic ILC subsets. The data shown are average of two donors. CD34$^+$CD1a$^-$ progenitor cells and NK cells were isolated from PNT and used for negative and positive controls, respectively.

all control transduced ILCs expressed c-Kit, only a fraction of the *in vitro* generated Id2$^+$ ILCs expressed c-Kit, whereas no CRTH2$^+$ ILC2 or NKp44$^+$ ILC3 were detected (Figure S1A in Supplementary Material). This CD5$^+$ ILC-like cell population was also generated on OP9 cells expressing the Notch ligands Jagged1 (Jag1), Jagged2 (Jag2) or delta-like 1(DL1), with the highest expansion rate when cells were cultured on OP9-Jag1 (Figures S1A,B in Supplementary Material), indicating that the Notch ligand Jag2 can affect *in vitro* expansion of Id2$^+$ ILCs, but Notch ligands have little effect on their phenotype. Id2$^+$(mCherry$^+$) Lin$^-$CD127$^+$CD161$^+$ cells can be divided into three populations based on their CD5 and α4β7 expression (**Figure 2C**). The Id2$^+$ Lin$^-$CD127$^+$CD161$^+$ cells expressing both CD5 and α4β7 lacked cell surface (s) expression of CD3, but expressed high intracellular (ic) CD3 staining, while the cells with CD5, but no α4β7 showed lower icCD3 staining. Our data indicate that whereas Id2 inhibits TCR rearrangement and expression, induction of intracellular expression of CD3 is not prevented by Id2. As ectopic expression of Id2 completely blocks development of T cells by preventing TCR rearrangements (10, 11), sCD3$^-$icCD3$^+$CD5$^+$ should be non-T cells. CD4, which is expressed on immature single positive T cell progenitors (31), was not detected on Id2$^+$CD5$^+$ ILC (Figure S1D in Supplementary Material). Id2-induced ILCs expressed higher levels of PLZF, Nfil3, and TOX transcripts when compared with CD34$^+$CD1a$^-$ progenitor cells, especially the CD5$^+$α4β7$^-$ ILC population, which had slightly higher expression of PLZF and Nfil3 compared to the CD5$^+$α4β7$^+$ population (**Figure 2D**). Nfil3 and TOX expression were not restricted to ILCs as high expression was also detected in both NK and T cells, but PLZF expression was hardly detected in T cells, consistent with the notion that PLZF expressing Id2$^+$Lin$^-$CD127$^+$CD161$^+$CD5$^+$ cells are indeed ILCs.

The expansion of NK cells by Id2 is strongly increased in the presence of IL-15 (**Figure 2B**). In contrast, no effect of IL-15 was observed on the expansion of the Lin$^-$CD1a$^-$CD5$^+$CD161$^+$CD127$^+$ ILC-like cell population (**Figure 2B**) indicating that differentiation and expansion of Lin$^-$CD1a$^-$CD5$^+$CD161$^+$CD127$^+$ cells are independent of IL-15. To substantiate this finding, we investigated whether the *in vitro* generated CD5$^+$ ILCs have the capacity to develop further into NK cells. We purified the Id2$^+$Lin$^-$CD5$^+$CD127$^+$CD161$^+$ cells generated on OP9-Jag1 and cultured these cells with OP9 cells with or without IL-15. We observed no upregulation of the NK cell markers CD94 or CD56. Furthermore, CD127 was not downregulated by IL-15 (Figure S1C in Supplementary Material). These results indicate that Id2$^+$Lin$^-$CD5$^+$CD161$^+$CD127$^+$ cells are not precursors of NK cells.

CD5$^+$ ILCs Are Present in the PNT and UCB

We next investigated the expression of CD5 on *ex vivo* isolated ILC subsets. We observed that on average 17% of the total thymic ILC population (Lin$^-$CD127$^+$CD161$^+$) expressed CD5 (**Figure 3A**). Similar percentages of CD5$^+$ ILCs were detected in UCB (**Figure 3B**). The CD5 expression level on ILCs was lower compared to T cells (Figure S2B in Supplementary Material). In

