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Innate lymphoid cell development, migration, and function

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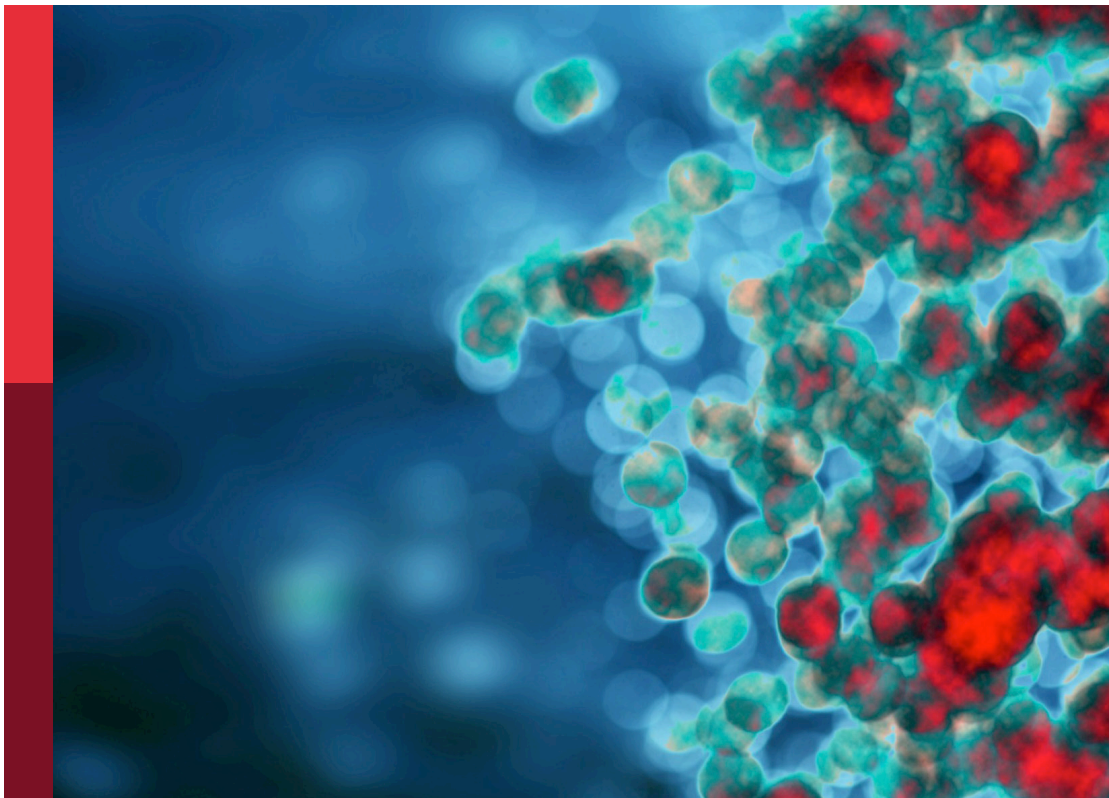

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Innate lymphoid cell development, migration, and function

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Editorial: Innate lymphoid cell development, migration, and function

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innate lymphoid cell (ILC), natural killer (NK), pathology, homeostasis, heterogeneity, progenitor

Editorial on the Research Topic
Innate lymphoid cell development, migration, and function

Innate lymphoid cells (ILCs) represent a subset of immune cells derived from a common lymphoid progenitor. They share characteristics with classical lymphoid T cell subsets but are devoid of the antigenic presentation requirement for their activation. These cells express transcriptional regulators and effector cytokines similar to T cell subpopulations and, based on their molecular expression profile are classified into three groups: ILC1 (including NK cells), ILC2 and ILC3 (including LTi cells) (1, 2). Typically, ILCs reside in peripheral tissues and function as sentinels for pathological insults while maintaining tissue homeostasis in steady state conditions. Over the last decade, a broad spectrum of physiological conditions where ILC cells have been shown to perform critical functions has been demonstrated and growing evidence suggest they play a key, non-redundant and fundamental role in the establishment, maintenance and resolution of immune responses.

The present Research Topic includes 10 reports on key processes controlled by different types of ILCs, ranging from their development to the regulation and contribution in homeostatic and pathogenic contexts.

A broad perspective on the role of ILC subsets in different pathological contexts is discussed in two reviews by Clottu et al. and Yang et al. The first group describe functional and quantitative differences observed in ILCs in autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, antineutrophil cytoplasm antibody-associated vasculitis and systemic sclerosis; they also suggest the use of these cells as potential diagnostic markers and therapeutic targets. On the other hand, Yang et al. discuss cumulative evidence linking different ILC groups with the protective and pathological aspects of myocardial infarction (MI), atherosclerosis, myocardial ischemia reperfusion-injury and repair and regeneration of hearth tissue after MI. They conclude that further

studies are required to dissect the contribution of different types of ILCs to be able to explore and validate their therapeutic potential in these pathologies. Lastly, a minireview by Nagashima and Iyoda expands on the possible role of ILC2s in the regulation and maintenance of renal physiology, discussing both positive and negative effects of these cells in different aspects of renal function and homeostasis maintenance.

NK cells were the first subset of ILCs described (3). They represent the cytotoxic subpopulation among ILC and accordingly, secrete perforin, granzyme and proinflammatory cytokines. A key feature of immune cells is their systemic distribution being regulated at the molecular level by different proteins coordinating migration, cytoskeletal changes, and adhesion. Myosins are some of the motor proteins involved in these processes and Cruz-Zárate et al. provide a comprehensive view of their potential role in NK cells by performing a broad analysis using public transcriptomic databases and evidence reported in other immune subsets. NK cell therapy is a promising field. Therefore, Ramos-Mejía et al. summarize the novel and functional attributes of NK cells as well as the integration of inhibitory and activation signals in different physiological contexts to exploit their therapeutic potential. Finally, by using hematopoietic specific genetic depletion of FOXO1 and FOXO3, Luu et al. explore how these transcription factors affect NK development and other non-cytotoxic ILC subsets and identify some of the molecular targets regulated by these molecules. They confirm FOXO1 and FOXO3 as transcriptional regulators controlling NK precursor commitment and development.

Growing evidence suggest a fundamental role of ILCs for tissue homeostasis. However, the current knowledge regarding the ontogeny and homing of ILC subsets to peripheral tissues, as well as specific markers able to discriminate between precursors with different origin and homing capacities *in vivo*, is still limited. In this Research Topic, Alisjahbana et al. identify CD5 as a marker of a particular subset of human ILCs present in blood and lung of humanized mice transferred with human CD34+ Hematopoietic Stem and Progenitor Cells (HSPCs) that seem to derive from a different progenitor, as suggested by its homing capacities. CD5 positive ILCs in the lung have a cytokine profile resembling ILC1s and phenotypic markers associated with ILC precursors. Given their presence in the blood, the authors propose that these cells could represent an ILC sentinel subset ready to migrate to the lung upon antigenic challenges. An important limitation to study human ILCs is the lack of efficient protocols to differentiate these cells *in vitro*. In this Research Topic, Bennstein et al. describe a method to differentiate IL-22-secreting ILC3 cells derived from CD34+ HSCPs supported by human mesenchymal stem cells (MSCs). This protocol allows for the generation of ILC3s with a molecular signature that faithfully recapitulates phenotypic hallmarks of bonafide IL-22-expressing ILC3 cells, including the expression of ROR-γt, AHR, ID2, and CD117, among others.

Finally, a detailed phenotypic characterization of ILC2s, their plasticity to respond and adapt to different microenvironments, as well as its tissue-specific heterogeneity is compiled by Olguín-Martínez et al., who emphasize the importance of using appropriate markers to identify those cells considering the pathological context and their anatomical localization. Such heterogeneity and plasticity is not restricted to ILC2s and should also be considered when studying other ILCs (4). An additional original research by the same authors uses a helminth infection mouse model to describe for the first time the phenotypic markers associated with IL-9-expressing ILC2s from the infected intestine, a tissue previously unappreciated given the difficulty to isolate and study viable ILCs. The authors then describe a protocol to generate bonafide murine ILC2s *in vitro* and identify IL-33 and the PKA pathway as key signals driving IL-9 expression and regulating functional responses in these cells.

The collection presented in this Research Topic highlights the progress made to understand and characterize the phenotype and function of ILCs in different contexts and tissues (5). It also provides new methods to study these cells *in vitro* and calls for the need of future studies to analyze their contribution and relevance in homeostatic and pathological conditions in order to explore their potential as therapeutic targets.

Author contributions

PL-L, the guest editor, and the co-editors JH-M and JCC invited expert contributions and handled peer review. PL-L wrote the editorial with input from JH-M and JCC. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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The Roles of Kidney-Resident ILC2 in Renal Inflammation and Fibrosis

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Innate lymphoid cells (ILCs) are a recently discovered lymphocyte population with high cytokine productive capacity. Type-2 ILCs (ILC2s) are the most studied, and they exert a rapid type-2 immune response to eliminate helminth infections. Massive and sustainable ILC2 activation induces allergic tissue inflammation, so it is important to maintain correct ILC2 activity for immune homeostasis. The ILC2-activating cytokine IL-33 is released from epithelial cells upon tissue damage, and it is upregulated in various kidney disease mouse models and in kidney disease patients. Various kidney diseases eventually lead to renal fibrosis, which is a common pathway leading to end-stage renal disease and is a chronic kidney disease symptom. The progression of renal fibrosis is affected by the innate immune system, including renal-resident ILC2s; however, the roles of ILC2s in renal fibrosis are not well understood. In this review, we summarize renal ILC2 function and characterization in various kidney diseases and highlight the known and potential contributions of ILC2s to kidney fibrosis.

Keywords: ILC2, renal fibrosis, CKD - chronic kidney disease, ILCreg, IL-33

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INTRODUCTION

Kidney fibrosis is a critical condition leading to kidney dysfunction and is a common characteristic of chronic kidney diseases (CKDs), which are increasing around the world (1). The major clinical issue in the progression of renal fibrosis is the loss of kidney function, which requires dialysis or kidney transplantation in end-stage renal disease (ESRD) (2). Kidney injuries, such as acute kidney injury (AKI) or glomerulonephritis, contribute to the progression of kidney fibrosis and CKD pathology. Environmental factors including metabolic syndrome, diabetes, and hypertension are also risk factors for renal disease onset and progression. Recently, an AKI-to-CKD continuum has been recognized as a clinical issue that contributes to fibrosis (3). Therefore, the establishment of therapies for renal fibrosis will improve quality of life not only for kidney disease patients but also for various tissue fibrosis patients.

Tissue fibrosis involves several causal factors, such as epithelial- and endothelial-mesenchymal transition and the immune system (4, 5). In the kidneys, fibroblasts in renal stroma transform to myofibroblasts by profibrotic factors such as TGF-β, PDGF, FGF2, and CTGF, and express the myofibroblast-unique markers α-SMA and fibronectin (6–8). These profibrotic factors are considered to derive from inflammation-induced infiltrating macrophages and migrating Tregs that repair tissue damage (9, 10). TGF-β derived from kidney-infiltrated M2 macrophages and Tregs enhances renal fibrosis (10–12). Furthermore, it has been reported that newly identified

innate lymphoid cells, ILCs, are associated with tissue fibrosis, including in the lung, liver, and intestine (13–16). In kidneys, ILC2s have a protective function against AKI and glomerulonephritis, but it remains unclear if they are involved in kidney fibrosis.

As an ILC2-activating cytokine, IL-33 is a member of the IL-1 family and has been recognized as an "alarmin" that is ubiquitously expressed in various tissue cells (17, 18). IL-33 protein is divided into three domains, a nuclear domain, central domain, and IL-1-like cytokine domain (19, 20). IL-33 is constitutively distributed in the nuclei of epithelial cells under basal conditions by binding the histone H2A-2B dimer and chromatin-binding motif within the nuclear domain (21). Upon the initiation of inflammation, stored full-length IL-33 is released quickly from nuclei, and infiltrated inflammatory cell-derived proteases act on the cleavage site in the central domain (22). Cleaved-IL-33 has high activity and binds with ST2 (IL-33 receptor)-expressing cells, leading to the induction of MyD88-IRAK-TRAF signaling for proliferation, survival, and cytokine production (18, 23). IL-33-ST2 signaling is upregulated by various kidney injuries and diseases, leading to the activation of ILC2s in the kidney (24–27); however, it likely depends on the amount of IL-33 whether ILC2s play a protective or progressive role in renal disease. Recent studies have demonstrated that renal ILC2s have pivotal roles in various kidney diseases and tissue fibrosis and repair (28–33), so that these cells are being focused on as a new therapeutic target. Here, we highlight recent findings on renal ILC, especially ILC2s, in kidney disease leading to kidney fibrosis.

ILC SUBSETS

ILCs lack antigen-specific receptors, like T-cell receptors (TCRs) and B-cell receptors (BCRs), and do not express classical immune cell lineage markers. ILCs are activated depending on cytokines in the surrounding tissue microenvironment and play pivotal roles in the protection against infection, inflammation, and in immune-homeostasis (34). ILCs are categorized into three groups depending on their function: ILC1, ILC2, and ILC3, which reflect the acquired immunity of helper T-cell subsets Th1, Th2, and Th17 respectively. T-bet-expressing ILC1 exerts type-1 immune responses for viral infection, GATA3-expressing ILC2s exert type-2 immune responses for helminth infection, and RORγt-expressing ILC3s exert type-17 immune responses for bacterial infection. However, it has been clarified that ILCs are also involved in the pathogenesis of a variety of diseases. In particular, pulmonary ILC2s have critical roles in asthma accompanied with steroid resistance (35, 36). The appropriate regulation of ILCs is therefore important for controlling various diseases and maintaining immune-homeostasis. Recently, a new subset of ILCs, regulatory ILCs (ILCreg), has been reported (37). ILCregs exert immune-suppressive functions by producing IL-10 and TGF-β, similar to Tregs. Although ILC1, 2, and 3 commonly develop from innate lymphoid cell progenitors (ILCP), ILCregs differentiate from common helper innate lymphoid progenitors (CHILP) in an Id3-dependent manner (37). As ILCregs do not express the Treg master regulator foxp3, it is unclear whether ILCregs are an independent subset like Tregs. However, ILCregs have the potential for unique phenotypes and functions in comparison with Tregs, and these will be further investigated in the future.

ILC2's FUNCTION AND REGULATION

ILC2s reside in various tissues such as lung, intestine, mesenteric fat associated lymphoid cluster (FALC), liver, skin, and kidney, and are mainly responsible for helminth elimination mediated by the type-2 immune response. Upon tissue damage by allergen and pathogen exposure, IL-33, IL-25, and TSLP, which are the strongest activating cytokines for ILC2 proliferation and cytokine production, are released from epithelial cells leading to rapid activation of ILC2s. Then, activated ILC2s secret large amounts of type-2 cytokines IL-5 and IL-13, and induce eosinophilic inflammation and mucosal hyperplasia. However, abnormal and sustained ILC2 activation elicits allergic diseases such as asthma, atopic dermatitis, and rhinitis (38–40), and thus it is clinically important to understand ILC2 regulation. In addition, ILC2s also produce amphiregulin (Areg) and IL-9, which contribute to remodeling and repair of tissue damage after inflammation. Areg produced from ILC2s promotes epithelial proliferation and differentiation for epithelial repair (41). Furthermore, IL-9-producing ILC2s help resolve inflammation in rheumatoid arthritis (42).

Although IL-33 and ST2 signaling are critical in ILC2 activation, other stimulations including common γ chain (γc) cytokines (IL-2, -7, -9, 15) and co-stimulatory molecules (ICOS, GITR, PD-1) are required for ILC2 regulation (43–45). Numerous studies have identified positive or negative regulators of ILC2s as follows: cytokines (IL-25, TSLP, IFN-γ, IL-27), neuropeptides (VIP, NMU, CGRP), neurotransmitters (catecholamine, acetylcholine), lipid mediators (prostaglandins and lipoxins from the arachidonic acid pathway), hormones (androgen and estrogen), and nutrients (vitamins A and D and butylate) (46–55). Since ILC2s are distributed in various tissues, it is assumed that tissue-specific regulatory mechanisms and factors of ILC2s exist. We previously reported that the oxidative-stress responder Nrf2 activates lung-ILC2s, and their activation ameliorates lung allergic inflammation (56). Oxidative stresses are frequently generated in kidney injury and disease, and thus renal ILC2s may be regulated by the Keap1-Nrf2 pathway. Taken together, ILC2s are regulated by various factors, and have diverse roles depending on the tissue environment.

RENAL ILC2 AND DISEASES

ILC2s are also resident throughout murine kidneys and are especially localized in renal vasculature. GATA3-expressing ILC2s are the main ILC subset (70~80% of ILCs) in murine kidney, while T-bet-expressing ILC1s and RORγt-expressing ILC3s are less than 10% of ILCs (2 7). Renal ILC2s constitutively express IL-5 and IL-13 under steady-state conditions, and almost

all of the expressed IL-5 is derived from ILC2s and not Th2 (57). Although ILC2s account for approximately 1% of total renal leukocytes, IL-33 expression is upregulated in several kidney disease models, indicating that renal ILC2s are potentially activated and exert unknown functions at both the acute and chronic phases (**Figure 1**).

AKI manifests as acute dysfunction of kidneys, inducing electrolyte imbalance, and is related to CKDs, fibrosis, and cardiovascular diseases. AKIs are caused by traumatic injury, reduced renal perfusion due to surgery, and various renal and vascular diseases (58). AKIs have been investigated using experimental AKI animal models induced by methods such as drugs, ischemia-reperfusion injury (IRI), and sepsis (59). Recently, it has been reported that ILC2s and IL-33 are associated with AKI pathogenesis. In a cisplatin-induced AKI model, Akcay et al. demonstrated that recombinant IL-33 administration exacerbates AKI, while soluble ST2, which binds IL-33 preferentially to neutralize its activity, ameliorates its pathogenesis (60). High-dose IL-33 administration induces AKI progression in a $CD4^+$ T cell-dependent manner. Conversely, low-dose IL-33 has a protective effect against AKI. IRI models are commonly used to identify the mechanisms responsible for AKI pathogenesis and show that the innate immune response has a critical role. Cao et al. found that pretreatment with IL-33 ameliorates renal damage and recovers kidney function in IRI-induced mice (29). Renal ILC2s are increased in Rag1-knockout mice by the administration of IL-33, and result in reduced tubular injury score and serum creatinine irrespective of acquired immunity. However, ILC reduction using an anti-CD90 antibody in Rag1-KO mice does not rescue tubular damage. Furthermore, adaptive transfer of *ex vivo* proliferated renal ILC2s ameliorates renal injury. These results indicate that abundant ILC2s in the kidney have a renal protective effect and improve kidney functions in AKI.

CKDs have different origins such as diabetes, hypertension, and immune and toxic responses (1). Various pathologies including chronic inflammation and renal fibrosis are associated with the underlying causes of CKDs. It has been clarified that renal ILC2s have important roles in both AKI and CKD pathology. IL-33 also relieves glomerular injury by lupus nephritis (61). Moreover, the type-2 immune response induced by IL-25 and the induction of M2 macrophages can alleviate renal damage in adriamycin-induced nephropathy, which is a widely used CKD model (28). These protective effects require eosinophils recruited by IL-5 produced from ILC2s, and IL-33 fails to protect kidney function despite ILC2 abundance in eosinophil-deficient ΔdblGATA mice. However, eosinophils are considered to be pro-inflammatory cells in various diseases, so it is unclear whether eosinophil accumulation is protective against renal damage without clarifying the mechanism. In addition, ILC2s are retained in murine kidneys for up to eight weeks by IL-33 administration for four consecutive days, so there is a clinical benefit to sustained activation of ILC2s for CKD therapy. Interestingly, IL-233, a fusion cytokine of IL-2 and IL-33,

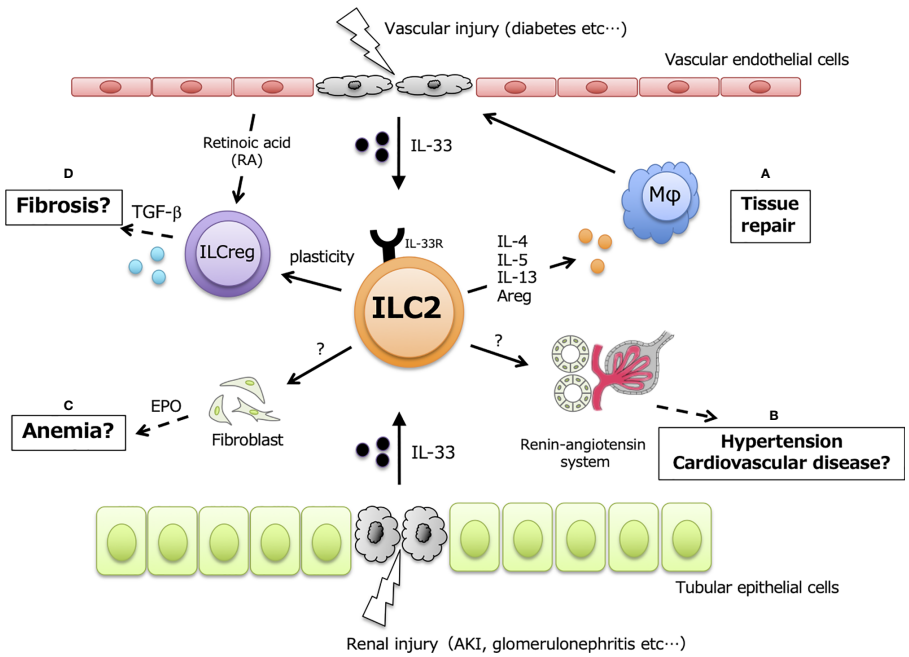

FIGURE 1 | Potential functions in renal ILC2. ILC2 have protective functions in kidney, but exerts positively or negatively effects in dependent on the amount of renal IL-33. Upon kidney injury and disease, IL-33 released from vascular endothelial cells and/or tubular epithelial cells leads to activate renal-resident ILC2, and secreted type2 cytokines possibly affects in following events; **(A)** tissue repair by inducing M2 macrophages, **(B)** hypertension and cardiovascular disease through renin-angiotensin system, **(C)** renal anemia mediated by EPO, **(D)** renal fibrosis *via the* plasticity for TGF-β producing ILCreg.

contributes to kidney protection from diabetic nephropathy (30). IL-233 attenuates hyperglycemia and proteinuria in BTBR.Cg-Lep$^{ob/ob}$ mice and has therapeutic potential for type-2 diabetic nephropathy. These findings imply that ILC2s are a potential therapeutic target in AKI and CKDs.