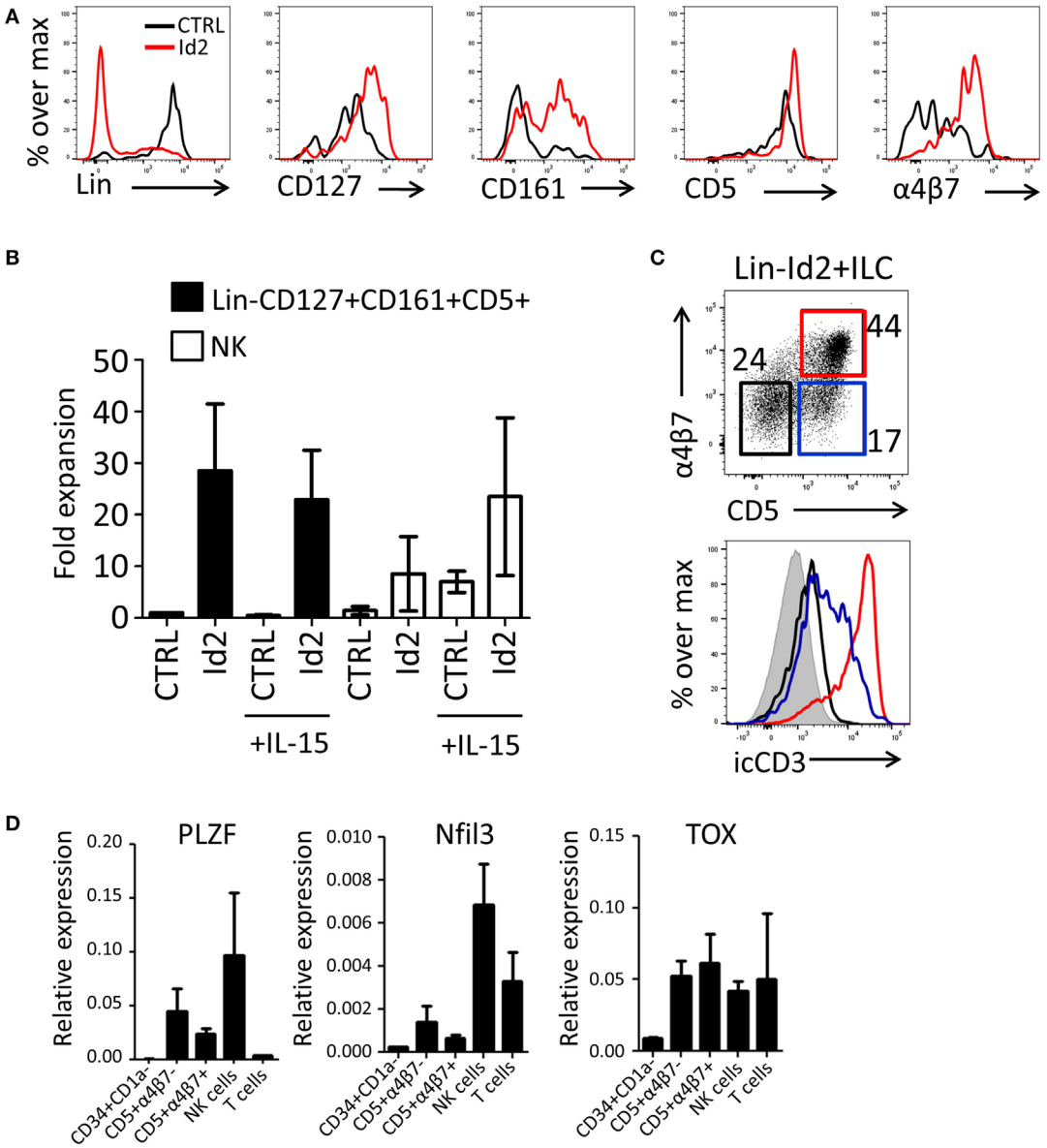

FIGURE 2 | Id2 induces development of Lin⁻CD127⁺CD161⁺CD5⁺ cells from PNT CD34⁺CD1a⁻ cells. **(A)** Cell surface molecule expression on control or Id2-transduced cells after 7 days cultured on OP9 cells. **(B)** Fold expansion of control or Id2-transduced Lin⁻CD127⁺CD161⁺CD5⁺ and NK cells with or without IL-15. The cell number of control transduced Lin⁻CD127⁺CD161⁺CD5⁺ cells in the culture without IL-15 was set as 1. NK cells were determined by their cell surface expression of CD56 and low CD127. The data shown are an average of three independent experiments at day 13 of OP9 coculture. **(C)** Intracellular CD3 (clone OKT3) staining of Id2⁺Lin⁻CD127⁺CD161⁺ cells generated on OP9-Jag1 after 7 days. The population is further divided based on the expression of surface CD5 and α4β7. icCD3 histogram indicated in red: CD5⁺α4β7⁺, blue: CD5⁺α4β7⁻, black: CD5⁻α4β7⁻, gray filled: isotype control. **(D)** qPCR analysis of promyelocytic leukemia zinc finger (PLZF), Nfil3, and TOX mRNA expression in Id2⁺Lin⁻CD127⁺CD161⁺CD5⁺ cells generated on OP9–Jag1 and sorted at day 7 of coculturing. PNT CD34⁺CD1a⁻ cells and NK cells were used as negative and positive controls, respectively. All qPCR values presented are relative to GAPDH expression.

contrast to *in vitro* generated Id2⁺CD5⁺ ILCs, a small proportion of *ex vivo* CD5⁺ ILCs expressed CRTH2 and NKp44 in addition to c-Kit, which indicates that CD5 can be expressed on all ILC subsets (**Figure 3B**). In addition, we observed a minor population of α4β7⁺ cells among CD5⁺ ILCs (Figure S2A in Supplementary Material). We tested CD5⁺ ILCs from thymus and UCB for ILC related gene expression by qPCR. Similar to CD5⁻ ILCs, CD5⁺ ILCs expressed Id2 and PLZF (**Figures 3C,D**). In addition, representative transcription factors for each ILC subset, namely T-bet (ILC1), GATA3 (ILC2) and RORγt (ILC3), were expressed at similar levels in the UCB CD5⁻ and CD5⁺ ILCs (**Figure 3D**). In contrast to CD5⁺ ILCs, CD5⁺CD1a⁺ thymic T cell progenitors lacked Id2 and PLZF (Figure S2C in Supplementary Material) consistent with the notion that CD5⁺ ILCs are not T cell precursors. We next considered the possibility that CD5⁺ ILCs are in fact mature T cells, which have downregulated their T cell receptor