In humans, ILCs (lineage$^-$ CD127$^+$ CD161$^+$ populations) account for 0.5% or fewer of total kidney lymphocytes (28). In contrast to murine kidneys, human ILC2s account for 40% or fewer of the ILCs in kidneys, and ILC3s are the main constituents. It is unclear how this difference in the ILC constitutions of human and mouse kidneys affects kidney homeostasis and disease pathogenesis. A recent study indicated that blood ILC2s are upregulated in ESRD patients (62); however, it is unclear if this elevation is related to ESRD pathogenesis. Moreover, it has also been reported that changes of blood ILC correlate with the severity of DN and LN (63, 64). Further investigation is required to determine whether human renal ILC2s are friend or foe in kidney diseases.

ILC2 CONTRIBUTE TO PROGRESSION OF RENAL FIBROSIS?

ILC2 also has been shown to affect various tissue fibrosis. ILC2 contributed collagen deposition *via* IL-25 leading to induce pulmonary fibrosis (13). IL-33 is a profibrotic cytokines, and promote the initiation and progression of lung fibrosis at ST2-dependent manner (65). Some study reported that liver-resident or cardiac tissue resident ILC2 are associated with hepatic or cardiac fibrosis, respectively (66, 67). Therefore, ILC2-IL33 axis is likely affect to promote tissue fibrosis.

Kidney fibrosis is characterized by aberrant accumulation of extracellular matrix (ECM), and then destruct robust kidney structure and function (68). Fibrosis is a part of normal response to restore tissue structure and environment. Upon kidney injury, damaged tubular and vascular epithelial cells, and infiltrated immune cells are released profibrotic factors with progression of renal damages, and then various signaling are activated leading to promote fibroblast to a-SMA positive myofibroblast transition. Persistent renal damages disrupt the balance of ECM production and degradation, and excessive ECM accumulation leads abnormal fibrosis, resulting kidney dysfunction. Progression of renal fibrosis elicit CKD exacerbation, and its pathology proceed irreversible course if kidney function less of a certain level, resulting in ESRD. In addition, renal stroma produces the erythropoietin (EPO) required for erythrocyte development, and its reduction induced by kidney fibrosis results in renal anemia. Thus, the overcome of renal fibrosis is a clinical importance in nephrology.

During chronic renal injury and inflammation, damaged renal and vascular endothelial cells also release IL-33 together with aberrant ECM production, and renal-resident ILC2 are thought to be activated (**Figure 2**). The commonly used method to study renal fibrosis is unilateral ureter obstruction (UUO)-model, and IL-33 increased in serum and urine in this model (24, 69). In fact, ST2+ innate immune cells are increased in UUO-model, and ILC2 numbers also increased in murine kidney (25). Furthermore, Liang et al. showed that renal IRI-induced fibrosis

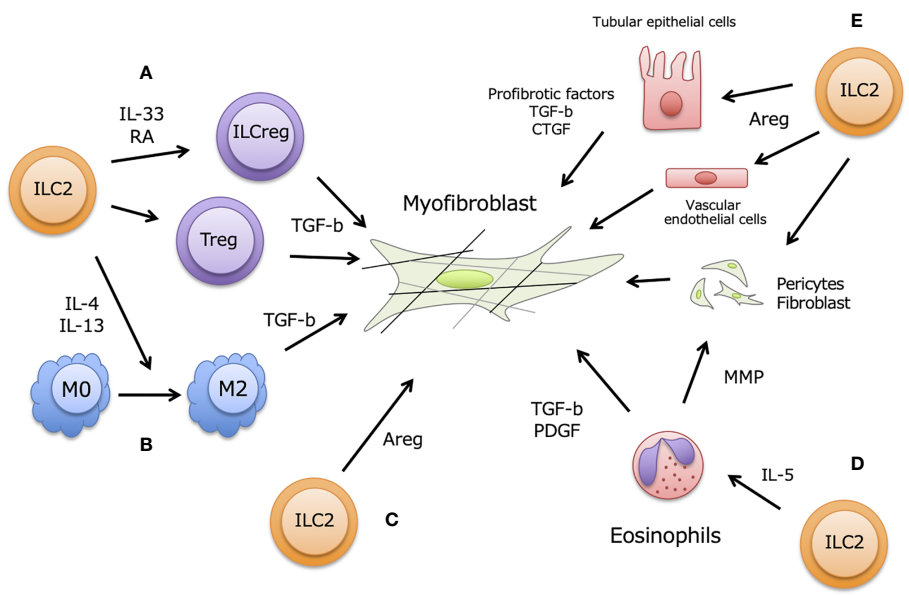

FIGURE 2 | ILC2 contribution for renal fibrosis. Renal fibrosis is partially induced by pro-fibrotic factors including TGF- β from M2 macrophages, Tregs, and ILCs, and fibroblasts accelerate trans-differentiation to myofibroblasts in renal stromal region. Activation of myofibroblast occurs excessive ECM deposition, leading to impair renal functions. Renal ILC2 possibly contribute to enhance fibrosis at following aspects of myofibroblast activation; **(A)** ILC2-ILCreg plasticity, **(B)** M2 macrophages differentiation, **(C)** direct effect by Areg production, **(D)** indirect effect *via* eosinophils induced by IL-5, **(E)** Effect of Areg to produce pro-fibrotic factors on tubular epithelial cells, vascular endothelial cells, fibroblasts and pericytes.

is accelerated by exogenous IL-33 treatment, and soluble ST2 ameliorated fibrosis (70). High-dose administration of IL-33 promoted renal fibrosis *via* AKI, but the inhibition of IL-33 was decreased AKI-induced renal fibrosis (60). While, low-dose and short-term IL-33 administration attenuate renal damages induce by IRI (28, 29). These may imply that modest IL-33 release is induced by mild renal damages at early time point to protect renal damages, while progressive renal destruction caused excessive and long-term IL-33 release leading to exacerbate renal damages and fibrosis. Taken together, renal ILC2 and adequate IL-33 has pivotal roles in kidney fibrosis.

ILC2s have been reported to produce amphiregulin (Areg) to recover pulmonary epithelial integrity at viral infection (71, 72). In kidney, ILC2-producing Areg also exerted protective function for renal damages by IRI, and directly contribute to repair renal tubular structure (29). Knock-out of Areg in ex-vivo cultured ILC2 using by CRISPER-Cas9 system could not restore tubular injury score and serum creatinine, and renal TECs apoptosis. This renoprotective effect is partially responsible for anti-inflammatory M2 macrophages induced by activated ILC2. While, Areg has function to progress tissue fibrosis including liver, lung, and kidney (73–75). Recent studies indicated that Areg-EGFR signaling enhanced renal fibrosis in proximal renal tubules (76, 77). In addition, type2 immune responses are also profoundly associated with fibrosis. BM-derived CD11c+ cell produces Areg in response to tissue damages (78), and these producing Areg induce fibroblast activation leading to promote pulmonary fibrosis. Moreover, Areg-producing pathogenic memory Th2 cells trained eosinophils to produce large amount of osteopontin, and accelerated pulmonary fibrosis (79). In addition, Liu et al. have been reported that ILC2 was negatively correlated with eGFR level in diabetic kidney disease patient with promoting renal fibrosis (63). Although it is not well understood whether Areg produced from ILC2 contribute to progress renal fibrosis, Areg expression is required for fibrosis induced by TGF-b overproduction. Further studies would be clarified the relationship among ILC2, Areg and TGF-b leading to reveal the roles of ILC2 in renal fibrosis. Areg is detected from serum and urine from CKD and AKI patients (77), and it will be as a novel therapeutic target and biomarkers in kidney fibrosis and kidney diseases including CKD and AKI.

A POSSIBLE NEW PLAYER ILCreg IN RENAL FIBROSIS

One cause of renal fibrosis is TGF-β signaling, which accelerates fibroblast transformation to myofibroblasts in renal stroma (80). Kidney-infiltrating macrophages and Tregs contribute to the progression of renal fibrosis *via* TGF-β production. Also, IL-4- and IL-13-producing ILC2s are associated with the progression of renal fibrosis through the induction of M2 macrophages (81). Moreover, Wang et al. reported that TGF-β signaling induces ST2 expression and contributes to the development of ILC2s from ILC2 progenitors (82). Therefore, the relationship among M2 macrophages, Tregs, and ILC2s is critical in renal fibrosis, and TGF-β plays a central role in these profibrotic networks.

The recently defined ILCreg subset resides in both human and mouse kidneys, and is a source of TGF-β in kidneys (83). Renal ILCregs suppress immune responses by secreting IL-10 and TGF-β, and they express CD25, ICOS, and transcriptional factor Id3, but not ST2 and KLRG1. *In vitro* cultured ILCregs produce large amounts of IL-10 and TGF-β and suppress the innate immune response of ILC1 and macrophages. Adaptive transfer of *ex vivo* expanded ILCregs to IRI-treated mice ameliorates kidney damage, so ILCregs have therapeutic potential for kidney disease. However, it is a concern that large amounts of TGF-β produced from ILCregs could enhance renal fibrosis, so additional studies are needed. Intriguingly, Morita et al. demonstrated that ILC2s are plastic and can develop into ILCregs in human nasal tissue. ILC2s stimulated by IL-33 and retinoic acid are transdifferentiated to ILCregs, producing IL-10 to suppress ILC2s and $CD4^+$ T cell proliferation (84). In addition, Nakamura et al. demonstrated that fibroblasts acquire retinoic acid-producing capacity in transitioning to myofibroblasts in several kidney injury models (85). These findings indicate that ILC2-to-ILCreg plasticity is common during kidney injury, leading to the progression of renal fibrosis. These are therefore potential therapeutic targets as TGF-β source cells, although the full contribution of ILCregs to renal fibrosis is still enigmatic.

CONCLUDING AND FUTURE PERSPECTIVE

The relationship of IL-33 and ILC2s has pivotal roles in renal immune homeostasis and kidney diseases leading to renal fibrosis. Although the functions of ILC2 in kidney diseases are gradually understood, there are still obscure in humans. However, it is intriguing that change of circulating ILCs correlate with the severity of some renal diseases. Moreover, it is interesting question what the difference means that ILC2 is dominant in murine kidney but ILC3 is dominant in human kidney, and it would be necessary to further investigate in detail. When these questions are clarified, it may be possible to elucidate the role and characteristics of ILC2 in the kidney and apply it to new therapeutic targets and clinical diagnosis in renal diseases.

AUTHOR CONTRIBUTIONS

RN and MI wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Tissue-Specific Molecular Markers and Heterogeneity in Type 2 Innate Lymphoid Cells

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Innate lymphoid cells (ILCs) are the most recently described group of lymphoid subpopulations. These tissue-resident cells display a heterogeneity resembling that observed on different groups of T cells, hence their categorization as cytotoxic NK cells and helper ILCs type 1, 2 and 3. Each one of these groups is highly diverse and expresses different markers in a context-dependent manner. Type 2 innate lymphoid cells (ILC2s) are activated in response to helminth parasites and regulate the immune response. They are involved in the etiology of diseases associated with allergic responses as well as in the maintenance of tissue homeostasis. Markers associated with their identification differ depending on the tissue and model used, making the study and understanding of these cells a cumbersome task. This review compiles evidence for the heterogeneity of ILC2s as well as discussion and analyses of molecular markers associated with their identity, function, tissue-dependent expression, and how these markers contribute to the interaction of ILC2s with specific microenvironments to maintain homeostasis or respond to pathogenic challenges.

Keywords: tissue, marker, heterogeneity, ILC2, function

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INTRODUCTION

Innate lymphoid cells are tissue-resident immune cells derived from lymphoid progenitors. They lack rearranged receptors to recognize specific antigens, therefore, their activation depends on cytokines present in the microenvironment (1). ILCs parallel the heterogeneity observed on T lymphocytes and are classified as their counterparts into cytotoxic NK cells and three main categories of helper ILCs: ILC1, ILC2, and ILC3 (2).

NK cells and ILC1s are subsets of ILCs that can be found in tissues such as liver, skin and gut, among others. They were initially classified within the same ILC1 group because of their expression of IFNγ and NK cell markers like Nkp46 in mice and Nkp44 in humans (1–3). Subsequent analyses determined that unlike helper ILCs, NK cells did not require the GATA-3 transcription factor for their development (4). Furthermore, the cytotoxic activity of NK cells was found to be strongly dependent on the transcription factor Eomes, whereas cytokine production by ILC1s mostly relied on the transcription factor T-bet (5, 6). Together, these findings lead to the separation of the ILC1 population into two independent groups (1, 2, 4–6).

Group 3 innate lymphoid cells were initially only composed of lymphoid tissue-inducer cells (LTis) or Lti-like cells (7–9), until a new cellular subset mainly found in mucosal tissues was identified. This new RORγt-dependent subset played a role in the immune response against infections (10) and together with LTis, it now represents type 3 ILCs (11). ILC3s express cytokines associated with type 3 immunity such as GM-CSF, IL-17 and IL-22 (12, 13).

Type 2 innate lymphoid cells or ILC2s are tissue-resident cells found mainly in the lung, intestine, skin and adipose tissue. Their function depends on the expression of the GATA-3 transcription factor (4, 14) and they are activated by alarmins such as IL-25, thymic stromal lymphopoietin (TSLP) and IL-33 derived from epithelial cells after tissue damage (15–17). ILC2s perform different effector responses in the immune system, both in inflammatory conditions as well as in the maintenance of homeostasis of different tissues.

Homeostasis of Adipose Tissue

ILC2s are present in white adipose tissue (WAT) where they contribute to homeostasis maintenance through constitutive cytokine secretion. Adipose tissue ILC2s are key producers of IL-5, which is essential for the recruitment and maintenance of eosinophils (18). They also express IL-13, important for the maintenance of M2 macrophages in this tissue (19). Recent work has shown that the expression of inducible costimulator ligand (ICOSL) and OX40 ligand (OX40L) by ILC2s, contributes to the accumulation of a group of regulatory T lymphocytes in visceral adipose tissue and perigonadal adipose tissue respectively (19–21). In addition, the production of methionine-enkephalin peptides from ILC2s induces the expression of uncoupling protein 1 (UCP1) in adipocytes, favoring the browning process of adipose tissue (22), while ILC2-derived IL-13 together with eosinophil-derived IL-4 activate IL-4 receptor α (IL-4Rα) signaling of adipocyte precursors in subcutaneous white adipose tissue to commit them to the beige adipocyte lineage (19, 23).

Immune Response Against Helminths

ILC2s mediate immunity against helminth parasites through the secretion of type 2 immune response cytokines when they are activated by alarmins such as IL-33 and IL-25 (1, 24, 25). In an infection mouse model using *Nippostrongylus brasiliensis (N. brasiliensis)*, ILC2-derived IL-9 was shown to be indispensable for the expulsion of worms, prompting muscle contraction, goblet cell hyperplasia, and mast cell hyperproliferation (24). In this context, autocrine IL-9, whose expression preceded that of IL-5 and IL-13 (26, 27), increased survival, proliferation, and expression of these cytokines and amphiregulin in lung ILC2s (27). In addition, IL-5-secreting ILC2s are associated with the recruitment of eosinophils, a population that contributes to the elimination of parasites (28). IL-4 and IL-13 derived from ILC2s induce the alternative activation of macrophages and the differentiation of Th2 lymphocytes; in turn IL-13 also acts by inducing smooth muscle contraction and epithelial turnover contributing to worm expulsion (24, 28), in addition to inducing the differentiation of goblet cells and mucus production (24, 28, 29). In the intestinal mucosa, the activation of ILC2s induces hyperplasia of Tuft cells, which increases the production of IL-25, generating a positive feedback loop with ILC2s (25, 29). Lastly, ILC2-derived IL-5 and IL-6 can regulate the production of antibodies by B lymphocytes in fat associated lymphoid clusters during helminth infection of the intestinal and lung barriers (19).

Tissue Repair

In addition to their roles in inflammation, ILC2s contribute to the maintenance of tissue integrity by inducing repairing mechanisms in damaged tissue after inflammatory processes. They accomplish this through expression of amphiregulin, a member of the epidermal growth factor family (1, 30, 31). Unlike other epithelial growth factor receptor (EGFR) ligands, amphiregulin not only induces a mitogenic signal but is also capable of inducing cell differentiation in a wide variety of cell types in different organs, following a signaling pattern that is like sustained activation of mitogen-activated protein kinases (MAPK) (32). Therefore, ILC2-derived amphiregulin could contribute to tissue repair in a wide range of tissues (32).

ILC2s in Disease

Allergic diseases such as asthma and atopic dermatitis have also been associated with ILC2s. In murine models of asthma, ILC2s have been observed to be the main source of type 2 cytokines responsible for the hyperproduction of mucus and recruitment of other leukocytes, which together with ILC2s are responsible for asthma symptoms (33, 34). In addition, a group of IL-5 and IL-13-producing resident ILC2s have been observed in the skin of healthy humans and increased in samples from patients with atopic dermatitis, as well as in a murine model of the same condition (15). The presence of hyperactivated ILC2s in other tissues including the nasal mucosa and the intestine has also been described and are linked to the development of pathologies such as allergic rhinitis and food allergy, respectively (35, 36).

Although alarmins have been reported to be the main activators of ILC2s, these cells can also respond to cytokines derived from the immune compartment like IL-2 and IL-7 (37). Recent studies have also shown a plethora of different molecules, besides cytokines, that can positively or negatively regulate the functions of ILC2s in different contexts, both *in vitro* and *in vivo*. Among these stimuli are lipid mediators (leukotrienes and prostaglandins), neuropeptides, hormones and diet components (38), demonstrating that the regulation of ILC2 function is tightly controlled by a variety of signals within the cellular microenvironment.

Recent studies in mice and humans suggest that the ILC2 population is extremely diverse. Markers typically used for their identification exhibit different expression profiles in a tissue-dependent fashion. Additional tissue-specific heterogeneity is found in ILC2 activation (39–44).

This review focuses on the molecular markers that provide ILC2s their identity and the receptors that regulate their function *in vivo*. We will discuss the phenotypic features and signaling pathways activated in specific tissues and how these allow ILC2s to interact with and be regulated by the environment within which they are embedded.

RECEPTORS OF EPITHELIAL CELL DERIVED CYTOKINES

Barrier epithelial cells produce a variety of cytokines that can modulate the immune system. These include IL-33, IL-25 and TSLP, a prototypical group of cytokines produced in response to specific stimuli such as tissue damage by allergen exposure, and helminth infection, which induce type 2 immune responses. These cytokines activate ILC2s (**Figures 1**–**3**), among different cells of the immune system, to induce their expansion, survival and expression of cytokines (45–47).

Suppressor of Tumorigenicity 2

ST2 was recognized as one of the first markers for the identification of ILC2s. ST2 also known as IL-1 receptor like 1 (IL1RL1) dimerizes with IL-1 receptor accessory protein (IL-1RAcP) to form the IL-33 receptor. Once activated by IL-33, the receptor signals through myeloid differentiation primary response gene 88 (Myd88), interleukin-1 receptor-associated kinases 1 and 4 (IRAK1 and IRAK4) and tumor necrosis factor receptor associated factor 6 (TRAF6) to trigger the activation of MAPK and Nuclear Factor kappa B (NFkB) (48). When activated, ST2 induces the proliferation of ILC2s and their secretion of IL-4, IL-5, IL-9 and IL-13 in a tissue, species and microenvironment-dependent manner (38).

In mice, ST2 is expressed in several immune cells including T cells (49, 50), macrophages (51), basophils and mast cells (52) among others. On ILC2s, it is mainly expressed in the lung, although it can also be found in bone marrow and adipose tissue, while its basal expression is limited in tissues such as the gut and skin (39, 41).

In the lung, IL-33 participates in the activation of most of the inflammatory processes in which ILC2s are involved (**Figure 1**); it does so by inducing the expression of IL-5 and IL-13, mainly in models of upper airway inflammation or helminth infection (53–55). In a sepsis model, ILC2 activation induced by IL-33 was reported to be important for maintaining epithelial cell viability through the secretion of IL-9, which inhibits pyroptosis of epithelial cells (56).

In WAT, the expression of ST2 on ILC2s is of great importance. IL-33/ST2 signaling activates ILC2s, maintaining tissue homeostasis and preventing obesity in mice and humans. This signaling is also important in the biogenesis of brown adipose tissue and therefore in thermoregulation. These processes seem to be regulated by the secretion of the cytokines IL-5 and IL-13, as well as methionine-enkephalin peptides, which directly induce the browning of adipocytes in WAT (18, 22, 23, 57).

ST2 expression in ILC2s and its precursors has also been reported in the bone marrow. IL-33 induces ILC2 egress from the bone marrow to populate tissues in the perinatal period of mice (58). In addition, ST2 signaling in response to IL-33 allows for local expression of ILC2-derived IL-5 in the bone marrow, which induces eosinophilia and promotes inflammation (59).

ILC2s from the small intestine and colon express ST2 in mice at steady state (**Figure 2**) (60–62). This expression is also seen in mesenteric lymphoid nodes where IL-33 induces a recently described population of inflammatory ILC2s (iILC2s) characterized by decreased expression of ST2 and CD25 (62). In the small intestine, IL-33 also induces iILC2s and upregulation of tryptophan hydroxylase 1 which results in decreased susceptibility to infection by helminths (62). In the colon, ILC2s expressed ST2 after intraperitoneal administration of IL-33, leading to their proliferation, a high expression of IL-5 and IL-13, and low IL-17 levels (63).

There are some reports on the effects of IL-33 in the skin and its participation in wound healing. However, the specific mechanism responsible for this function has not been elucidated (**Figure 3**), although it could be explained by the known tissue repairing properties of ILC2s (64). IL-33 has been reported in skin-associated pathologies such as atopic dermatitis and psoriasis (16, 65, 66). The relevance of IL-33 in the immune response against helminth infection in humans remains unclear, possibly due to the limitations that exist in studying human ILC2s. Nevertheless, some studies suggest that this pathway works similarly to skin pathologies and adipose tissue homeostasis in mice (16, 22).

ILC2s also reside in the central nervous system (brain, spinal cord and meningeal space) under steady state; these ILC2s express ST2 and expand in response to IL-33 (67, 68). Brain meningeal ILC2s increase their expression of IL-5 and IL-13 in response to spinal cord injury in an IL-33-dependent manner and contribute to recovery (67). Additionally, in an experimental autoimmune encephalitis model, IL-33 activated CNS ILC2s ameliorate the disease by antagonizing the Th17 response (68).

IL-33 is an alarmin released in conditions of tissue damage. Hence, expression of ST2 on ILC2s within tissues such as skin and lung that are damaged during a parasitic infection or allergic process is crucial and provides a key mechanism for restoring homeostatic conditions. Additionally, the relevance of the ST2/IL33 axis in the control of other physiological responses is demonstrated by the specific functions it regulates in adipose tissue, where IL-33 derived from stromal cells coordinates key developmental and adaptational processes (69, 70).