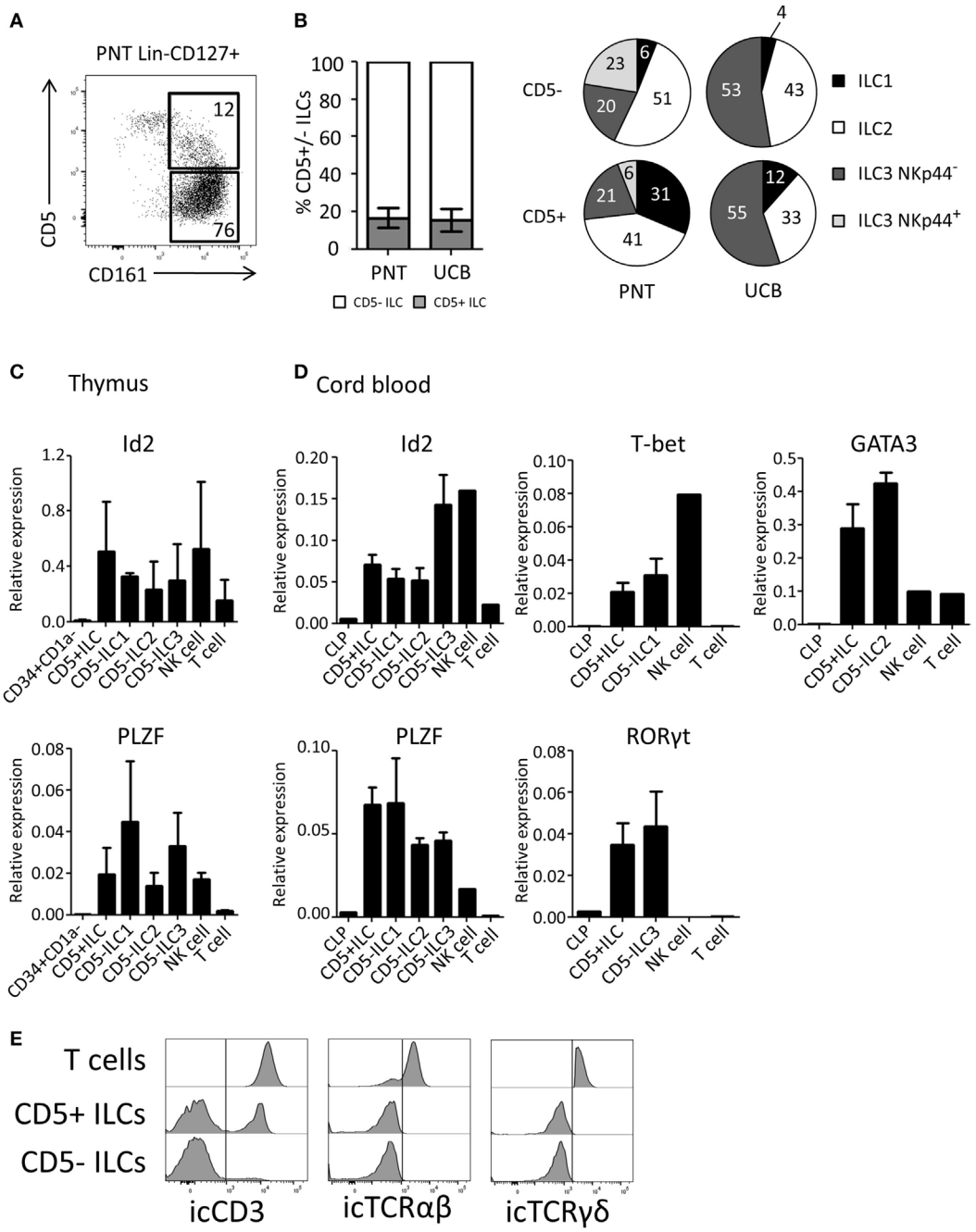

FIGURE 3 | CD5⁺ ILCs are present in the postnatal thymus (PNT) and umbilical cord blood. **(A)** Flow cytometry of PNT Lin⁻CD127⁺ showing CD5⁺ ILCs **(B)** Percentage of CD5⁺ cells within total innate lymphoid cell (ILC) population (Lin⁻CD127⁺CD161⁺) in PNT and umbilical cord blood (UCB). Pie chart indicates the proportion of ILC subsets in the CD5⁺ or CD5⁻ fraction of PNT and UCB. Summary of PNT $n = 4$ and UCB $n = 5$ **(C)** Id2 and PLZF mRNA expression levels CD5⁺ ILCs and CD5⁻ ILC subsets isolated from PNT were determined by qPCR. CD34⁺CD1a⁻ progenitors, NK cells and T cells were isolated from the thymus and used as a reference. The data shown are average of three donors. **(D)** Id2, PLZF, T-bet, Gata3, and RORγt mRNA expression levels in CD5⁺ ILCs and CD5⁻ ILC subsets isolated from UCB were determined by qPCR common lymphoid progenitor (CLP), NK cells, and T cells isolated from UCB were used as a reference. The data shown are average of three donors. All qPCR values presented are relative to GAPDH expression. **(E)** UCB CD5⁺ and CD5⁻ ILCs were intracellularly stained with anti-CD3 (clone OKT3), anti-TCRαβ, and anti-TCRγδ antibodies. T cells isolated from UCB were used as positive control. icTCRγδ histogram shown is gated on icTCRγδ positive fraction of control T cells.

as CD5 is commonly known to be expressed on mature T cells (32). We found, however, that although part, but not all, of CD5⁺ ILCs from UCB expressed icCD3, no icTCRαβ or icTCRγδ expression was detected (**Figure 3E**), enforcing the notion that CD5⁺CD127⁺CD161⁺ cells that also express Id2 and PLZF (which was not expressed in UCB T cells) are ILCs and not T cells.

Thymic and Cord Blood CD5$^+$ ILCs Are Functionally Immature

To assess their ability to produce cytokines, CD5$^+$ ILCs from thymus were sorted and stimulated with PMA/Ionomycin and analyzed for cytokine gene expression by qPCR (**Figure 4A**). CD5$^-$ ILC subsets were also isolated from the thymus and used as a reference. Notably, CD5$^+$ ILCs did not express any of the ILC signature cytokine genes after stimulation, whereas CD5$^-$ ILC1 expressed IFNγ, CD5$^-$ ILC2 expressed IL-5 and CD5$^-$ ILC3 expressed IL-17A and IL-22. All ILCs, including CD5$^+$ ILCs, expressed IL-2, but at lower levels compared to T cells isolated from tonsils (Figure S3A in Supplementary Material). We further investigated the expression of cytokine-encoded genes by UCB CD5$^+$ ILCs. Similarly to thymic CD5$^+$ ILCs, UCB CD5$^+$ ILC either did not respond to stimulation or expressed lower levels of cytokine genes than CD5$^-$ ILC (**Figure 4B**). ILC3 related cytokine gene expression was low in both CD5$^+$ and CD5$^-$ ILC, which is in line with our observation that adult peripheral blood ILC3s lack the ability to produce significant amounts of IL-17A and IL-22 upon activation with PMA and ionomycin (Figure S3B in Supplementary Material). IL-2 expression was also low in all UCB ILCs compared to T cells from tonsil (Figure S3A in Supplementary Material). Hence, CD5$^+$ ILCs appear to be functionally immature as compared to CD5$^-$ ILCs.

UCB CD5$^+$ ILCs Differentiate into CD5$^-$ ILCs

Given that CD5$^+$ ILCs presented as functionally immature ILCs upon stimulation, we asked whether CD5$^+$ ILCs may be precursors of CD5$^-$ ILCs. To test the differentiation capacity of CD5$^+$ ILCs, total CD5$^+$ ILCs were highly purified from cord blood and cultured for 7 days in the presence of IL-2 and IL-7. We observed that part of CD5$^+$ ILCs lost CD5 expression (**Figure 5A**). This differentiation was also induced when the cells were cultured with IL-1β, TNFα, and IL-6, cytokines typically elevated in the inflammatory state. Even though the cells were isolated at high purity, we still cannot exclude the possibility that CD5$^-$ ILCs appearing in those culture conditions are derived from a minor contamination of CD5$^-$ ILCs. Thus, we deposited single CD5$^+$ ILCs in wells of a microtiter culture plate and generated clones in the presence of irradiated allogenic peripheral blood mononuclear cells and JY cells (feeder cells), IL-2 and IL-7. After 3 weeks of culturing, 65 out of 360 wells showed cell growth equivalent to 17% plating efficiency, making it highly likely that the starting cell number was indeed 1 cell per well as previously demonstrated (33). Among those 65 clones, 5 clones exhibited sCD3 expression (data not shown) and were excluded from further analysis. There were three types of CD5$^+$ ILC-derived clones observed; those which remained CD5$^+$, those which gave rise to both CD5$^+$ and CD5$^-$ ILCs, and those which became CD5$^-$ ILCs (**Figure 5B**). The frequency of those three phenotypes was equally distributed among the clones. This result strongly indicates that CD5$^+$ ILCs can indeed differentiate into CD5$^-$ ILCs, and confirmed that CD5$^-$ ILCs appearing in differentiation and proliferation assays were derived from CD5$^+$ ILCs.

UCB CD5$^-$ ILC2s Are Functionally Competent and Derive from CD5$^+$ ILC2s

To assess the functionality of CD5$^-$ ILCs derived from CD5$^+$ precursors, we cultured highly purified cord blood CD5$^+$ ILC2 with feeder cells in the presence of IL-2 and IL-7 (**Figure 6A**) and after 2 weeks determined the production of cytokines and expression of cytokine transcripts by CD5$^+$ and CD5$^-$ ILC2s following PMA/ionomycin stimulation. A higher frequency of cytokine-producing cells in the CD5$^-$ fraction compare to the CD5$^+$ fraction was observed and cytokine gene expression levels of CD5$^+$ ILC2s and CD5$^-$ ILC2s, derived from CD5$^+$ ILC2s, were significantly different (**Figures 6A,B**). To further corroborate that CD5$^+$ ILC2 can differentiate into mature CD5$^-$ ILC2 we performed single cell cloning experiments starting from ILCs that expressed CRTH2$^+$ (**Figure 6C**). Consistent with the previous experiments, most of the clones had lost CD5 and were able to produce type 2 cytokines upon stimulation with PMA and ionomycin. In addition to type 2 cytokines, we have also tested type 1 (IFN-γ) and type 3 (IL-17A and IL-22) cytokines. There were no IFN-γ nor IL-17A producing clones observed, suggesting that CD5$^+$ LC2s are ILC2 committed precursors. We observed that many clones produced IL-22 consistent with earlier observations on cultured ILC2 from peripheral blood (34). These results together indicate that CD5$^+$ ILC2s can differentiate into functionally competent CD5$^-$ ILC2.