IL-17 Receptor B

IL-17RB together with IL-17RA conform the receptor for IL-25 (IL-17E) (71), an alarmin of the IL-17 family that activates ILC2s. Studies evaluating the heterogeneity of the ILCs have found IL-17RB to be a marker almost exclusively restricted to the gut; however, some expression has also been observed in the lung (39, 41).

ILC2s express higher levels of IL-17RB in the small intestine (**Figure 2**) compared to other tissues such as lung, skin and fat; this allows a communication with Tuft cells that produce IL-25 and enables ILC2 expression of IL-5 and IL-13 in homeostasis. Furthermore, Tuft cell numbers increase during helminth infections, in turn secreting more IL-25 to promote a more efficient activation of ILC2s during the defense response against these pathogens (72–75).

In the lung, expression of IL-17RB has been described in a particular population: inflammatory ILC2s (iILC2s) (**Figure 1**). These cells are characterized by low ST2 and high KLRG1 expression and can produce IL-5 and IL-13 in response to IL-25. In different *in vitro* and *in vivo* models, iILC2s give rise to natural ILC2s (nILC2s), which are characterized by higher expression of ST2 (17). However, it has also been reported that

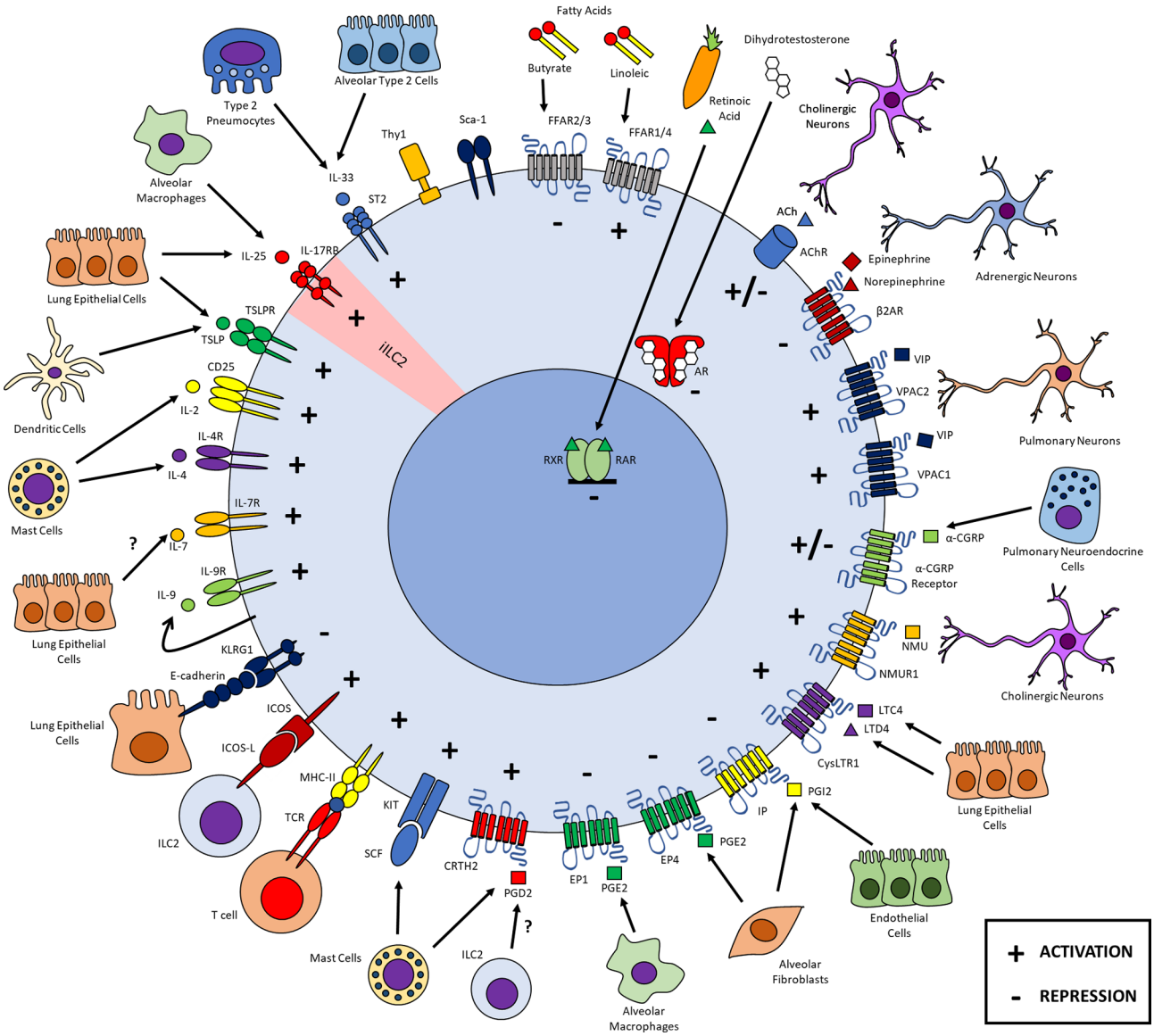

FIGURE 1 | Lung ILC2s. Expression of different markers on pulmonary ILC2s and possible cellular interactions within this tissue. Lung ILC2s interact with the epithelium and different cell populations of the immune and the nervous systems. Cytokine receptors transduce signals that activate ILC2s, as does the immune checkpoint molecule ICOS and MHCII. Lipid mediators PGD2, LTC4 and LTD4 induce positive signals in ILC2s while PGE2 and PGI2 negatively regulate their function. The neuropeptides NMU and VIP positively regulate ILC2s while catecholamines drive a negative regulation and CGRP and ACh displays a dual function. Dihydrotestosterone and Klrg1 repress lung ILC2s. Diet derived factors like retinoic acid and butyrate inhibit ILC2 function while linoleic promotes it. iILC2 inflammatory ILC2, SCF stem cell factor, PG, prostaglandin; LT, leukotriene; NMU, neuromedin U; CGRP, calcitonin gene related peptide; VIP, vasoactive intestinal peptide; ACh, acetylcholine; RAR, retinoid acid receptor; RXR, retinoid X receptor.

IL-33 stimulation can promote nILC2s to become iILC2s, thus generating a population that is important for the correct immune response against helminth infection (62).

IL-17RB-expressing ILC2s are also relevant in the skin (**Figure 3**). An increased expression of this receptor on ILC2s has been reported in patients with atopic dermatitis (16). Additionally, in mice, keratinocyte-derived IL-25 activates ILC2s, promoting IL-13 expression, Th2 cell accumulation and epidermal hyperplasia in allergic skin inflammation (76).

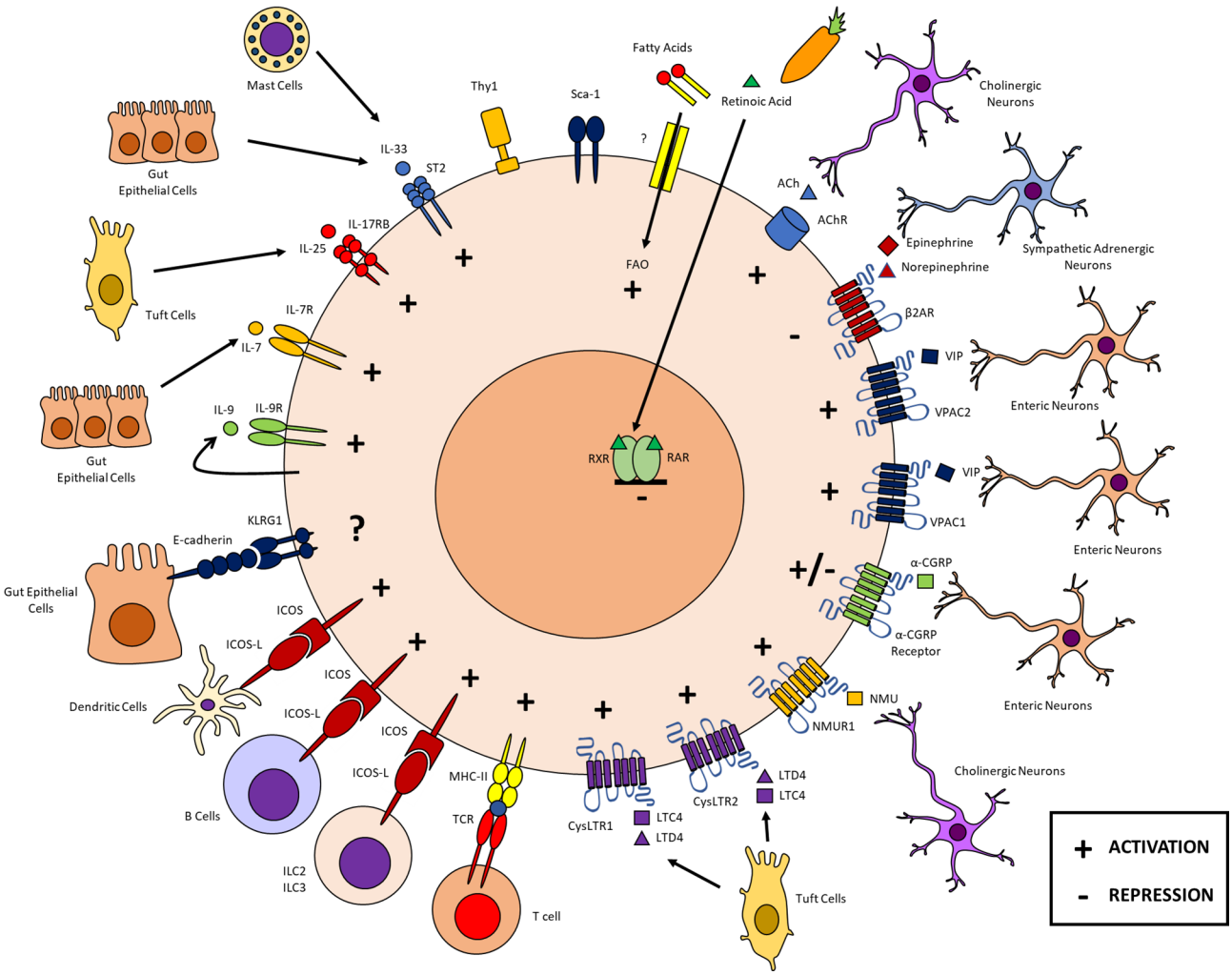

FIGURE 2 | Gut ILC2s. Expression of different markers in intestinal ILC2s and cellular interactions within this tissue. In the gut, ILC2s interact with other immune cells, neurons, epithelial cells, among other populations. All cytokine, leukotriene, and neuropeptide receptors mentioned in this review, except the β2AR, provide signals that activate ILC2s in the gut. CGRP both positively and negatively regulates these cells. In the gut, expression of MHCII, ICOS and its ligand favors the function of ILC2s. The function of ILC2s in this tissue depends on fatty acid oxidation, while retinoic acid inhibits ILC2s. PG, prostaglandin; LT, leukotriene; NMU, neuromedin U; CGRP, calcitonin gene related peptide; VIP, vasoactive intestinal peptide; RAR, retinoid acid receptor; RXR, retinoid X receptor; fatty acids oxidation.

TSLP Receptor

TSLP is a pleiotropic cytokine originally isolated from a murine thymic stromal cell line and characterized as a lymphocyte growth factor (77). It was later shown to activate ILC2s and the expression of the TSLP receptor has been used as a marker to identify these cells. TSLP binds to its receptor TSLPR and forms a complex that subsequently binds to the IL-7 receptor alpha chain (IL-7Rα) to initiate a JAK/STAT5 dependent signaling pathway (77).

Although the transcript for this receptor has been detected in murine ILC2s from lung, gut, adipose tissue, skin and bone marrow (39), there are no reports directly showing the expression of the TSLP receptor at the protein level in any of these tissues except for the lung in mice (78, 79) and the skin in humans (16).

In the lung, ILC2s respond to TSLP (**Figure 1**) in allergy and viral infection. TSLP is important to potentiate the effects of IL-33 on ILC2s such as proliferation and IL-5 and IL-13 production (46, 78, 79). In addition, TSLP can promote ST2 expression on ILC2s; in turn, IL-33 increases TSLPR expression, initiating a positive feedback loop potentiating the alarmin responsive capacity of ILC2s (79). Regardless of IL-33, TSLP plays an essential role in papain-induced airway inflammation by stimulating ILC2s *in vivo* to promote production of type 2 cytokines (80).

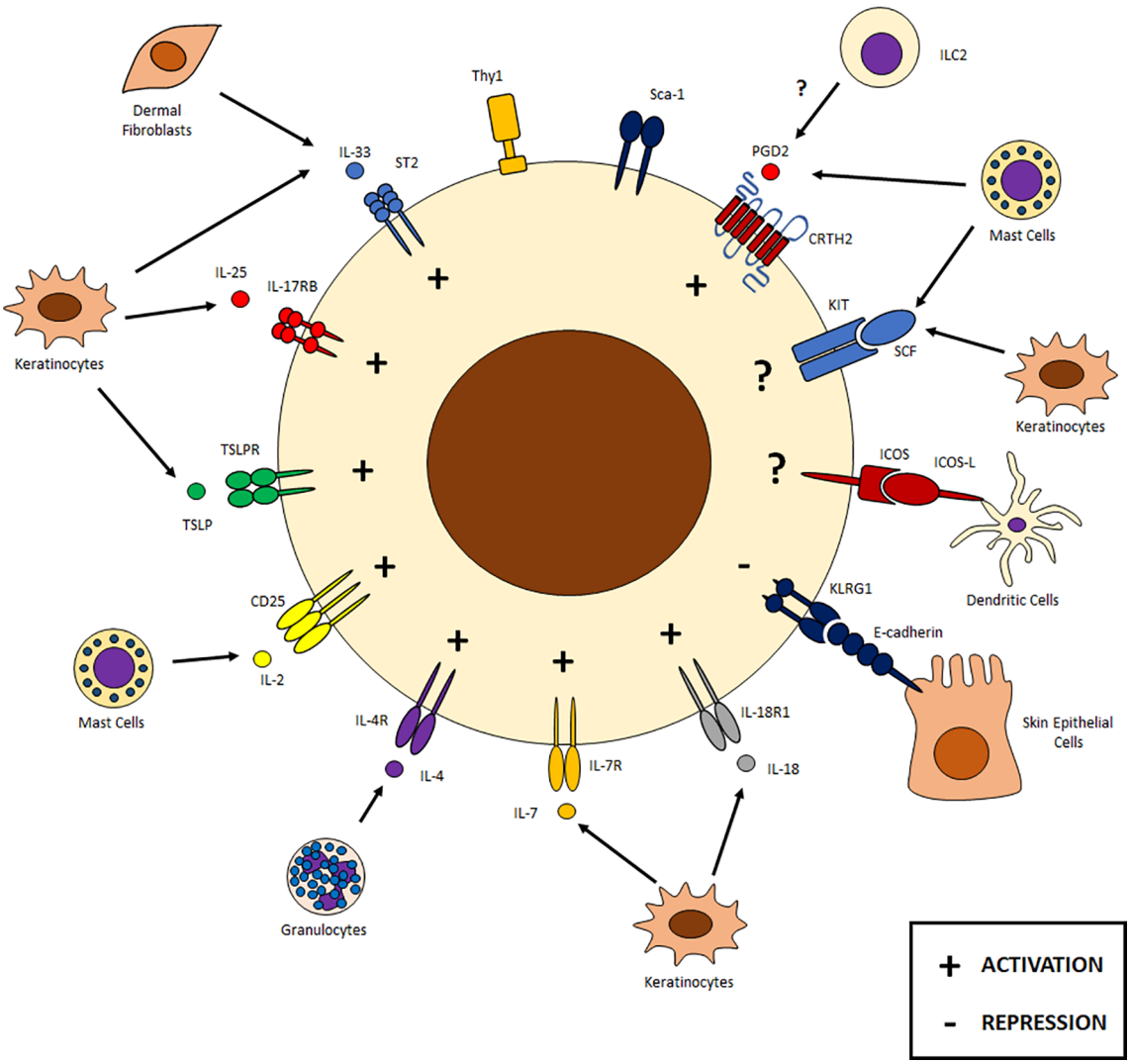

FIGURE 3 | Skin ILC2s. Expression of different receptors on skin ILC2s and cellular interactions within this tissue. ILC2s in the skin interact with different cell populations, including epithelial cells and other components of the immune system. Signals from IL-33, IL-25, TSLP, IL-2, IL-4, IL-7, IL-18 and PGD2 receptors favor ILC2 function in this tissue. Klrg1 on ILC2s can interact with E-cadherin to negatively regulate these cells in the skin. Other receptors such as KIT and ICOS have also been reported in skin ILC2s although their specific function has yet to be determined. SCF, stem cell factor; PG, prostaglandin; LT, leukotriene.

In the skin, ILC2s have been reported to be important players in the induction of inflammatory processes such as atopic dermatitis in both humans and mice. In a murine model of dermatitis, an IL-33-independent inflammatory process has been reported, where TSLP is essential for the induction of ILC2 proliferation (15). In humans TSLP induces CD1a expression in the skin, which promotes the production of IFNγ, IL-22 and IL-13 by CD1a-reactive T cells, and a defense response against some types of bacteria (81).

TSLP is primarily expressed in lung and gut epithelial cells, as well as in skin keratinocytes (77); consequently, the role of this cytokine in ILC2s from these tissues is being actively investigated. Most reports agree that TSLP works in cooperation with IL-33 to enhance cytokine expression and proliferation responses in ILC2s in the lung. In the skin, TSLP appears to play an important role since it induces responses independent from other alarmins (46, 78, 79). Interestingly, there are not yet to be reports on the function of TSLP in the gut.

γ-COMMON CYTOKINE RECEPTORS

IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are members of a family of cytokines that regulate biological functions in different cell types of

the immune system. They share the common gamma chain (γc) receptor, a subunit initially discovered as the third component of the IL-2 trimeric receptor (IL-2Rγ or CD132) (82). ILC2s have been reported to be a major source of IL-9 and under certain stimuli can also produce IL-4. They have also been shown to express IL-9 and IL-4 receptors, suggesting a potential autocrine regulation of these mediators on the cells. Specific subunits of the IL-2 and the IL-7 receptors belonging to this family of cytokines are used as markers for ILC2s on different tissues.

IL-2 Receptor Alpha

The high affinity IL-2 receptor is made up of 3 subunits: IL-2Rα (CD25), IL-2Rβ (CD122) and the γc chain (CD132). IL-2 binds to CD25 and this complex recruits CD122 and CD132 to initiate signaling leading to STAT5 activation (83).

CD25 is used as a marker for ILC2s identification in most tissues in mice, including bone marrow, lung, gut, adipose tissue and skin (39–41). In humans, it is found on ILC2s from the lung, tonsils, spleen, bone marrow and peripheral blood (42, 43).

The expression levels of CD25 in the lung vary depending on the *in vivo* model used. IL-33-activated ILC2s uniformly express high levels of CD25, while exposure to house dust mite (HDM) generates a model of allergic inflammation in which low expression of CD25 is observed on ILC2s without affecting their ability to produce type 2 cytokines (84). CD25-expressing ILC2s have also been reported to proliferate in response to IL-2 produced by mast cells, thus contributing to lung inflammation in allergy models (85). In humans, it has been observed that retinoic acid increases the expression of CD25 among other markers, and in combination with IL-2 induces these cells to acquire a regulatory phenotype by promoting the expression of inhibitory receptors such as CTLA4 and cytokines such as IL-10 (86).

In the skin, CD25 is expressed by ILC2s (**Figure 3**) and this expression increases in models of allergic inflammation (87). Dermal ILC2s require IL -7 for survival and can respond to IL-2, resulting in production of IL-5, and contributing to the induction of dermatitis (88).

Finally, recent reports suggest that intrahepatic ILC2s express CD25 both basally and in inflammatory conditions, allowing this subset to be activated by T lymphocytes, the intrahepatic sources of IL-2 (89, 90).

IL-4 Receptor Alpha

IL-4 binds its receptor IL-4Rα to form a complex that subsequently recruits the γc chain in lymphoid cells, or the IL-13Rα1 in myeloid or non-hematopoietic cells. In both cases, activation of the IL-4 receptor complex leads to signaling dependent on STAT6 activation (91).

The IL-4 receptor alpha and the γc chain are expressed in mouse and human lung ILC2s (**Figure 1**) (92–94). *In vitro* stimulation of murine ILC2s with IL-4 increases the expression of IL-5, IL-13, IL-9, CCL11, CCL5 and CCL3 induced by IL-33 and IL-2. In a papain-induced inflammation lung model, deletion of IL-4 specifically in basophils results in decreased numbers of ILC2s and their production of IL-5 and IL-13, along with diminished eosinophilic inflammation induced by protease allergens (93).

Skin ILC2s also express IL-4Rα (**Figure 3**) and the proliferation and inflammatory responses of these cells are dependent on basophil-derived IL-4 in a murine model of atopic dermatitis (95).

The IL-4 receptor is also expressed in peripheral blood ILC2s in humans. Stimulation with this cytokine is important for the increase in ILC2 numbers and the maintenance of the CRTH2 expression marker (94). CRTH2 acts as the PGD2 receptor whose activation cooperates with IL-25 and IL-33, enhancing ILC2 functions such as migration and expression of Th2 cytokines (96, 97).

As described in these reports, the main sources of IL-4 are mast cells, basophils, eosinophils, T lymphocytes and under specific stimuli, ILC2s (91). Importantly, these cell populations partner with ILC2s in type 2 immune inflammatory responses.

IL-7 Receptor Alpha

IL-7Rα or CD127 forms the IL-7 receptor in conjunction with the γc chain. IL-7Rα is also a subunit of the TSLP receptor. Given that the importance of TSLP was previously discussed, we will mainly focus on the relevance of IL-7Rα as an IL-7 receptor on ILC2s in this section.

IL-7 is necessary for the expression of NFIL3, a transcription factor required for the expression of Id2 and the generation of the common helper innate lymphoid progenitors (CHILP) (98). IL-7 contributes to the development of more committed precursors to certain ILC lineages, however the molecular mechanisms involved are currently unknown. CD127 is expressed in all helper ILC populations, and a recent study suggests that this expression works as a mechanism to regulate IL-7 activity in other lineages by restricting its availability in the niche (99). CD127 expression in the ILC2 group has been reported in several tissues including lung, gut, skin (**Figures 1**–**3**), and adipose tissue, among others (39–44, 100).