DISCUSSION

Our data show that expression of Id2 in human progenitor cells favors development of ILCs while inhibiting T cell and pDC development (10, 11, 14, 15, 17). One of the mechanisms by which Id2 inhibits T cell development is preventing TCR gene rearrangements (10). As CD5 was described to be a marker that distinguishes T cells from ILCs (35) it was unexpected to observe that under conditions in which TCR gene rearrangements and thus T cell development is blocked, CD5$^+$ ILCs are generated. These CD5$^+$ ILCs cannot be T cells that have downregulated the expression of TCR, because Id2 prevents TCR gene rearrangements (10). CD5 expression on ILCs that express ectopic Id2 is not an *in vitro* artifact, because CD5$^+$ ILCs were also found when isolated *ex vivo* from thymus and UCB. These cells are distinct from CD5$^+$ T-cell progenitors as they express Id2, which precludes T-cell development, and lack expression of CD1a (12). Moreover, CD5$^+$ ILC expressed markers that are characteristic of ILCs, including CD127, CD161, Id2, and PLZF. As it was shown that Id2 promoted expression of PLZF during invariant NKT cell development in the mouse (36), our findings support the notion that Id2 may regulate the expression of PLZF to drive human ILC lineage development, but it should be noted that PLZF may be differentially regulated in mouse and human ILC, because unlike mouse ILCs (24) PLZF is also expressed in mature ILCs in humans (37). A proportion of CD5$^+$ ILCs express icCD3, similar to the CD5$^+$ ILC generated from Id2 overexpressing progenitor cells, but did not express TCRαβ or TCRγδ proteins in the cytoplasm consistent with the fact that Id2 inhibits TCR gene rearrangement. Together with our data of Id2 overexpression, we conclude

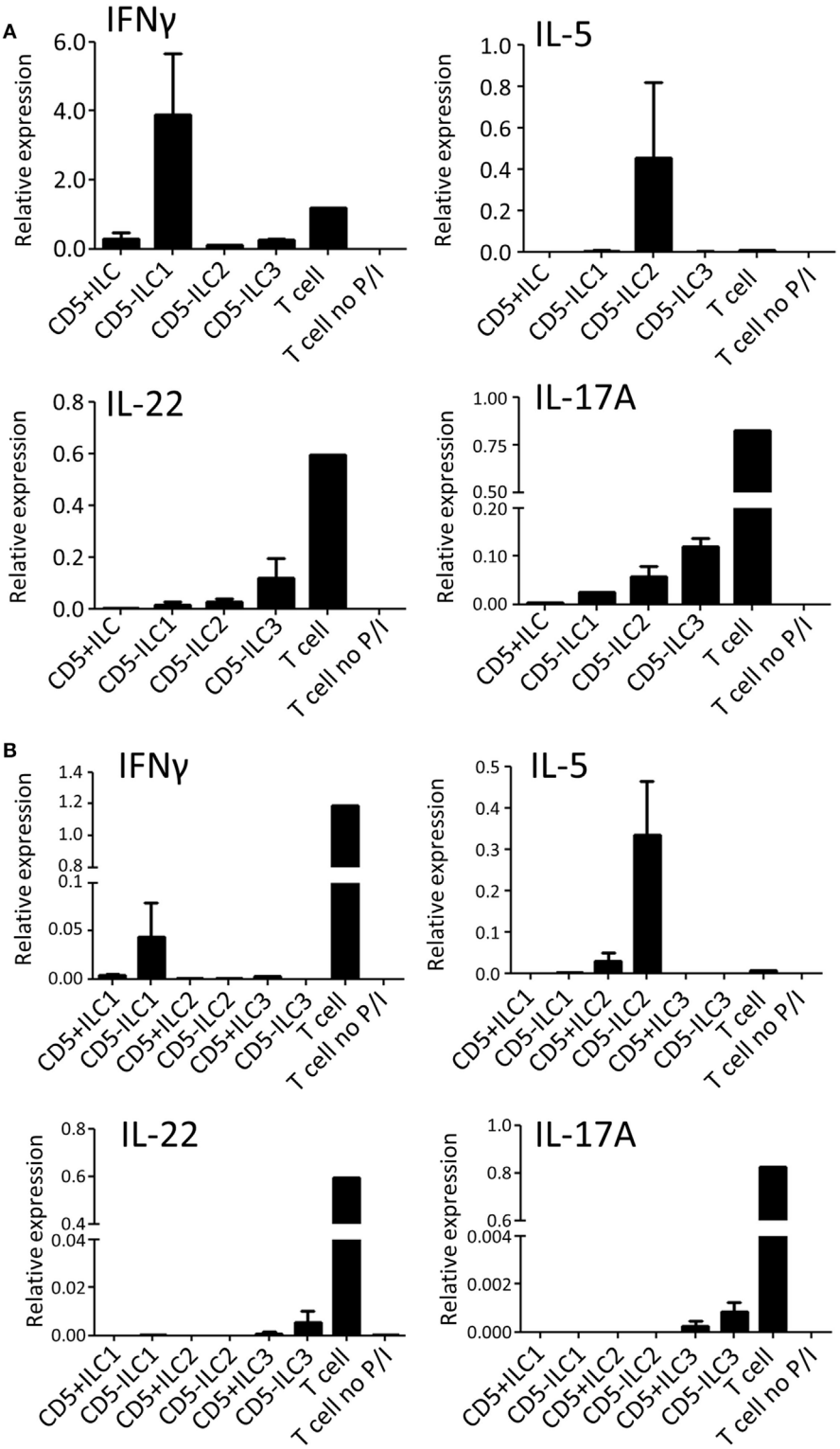

FIGURE 4 | CD5⁺ ILCs display a functionally immature phenotype. **(A)** qPCR analysis of cytokine mRNA expression levels of total PNT CD5⁺ ILC compared to CD5⁻ ILCs after 6 h PMA/ionomycin (P/I) stimulation. Tonsil T cells were used as stimulated and unstimulated references. The data shown are average of two independent experiments. **(B)** qPCR analysis of cytokine mRNA expression levels of UCB CD5⁺ ILC subsets compared to CD5⁻ ILC subsets after 6 h P/I stimulation. Tonsil T cells were used as stimulated and unstimulated references. The data shown are average of two independent experiments. All qPCR values presented are relative to GAPDH expression.

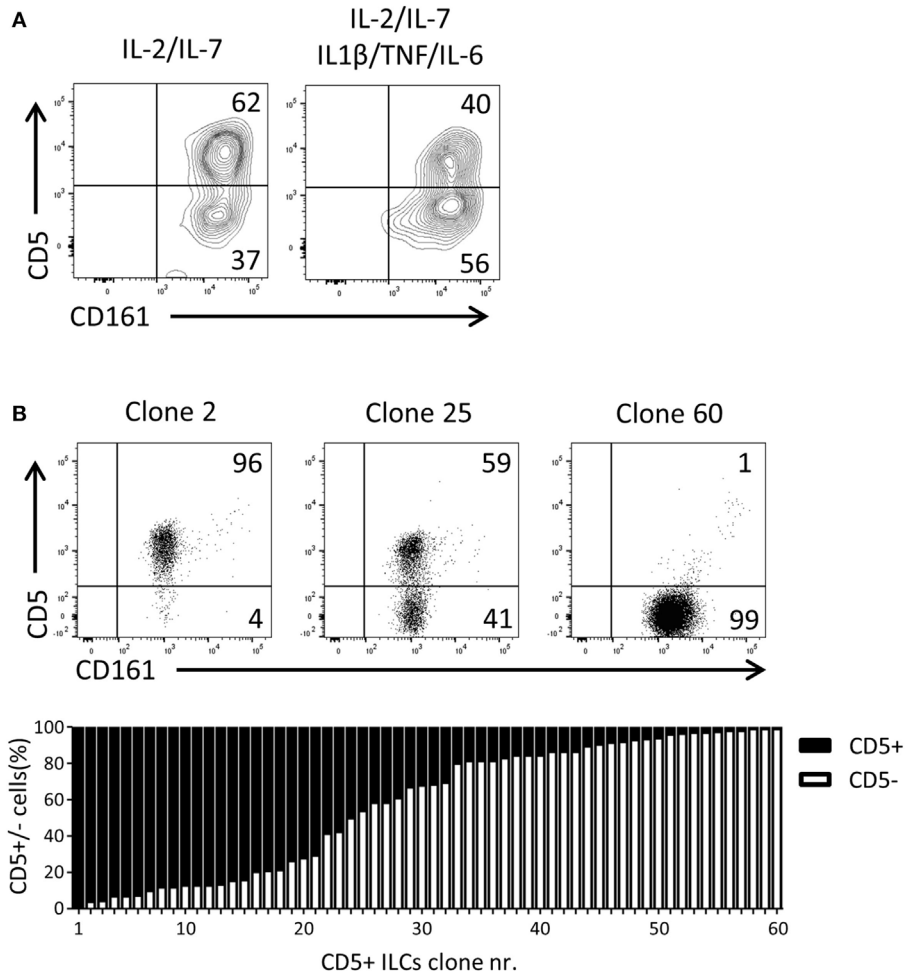

FIGURE 5 | UCB CD5$^+$ ILCs differentiate into CD5$^-$ ILCs. **(A)** Flow cytometry analysis of CD5$^+$ ILC phenotype after 7 days in culture with indicated cytokines. The plots showed here is pre-gated for CD3$^-$CD94$^-$ cells. CD5$^+$ ILCs were isolated from UCB and cultured with IL-2 (10 U/ml) and IL-7(10 ng/ml) or IL-2, IL-7, IL-1β (50 ng/ml), IL-6 (50 ng/ml), and TNFα (50 ng/ml). The data shown are a representative of four independent experiments. **(B)** Flow cytometry analysis of three representative CD5$^+$ ILC clones (top), frequency of CD5 expression (bottom).

that CD5$^+$ ILCs are not T cells in disguise, but rather are *bona fide* ILCs. Further confirmation is provided by the observation that almost all clones derived from CD5$^+$ ILCs did not express the TCR/CD3 complex on their cell surface. *In vitro* generated Id2$^+$CD5$^+$ ILCs did not further differentiate into NK cells in the presence of IL-15, a cytokine known to be essential for NK cell development (38, 39), suggesting that Id2$^+$CD5$^+$ ILCs are downstream of bipotent NK/ILCp. About half of the *in vitro* generated CD5$^+$Id2$^+$ ILC expressed α4β7 that is also expressed on mouse common ILCp (23), suggesting that CD5 is induced early in ILC development.