Besides the role of IL-7 in the development of ILC2s, this cytokine can control other cellular functions in specific tissues. For example, in the stomach, an organ in which ILC2s have been recently described, CD127 expression on these cells is higher compared to other tissues. In addition, ILC2s can respond to IL-7 both *in vitro* and *in vivo* by proliferating and producing IL-5 and IL-13 (101).

In the lung, CD127 is critical for the induction of natural and inflammatory ILC2s. Mice deficient in this receptor showed a complete absence of ILC2s, exhibiting the same phenotype observed in mice deficient of the γc chain (17). These data suggest that the phenotype observed in those mice could be associated with a deficiency in IL-7 signaling. Accordingly, IL-7 or γc chain deficiency in the skin also results in a total absence of dermal ILC2s (88).

IL-7 is expressed in both immune and non-immune cells including stromal cells, keratinocytes, gut epithelial cells, follicular dendritic cells, macrophages, monocytes and B cells (102); therefore, they could serve as primary sources of IL -7 for ILC2s in different tissues.

IL-9 Receptor Alpha

IL-9 is a pleiotropic cytokine associated with classical type 2 immune responses. Overexpression of this cytokine is associated

with mastocytosis, eosinophilia, increased production of mucus, airway hyperreactivity and resistance to helminth infections (103, 104). Binding of IL-9 to IL-9Rα induces the heterodimerization of this receptor with the γc chain, leading to activation of Jak1 and Jak3 kinases, which induces receptor phosphorylation and activation of STAT1, STAT3 and STAT5 (103).

Lung ILC2s express high levels of the IL-9 receptor (**Figure 1**) in a context of airway inflammation in a papain administration model where stimulation with this cytokine potentiates the expression of IL-5, IL-6 and IL-13 in these cells (105). In an infection model with *N. brasiliensis*, ILC2s highly express IL-9 (27, 106), while mice deficient in IL-9 receptor have reduced numbers of ILC2s as well as impaired cytokine expression, deficient parasite clearance and inefficient repair of damaged tissue (27). Lastly, stimulation of lung ILC2s with IL-9 *in vitro* increases their survival and expression of IL-5 and IL-13 (27), indicating the importance of this cytokine on ILC2 function.

Expression of IL-9Rα on ILC2s has been reported in the small intestine (**Figure 2**), however, its direct function has not been studied (41, 107).

ILC2s are characterized by expressing IL-9. In fact, the main source of IL-9 in models of lung inflammation with papain are ILC2s themselves (105). In addition, ILC2s are the main source of IL-9 in the resolution phase of arthritis and the deficiency of this cytokine reduces the numbers of ILC2s (108). Finally, in helminth infection, both ILC2 and Th9 cells can produce this cytokine in the lung and small intestine (104). These reports suggest an autocrine regulation of the ILC2 functions mediated by IL-9.

OTHER CYTOKINE RECEPTORS: IL-18 RECEPTOR 1

A recent study aimed to determine the tissue-specific expression profiles of ILC2s in mice through single cell RNAseq analysis, revealed the presence of the IL-18 receptor 1 (a cytokine of the IL-1 family) specifically in skin ILC2s (39).

IL-18 is important for the *in vivo* function of ILC2s. Mice deficient for this cytokine challenged with MC903 exhibit defects in the proliferation and activation of skin ILC2s (39). Furthermore, ILC2 skin cultures showed increased expression of IL-5 and IL-13 in the presence of IL-18 (39). Although IL-18R1 expression was also observed in a small population of lung ILC2s, the difference in the frequency and expression of this marker suggests a greater relevance of IL-18 signaling in skin ILC2s (**Figure 3**).

CELL-CELL INTERACTION MOLECULES

Since their discovery, ILC2s have been characterized for expressing different surface molecules that allow them to interact directly within their group and with other cells in the microenvironment (109). These interactions play an essential role in the regulation of ILC2 function in different inflammatory contexts.

Killer Cell Lectin-Like Receptor Subfamily G Member 1

Klrg1 is important for ILC2 cell-cell interaction and is used as a marker to identify these cells *in vivo*. It is an inhibitory receptor originally associated with NK cells and able to interact with proteins of the cadherin family (110). Several studies have described the expression of Klrg1 on ILC2s from different tissues in both mice and humans. In mice, Klrg1 transcripts have been reported in lung, gut and adipose tissue (39–41); while in humans, this molecule has been reported in blood, bone marrow, spleen, lung, tonsils and colon (42–44).

In murine models, Klrg1 has been described as a maturation marker in small intestine ILC2s (**Figure 2**), identifying IL-5 and IL-13 producers in this tissue (107). In the lung, Klrg1 expression has been reported in basal conditions (**Figure 1**) (111, 112). Its role as a negative regulator was recently demonstrated in irradiated mice that received bone marrow from Klrg1-deficient mice together with wild type bone marrow. Klrg1-deficient ILC2s presented a competitive advantage, increasing their ratio in the lung with no differences observed in their proliferation or cytokine production compared to wild type ILC2s (113). Upon IL-25 stimulation, a distinct population of inflammatory ILC2s whose expression of Klrg1 is much higher, emerges. The induction of this population has been observed at specific time points in the lung during models of helminth infection, where they orchestrate the immune response against this class of pathogens (17, 114).

Regarding the skin, Klrg1 expression has been observed under allergic conditions (**Figure 3**) (87). Patients with atopic dermatitis show increased expression of Klrg1 and the interaction of activated ILC2s with E-cadherin results in a down regulation of GATA-3, IL-5, IL-13, amphiregulin and reduced ILC2 proliferation; suggesting a negative regulatory role for Klrg1 in skin ILC2s (16). In bone marrow derived ILC2s, Klrg1 deficiency restores the decreased proliferation observed by the effect of E-cadherin (113). This suggest that Klrg1 could have the same function in other tissues, especially considering that E-cadherin is a protein widely expressed in all epithelial tissues.

The Inducible T Cell Costimulator

ICOS is a costimulatory molecule belonging to the CD28 superfamily (115) whose expression has been associated with different populations of immune cells. This molecule is used as a marker for ILC2s in different models.

ICOS expression in mouse ILC2s has been reported in tissues such as bone marrow, lung, adipose tissue, gut and skin (39). In humans, the expression of ICOS seems to be restricted to tissues such as the lung, tonsils and skin (42).

In the lung and small intestine, signaling mediated by ICOS and its ligands is important for ILC2 survival and proliferation at steady state and inflammation; in this last condition, it is also important to produce IL-5 and IL-13 (116–118). In the lung, ILC2s express the ICOS ligand (ICOS-L), thus providing a path to interact with each other and enhance their activation (**Figure 1**) (117). Although its role has not been studied in skin, ICOS has been shown to be expressed on ILC2s on this tissue (**Figure 3**) (119) and increased in allergic conditions (87).

Similarly, a specific function for ICOS has not been described in adipose tissue, however, visceral adipose tissue ILC2s express ICOS-L and interact with regulatory T cells to help maintain homeostasis (20).

ICOS and ICOS-L expression is also detected in human peripheral blood ILC2s, along with an increased presence when these cells are activated (117). Additionally, there are reports of ICOS expression on ILC2s from nasal polyps which is reduced in conditions of chronic rhinosinusitis; however, its specific function there has not been determined (120).

Altogether, the expression of ICOS and ICOS-L in different tissues suggest that the function of this marker might be to enhance ILC2 activation and allow communication within that group as well as with other cells in different tissues, including regulatory T cells and dendritic cells.

Class II Major Histocompatibility Complex

Antigen presentation by MHC-II molecules is critical for the maintenance of self-tolerance and the initiation of an effective immune response. These molecules are constitutively expressed on professional antigen presenting cells (APCs) such as dendritic cells, macrophages, B cells and thymic epithelia (121). Recent studies have shown that ILC2s are capable of presenting antigens and the expression of MHC-II in these cells has been reported in tissues such as the lung, small intestine and colon (63, 74, 105, 122, 123).

A fraction of lung ILC2s express high levels of MHC-II (**Figure 1**) and present antigens, inducing T cell proliferation and differentiation towards a Th2 phenotype. In turn, T cells secrete IL-2, inducing ILC2 proliferation and expression of IL-5 and IL-13 (122). Expression of MHC-II in lung ILC2s is also induced during *N. brasiliensis* infection and is dependent on STAT6 activation (123).

In the gut, MHC-II expression has been reported in the small intestine and colon (**Figure 2**) (63, 74). Small intestine ILC2s express MHC-II in *Trichinella spiralis* infection, which allows their interaction with CD4+ T cells for the induced expression of type 2 cytokines in response to IL-25 (74).

In humans, increased expression of MHC-II has been reported in peripheral blood ILC2s of patients with acute exacerbation of chronic obstructive pulmonary disease (AECOPD). Cocultures of these ILC2s with Th2 cells showed an increased expression of IL-4, IL-5 and IL-13 that is dependent on MHC-II-mediated interaction (124).

Hence, the MHC-II-mediated interaction between T lymphocytes and ILC2s plays an additional role in the immune response against helminth parasites. IL-2 derived from T cells induces proliferation of ILC2s and enhances the expression of type 2 cytokines, contributing to the elimination of parasites and demonstrating a cooperation between these two cell populations (125).

Growth Factor Receptor: KIT

The stem cell factor (SCF) also called mast cell growth factor or KIT-ligand was described several years ago as being of great importance in the physiology and pathology of the skin. Its receptor, KIT, is expressed in mast cells and melanocytes (126). Subsequent studies reported this ligand-receptor pair in other tissues and currently the expression of KIT has been widely described in different groups of ILCs.

KIT is a type 3 tyrosine kinase receptor. Interaction with its ligand occurs in different tissues where it can regulate processes such as cell survival, proliferation, migration and differentiation (127). This receptor is expressed on ILC2s and ILC3s. On ILC2s, it is expressed in different tissues like lung and skin; however, its function has not been fully characterized in all of them (42).

KIT is expressed in human peripheral blood ILC2s, where it allows the distinction between two cell populations. ILC2s with higher levels of KIT expression acquire the ability to secrete cytokines associated with ILC3s, while the ones with low expression express high levels of type 2 cytokines, possibly corresponding to mature and lineage committed ILC2s (128, 129). These KIT+ cells are increased under conditions of helminth parasite infections and can also produce IL-13 (130). TSLP, IL-25 and IL-33 signaling *in vitro* can promote KIT expression on ILC2s (131).

In mice, KIT is also heterogeneously expressed on ILC2 populations in the lung, exhibiting high or low expression of this marker in the context of allergies and viral infections where these cells are important producers of IL-5 and IL-13 (33, 132–134). SCF is also important for the increase of ILC2 numbers in a context of allergic inflammation, and induces the expression of IL-4, IL-5, IL-9, IL-13 and TGFβ in these cells (132).

In humans, heterogeneous KIT expression is observed under homeostatic conditions in the skin (**Figure 3**), and cells expressing this protein increase in patients with psoriasis, concomitant with the acquisition of an ILC3 phenotype (129); however, dermal ILC2s maintain their potential since upon stimulation with IL-33 and TSLP they are still capable of producing IL-13 (135). In addition, ILC2s from peripheral blood that express KIT also express RORγt, although at lower levels than ILC3s, and can produce IL-17. Furthermore, a fraction of this cells has been found to express skin homing markers such as CCR10 and cutaneous lymphoid antigen (CLA) (129, 135); therefore, it is proposed that KIT^+ ILC2s could migrate to the skin and contribute to chronic inflammation in pathological conditions such as psoriasis (129).

Expression of KIT has also been described in other mice tissues. The first reports of ILC2s in fat-associated lymphoid clusters were phenotypically defined as KIT positive cells, able of express type 2 cytokines important in the defense against helminth parasites (37). KIT expression in functional ILC2s has also been shown in mesenteric lymph nodes, spleen, liver, and corneal limbus (136–138).

Expression of the KIT ligand is associated with different cell types including fibroblasts, keratinocytes (126) and stromal cells. In humans, this factor is found in mast cells from lung and skin (139), suggesting that ILC2s could interact with these cells through this signaling pathway.

LIPID MEDIATOR RECEPTORS

In addition to the classic activators of ILC2s, it has been shown that lipid mediators like prostaglandins and leukotrienes derived from arachidonic acid can regulate the function of these cells by positively or negatively controlling their activation (140, 141).

Chemoattractant Receptor-Homologous Molecule Expressed on Th2 Cells

CRTH2, the receptor for prostaglandin D2, is one of the most common prostaglandin receptors used for ILC2 identification in humans. CRTH2 is a Gi protein coupled receptor that regulates the function of cells by lowering cAMP levels and inducing calcium mobilization (142).

In humans, CRTH2 positive ILC2s can be found in fetal and adult lung and intestinal tissues, as well as in adult nasal polyps and peripheral blood (143). Another report indicates that this marker is highly expressed in human and murine peripheral blood ILC2s in the context of helminth infections or upon activation with alarmins, where the CRTH2/PGD2 pathway is important for the accumulation of ILC2s in the lung (**Figure 1**) (144, 145).

Teunissen et al. described the presence of CRTH2 positive ILC2s in healthy human skin samples (**Figure 3**). It has also been reported that CRTH2+ ILC2s from peripheral blood express skin-homing markers such as cutaneous lymphocyte antigen (CLA) (135). PGD2 is important in skin where it acts as a synergic signal with IL-25 and IL-33 for the migration and induction of cytokine expression (96). Ex vivo activation of skin ILC2s with PGD2 induces the expression of cytokines associated with the type 2 response as well as pro-inflammatory cytokines such as IL-3, IL-8, IL-21, GM-CSF and CSF-1. IL-3, GM-CSF and CSF-1 contribute to myeloid cell differentiation, while IL-8 and IL-21 have been associated with the recruitment of neutrophils. Together, these signals could contribute to the allergic inflammation induced by ILC2 (96).

The CRTH2 pathway has also been reported in lymphoid tissue. Interestingly, tonsillar ILC2s express not only the CRTH2, but can also produce PGD2 when activated by IL-2, TSLP, IL-25 and IL-33. This apparent autocrine loop has been suggested to be essential for the expression of IL-5 and IL-13 and for the increased expression of GATA-3 and CD25 on these cells (97).

CRTH2 expression in murine ILC2s has been described in the lung (**Figure 1**); however, this receptor is more commonly used in the analysis of human ILC2s and consequently, most of the evidence showing the importance of the expression of this marker is restricted to human studies. Regardless of whether CRTH2 is important for murine ILC2 regulation or not, a common feature in both species is that one of the main sources of PGD2 are mast cells, providing a potential direct interaction between those two cell types. Other sources of PGD2 such as Tuft cells have been characterized in the small intestine in helminth infection (146) or dendritic cells in skin (147). Finally, the interesting observation that ILC2s can produce PGD2 themselves also hints to a possible autocrine function of this signal on ILC2s that requires further characterization. These results show that through the PGD2-CRTH2 pathway, ILC2s can interact with different immune cells at different anatomical sites to be activated, thus enhancing their effector functions in the type 2 immune response.

E-Type Prostanoid Receptors (EP1, EP2 and EP4)

Three out of the four existing prostaglandin E2 (PGE2) receptors have been reported to be expressed on ILC2s (148, 149). These receptors are coupled to different G proteins. EP1 is associated with Gq proteins and therefore induces calcium mobilization while EP2 and EP4 are associated with Gs proteins, hence promoting increases in cAMP (150). Tonsillar and peripheral blood human ILC2s express EP2 and EP4 receptors. EP2 and EP4 play a protective role in the lung during allergic inflammation due to their inhibitory effect on ILC2 proliferation and GATA-3 and CD25 expression, as well as in IL-5 and IL-13 induction, all of this in response to activation with IL-2, TSLP, IL-25 and IL- 33 (149).

EP1 and EP4 transcripts have been observed in ILC2s isolated from murine lung (**Figure 1**). In addition, an EP4-dependent inhibition induced by prostaglandin E2 has been reported in ILC2s upon activation induced by IL-33 *in vitro* (148). Furthermore, it was observed that the administration of PGE2 attenuates inflammation induced by IL-33 *in vivo*, affecting the expansion of the ILC2 population and accordingly, this inflammation was exacerbated in EP4 deficient mice (148). The production of PGE2 in the lung by alveolar fibroblasts and alveolar macrophages has been widely reported (151, 152), therefore it is not surprising that ILC2s in this tissue express receptors for that mediator. Even though there have not been additional reports of PGE2 regulating ILC2s in other tissues, we cannot rule out that it might impact their function in other compartments.

Prostaglandin I2 Receptor

The prostaglandin I2 receptor also called prostacyclin receptor or IP is a Gs protein-coupled receptor that increases cAMP levels (150) and can reduce allergic inflammation in different animal models (153, 154).

Expression of IP on ILC2s has been reported in the lung only (**Figure 1**). Stimulation of the IP pathway was shown to be important for the regulation of IL-5 and IL-13 secretion in lung ILC2s *in vivo* in an IP-deficient mouse model of allergic inflammation (155, 156). However, analogs of the IP ligand, PGI2, can regulate ILC2s differentiated from bone marrow by a mechanism involving inhibition of IL-5 and IL-13 production, in response to IL-33, in an IP-dependent manner (155).

Cysteinyl Leukotriene Receptors

Leukotrienes (LTs) are mediators derived from arachidonic acid involved in self-defense systems, but overproduction causes a variety of inflammatory diseases (157). Leukotrienes (LT) C4, D4 and E4 function as ligands for cysLTRs. So far, five CysLT receptors have been identified: CysLTR1, CysLTR2, P2Y12, GPR99, and GPR17; but only CysLTR1 and CysLTR2 have been reported on ILC2s.

LTD4 has higher affinity than LTC4 to cysLTR1; cysLTR2 is similarly related to these two leukotrienes, with E4 having the lowest affinity for this receptor. CysLTR1 and CysLTR2 are coupled to Gq and Gi proteins, so they exert their action by activating PKC and producing calcium mobilization, or decreasing cAMP levels, respectively (157).

CysLTR1 is expressed in lung and bone marrow ILC2s, where LTD4 induces IL-5 and IL-13 expression similarly to IL-33, in a cysLT1R-dependent manner (158, 159). Interestingly, LTD4

induces the expression of IL-4 in lung ILC2s *in vitro* (158), while *in vivo*, LTD4 administration increases the production of IL-5, IL-13 and the proliferation of ILC2s in a model of allergic inflammation (158). Accordingly, studies on type 2 inflammation models using cysLTR1 or LTC4 deficient mice revealed suppressive effects of these responses in ILC2s (159). Also in the lung, ILC2s are activated by leukotriene C4 in synergy with IL-33, resulting in increased proliferation and expression of IL-5 and IL-13 *in vivo*, in a cysLTR1 and NFAT-dependent manner (160). In addition to the production of IL-5 and IL-13, the activation of ILC2s with IL-7 and IL-33 plus LTC4 or LTD4, can induce IL-17 expression on these cells (161).

cysLTR1 and cysLTR2 are expressed similarly on ILC2s in lamina propria of the small intestine (162). *In vitro* activation of these intestinal ILC2s with LTC4 and LTD4 increases their expression of IL-13 (162). The action of these leukotrienes *in vivo* is important for the activation of ILC2s and the control of helminth infections, but irrelevant in controlling infections by protists (162).

The expression of LTC4 and LTD4 in the lung and small intestine is carried out by epithelial cells (**Figures 1**, **2**). In the intestine, it is specifically produced by Tuft cells (163, 164), whose direct production of LTC4 is important in regulating the function of ILC2s (162). However, leukotriene expression has also been reported in the hematopoietic compartment, associating neutrophils, eosinophils, basophils, mast cells, and macrophages as potential sources (165).

NEUROPEPTIDE RECEPTORS

It is known that the immune system is in constant communication with the nervous system, and both are capable of perceiving and responding to external stimuli. This communication relies on cell-cell interactions, soluble mediators, and occurs in different tissues (166, 167). Recent studies have shown that ILC2s can express receptors for several neuropeptides that can positively or negatively regulate their different functions (167).

Neuromedin U Receptor 1

Neuromedin U (NMU) is a neuropeptide associated with cholinergic neurons and expressed in different tissues. This neuropeptide exerts its action through NMUR1 and NMUR2 receptors (168), which belong to the 7 transmembrane family and are coupled to Gq proteins, inducing mobilization of intracellular calcium upon their activation (168). ILC2s of small intestine and lung express high levels of the NMUR1 gene (**Figures 1**, **2**) but do not express NMUR2 (114, 169), supporting other reports of NMUR2 expression being restricted to the central nervous system (168).

In the lung, NMU can activate ILC2s by inducing the expression of IL-5 and IL-13 alone or in combination with IL-25 (169, 170). In addition, NMU activation induces proliferation of ILC2s and their expression of amphiregulin and CSF-1; all these responses are dependent on NFAT activation (169). Expression of NMU in the lung is increased in response to helminth infection and ablation of this pathway leads to an inefficient control of the infection (169). NMU can increase the ILC2 response by inducing type 2 inflammation following allergen challenges (170). Interestingly, NMU increases the expression of the neuropeptide calcitonin gene related peptide alpha (α-CGRP) and its receptor, however this same treatment decreases the expression of NMUR1 (114).

Gut ILC2s also express the NMUR1 receptor. Its activation in this tissue induces similar effects as those in the lung in terms of the induced expression of IL-5, IL-13, amphiregulin, CSF-1 and IL-9, leading to proliferation and promotion of a defense response against helminth infections (169, 171).

These pathways are important to ILC2 biology since they allow these cells to communicate with the nervous system. In the small intestine, enteric cholinergic neurons are thought to be the source of NMU, while in the lung it is assumed that local cholinergic neurons could be working as sources of this molecule (169).

Calcitonin Gene Related Peptide Alpha Receptor

Calcitonin gene related peptide alpha is a highly potent vasoactive peptide characterized by having protective effects at the cardiovascular level. Its receptor is composed of 2 subunits, the calcitonin like receptor (CLR) and the transmembrane protein RAMP1 (172). Downstream, this receptor is coupled to Gs and Gq proteins depending on the cell type in which they are expressed (172). Like NMUR1, the expression of the α-CGRP receptor has been identified in ILC2s from lung and gut (**Figures 1**, **2**).

In the lung, ILC2s express both subunits of the α-CGRP receptor with regulatory effects on the function of these cells (173). In culture, α-CGRP increases IL-5 production together with IL-7, IL-25 and IL-33 but only at early time point stimulations. At later times cell proliferation and the levels of IL-5, IL-13 and amphiregulin decrease (114, 173, 174). *In vivo*, intranasal administration of α-CGRP counteracts the effects of IL-33 on ILC2 activation, which is consistent with the exacerbated inflammatory response of ILC2s in RAMP1 deficient mice (114, 173).