Close inspection of CD5 expression on thymic and UCB ILCs revealed that CD5 is also expressed on a minority of ILCs that have hallmarks of ILC2 and ILC3. *Ex vivo* CD5$^+$ ILC1, ILC2, and ILC3 were unable to produce cytokines after stimulation with PMA and ionomycin, suggesting that they are functionally immature. Indeed, functional studies and cloning experiments clearly demonstrate that CD5$^+$CRTH2$^+$ ILC2, which produced very low amounts of type 2 cytokines can differentiate into CD5$^-$ ILC2 capable of producing high amounts of cytokines. Together, our data indicate that CD5 is expressed on early ILC progenitor cells and on immature ILC1, ILC2, and ILC3. Functional maturation is accompanied by downregulation of CD5. A recent report presented evidence for the existence of c-Kit + multipotent ILCp in peripheral blood, which differentiates into all cytokine-producing ILC subsets (26). These cells were selected for absence of CD5. However, because CD5$^+$ ILCs express one log less CD5 than T cells (Figure S2B in Supplementary Material), it might be possible that Lim et al. by adding anti-CD5 to their antibody cocktail to remove non-ILCs, depleted CD5$^+$ T cells, but not CD5$^+$ ILC.

CD5$^+$ ILCs are present in the adult peripheral blood, spleen, lung, and bone marrow (data not shown) indicating that immature CD5$^+$ ILCs are not restricted to infants. We observed that CD5$^+$ ILCs were not present in inflamed nasal polyps (data not shown) of patients suffering chronic rhinosinusitis with nasal polyps in which ILC2 accumulate (30, 34), suggesting that immature CD5$^+$