In the small intestine, ILC2s express the components of the α-CGRP receptor at steady state; however, this expression decreases under conditions of type 2 inflammation. *In vitro*, α-CGRP reduces the proliferation of ILC2s as well as the production of IL-13 while increasing IL-5 levels at early time points (175). *In vivo*, α-CGRP antagonizes IL-25-dependent activation of ILC2s (175).

Different cells have been identified to express α-CGRP in the lungs, among them are pulmonary neuroendocrine cells (PNEC) (174), sensory neurons, endothelial cells, and ILC2s themselves (114, 173). The source of α-CGRP in homeostasis *in vivo* was described in the gut as enteric neurons that express choline acetyltransferase (175). All these sources have been characterized by being located spatially close to ILC2s in their respective tissues, further supporting the potential relevance of this neuropeptide for ILC2 regulation and function.

Vasoactive Intestinal Peptide Receptors 1 and 2

The vasoactive intestinal peptide (VIP) was initially characterized as a potent vasodilator widely distributed in the central and peripheral nervous system, as well as in the digestive, respiratory and cardiovascular systems. Both VIP receptors, VPAC1 and VPAC2, signal through adenylate cyclase and activate the PKA pathway (176).

VPAC1 and VPAC2 are expressed in intestinal ILC2s (**Figure 2**) and their activation induces an increase in the expression of IL-5, which regulates the recruitment of eosinophils in basal conditions (177).

Both receptors are also expressed in lung ILC2s (**Figure 1**) and similarly to what has been described in the intestine, VIP-mediated activation increases IL-5 expression (177, 178). IL-5 then stimulates nociceptor neurons in the tissue to produce VIP, thus forming a positive regulatory loop with ILC2s in a context of allergic inflammation (178).

Like other neuropeptides, VIP is mainly expressed by neurons that innervate these tissues; therefore, the expression of all these receptors and their ligands licenses a complex system for the regulation of ILC2 function controlled by the nervous system.

Beta 2 Adrenergic Receptor

Upon ligand binding, adrenergic receptors can activate G proteins. In the case of β2AR, the binding of adrenaline and norepinephrine induces the activation of Gs proteins that in turn increase cAMP levels and activate the PKA pathway. It should be noticed that the same receptor has also been reported to activate Gi proteins (179).

The expression of beta 2 adrenergic receptor (β2AR) has been described in ILC2s of the small intestine, colon and lung (**Figures 1**, **2**) (180). In the small intestine and lung, binding to its ligand results in a negative regulation of ILC2 activation. β2AR-deficient mice showed increased ILC2 proliferation in a helminth infection model while β2AR agonist treatment of small intestine ILC2s decrease cytokine expression (180). Similar effects were observed in the lung using sterile inflammation models such as intranasal administration of IL-33 or challenge with allergens (180).

Expression of the β2AR ligands have been reported in both lung and small intestine. Sympathetic adrenergic neurons that are in regions anatomically relevant within these tissues may be inhibiting the action of ILC2s by secreting epinephrine and norepinephrine (180).

Acetylcholine Receptors

Acetylcholine (ACh) exerts its effects activating two groups of receptors: nicotinic and muscarinic. The nicotinic receptors are pentameric and function as sodium ion channels that activate neurons (181), while muscarinic are G protein-coupled receptors classified in 5 types (M1-M5): M1, M3 and M5 are activator receptors associated with $G_{q/11}$ proteins. while M2 and M4 are inhibitory receptors associated with $G_{i/o}$ proteins (182).

The α 7 nicotinic acetylcholine receptor (α7nAChR) is an excitatory synaptic receptor that serves as a therapeutic target in different neurological and inflammatory disorders (183). Expression of α7nAChR has been reported in lung ILC2s after intranasal administration of IL-25 and IL-33. Acetylcholine receptor agonists can attenuate the type 2 immune response in pulmonary allergic inflammation models (184). Treatment with agonists of this receptor *in vitro* and *in vivo*, decreases the expression of IL-5 and IL-13 induced by IL-33. Cholinergic activation has also been reported to inhibit the function of ILC2s in airway hyperreactivity models and in challenges with *Alternaria* extract, by inhibiting GATA-3 expression and NFKb activation (183). Contrastingly, in *N. brasiliensis* infection, the administration of ACh agonists, increases the number of IL-5 and IL-13 producing ILC2s in the lung (185).

Small intestine ILC2s express transcripts of the Chrm4 and Chrm5 subunits, associated with muscarinic receptors, as well as the Chrna2, Chrna5, Chrna9, Chrna10, Chrnb1 and Chrnb2 subunits, associated with nicotinic receptors (185). *In vitro* stimulation of these ILC2s increases the expression of IL-5 and IL-13 and experiments with specific inhibitors of both types of receptors, showed that both contribute to the activation of ILC2s mediated by ACh (185).

In addition to cholinergic neurons, it has been proposed that ILC2s produce ACh since the enzyme choline acetyltransferase (ChAT) is expressed in ILC2 of the lung and small intestine during *N. brasiliensis* infection (185, 186). This implies, in addition to an interaction with neurons, an autocrine loop of regulation of the ILC2s through ACh.

5-Hydroxytryptamine 1B Receptor

5-Hydroxytryptamine (serotonin) is perhaps best known as a neurotransmitter that modulates neural activity and a wide range of neuropsychological processes. The 5-HT1B receptor is a receptor with 7 transmembrane domains that is coupled to Gi proteins, therefore its activation results in the inhibition of adenylate cyclase (187).

A recent study showed that ILC2s of mesenteric lymph nodes express the 5-TH1B receptor and tryptophan hydroxylase 1 (Tph1), the limiting enzyme in serotonin biosynthesis (62). Induction of Tph1 in helminth infection is dependent on IL-33 and is important for the generation of iILC2s and the immune response against the parasites (62).

Although it has been reported that the main source of serotonin in the gastrointestinal tract are enterochromaffin cells (188), the finding that ILC2 express Tph1 postulates them as additional sources of this neurotransmitter. In addition, expression of the 5-HT1B receptor in these cells suggests that serotonin can play a role as an autocrine regulator of ILC2s like other mediators such as acetylcholine or IL-9.

SEX HORMONES: ANDROGEN RECEPTOR

Cumulative evidence shows a higher prevalence of asthma in women compared to men (189). This assertion was the starting point to investigate the mechanisms behind this gender-bias,

including the study and characterization of sex hormones and how they could be acting on the immune cells associated with this type of inflammation. In general, it has been observed that females have a higher number of ILC2s compared to males (189–191), which could contribute to their predisposition to asthma.

The expression of AR in ILC2 precursors was identified while studying the function of ILC2s in the bone marrow. Subsequent culture of these precursors in the presence of dihydrotestosterone (DHT) showed that this hormone can inhibit ILC2 expansion *in vitro* while an AR antagonist is capable of reversing this effect (190).

In the lung of gonadectomized mice, DHT tests performed *in vivo* and *in vitro* showed that androgens are capable of inhibiting IL-2 dependent proliferation of ILC2s as well as expression of GATA-3, RORα, CD25, KLRG1, IL-5 and IL -13 (189–191). Another study showed that activation of the AR increases Klrg1 expression, which has an inhibitory function when interacting with E-cadherin (113). In androgen receptor deficient mice, ILC2s were regulated in the lung and bone marrow (189–191).

NUTRIENTS

Given that ILCs participate in the maintenance of tissue homeostasis, it is not surprising that these cells play a role in the regulation of metabolic processes and glucose tolerance (192). How the function of ILCs is regulated by the availability of nutrients and factors derived from diet has been a topic of study in recent years (192).

Vitamin A

Vitamin A deficiency (VAD) in mice leads to increased expression of IL-5, IL-13 and IL-4 by ILC2s in the small intestine upon helminth infection. In addition, VAD increases proliferation and differentiation of ILC2s and their precursors by augmenting IL-7 receptor expression on these cells, enhancing their responsiveness to IL-7 (193). This deficiency can also lead to a decrease in ILC3 numbers and IL-17 and IL-22 expression (193). These effects occur *via* retinoic acid (RA), a vitamin A metabolite that negatively regulates ILC2s while favoring the function and expansion of ILC3s as a result of the expression of the alpha receptor of RA (RARα) on both cell types (193). This has also been observed in lung, where vitamin A deficiency increases the type 2 immune response, ILC2 activation and the severity of a lung cancer model (194).

The effect of retinoic acid on peripheral blood ILC2s in humans is opposite to that observed in murine tissues. *In vitro* stimulation of ILC2s with retinoic acid synergizes with other cytokines such as IL-7 and IL-33 to enhance IL-5 expression, while in combination with IL-2, IL-7, TSLP, IL-25 and IL-33 it increases the expression of IL-13 (195). Finally, it was also reported that treatment with retinoic acid and IL-2 induces the expression of the gut-homing integrin α4β7 (195). Hence, the effects of retinoic acid differ in humans and mice. Whereas in humans the retinoic acid activates ILC2s in mice the evidence suggests a direct effect through the receptor of this metabolite that regulates the function of ILC2s.

Fatty Acids

Several reports indicate that ILC2s have a higher fatty acid internalization compared to other cell populations such as regulatory T cells, ILC1s and ILC3s; this specific feature persists in different tissues including the small intestine, lung, skin and mesenteric adipose tissue (196). In the small intestine, the accumulation of ILC2s and production of IL-13 upon helminth infection or malnutrition conditions are dependent on fatty acids oxidation (FAO), as a process required to obtaining energy (196). Lung ILC2s express the free fatty acid receptors FFAR1 and FFAR4 (**Figure 1**), and *in vitro* addition of linoleic acid to IL-33 activated ILC2s, increases the expression of IL-5 (197).

Butyrate, a short-chain fatty acid (SCFA) produced by the fermentation of dietary fibers by the commensal microbiota, has been reported to work as a regulator of ILC2 function *in vivo* (198, 199). ILC2s can also be regulated by butyrate *in vitro*, having inhibitory effects on IL-33-induced expression of GATA-3, IL-5, IL-13 and GM-CSF, as well as on ILC2 proliferation (198, 199). Interestingly, this fatty acid is capable of inducing IL-17 production in ILC2s *in vitro*, decreasing in turn the expression of IL-5 and IL-13. Of note, ILC2s can express low levels of IL-17 in the absence of butyrate, so this SCFA only increases this capacity (198). In an airway hyperreactivity model, the transfer of ILC2s treated with butyrate fails to recruit eosinophils, recruiting neutrophils to the lungs instead (198). Finally, oral butyrate administration decreases ILC2 accumulation in models of lung inflammation (198, 199).

Together, these reports indicate that direct contact with metabolites derived from the diet can modulate the function of ILC2s, not only in the gastrointestinal tract but also in other tissues and this regulation is necessary for the control of the immune response promoted by these cells in different inflammatory contexts.

ILC2s IN TISSUES

While the study of ILC subsets is clearly an area of huge interest in the scientific community, most of cumulative data so far on ILC2s are limited to lung, skin and intestine, which are the main sites of inflammation in the canonical models for the study of these cells.

Lung ILC2s

Many cytokine receptors are expressed in the lung ILC2s (**Figure 1**). This includes receptors for IL-33, IL-25, TSLP, IL-2, IL-4, IL-7, IL-9 and SCF, all of which activate or enhance the activation of ILC2s. Cell-cell interaction molecules also regulate ILC2s in the lung; ICOS provides activation signals while Klrg1 could act as a negative regulator as recently suggested (113). Lung ILC2s also express lipid mediator receptors that transduce signals for prostaglandins and leukotrienes. The activation of CRTH2 and cysLTR1 favor the function of lung ILC2s while signaling through PGE2 and PGI2 receptors negatively regulate them. The neuropeptides NMU and VIP activate ILC2s through

their cognate receptors, while some catecholamines have the opposite effect, and CGRP, acetylcholine and fatty acid derivatives provide both positive and negative regulation to ILC2 function. Finally, among factors derived from the diet, retinoic acid functions as a negative regulator of these cells. All these signals allow ILC2s to interact both with other cells of the immune, nervous and epithelial system, as well as with other ILC2s through autocrine regulation by IL-9 or ACh in the lung (**Figure 1**).

Gut ILC2s

ILC2 communication with the epithelium, and the nervous and immune systems also occurs in the gut (**Figure 2**). In this tissue, the cytokines IL-33, IL-25, IL-7, and IL-9, leukotrienes C4 and D4, and neuropeptides NMU, VIP and ACh contribute to the activation of ILC2s through their cognate receptors, as does the signaling mediated by ICOS and its ligand ICOS-L. Metabolism of fatty acids also favors gut ILC2s, however, to our knowledge there are no reports indicating that it occurs through a receptor. Similarly to the lung, a dual function of CGRP has been reported in the gut, while the B2AR receptor and retinoic acid are related to a negative regulation of ILC2s in this tissue. Klrg1 is also expressed on ILC2s in the gut, although its specific function in this tissue has yet to be characterized (**Figure 2**). Although the interaction of gut ILC2s with different cell groups within the immune, epithelial, and nervous systems are important, a particular interaction that should be noted is that of small intestine ILC2s with Tuft cells. IL-17RB expression is critical in small intestine ILC2s and Tuft cells are the main source of IL-25 and lipid mediators that can activate ILC2s including leukotrienes and prostaglandins.

Skin ILC2s

Unlike the two previous tissues, Klrg1 in skin has been characterized as a negative regulator of ILC2 function since the interaction of ILC2s with E-cadherin results in a down regulation of the expression of GATA-3 and type 2 cytokines. Conversely, IL-33, IL-25, TSLP, IL-2, IL-4, IL-7, IL-18, and PGD2 provide activating signals to ILC2s through their receptors. The expression of these receptors allows ILC2s to communicate with different cells of the skin microenvironment like fibroblasts and keratinocytes, which are sources of alarmins in the skin, as well as with cytokine-producing granulocytes such as mast cells (**Figure 3**).

CONCLUDING REMARKS

This review summarizes the most current reports associated with the function of a large number of markers, ligands and signals that could help in our understanding of the intricate way ILC2s are regulated and perform their functions *in vivo*, both in homeostasis and in inflammatory models. We are aware that this is a growing field, and new signals able to regulate these cells are being constantly identified. The main focus was on widely described receptors that activate ILC2s as well as recent discoveries of other signals capable of regulating these cells in contexts of inflammation and infection while excluding markers that although are of great importance, have already been covered in other reviews (109, 140, 200–202).

In this review we focus on markers described in both mice and humans, however there is more information associated with ILC2s from tissues in mice given the existing limitations for its study in humans.

In addition to describing the function of different receptors expressed on ILC2s, this review seeks to be a tool to facilitate the use of markers for ILC2 identification in different study models in a tissue-dependent manner. Therefore, despite not knowing the function of some of the molecules mentioned, in **Table 1** we summarize their expression reported at the protein level in different models. In addition to the lung, gut and skin ILC2 markers, we include markers for their identification in adipose tissue and bone marrow. ILC2s have been reported to be present at different anatomical locations within adipose tissue and are important for the maintenance of its homeostasis. Multiple studies of ILC2 precursors have been carried out in the bone marrow and the ILC2s from this lymphoid organ can contribute to the different responses of these cells.

CD90 and Sca-1 are a couple of markers widely expressed on ILC2s commonly used to identify these cells in most tissues. However, their role in the function of ILC2s is not entirely clear and additional studies are required to fully understand their importance *in vivo* (**Figures 1**–**3** and **Table 1**).

Establishing a panel of specific markers for the identification of ILC2s in different tissues is a complicated task. For example, early reports suggested that alarmin-dependent activation of ILC2s could be tissue-specific. ST2 was found to be expressed on ILC2s of the lung, while in the intestine, the response to IL-25 appeared to be more relevant than IL-33, given the expression of IL-17RB in this tissue. However, current evidence suggests that ILC2s from different tissues have the potential to express the same markers and their profiles are established as a result of modulation by the microenvironment, challenges and interaction with other cell types.

Another complication when selecting a marker to track ILC2s during the course of a study is that their activation modulates the expression of the different markers. In *N. brasiliensis* infection, iILC2s are induced during a specific window of time in the lung and small intestine. These cells are characterized by expressing IL-17RB but not ST2 or CD25. In the lung, iILC2s subsequently give rise to natural ILC2s with increased ST2 expression. For this reason, it is critical to understand how the expression of a marker is expected to change in a specific tissue on different models.

The information presented in this review has been compiled in **Table 1**. At steady state, the markers shared by ILC2s in most tissues include Thy1, IL-7Ra and IL-2Ra. In lung ILC2s, ST2 is one of the more consistently reported markers, while the basal expression of IL-17RB is more commonly used to identify gut ILC2s. Although there are few reports about IL-18R1, it could be an option to identify skin ILC2s, given its differential expression with respect to ILC2s from other tissues. Finally, Klrg1 has been widely reported in the lung and gut ILC2s, not so in the skin.

We would like to emphasize that we do not rule out that some other markers not listed here might be used to identify ILC2s.

Future studies could provide additional markers to identify ILC2s in a tissue specific manner, especially those involved in the interaction of these cells with other cell types including epithelial and neural. Another issue to consider is the shared expression of markers such as Thy1 or IL-7Ra with other leukocytes, including other groups of ILCs. Therefore, until specific ILC2 markers are found, the use of ST2, IL-17RB, IL-18R1 or IL-2Ra will require additional lineage cocktails for negative selection.

Even though most of studies have characterized ILC2s in the lung, skin and intestines, new studies indicate that these cells are important in many other organs, performing functions in both homeostasis and inflammation. ILC2s are also located in the stomach, liver and central nervous system. Therefore, it is likely that future studies will describe the presence of ILC2s and novel markers for their identification in different tissues, supporting the heterogeneity of their subsets in a tissue and environmental specific manner.

Interestingly and as mentioned throughout this review, growing evidence suggests that the same pathway can fulfill different functions depending on the context. Therefore, the availability of different signals as well as their sources can substantially vary among distinct tissues. Data discussed in this review, together with cumulative evidence related to particular signals in different tissue-specific microenvironments can help us understand not only how ILC2 are activated but also how they function as part of a more complex system.

Altogether, the information compiled in this review highlights the complexity of ILC2s, which are an extremely important part of the immune response. Not only are ILC2s initiators of inflammatory processes and aid in the maintenance of tissue homeostasis, but they also link the immune system to other systems, allowing a timely and efficient response of the entire organism to different challenges. We also would like to stress that the heterogeneity of ILC2s is highly important and the selection of markers used to identify these cells

TABLE 1 | Expression of markers associated with the identification and regulation of ILC2s in different tissues and study models.

Tissue	Marker	Models	References
Lung	ST2	Basal conditions (mouse and human)	(9, 17, 33, 39, 54, 79, 85, 86, 112, 117, 119, 122, 123, 134, 144, 145, 177, 183, 186, 191, 199)
		Allergic inflammation (papain administration)	(53, 105)
		Helminth infection (*N. brasiliensis*)	(54, 92, 123, 185, 186)
		Cecal ligation and puncture sepsis model	(56)
		Intraperitoneal administration of IL-33	(17, 145)
		Intraperitoneal administration of IL-25	(17, 173)
		Intranasal administration of TSLP	(79)
		Intranasal administration of IL-33	(112, 145, 148, 183)
		A. fumigatus infection	(85)
		Respiratory syncytial virus infection	(134)
		Alternaria alternata challenge	(148, 199)
	IL-17RB	Basal conditions (mouse and human)	(58, 94, 186)
		Intraperitoneal administration of IL-25	(17)
		Helminth infection (*N. brasiliensis*)	(186)
	TSLPR	Basal conditions	(78–80)
		Respiratory syncytial virus infection	(78)
		Intranasal administration of IL-33	(79)
	IL-2Rα	Basal conditions (mouse and human)	(33, 58, 84, 85, 93, 94, 107, 112, 119, 122, 177, 183, 189–191)
		Allergic inflammation (HDM* administration)	(84)
		Helminth infection (*N. brasiliensis*)	(27, 92)
		Intranasal administration of IL-33	(84, 148, 183)
		A. fumigatus infection	(85)
		Allergic inflammation (papain administration)	(93, 105)
		Alternaria alternata challenge	(148, 197)
	IL-4Rα	Basal conditions (mouse and human)	(93, 94)
		Allergic inflammation (papain administration)	(93)
		Helminth infection (*N. brasiliensis*)	(92)
	IL-7Rα	Basal conditions (mouse and human)	(19, 33, 58, 61, 84, 86, 93, 118, 119, 122, 144, 145, 177, 185, 189, 199)
		Helminth infection (*N. brasiliensis*)	(27, 92, 185)
		Intraperitoneal administration of IL-25	(17)
		Allergic inflammation (HDM* administration)	(84)
		Allergic inflammation (papain administration)	(93)
		Intraperitoneal administration of IL-33	(145, 185)
		Intranasal administration of IL-33	(145, 180, 183)
		Alternaria alternata challenge	(199)
	IL-9Rα	Basal conditions (human)	(94)
	IL-18R1	Basal conditions	(39)
	Klrg1	Basal conditions (mouse and human)	(19, 84, 86, 112, 114, 118, 156, 170, 177, 190, 191)
		Intraperitoneal administration of IL-25	(17, 173)
		Intranasal administration of IL-33	(84, 112, 156, 170)

(Continued)