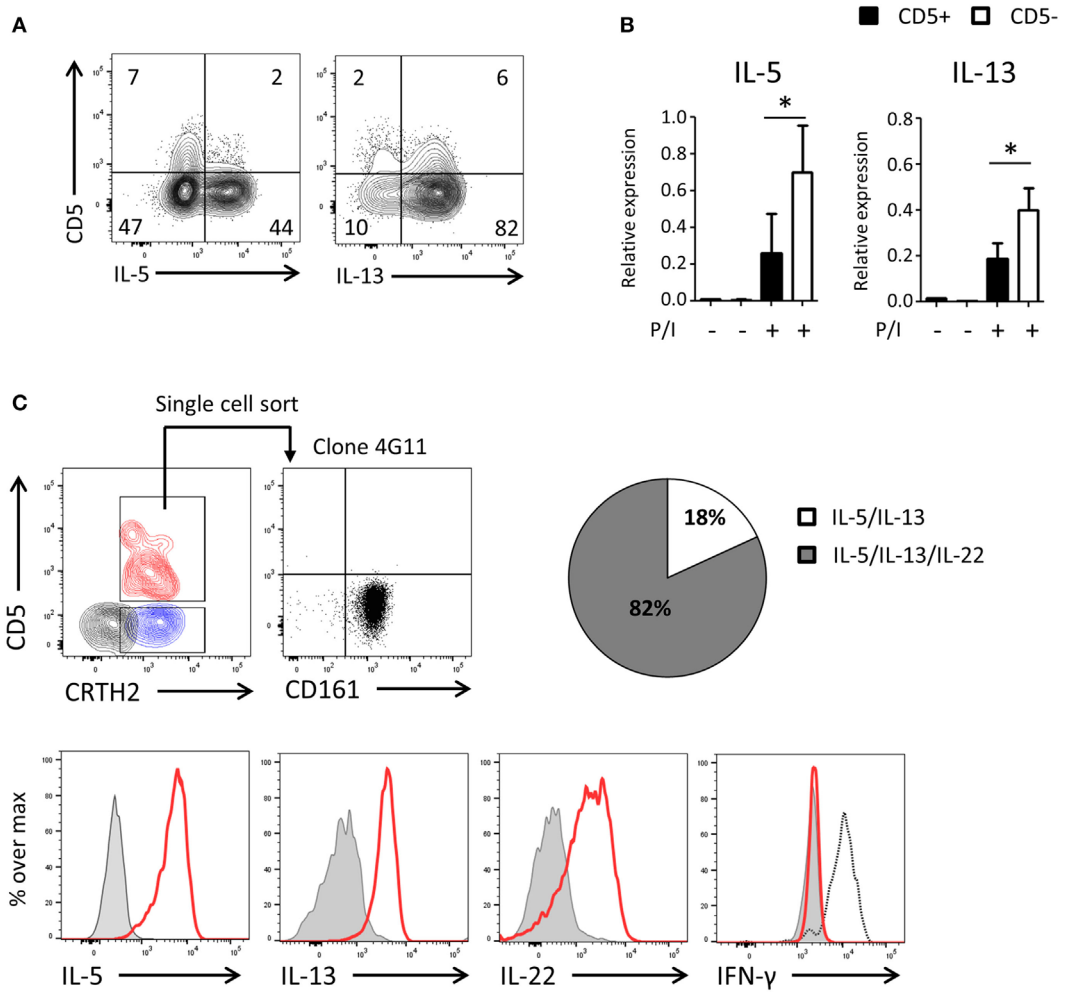

FIGURE 6 | UCB CD5⁻ ILC2s are functionally competent and derive from CD5⁺ ILC2s. **(A)** Cytokine production of bulk CD5⁺ ILC2 after 2 weeks of coculturing with the irradiated allogenic peripheral blood mononuclear cells and JY cell (feeder cells) with IL-2 (100 U/ml) and IL-7 (10 ng/ml). Total cells were stimulated with P/I for 6 h. **(B)** qPCR analysis of cytokine expression level of CD5⁺ ILC2 derived CD5⁺ ILC2s and CD5- ILC2s. Cells were sorted from the feeder cells coculture with IL-2 (100 U/ml) and IL-7 (10 ng/ml) after 2 weeks and unstimulated or stimulated with P/I for 6 h ($n = 5$). *$P < 0.05$ (Student's *t*-test) **(C)** Single CD5⁺ CRTH2⁺ ILC2 cell was sorted and cultured in feeder cells with IL-2 (100 U/ml) and IL-7 (10 ng/ml) for 2 weeks and cytokine production was evaluated after stimulated with P/I for 6 h. Counter plot (left top): CD5⁺ CRTH2⁺ ILC2 (red), CD5⁻CRTH2⁺ ILC2 (blue) and CD5⁻ CRTH2⁻ ILC1 (black). Dotplot (right top): CD5 and CD161 expression on one representative clone (4G11). Histogram: clone (red), isotype control (gray filled), and IFN-γ positive control (black dashed). Pie chart indicate the frequency of clones producing IL-5 and IL-13 or IL-5 and IL-13 and IL-22.

ILCs fully differentiate into mature CD5⁻ ILCs under inflammatory conditions. This notion is supported by our observation that, pro-inflammatory cytokines IL-1β, IL-6, and TNF-α promoted differentiation of CD5⁺ ILCs into CD5⁻ ILCs *in vitro*.

It has been reported that SCID patients with IL-2 receptor gamma or JAK3 deficiency not only lack T cells, B cells, and NK cells, but also ILC subsets. After hematopoietic stem cell transplantation in these patients, T cells, B cells, and ILC1—but no other ILC subsets including NK cells—were reconstituted (40). Interestingly, these reconstituted ILC1 expressed CD5. Given our suggestion that CD5 expression represents an immature stage of ILCs, this may indicate that the CD5⁺ ILC1s in these patients are arrested from further differentiation into mature ILCs. Recently CD4⁺ ILC1 were found in peripheral blood (41). We confirmed those findings and found that those CD4⁺ ILC1 also expressed CD5 [(42) and Nagasawa, unpublished observation]. However, in contrast, the cord blood and thymic CD5⁺ ILCs described here, which express CD161, lacked CD4. The relationship between the immature CD5⁺ ILCs described here and the CD4⁺ CD5⁺ ILC1 described previously has yet to be fully established.

ETHICS STATEMENT

The use of postnatal thymus (PNT) tissue and umbilical cord blood (UCB) was approved by the Medical Ethical Committee of the Academic Medical Center. Thymocytes were obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery and UCB was collected

with informed consent of the patients in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MN designed the study, did experiments, analyzed the data, and wrote the manuscript; KG did experiments, analyzed the data, and wrote the manuscript; BB analyzed the data and wrote the manuscript; HS designed the study, analyzed the data, and wrote the manuscript; and the all authors critically read the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01047/full#supplementary-material.

FIGURE S1 | **(A)** Control or Id2$^+$(mCherry$^+$) cells cultured on OP9, OP9–Jag1, OP9–Jag2, and OP9–DL1 were gated as CD1a$^-$CD3$^-$CD4$^-$CD8$^-$CD94$^-$BDCA2$^-$ and further analyzed for their expression of CD127, CD161, CD5. c-Kit, CRTH2, and NKp44 expression were analyzed on Id2$^+$Lin$^-$CD127$^+$CD161$^+$CD5$^+$ cultured on OP9 cells. The data shown here are representative of two independent experiments at day 14. **(B)** Fold cell expansion rate of mCherry$^+$Lin$^-$CD127$^+$CD161$^+$CD5$^+$ cells. Control (CTRL) or Id2-transduced PNT CD34$^+$CD1a$^-$ progenitor cells were cocultured with OP9 cells expressing/not expressing Notch ligands [Jagged1 (Jag1) or Jagged2 (Jag2) or Deltalike1 (DL1)] for 2 weeks, the expansion rate was determined at day 7 and day 14 after start of the culture. **(C)** Id2$^+$Lin$^-$CD127$^+$CD161$^+$CD5$^+$ cells generated on OP9-Jag1 were sorted at day7 and cultured for a further 7 days on OP9 cells with IL-7, SCF, and Flt3L, with or without IL-15, and analyzed for their NK cell markers. Data shown is one representative of two independent experiments. **(D)** CD4 staining of Id2$^+$Lin$^-$CD127$^+$CD161$^+$ cells. Data shown are one representative of two independent experiments.

FIGURE S2 | **(A)** Flow cytometry of thymic innate lymphoid cells (ILCs) showing the expression of CD5 and α4β7. **(B)** Flow cytometry of CD161 MACS-enriched cord blood ILCs (red) and T cells (black) showing the expression of CD5. **(C)** qPCR analysis of Id2 and promyelocytic leukemia zinc finger (PLZF) mRNA expression levels in thymic CD34$^+$CD1a$^+$ cells. NK cells and T cells isolated from the thymus were used as a reference. The data shown are average of three donors.

FIGURE S3 | **(A)** qPCR analysis of IL-2 gene expression level of total PNT CD5$^+$ ILC compared to CD5$^-$ innate lymphoid cells (ILCs) after P/I stimulation. Tonsil T cells were used as stimulated and unstimulated references. **(B)** qPCR analysis of cytokine mRNA expression levels in adult peripheral blood CD5$^+$ ILCs compared to CD5$^-$ ILC subsets after P/I stimulation. The data shown are average of four donors. All the qPCR values presented are relative to GAPDH expression.

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