TABLE 1 | Continued

Tissue	Marker	Models	References
		Allergic inflammation (HDM* administration)	(84)
		Helminth infection (*N. brasiliensis*)	(17, 92, 123)
		Intranasal administration of IL-25	(170)
	ICOS	Basal conditions (mouse and human)	(33, 94, 116, 119, 122, 123, 177, 199)
		Intranasal administration of IL-33	(84)
		Allergic inflammation (HDM* administration)	(84)
		Helminth infection (*N. brasiliensis*)	(92, 123)
		Allergic inflammation (papain administration)	(116)
		Alternaria alternata challenge	(199)
	ICOS-L	Basal conditions	(20)
	MHC-II	Basal conditions	(122)
		Helminth infection (*N. brasiliensis*)	(123)
		Allergic inflammation (papain administration)	(105)
	KIT	Basal conditions	(33, 84, 133, 134)
		Influenza A virus infection	(133)
		Respiratory syncytial virus	(134)
		Intraperitoneal administration of IL-25	(17)
		Allergic inflammation (HDM* administration)	(84)
	CRTH2	Basal conditions (human)	(94)
	IP	Basal conditions	(155)
	CysLTR1	Basal conditions	(158)
		Alternaria alternata challenge	(158)
	NMUR1	Basal conditions	(170)
		Intranasal administration of IL-25	(170)
	α7nAChR	Intranasal administration of IL-33	(183)
		Intranasal administration of IL-25	(183)
	Thy1	Basal conditions (mouse and human)	(33, 39, 56, 85, 86, 93, 117, 143–145, 158, 177, 183, 189–191, 193, 197, 199)
		Cecal ligation and puncture sepsis model	(56)
		Intraperitoneal administration of IL-25	(17)
		Helminth infection (*N. brasiliensis*)	(17, 27)
		A. fumigatus infection	(85)
		Allergic inflammation (papain administration)	(93, 105)
		Intraperitoneal administration of IL-33	(145, 190)
		Intranasal administration of IL-33	(145, 180)
		Alternaria alternata challenge	(158, 197, 199)
	Sca-1	Basal conditions (mouse and human)	(33, 86, 107, 133, 156)
		Intraperitoneal administration of IL-25	(17)
		Allergic inflammation (papain administration)	(105)
		Intranasal administration of IL-33	(105, 156)
		Intranasal administration of IL-25	(105)
		Influenza A virus infection	(133)
		Alternaria alternata challenge	(197)
Gut	ST2	Basal conditions	(60–62, 72, 107, 118)
		Intraperitoneal administration of IL-33	(63)
		Helminth infection (*T. spiralis*)	(74)
	IL-17RB	Basal conditions	(39, 61, 72)
		Tritrichomonas colonization	(72)
		Helminth infection (*T. spiralis*)	(74)
	IL-2Rα	Basal conditions	(63, 107, 112, 118, 119)
		Intraperitoneal administration of IL-33	(63)
	IL-7Rα	Basal conditions	(61, 107, 112, 118, 119, 171, 177)
		Intraperitoneal administration of IL-33	(63)
		Helminth infection (*T. spiralis*)	(74)
	Klrg1	Basal conditions	(60–62, 72, 107, 114, 118, 119, 162, 171, 177, 180)
		Tritrichomonas colonization	(72)
		Helminth infection (*T. spiralis*)	(74)
		Helminth infection (*N. brasiliensis*)	(171)
	ICOS	Basal conditions	(118, 119, 177)
	MHC-II	Basal conditions	(74)
		Helminth infection (*T. spiralis*)	(74)
		Intraperitoneal administration of IL-33	(63)
	KIT	Basal conditions	(107)
		Intraperitoneal administration of IL-33	(63)

(Continued)

TABLE 1 | Continued

Tissue	Marker	Models	References
	CRTH2	Basal conditions	(143)
	NMUR1	Basal conditions	(171)
		Helminth infection (*N. brasiliensis*)	(171)
	Thy1	Basal conditions	(39, 72, 107, 193)
		Intraperitoneal administration of IL-33	(63)
	Sca-1	Basal conditions	(107)
Skin	ST2	Basal conditions	(15, 64, 96)
		Wounding model	(64)
		Induced expression of IL-33 in skin	(65)
		Atopic dermatitis (MC903 administration)	(15, 95)
		Atopic dermatitis (human)	(15, 16, 95)
	IL-17RB	Basal conditions	(58)
		Atopic dermatitis (human)	(16)
	TSLPR	Atopic dermatitis (human)	(16)
	IL-2Rα	Basal conditions (mouse and human)	(15, 16, 58, 64, 87, 112)
		Wounding model	(64)
		Atopic dermatitis (MC903 administration)	(15, 95)
		Atopic dermatitis (human)	(15, 95)
		Allergic inflammation (DDAC** administration)	(87)
	IL-4Rα	Basal conditions	(95)
	IL-7Ra	Basal conditions (mouse and human)	(15, 16, 58, 81, 87, 96, 112, 119, 135)
		Atopic dermatitis (human)	(16)
		HDM* administration (Human)	(81)
		Allergic inflammation (DDAC** administration)	(87)
	IL-18R1	Basal conditions	(39)
	Klrg1	Basal conditions	(95)
		Atopic dermatitis (human)	(16)
	ICOS	Basal conditions (mouse and human)	(15, 16, 87, 88, 119)
		Allergic inflammation (DDAC** administration)	(87)
	KIT	Basal conditions (mouse and human)	(15, 16, 129, 135)
	CRTH2	Basal conditions (human)	(16, 81, 96, 129, 135)
		HDM* administration (Human)	(81)
		Allergic inflammation (DDAC** administration)	(87)
		Psoriasis (human)	(135)
	Thy1	Basal conditions	(15, 39, 64, 88, 193)
		Wounding model	(64)
	Sca-1	Basal conditions	(15)
		Induced expression of IL-33 in skin	(65)
Adipose tissue	ST2	Basal conditions (mouse and human)	(18, 22, 37, 39, 70)
		Intraperitoneal administration of IL-33	(22)
	IL-2Rα	Basal conditions (mouse and human)	(18, 22, 37)
		Intraperitoneal administration of IL-33	(18)
	IL-7Ra	Basal conditions (mouse and human)	(18, 22, 37, 61)
	Klrg1	Basal conditions	(18, 114)
	ICOS-L	Basal conditions	(20)
	KIT	Basal conditions	(18, 37)
	Thy1	Basal conditions	(18, 37, 39, 70)
	Sca-1	Basal conditions	(18, 37)
Bone marrow	ST2	Basal conditions	(39, 59, 84, 191)
		Intranasal administration of IL-33	(59, 84)
		Allergic inflammation (HDM* administration)	(84)
	IL-17RB	Basal conditions	(39, 58)
		Allergic inflammation (*A. alternata*)	(58)
	IL-2Rα	Basal conditions	(58, 59, 84, 190)
		Allergic inflammation (*A. alternata*)	(58)
		Intranasal administration of IL-33	(59, 84)
		Allergic inflammation (HDM* administration)	(84)
		Intraperitoneal administration of IL-33	(145)
	IL-7Ra	Basal conditions	(33, 58, 59, 84, 191)
		Allergic inflammation (*A. alternata*)	(58)
		Intranasal administration of IL-33	(59, 84)
		Allergic inflammation (HDM* administration)	(84)
	Klrg1	Basal conditions	(191)

(Continued)

TABLE 1 | Continued

Tissue	Marker	Models	References
		Intranasal administration of IL-33	(84)
		Intraperitoneal administration of IL-33	(145)
	ICOS	Basal conditions	(84)
		Intranasal administration of IL-33	(84)
		Allergic inflammation (HDM* administration)	(84)
	KIT	Basal conditions	(33)
	CysLTR1	Basal conditions	(158)
	Thy1	Basal conditions	(39, 158, 191)
		Intraperitoneal administration of IL-33	(145)
	Sca-1	Basal conditions	(33, 190, 191)
		Intraperitoneal administration of IL-33	(145)

HDM, House dust mite.
***DDAC, Didecyldimethylammonium chloride.*

when working on a specific model should be carefully considered. Acknowledging the differential expression profile of ILC2s specific to each tissue resident population could help our understanding of ILC2s and reconcile apparent controversies between reports of their functions *in vivo*.

AUTHOR CONTRIBUTIONS

EO-M conceived the idea, wrote and discussed the review. BR-M revised the manuscript. PL-L conceived the idea, discussed, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Innate Lymphoid Cells and Myocardial Infarction

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Myocardial infarction results from obstruction of a coronary artery that causes insufficient blood supply to the myocardium and leads to ischemic necrosis. It is one of the most common diseases threatening human health and is characterized by high morbidity and mortality. Atherosclerosis is the pathological basis of myocardial infarction, and its pathogenesis has not been fully elucidated. Innate lymphoid cells (ILCs) are an important part of the human immune system and participate in many processes, including inflammation, metabolism and tissue remodeling, and play an important role in atherosclerosis. However, their specific roles in myocardial infarction are unclear. This review describes the current understanding of the relationship between innate lymphoid cells and myocardial infarction during the acute phase of myocardial infarction, myocardial ischemia-reperfusion injury, and heart repair and regeneration following myocardial infarction. We suggest that this review may provide new potential intervention targets and ideas for treatment and prevention of myocardial infarction.

Keywords: myocardial infarction, innate lymphoid cells, myocardial ischemia-reperfusion injury, regeneration and repair after myocardial infarction, the acute phase of myocardial infarction

INTRODUCTION

Myocardial infarction (MI) is one of the main causes of death worldwide, is characterized by high morbidity and mortality, and poses a serious threat to human health (1). The pathophysiological basis of MI is atherosclerosis (AS), which is a chronic inflammatory disease characterized by formation of fibrofatty lesions (2). The 'Canakinumab Anti-inflammatory Thrombosis Outcomes Study' (CANTOS) demonstrated the clinical benefits of suppressing inflammation (3). In the immune microenvironment of AS, interactions between innate immune, adaptive immune, and non-immune cells promote progression of AS (4). Innate lymphoid cells (ILCs) are a family of immune cells recently shown to be implicated in inflammation, metabolism and tissue remodeling (5), and cardiovascular diseases such as AS (6–8), pericarditis (9), cardiac fibrosis (10)and MI (11). Gong et al. summarized the roles of helper ILCs in inflammation-associated cardiovascular disease

(12). However, the specific roles of ILCs in MI are still unclear. To identify potential intervention targets and propose ideas for treatment and prevention of MI, this review explores the relationship between ILCs and MI during the acute phase of MI, myocardial ischemia-reperfusion injury, and heart repair and regeneration following MI.

TYPES OF ILCS

ILCs are mainly distributed in the intestines, lungs and other mucosal layers that are in direct contact with the environment, and participate in the immune system's first line of defense against pathogens. ILCs lack cell surface molecules that recognize other lymphocytes and cannot recognize antigens. They produce cytokines such as interferon-γ (IFN-γ), interleukin (IL)-4, IL-5, and IL-13, which play an important role in tissue homeostasis and inflammation (5). The types and functions of ILCs are shown in **Table 1**. Depending on their killing ability, ILCs can be divided into cytotoxic ILCs, namely conventional natural killer (NK) cells, and auxiliary ILCs such as type 1 (ILC1), 2 (ILC2), and 3 (ILC3) ILCs. Based on differences in expression of key transcription factors and cytokines, ILCs are divided into three subgroups: Groups 1, 2, and 3. Group 1 ILCs (including ILC1s and NK cells) are functionally similar, express the T-box transcription factor T-bet, produce IFN-γ and tumor necrosis factor (TNF), and react to tumors and intracellular pathogens such as viruses. Group 2 ILCs include cytokine-producing T_h2 cells, whose development and function depend on GATA binding protein 3 (GATA3) and retinoic acid receptor-related orphan receptor-α (RORα), and ILC2s, which mainly act on extracellular parasites and allergens and participate in repair of tissue and organ damage. Group 3 ILCs include ILC3s and lymphoid tissue inducer (LTi) cells, which produce IL-22 and IL-17 cytokines to resist bacteria, fungi and other extracellular microorganisms (13).

Recently, ILCregs were identified as belonging to a new distinct regulatory subset of ILCs. ILCregs suppress activation of ILC1s and ILC3s *via* secretion of IL-10 and are inhibitory during innate intestinal inflammation (14). ILCregs can also promote colorectal tumor growth from ILC3 transdifferentiation. Blockade of TGF-β signaling disrupts conversion of ILCregs and inhibition of tumor growth (15). The roles of NK cells in MI have been reviewed elsewhere (16) and therefore are not discussed here.

THE DISTRIBUTION OF ILCS IN THE HEART AND SURROUNDING TISSUES

Cardiac ILCs are a type of static and undifferentiated tissue-resident cell that lack a clear immunophenotype, have unique surface markers and self-renew quickly when disturbed (17). Most research on the tissue distribution of ILCs has focused on ILC2s. Perry et al. (18) reported that NH cells (one type of ILC2) were present in aortic perivascular adipose tissue. These adipose tissue-resident NH cells could be stimulated by IL-33 to produce IL-5. Engelbertsen et al. (19) demonstrated that ILC2s were present in the aorta and in perivascular adipose tissue. Newland et al. (7) showed that ILC2s were present in para-aortic adipose tissue and lymph nodes. Wu et al. reported auxiliary ILCs (ILC1s, ILC2s and ILC3s) in the aorta in response to a high fat diet (10). Gao et al. (6), Deng et al. (17) and Chen et al. (10) reported expression of ILC2s in the heart. Bracamonte-Baran et al. (20) found that in the hearts of healthy humans and mice, non-cytotoxic ILCs were predominantly a type 2-committed population with progenitor-like features and could differentiate into conventional ILC2s during myocarditis and ischemia.

THE ROLE OF ILCS IN VARIOUS STAGES OF MI

It is known that the immune system is activated during myocardial injury. In the coronary arteries, exposed atherosclerotic plaques promote recruitment and entry of cells of the innate immune system into heart tissue, prompting release of cytokines and aseptic inflammation in damaged heart tissue (21). Immune cells participate in the cleanup of necrotic debris,

TABLE 1 | Types and functions of the ILCs.

Type	Group 1 ILC		Group 2 ILC	Group 3 ILC		ILCreg
	NK Cell	ILC1s	ILC2s	ILC3s	LTi	
Transcription factors	T-bet		RORα and GATA3	RORγt		Id3 and Sox4
Secretion	IFN-γ and TNF		IL-4, IL-5 and IL-13	IL-17A and IL-22		IL-10 and TGF-β1
Function	Anti-tumor and anti-viral functions, immune regulation, hypersensitivity, autoimmune diseases, etc.	Defense against parasites and intracellular bacteria, killing tumor cells.	Respond to extracellular parasites and allergens; participating in repair of tissue and organ damage, treatment of respiratory diseases, allergic inflammation, etc.	Defense against bacteria, fungi and other extracellular microorganisms, maintain intestinal stability.		Regulate congenital intestinal inflammation.

and at the same time initiate a repair response in the myocardium. However, excessive activation of immune cells in heart tissue may lead to cardiomyocyte apoptosis and fibrosis (22). In this section, we summarize the roles ILCs play in the acute phase of MI, myocardial ischemia-reperfusion injury, and repair and regeneration of heart tissue after MI.

THE ROLE OF ILCs IN AS

As is well known, AS is the pathologic basis for MI. Several studies have reported roles for ILCs in AS. For ILC1s, Wu et al. (10) showed that ILC1s can aggravate AS. When depleted of ILC1 cells, the atherosclerotic lesions shrank in $Apoe^{-/-}Rag1^{-/-}$ mice, and this effect could be rescued through adoptive transfer of ILC1s. In the case of ILC2s, IL-5 and IL-13 are cytokines that play a central role in their function. Perry et al. (18) demonstrated a role for the ILC2-IL-5-Bl-IgM axis in AS. IL-33 stimulated NH cells to secrete IL-5; IL-5 then stimulated B-1a B cell proliferation to produce IgM natural antibody, which is an atheroprotective factor. Engelbertsen et al. (19) demonstrated that expansion of CD-25-expressing ILCs could reduce AS, and was associated with reduced VLDL and increased IL-5. Global depletion of ILCs did not affect lesion size, indicating that different subsets of ILCs may have different roles in AS, consistent with the report of Wu (10). Newland et al. (7) showed that genetic ablation of ILC2 could accelerate development of AS, which could be prevented by reconstitution with ILC2 (wild type) but not $IL5^{-/-}$ or $IL13^{-/-}$. They found that ILC2-derived IL-5 and IL-13, and especially IL-13, were critical for control of AS progression, partly through M2 polarization. Therefore, the ILC2s-IL-13-M2 axis could be another pathway for ameliorating AS. Gao et al. (6) reported that Treg-ILC2s-IL13 could improve AS. Tregs may play a partially protective role against AS by expanding the number of ILC2s and thereby increasing IL-13 production. We are not aware of any studies on ILC3s and ILCregs in AS. ILC3s can secrete IL-17 and IL-22, but reports of the roles of these two cytokines in AS are inconsistent (23). In addition, given the small numbers of ILC3s, their role in AS is unclear. ILCregs can produce IL-10 and TGF-β, which are protective factors for AS. Therefore, ILCregs may have the potential to improve AS, but further studies are needed.

THE ROLE OF ILCS IN THE ACUTE PHASE OF MI

During acute MI, coronary blood flow is suddenly blocked and the myocardium goes from ischemic to necrotic. Although several types of immune cells are involved in this process, the roles and mobilization of ILCs as "front-line" innate immune cells in acute MI are not clear.

Recently, Li et al. (24) described levels of ILCs in the peripheral blood in the setting of acute ST-segment elevation myocardial infarction (STEMI). They consecutively enrolled 176 STEMI patients and 52 control patients, initially collecting blood samples after the diagnosis and prior to medical therapy, and following with serial samples at days 3, 5 and 14 after the onset of STEMI. During this period, the proportion of total ILCs and ILC1s increased compared with controls; while the proportion of ILC2s decreased significantly. In addition, patients were followed for up to 23 months. They found that ILC1s were an independent predictor of major adverse cardiovascular events. In addition, RNA sequencing performed on ILC1s showed that IFN-γ, TNF-α, vascular cell adhesion molecule 1 (VCAM1), and matrix metallopeptidase 9 also increased. These results suggested the possibility of using ILCs as a disease marker in the future.

Several papers have focused on ILC2s in AS (7, 10, 11), but few in MI. Bracamonte-Baran (20) reported the presence of ILC2s during the early stages of ischemic injury. During ischemia, cardiac ILCs could differentiate into ILC2s, but not ILC1s or ILC3s. Importantly, they demonstrated that the ILC2s resulted from local proliferation rather than infiltration of circulating ILCs. Yu et al. (11) demonstrated that low-dose IL-2 injection could activate ILC2s in acute coronary syndrome (ACS). Individuals received low or medium-dose IL-2 or placebo daily for 5 days. The placebo had no effect on ILC2s, meanwhile, IL-2 injection could decrease ILC2 canonical surface markers among isolated peripheral blood mononuclear cells (PBMCs) and increase serum IL-5 titers, indicating activation of ILC2s. IL-5 plays an anti-atherosclerotic effect by increasing the titer of a natural IgM antibody specific to the oxidized LDL epitope (25). IL-13, another cytokine secreted by ILC2s, was reported to be significantly increased in the myocardium after MI, with a peak on day 3 (26). IL-13 could improve cardiac function by recruiting more monocytes/macrophages and inducing M2 macrophages (27). IL-13 could also reduce cardiac scar area (28), increase cardiomyocyte cell cycle activity (28) and even facilitate cardiac regeneration (29). It has been reported that IL-13 may also be a prognostic marker of acute myocardial infarction (AMI) (30). These results indicate that ILC2s may play vital roles in the acute phase of MI.

There are as yet no reports of a role for ILC3s in MI. However, the relationship between ILC3-secreted IL-17 and MI has been well documented (31). What's more, expression of another ILC3-produced cytokine, IL-22, was elevated in ACS (23). Tang et al. (32) reported that IL-22 could prevent LV dysfunction and heart failure after acute MI. The question of whether ILC3s have any effect on MI requires further research. The same question arises for ILCregs in terms of the known roles of IL-10 and TGF-β in MI (14).

THE ROLE OF ILCS IN MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

Despite the increasing use of coronary reperfusion in interventional therapy, morbidity and mortality after STEMI remain high. The mechanisms of myocardial ischemia-reperfusion injury (MIRI) are unclear. Apart from

cardiomyocyte cell death, coronary microvascular injury is also an important component of MIRI (33, 34). Manifestations of coronary microvascular injury during MIRI range from reversible edema to capillary destruction with intramyocardial hemorrhage. Cardioprotection refers to all measures and interventions to reduce myocardial ischemia and reperfusion injury. Ischemic conditioning, including remote ischemic conditioning, was reported to reduce inflammation, and was considered to be one of the main methods of cardioprotection (34, 35). There is currently no research directly connecting ILCs to MIRI, but there are some reports characterizing ILCs in ischemia-reperfusion injury to other organs. Kang et al. (36) reported that ILC1s increased in high-fat diet mice following liver ischemia-reperfusion injury (IRI), with an increased production of IFN-γ and TNF-α. Cao et al. (37) reported that ILC2s could prevent renal injury in mice subjected to IRI, and that this effect was associated with M2 macrophage induction and ILC2 production of amphiregulin. Subsequently, the same group showed that ILCregs could suppress innate immunity and reduce renal IRI (38). IL-2/IL-2 antibody complexes (IL-2C) could promote ILCreg expansion *in vivo* and prevent renal IRI in $Rag^{-/-}$ mice. Using anti-CD25 antibody to deplete ILCregs abolished these beneficial renal effects. Adoptive transfer of ILCregs improved renal function. For ILC3s, Eggenhofer et al. (39) reported that NCR^{+} ILC3s could protect against hepatic IRI through IL-22. Severe hepatic IRI in NCR^{+} ILC3-deficient mice can be reversed with adoptive transfer of IL-22-producing NCR^{+} ILC3s. What's more, Geha et al. (40) reported that ILC derived IL-17A was essential for intestinal IRI.

Studies in a mouse model of MIRI demonstrated that the inflammatory cytokines TNF-α, IL-1β and IL-6 were rapidly up-regulated after reperfusion, but then decreased 6 to 24 hours later. 24–72 hours after MIRI, expression of pro-inflammatory cytokines decreased significantly, and levels of fibrotic mediators such as TGF-β and IL-10 showed a continuous and rapid increase, reflecting suppression of acute inflammation and transition to cardiac repair and proliferation (41, 42). Recently, Pluijmert et al. (43) summarized the multifactorial and dynamic process of the inflammatory response in MIRI, including TNF-α, IL-4, IL-13, IL-10 and TGF-β. These results suggested that cytokines, especially those related to ILCs, play important roles in MIRI (22).

IL-33 is a member of the IL-1 family that plays an important role in innate immunity. The IL-33/ST2 signaling pathway mainly acts to reduce cardiac hypertrophy, ventricular dilatation and cardiac fibrosis under mechanical stress. IL-33 can stimulate secretion of IL-5, IL-13, BMP-7, IL-10 and G-CSF in ILC2s through the ST2 receptor, and has a protective effect on heart tissue damage. In acute heart injury, IL-13 and BMP-7 promote an IL-33-mediated anti-fibrosis response, immune cell recruitment and tissue repair (10). Studies have shown that circulating levels of IL-33 and soluble ST2 (sST2) are related to the severity of ACS. The level of IL-33 in serum is significantly lower in patients with ACS compared to those with stable angina pectoris. The level of sST2 is negatively correlated with left ventricular ejection fraction and prognosis of MI patients, and reflects the severity of MI (44). In a rat MIRI model, subcutaneous injection of IL-33 can significantly reduce infarct size and myocardial fibrosis. IL-33 exerts a cardioprotective effect by binding to the ST2 receptor (45). The IL-33/ST2 system is closely associated with ILC2s, and may be a potential target for predicting severity and prognosis of ACS and treatment of MIRI (44). Koeppen et al. (46) demonstrated that amphiregulin can suppress MIRI. It has also been shown that ILC2s can produce amphiregulin (37, 47). The IL-33-ILC2-AREG pathway could protect against intestinal inflammatory diseases (48). So, it is possible that the ILC2s-AREG pathway is involved in MIRI, although further research is needed. Liao et al. (49) demonstrated that IL-17A could promote cardiomyocyte apoptosis and neutrophil infiltration after MIRI *in vivo*. IL-17A knockout or anti-IL-17A monoclonal antibody treatment may significantly improve MIRI *via* reduced infarct size, reduced cardiac troponin T levels, and improved cardiac function. Whether ILC3s can affect MIRI through IL-17A is unknown.

In short, although critical evidence is lacking for an important role of ILCs in MIRI, ILC2s may be the most promising candidate.

THE ROLE OF ILCS IN REPAIR AND REGENERATION AFTER MI

The hearts of adult mammals cannot regenerate. After MI, necrotic areas gradually become fibrotic to form non-contractile scars, leading to heart failure and even death (50). Innate immune cells (such as macrophages, mast cells and ILCs) curb inflammation and regulate the balance between cardiac tissue repair and regeneration and scar formation by removing dying cells and promoting cardiomyocyte replacement (51). After myocardial ischemia injury, innate immune cells residing in the myocardium are immediately activated. Resident cardiac macrophages have been shown to initiate apoptosis after 2 hours, and circulating monocytes to infiltrate into the injury site and differentiate into macrophages (52). M1 macrophages are responsible for degradation of the extracellular matrix and removal of cell debris. M2 macrophages secrete anti-inflammatory cytokines and promote angiogenesis and collagen deposition (51). Mast cells release histamine to trigger vascular permeability and migration of circulating innate immune cells.

ILCs and macrophages are both resident-tissue cells. ILCs can interact with macrophages through different signaling pathways. ILC2s can secret IL-4 and IL-13, which promote polarization of macrophages into the M2 phenotype through STAT6 by inducing phosphorylation and promoting transcription (53). This anti-inflammation macrophage phenotype has important roles in wound repair and resolution (54), which is also important for myocardial injury. Hams et al. (55) summarized the roles of ILCs in fibrosis with potential interactions between ILCs and macrophages. Kim (56) also discussed how ILCs coordinate the polarization of lung macrophages through cytokine secretion and ILC-macrophage interactions. So, it's

possible that ILCs can communicate with macrophages *via* IL-4/IL-13 secretion and direct cell–cell contact. Together, they coordinate the removal of damaged cells, the remodeling of the tissue matrix, and the recruitment of additional immune cells from the blood to promote recovery of heart function after MI (**Figure 1**) (51).

Recently, Yu et al. (11) reported that ILC2s could promote cardiac healing and recovery of ventricular function after MI. They found that ILC2 levels increased after MI in an ST2-dependent manner. In addition, they suggested that ILC2s can shift scar formation toward less intrusive remodeling in the early recovery period. In terms of mechanism, they found that the IL-2 axis was a major upstream regulator of ILC2s and was also central to ILC2 function following MI.

Cardiac fibrosis is an important process following MI. Chen et al. (10) reported that IL-33 treatment expanded cardiac ILC2s and elicited protective effects against catecholamine-induced cardiac fibrosis with reduced cardiomyocyte death, immune cell infiltration, tissue fibrosis, and improved myocardial function.

For the ILC2-related cytokine IL-5, Xu et al. (57) reported that it facilitates recovery of cardiac function post-MI by promoting eosinophil accumulation and subsequent $CD206^{+}$ macrophage polarization *via* the IL-4/STAT6 axis. The ILC2-related cytokine IL-13 could regulate leukocyte recruitment and induce differentiation of M2-like monocytes/macrophages, and could promote recovery of cardiac function after MI by secreting anti-inflammatory cytokines to stimulate new blood vessel formation and collagen deposition, thereby enhancing wound healing in the infarct area (26).

In brief, the function of ILC2s in the post-MI period is becoming clear. More evidence is needed to address the roles of other ILCs following MI.

DISCUSSION AND PROSPECTS

In this review, we have summarized the complex relationships between ILCs and MI, and discussed evidence for their involvement in the acute phase of MI, MIRI, and repair and regeneration after MI. We also briefly described the spatiotemporal distribution of ILCs in MI. We suggested possible mechanisms by which ILCs play a role in MI by summarizing ILC-related cytokines and the involvement of ILCs in other similar diseases.

Functional analysis of the effects of different ILC subgroups found that ILC1s are involved in progression of AS, ILC2s exert an anti-atherosclerotic effect, while the effect of ILC3s on AS is still controversial (6–8). However, the roles and mechanisms of ILCs in MI have not been fully elucidated.

In patients with AMI (44), an increase in ILC1s that produce IFN-γ can be detected within 12 hours of the onset of symptoms, which is associated with a poor clinical prognosis. In the meantime, the proportion of ILC2s decreased significantly when compared to control group. ILC2s were also found to be

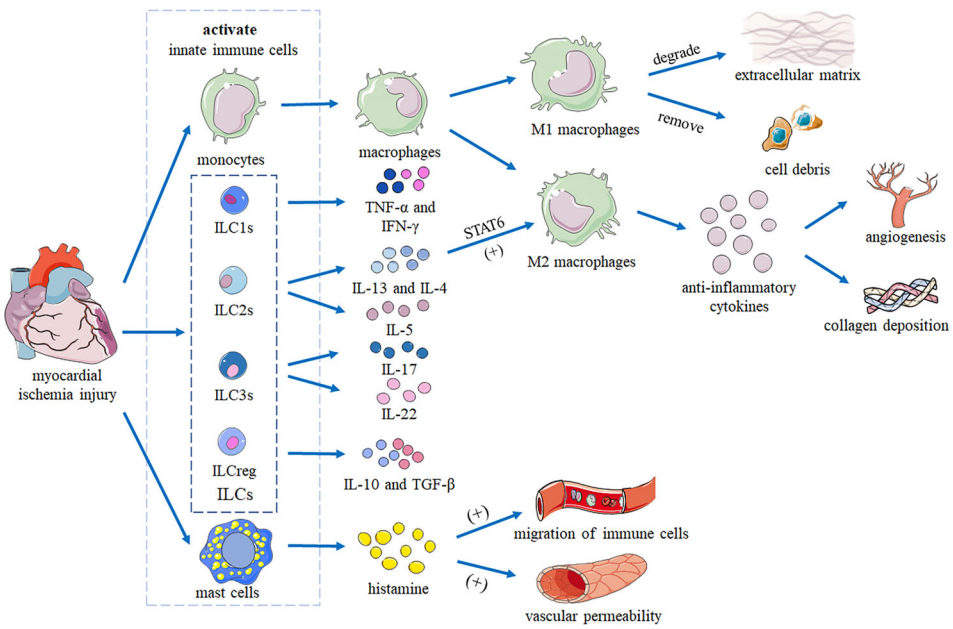

FIGURE 1 | Heart repair by innate immune cells following myocardial ischemia injury. After myocardial ischemic injury, innate immune cells residing in the myocardium are immediately activated. Monocytes infiltrate the injury site and differentiate into macrophages. M1 macrophages are responsible for degrading the extracellular matrix and removing cell debris. M2 macrophages secrete anti-inflammatory cytokines to promote angiogenesis and collagen deposition. IL-13 and IL-4 produced by ILCs promote polarization of macrophages into the M2 phenotype by activating STAT6. Mast cells release histamine to trigger vascular permeability and migration of innate immune cells. Together, these cells coordinate removal of damaged cells, remodeling of the tissue matrix, and recruitment of additional immune cells from the blood to promote recovery of cardiac function.

involved in the early stages of ischemic injury (20). Yu et al. (9) demonstrated that low-dose IL-2 injection could activate ILC2s in ACS.

Despite the increasing use of coronary reperfusion in interventional therapy, morbidity and mortality after STEMI are still high. This may be related to damage to coronary microvascular cells during MIRI (33). Manifestations of coronary artery injury during MIRI range from reversible edema to capillary destruction with intramyocardial hemorrhage. Ischemic conditioning, which refers to short-term ischemia-reperfusion in the heart or other organs to reduce the area of MI and coronary microvascular damage (34), involves several immune factors and is one of the main methods of cardioprotection (35). Minimizing inflammation requires removal of cell debris and promotion of healing following MI, while unlimited inflammation impairs healing and induces adverse cardiac remodeling (58). In remote ischemic conditioning, the spleen is immediately activated *via* the vagus nerve, releasing myocardial protective factors and reducing infarct size (59). As we know, the spleen is one of the main sources of ILCs (60), so it is possible that splenic ILCs could play a role in remote ischemic conditioning.

Though not direct evidence for ILCs in MIRI, several groups (22, 41–43) have reported roles for ILCs in IRI and ILC-related cytokines in MIRI, suggesting that ILCs can play important roles in MIRI, with ILC2s being the most promising candidate.

The presence of ILCs in and surrounding the heart (18) enables these innate immune cells to function as "local fire captains" in cardiovascular diseases, consistent with their tissue-resident character. Changes in numbers of ILCs among PBMCs in STEMI patients, and their association with clinical prognosis, makes them a potential disease biomarker. However, Bracamonte-Baran et al. demonstrated that increases in ILC2 levels resulted from local proliferation rather than infiltration of circulating ILCs. The origin of ILCs requires further research.

It is known that cardiomyocytes are non-regenerative cells. So human embryonic stem cells and human induced pluripotent stem cells may have great potential in treatment of MI (61). Similarly, ILC progenitor cells can differentiate into different ILCs under certain conditions (62). They have been shown to differentiate into ILC2s but not ILC1s and ILC3s during ischemia (20). More research is needed into the role of ILC differentiation in MI.

Recently, Yu et al. (11) reported that ILC2s could promote cardiac healing and recovery of ventricular function after MI through the IL-2 axis. Chen et al. (10) reported that cardiac ILC2s provide protection from cardiac fibrosis and improve myocardial function. Therefore, ILC2s are involved in the entire process of MI, from AS and the early stage of cardiac injury to tissue remodeling and post-MI repair. More studies are however needed to better elucidate the roles of other ILCs in MI.

For future studies of ILCs, more suitable animal models such as tissue-specific gene knockout mice, and over-expression methods like accessible adoptive transfer systems, need to be established. In clinical research, not only patients with acute MI can be recruited, but also patients with stable angina, unstable angina and non-STEMI to further clarify the role of ILCs. In addition, in the era of single cell transcriptomics, big data analysis of ILCs should be explored. What's more, communications between ILCs and other cells should be further investigated. Finally, the role of ILC plasticity in MI should be addressed, as should the role of ILCregs in MI.

In conclusion, ILC2s play important roles in MI, but more research is needed to further explore the mechanisms and roles of other ILCs in MI and to provide better treatment and prevention.

AUTHOR CONTRIBUTIONS

The paper presented was performed in collaboration with all authors. WY wrote the manuscript. JL edited the manuscript. JZ prepared the figure and table. YZ and SJ participated substantially in the organization and coordination of the paper. SH and DL devised the concept and supervised the whole study. All authors contributed to the article and approved the submitted version.

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CD5 Surface Expression Marks Intravascular Human Innate Lymphoid Cells That Have a Distinct Ontogeny and Migrate to the Lung

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Innate lymphoid cells (ILCs) contribute to immune defense, yet it is poorly understood how ILCs develop and are strategically positioned in the lung. This applies especially to human ILCs due to the difficulty of studying them *in vivo*. Here we investigated the ontogeny and migration of human ILCs *in vivo* with a humanized mouse model ("MISTRG") expressing human cytokines. In addition to known tissue-resident ILC subsets, we discovered CD5-expressing ILCs that predominantly resided within the lung vasculature and in the circulation. $CD5^+$ ILCs contained IFNγ-producing mature ILC1s as well as immature ILCs that produced ILC effector cytokines under polarizing conditions *in vitro*. $CD5^+$ ILCs had a distinct ontogeny compared to conventional $CD5^-$ ILCs because they first appeared in the thymus, spleen and liver rather than in the bone marrow after transplantation of MISTRG mice with human $CD34^+$ hematopoietic stem and progenitor cells. Due to their strategic location, human $CD5^+$ ILCs could serve as blood-borne sentinels, ready to be recruited into the lung to respond to environmental challenges. This work emphasizes the uniqueness of human $CD5^+$ ILCs in terms of their anatomical localization and developmental origin compared to well-studied $CD5^-$ ILCs.

Keywords: innate lymphoid cells (ILC), lung, migration, ontogeny, humanized mice

INTRODUCTION

Innate lymphoid cells (ILCs) are an emerging family of immune cells with diverse roles in tissue homeostasis, barrier maintenance, host-microbiota crosstalk, and immunity to pathogens (1–5). As innate counterparts of $CD4^+$ T helper cells, ILCs are divided into three subsets based on signature transcription factors and effector cytokines: (1) ILC1s express T-BET and produce interferon gamma (IFNγ); (2) ILC2s express GATA-3 and produce interleukin-5 (IL-5) and IL-13; (3) ILC3s

Abbreviations: HSPCs, human hematopoietic stem and progenitor cells; IFNg, interferon gamma; IL, interleukin; ILCs, innate lymphoid cells; ILCPs, innate lymphoid cell precursors; IV, intravenous; Lin, lineage; NK, natural killer.

express RORγt and produce IL-17 and/or IL-22. ILCs require IL-7 for their development and thus are characterized by the expression of IL-7Rα (CD127), while lacking lineage (Lin) markers that define other cell lineages, such as related cytotoxic natural killer (NK) cells (6–8).

One aspect of ILC biology that is difficult to study in human *ex-vivo* samples, is the migration of ILCs despite the importance of ILC migration in tissue homeostasis and inflammation (9, 10). Mouse studies have shown that ILCs are able to move within tissues and to traffic to organs *via* the blood during development and inflammation (11–16). In contrast, the migration of human ILCs and their tissue niches remain much less explored. To overcome this limitation, we are using humanized mouse models, which allow studies of the human immune system in an *in-vivo* setting (17–19). In particular, we previously developed a humanized mouse model, named "MISTRG", which expresses the human cytokines and proteins M-CSF, IL-3/GM-CSF, SIRPα, and TPO through gene knock-in (20–22). After transplantation with human hematopoietic stem and progenitor cells (HSPCs), MISTRG mice support the reconstitution of a human immune system in the mouse host, including the development of human NK cells (21). Human ILCs other than NK cells are also present in MISTRG mice and therefore this model offers the opportunity to study the biology of human ILCs *in vivo*.

In mice, ILCs are considered to be mostly tissue-resident (23), whereas circulating ILC subsets, mainly ILC2s and CD117$^+$ ILC precursors (ILCPs), have been shown to be present in humans (24–26). This led to the concept of local ILC-poiesis, where circulating ILCPs migrate into tissues to give rise to mature ILC subsets (27). However, it is unclear whether additional human ILCPs exist in the circulation.

CD5 is a cell surface protein mainly expressed by T lymphocytes and has an inhibitory function in antigen receptor signaling (28, 29). Accordingly, CD5 has been used in several studies to exclude T cells when analyzing ILCs (24, 26, 30–32). However, it has been discovered that ILC1s express T cell-associated molecules, including CD5 (33–38). Subsequently, CD5$^+$ ILCs were found to be present in human thymus and umbilical cord blood (34). Furthermore, the Spits' laboratory demonstrated that immature CD5$^+$ ILC2s were able to differentiate into mature cytokine-producing ILC2s *in vitro* (34). More recently, ILC1-like cells were identified in human cord blood that expressed CD5 and contained precursors for mature KIR$^+$NKG2A$^-$ NK cells (39). These studies indicate that human CD5$^+$ ILCs are heterogeneous and may be functionally distinct from conventional CD5$^-$ ILCs that have been extensively studied. However, the development and migration of human CD5$^+$ ILCs remains poorly understood.

Here we investigated human CD5$^+$ ILCs in humanized mice *in vivo* with a focus on the lung. We demonstrate that CD5$^+$ ILCs derived from CD34$^+$ HSPCs are distinct from well-studied CD5$^-$ ILCs in terms of their developmental origin, migration, and tissue localization. Specifically, we identified CD5-expressing human ILCs in the lung that had a predominantly intravascular localization. CD5$^+$ ILCs followed a different developmental path than conventional ILCs as they occupied a distinct anatomical niche and likely originated from the thymus, liver and/or spleen. Finally, circulating CD5$^+$ ILCs were composed of not only mature ILC1s, but also actively dividing immature ILCs that produced different ILC effector cytokines *in vitro*. Due to their intravascular localization, human CD5$^+$ILCs could function as blood-borne sentinels that are poised to respond to local and systemic environmental challenges.

MATERIALS AND METHODS

Mice

MISTRG mice homozygous for the human genes encoding M-CSF, IL-3/GM-CSF, SIRPα, and TPO in the *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ background were previously described (21). MISTRG mice were used under Material Transfer Agreements with Regeneron Pharmaceuticals and Yale University. For this study, we used an improved version of MISTRG mice with a *SIRPA* knock-in allele (40), instead of *SIRPA* transgene as in the original MISTRG mice. For transplantation with human CD34$^+$ cells, MISTRG mice heterozygous for *SIRPA* knock-in were used (both males and females). Heterozygous mice were derived from breeding MISTRG mice (homozygous for *SIRPA*) with MITRG mice (lacking the *SIRPA* knock-in allele) (21). MISTRG mice were re-derived by embryo transfer at Karolinska Institutet and maintained in individually ventilated cages under specific pathogen-free conditions without any prophylactic antibiotics. All mouse experiments were performed in accordance with protocols approved by the Linköping Animal Experimentation Ethics Committee (#29-15, 03127-2020).

Human Tissues

Umbilical cord blood and buffy coats were obtained from Caesarean sections and the Blood Bank at Karolinska University Hospital Huddinge, respectively. Human lung and spleen tissue was obtained from deceased organ donors through the Transplantation Clinic at Karolinska University Hospital Huddinge, where the lungs were not used for lung transplantation. Donors included 3 males and 2 females who were 25-83 years old. Causes of death were cardiac arrest, hypoxic brain damage, intracranial hemorrhage as well as subdural and subarachnoid hematoma. The collection of all human tissues was approved by local Ethical Review Boards at Karolinska Institutet (#2006/229-31/3, 2015/1368-31/4, 2015/2122-32, 2016/1415-32, 2019-05016). Informed consent was obtained from all tissue donors following verbal and written information and the investigations were conducted according to the Declaration of Helsinki.

Cell Isolation From Human Blood and Tissues

Peripheral blood mononuclear cells were isolated from cord blood and buffy coats using density gradient centrifugation with Lymphoprep (Fisher Scientific). For isolation of mononuclear cells from human lungs, the lung tissue was cut into small pieces and digested for 30 minutes at 37°C in RPMI 1640 (supplemented with 100 U/ml Penicillin, 50 μg/ml streptomycin, 1 mM

L-glutamine) containing 0.25 mg/mL collagenase II (Sigma), and 0.2 mg/mL of DNAse I (Roche). Digested tissue was washed with RPMI 1640 supplemented with 10% fetal calf serum (FCS) as well as 100 U/ml Penicillin, 50 ug/ml streptomycin, and 1 mM L-glutamine. Cells were then filtered through 70 µm and 40 µm cell strainers with a syringe plunger. Finally, after washing, mononuclear cells were obtained by density gradient centrifugation with Lymphoprep. For isolation of mononuclear cells from human spleens, a 2-3 cm piece of spleen was mechanically mashed and passed through a cell strainer.

Transplantation With Human CD34$^+$ Cells

For transplantation with human HSPCs, CD34$^+$ cells were isolated from pooled cord blood by density gradient centrifugation and positive immunomagnetic selection using a CD34$^+$ microbead kit (Miltenyi Biotec). Newborn MISTRG mice (3-5 days old) were transplanted with 1 x 10^5 human CD34$^+$ cells (usually >90% purity) by intrahepatic injection as previously described (22). HSPCs were pooled from several donors for transplantation. Mice did not receive any irradiation as pre-conditioning before transplantation. At ~7 weeks post-transplantation, blood was collected to determine engraftment of MISTRG mice with human CD45$^+$ hematopoietic cells by flow cytometry. Mice were generally used for experiments at 7-12 weeks after transplantation with human CD34$^+$ cells, except for the kinetics experiments where mice were analyzed also at 3-5 weeks post-transplantation.

Isolation of Immune Cells From MISTRG Mice

Lungs were perfused with cold PBS, minced into small pieces, and digested for 60 minutes at 37°C in digestion media composed of RPMI 1640 supplemented with 5% FCS, 0.2 mg/mL collagenase IV (Sigma), and 0.02 mg/mL of DNAse I (Sigma). After digestion, cells were mechanically dissociated by sequentially passing them through 18G and 20G needles attached to a syringe. Cells were then filtered and subjected to density gradient centrifugation with Lymphoprep (Fisher Scientific). Bronchoalveolar lavage (BAL) fluid was collected by inflating the lungs three times with 0.8 mL PBS *via* a catheter inserted into the trachea. BAL fluid was then centrifuged, the pellet resuspended in RPMI 1640/5% FCS, and BAL cells purified for flow cytometry by density gradient centrifugation. Livers were excised, mechanically crushed into small pieces, and digested for 60 minutes at 37°C in digestion media composed of RPMI 1640 supplemented with 5% FCS, 0.2 mg/mL collagenase IV (Sigma), and 0.02 mg/mL of DNAse I (Sigma). After washing with RPMI 1640/5% FCS and low-speed centrifugation (300 rpm for 3 minutes at 4°C) to remove hepatocytes, the supernatant was saved. Cells were then pelleted by centrifugation (1,700 rpm for 10 minutes at 4°C) and subjected to density gradient centrifugation with 27.5% Optiprep (Abbott Rapid Diagnostics) and red blood cell lysis. Blood was taken by cardiac puncture and diluted in 200 units/mL heparin (Sigma). Erythrocytes were removed using red blood cell lysis buffer (obtained from Karolinska University Hospital) and the remaining immune cells stained for flow cytometry analysis. Spleens and thymi from MISTRG mice were mashed using a syringe plunger and passed through a 70 µm filter before red blood cell lysis. To isolate bone marrow cells from MISTRG mice, hind legs were harvested and the bones were cleaned. After cutting the bone ends off, bone marrow cells were flushed out using a syringe, filtered, and treated with red blood cell lysis buffer. Isolated single cells were washed with RPMI 1640, counted, and then either stained directly for flow cytometry or stored overnight in R10 media (RPMI 1640 with 10% FCS and 1% L-glutamine) at 4°C prior to intracellular cytokine staining.

Intravascular Cell Labelling

HSPC-engrafted MISTRG mice were injected intravenously with 2 µg of PE-conjugated anti-human CD45 antibody (Biolegend, clone HI30) to label human hematopoietic cells in the blood and the lung vasculature. Mice were sacrificed 5 minutes after injection and lungs harvested without prior perfusion. Lung immune cells were isolated as above and stained with APC-Cy7-conjugated anti-human CD45 antibody and other antibodies *ex vivo* for flow cytometry as described below. As a positive and negative control, cells from blood and BAL fluid were used, respectively. Only lung samples from mice with successful intravascular labelling (>90% CD45-PE$^+$ in blood) were used for analysis.

Flow Cytometry

Single-cell suspensions from blood and tissues of MISTRG mice or from human samples were stained with fluorochrome- or biotin-labeled antibodies (see **Supplementary Table 1**) in 100 µl FACS buffer (PBS/2% FCS) for 30 minutes on ice, followed by secondary staining with streptavidin-Brilliant Violet 711 (BD Biosciences) for 20 minutes on ice. After surface staining, cells were stained with fixable viability dye-eFluor506 (eBioscience) according to the manufacturer's instructions. For detection of intracellular proteins, cells were first stained with surface antibodies and viability dye as above. Then cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining kit (eBioscience) according to manufacturer's protocol before staining with antibodies against transcription factors or Ki67. For intracellular CD3 and TCRab staining, cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Matched isotype antibodies were used as controls. For intracellular cytokine staining, cells were plated into 24-well plates at a cell density of 2.5-5 x 10^6 cells/ml (5-10 x 10^6 cells per well). Cells were then stimulated for 3 hours at 37°C with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 1 µg/ml ionomycin (Sigma) in R10 medium containing a 1:1,000 dilution of Golgi Plug (BD Biosciences). After surface and viability staining, cells were fixed, permeabilized, and intracellular cytokine staining performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Stained cells were acquired on a LSR II Fortessa flow cytometer (BD Biosciences), and data were analyzed with FlowJoV10 software.

In-Vitro Differentiation of Human ILCs

Peripheral blood mononuclear cells from buffy coat were enriched for ILCs using a negative selection protocol for CD3, CD14, CD16, and CD19 using Mojosort Streptavidin Nanobeads (Biolegend) as described by Krabbendam et al. (41). $CD5^-CD7^+$ and $CD5^+CD7^+$ ILCs were then purified from $CD127^+Lin^-CD3^-TCR\alpha\beta^-CD94^-$ cells using a MA900 cell sorter (Sony Biotechnology). Each ILC subset (1,000 cells per well) was co-cultured with OP9-DL1 cells (3,000 per well) in a 96-well U-bottom plate. OP9-DL1 cells were maintained in IMDM/10% FCS with penicillin/streptomycin before co-culture with ILCs. Cells were co-cultured for one week in IMDM supplemented with Yssel's media and 2% human serum, together with 20 ng/ml IL-2 and 20 ng/ml IL-7 (Miltenyi Biotech). For polarizing conditions the following cytokines (Peprotech) were added (all at 20 ng/ml unless stated otherwise) (1) ILC1s: IL-1β and IL-12; (2) ILC2s: IL-4, IL-25, IL-33, and TSLP; (3) ILC3s: IL-1β and IL-23. Cytokines were replenished at day 5 of culture. At day 7 of culture, cells were harvested, stimulated with PMA and ionomycin and stained for intracellular cytokines as described above.

Quantification and Statistical Analysis

Statistical parameters including number of biological replicates and repeat experiments, data dispersion and precision measures (mean and standard error of the mean (SEM)), and *P* values for statistical significance ($\alpha = 0.05$) are reported in Figures and Figure Legends. Student's *t* test was used to determine statistical significance between two groups. Multigroup comparisons were performed using one-way ANOVA followed by *post hoc* testing using Tukey' Multiple Comparison Test. Statistical analysis was performed using GraphPad Prism 8.

RESULTS

Human CD5+ ILCs Are Present in the Lung and Peripheral Blood

We previously showed that MISTRG mice transplanted with human HSPCs harbor human NK cells in several tissues, including the lung (21). In contrast, human ILC subsets derived from HSPCs have not been characterized in MISTRG mice. To investigate human lung ILCs *in vivo*, we transplanted newborn MISTRG mice with human $CD34^+$ cells containing HSPCs (**Figure 1A**), as in our earlier studies (21, 22, 42). Apart from $CD127^-CD94^+$ NK cells, MISTRG mice engrafted with human $CD34^+$ HSPCs harbored different types of $CD127^+CD94^-$ ILCs in the lung that did not express TCRαβ and CD3 on the cell surface and lacked the Lin markers CD11c, CD14, CD19, CD34, CD123, and FceRI (**Figure 1B**). These $CD127^+CD94^-$ ILCs consisted of $CD117^+$ ILCPs/ILC3s (24, 43–45), as well as $CRTH2^+$ ILC2s (46, 47) and $CD117^-CRTH2^-$ ILC1s (48, 49). Furthermore, flow cytometry revealed a population of human $CD127^+CD3^-TCR\alpha\beta^-CD94^-$ ILCs in the lung of HSPC-engrafted MISTRG mice that expressed both CD5 and CD7 on the cell surface (**Figure 1C**). $CD5^+CD7^+$ ILCs were also present in the human lung, although they were less prevalent than in HSPC-engrafted MISTRG mice (**Figure 1D** and **Supplementary Figure 1A**). In contrast to ILCs, human $CD127^-CD94^+$ NK cells lacked CD5 expression (**Supplementary Figure 1B**). Consistent with previous reports (34, 39), we detected $CD5^+CD7^+$ ILCs also in human cord blood (**Figure 1E**). Human $CD5^+CD7^+$ ILCs were prevalent in adult blood, as reported previously (35, 50), and they were also present with a similar frequency in the blood of HSPC-engrafted MISTRG mice (**Figure 1E**). Similar to earlier studies (34, 35, 39, 50), $CD5^+CD7^+$ ILCs expressed intracellular CD3, but lacked intracellular TCRαβ protein (**Supplementary Figure 2**), confirming that they were distinct from T cells. These data demonstrate that human $CD5^+CD7^+$ ILCs represent a sizeable population in the circulation and the lung, prompting us to further investigate their biology.

CD5+ ILCs in the Lung Contain Both Mature ILC1s and Functionally Immature ILCs

Further flow cytometric analysis showed that conventional $CD5^-CD7^+$ ILCs in the lungs of engrafted MISTRG mice mainly comprised $CD117^+$ ILCPs/ILC3s and $CRTH2^+$ ILC2s (**Figure 2A**). In contrast, $CD117^-CRTH2^-$ ILC1s were more abundant among human $CD5^+CD7^+$ ILCs (**Figure 2A**). To gain additional insights into the identity and heterogeneity of human $CD5^+CD7^+$ ILCs, we performed intracellular staining for signature transcription factors. This revealed that $CD5^+CD7^+$ lung ILCs were distinct from ILC2s and ILC3s, because they lacked expression of the lineage-defining transcription factors GATA3 and RORγt (**Figure 2B**). Consistent with containing cells with an ILC1 surface phenotype ($CD117^-CRTH2^-$), the $CD5^+CD7^+$ ILC subset expressed T-BET, although less than NK cells (**Figure 2B**). These data suggested that human $CD5^+CD7^+$ ILCs in the lung contained some mature ILC1s. To explore this possibility, we performed intracellular cytokine staining after stimulation with PMA and ionomycin *in vitro*. These experiments confirmed that a fraction of $CD5^+CD7^+$ ILCs produced the ILC1 signature cytokine IFNγ (**Figures 2C, D**). In contrast, the frequency of IL-22-/IL17A-expressing ILC3s was very low in the lungs of HSPC-engrafted MISTRG mice (**Figures 2C, D**), consistent with a lack of $NKp44^+$ ILCs (**Figure 2E**) that normally produce IL-22 (51, 52). These results are in accordance with previous data, reporting that $NKp44^+$ ILC3s are rare in the human lung at steady state (30). $CD5^+CD7^+$ ILCs also contained some mature ILC2s producing IL-13, but their frequency was lower than within the $CD5^-CD7^+$ ILC population (**Figures 2C, D**). Combined, these data support the notion that $CD5^+CD7^+$ ILCs consisted not only of mature ILC1s, but also of functionally immature ILCs that do not produce effector cytokines.

Human CD5+CD7+ ILCs Have an Immature Surface Phenotype and Are Actively Dividing

The above findings raised the possibility that $CD5^+CD7^+$ ILCs represented an immature ILC population. To further

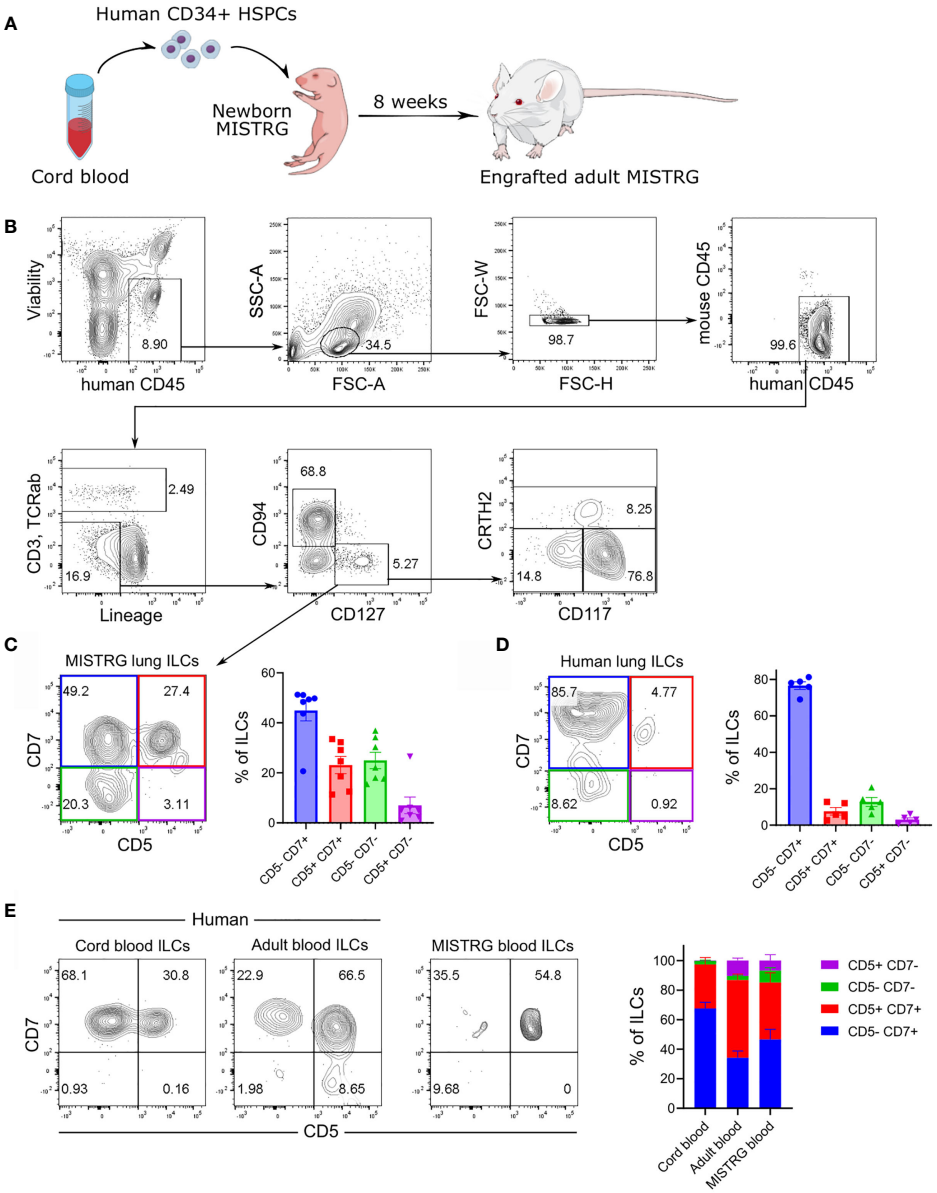

FIGURE 1 | Human CD5+ ILCs are present in the lung and peripheral blood. **(A)** Engraftment of newborn MISTRG mice with human CD34+ HSPCs from cord blood. **(B)** Gating strategy for the flow cytometric analysis of human ILCs in the lung of HSPC-transplanted MISTRG mice. Total ILCs were gated as viable human CD45+CD3-TCRαβ-CD94-CD127+ cells that were negative for lineage (Lin) markers (CD11c, CD14, CD19, CD34, CD123, FceRI) and mouse CD45. **(C, D)** CD5 and CD7 surface expression on human lung ILCs. Frequencies of CD5/CD7-expressing ILC subsets in the lung of HSPC-engrafted MISTRG mice (n = 7) and in human lung tissue (n = 5) are shown in **(C, D)**, respectively. **(E)** Frequencies of CD5/CD7-expressing ILC subsets in human cord blood, human adult blood, and in the blood of HSPC-transplanted MISTRG mice was determined by flow cytometry (n = 7-11). Data are represented as mean ± SEM. Data are from at least three independent experiments. **(A)** was adapted from Mind the Graph.

characterize this subset, we examined surface markers related to ILC maturation status. Flow cytometry showed that CD5+CD7+ ILCs in the lung of HSPC-engrafted MISTRG mice had intermediate CD127 expression, but did not express the mature ILC markers HLA-DR (**Figures 3A, B**) and NKp44 (**Figure 2E**). Instead, CD5+CD7+ ILCs mostly expressed CD45RA (**Figures 3A, B**), a surface protein found on naïve T cells as well as ILCPs and resting ILCs (24, 33, 53). These results indicated that CD5+CD7+ ILCs consisted of immature or naïve-like cells. To further explore this possibility, we investigated whether CD5+CD7+ ILCs from the lung of HSPC-engrafted MISTRG mice can proliferate. Staining for the proliferation marker Ki67 demonstrated that this subset contains actively dividing cells in steady state (**Figure 3C**). Finally, we determined

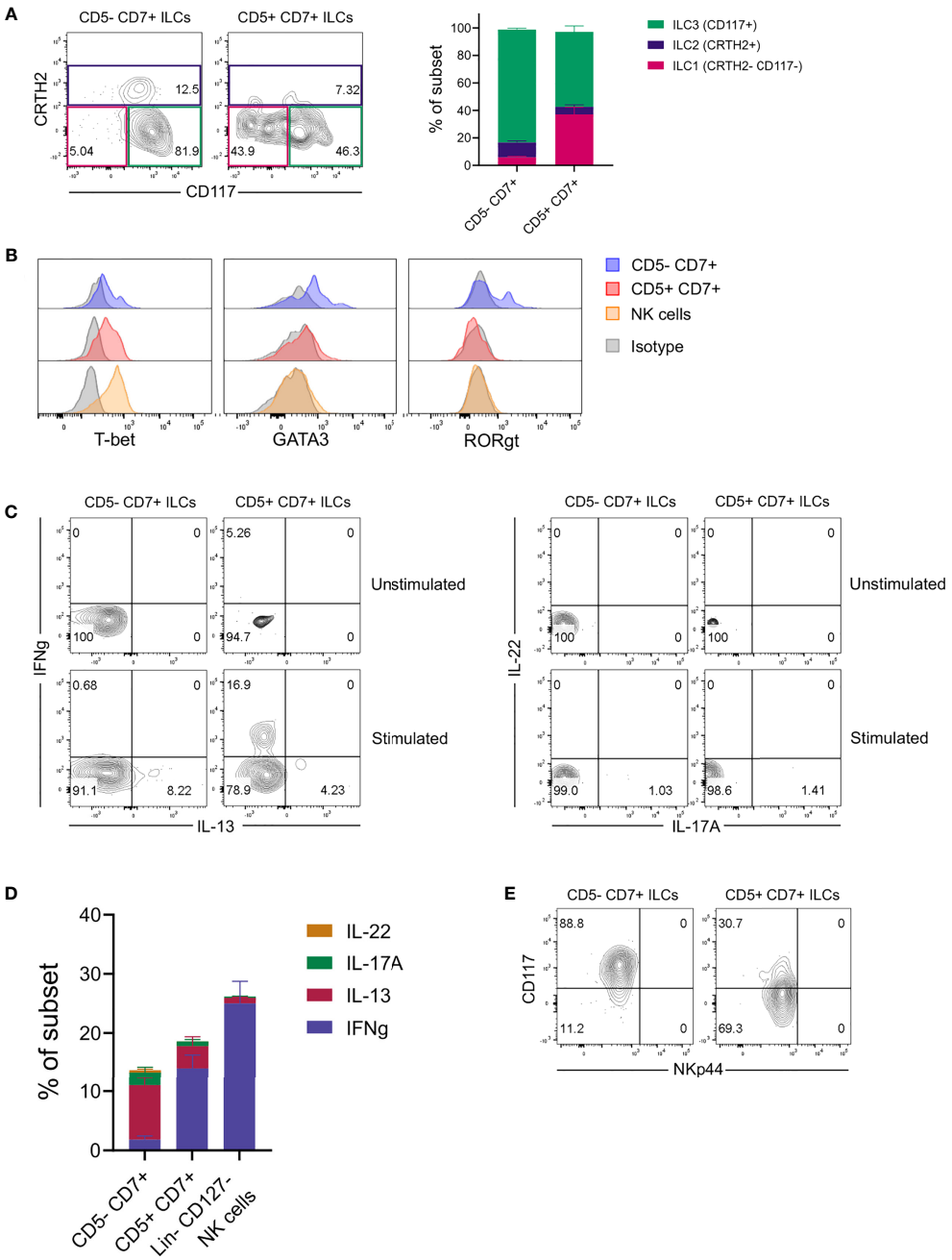

FIGURE 2 | CD5⁺ ILCs in the lung contain both mature ILC1s and functionally immature ILCs. **(A)** Frequencies of ILC1s (CD117⁻CRTH2⁻), ILC2s (CRTH2⁺), and ILC3s (CD117⁺CRTH2⁻) among ILC subsets based on expression of CD5 and CD7 in the lung of HSPC-engrafted MISTRG mice (n = 7). ILCs were gated as human CD45⁺Lin⁻CD3⁻TCRαβ⁻CD94⁻CD127⁺ cells as in **Figure 1B** and then as CD5⁻CD7⁺ or CD5⁻CD7⁺ ILCs as in **Figure 1C**. **(B)** Intracellular expression of signature transcription factors (T-BET, GATA3, RORγt) in human CD5⁻CD7⁺ ILCs, CD5⁺CD7⁺ ILCs, and NK cells isolated from the lung of HSPC-engrafted MISTRG mice. ILCs were gated as human CD45⁺Lin⁻CD3⁻TCRαβ⁻CD127⁺ cells and NK cells were gated as human CD45⁺Lin⁻CD3⁻TCRαβ⁻CD127⁻CD56⁺ cells. Matched isotype antibodies were used as controls. **(C)** Intracellular IFNγ and IL-13 as well as IL-17A and IL-22 production by human CD5⁻CD7⁺ and CD5⁻CD7⁺ lung ILCs from HSPC-engrafted MISTRG mice. Cells were stimulated with PMA/ionomycin or left unstimulated. **(D)** Frequencies of effector cytokine-producing producing cells in each CD5/CD7-expressing ILC subset and NK cells following PMA/ionomycin stimulation (n = 5). NK cells were gated as human CD45⁺Lin⁻CD3⁻TCRαβ⁻CD127⁻ cells. **(E)** NKp44 surface expression on human CD5⁻CD7⁺ ILCs and CD5⁺CD7⁺ ILC in the lung of HSPC-engrafted MISTRG mice. ILCs were gated as human CD45⁺Lin⁻CD3⁻TCRαβ⁻CD94⁻CD127⁺ cells as in **Figure 1B**. Data represent mean ± SEM and are representative of at least two independent experiments.

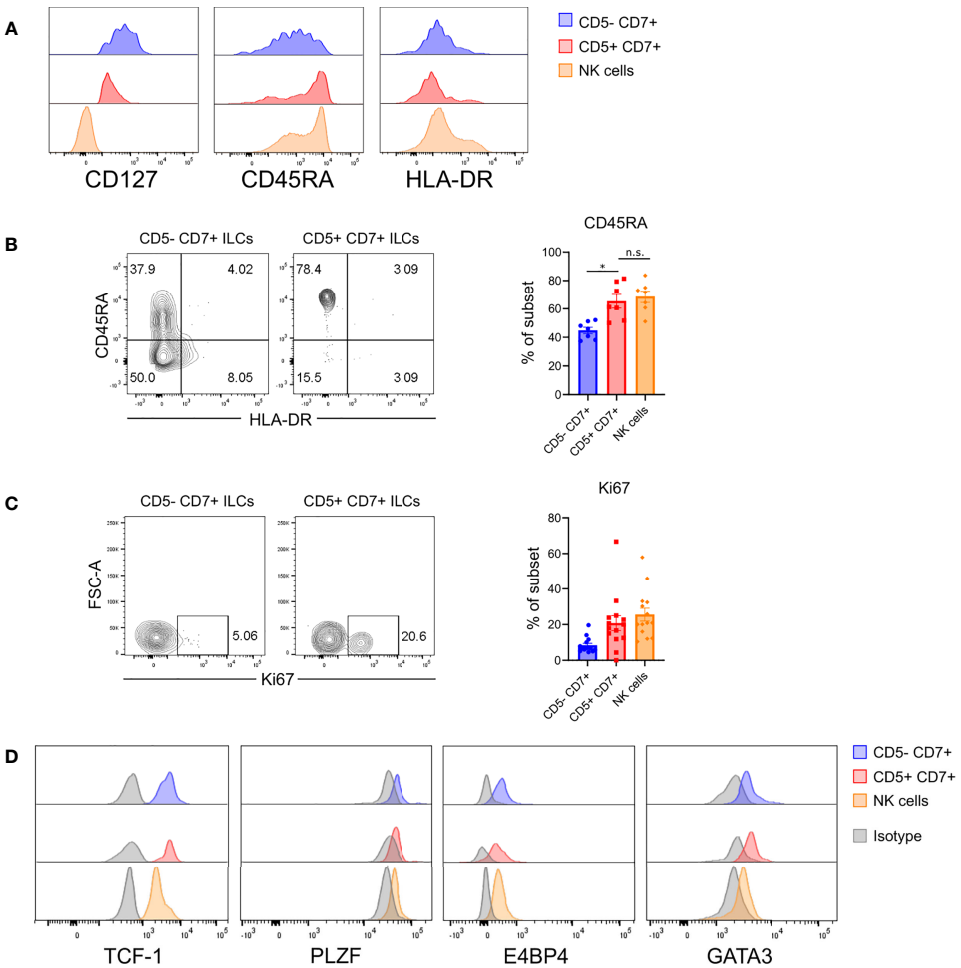

FIGURE 3 | Human CD5$^+$CD7$^+$ ILCs have an immature surface phenotype and are actively dividing. **(A)** Surface expression of CD127, CD45RA, and HLA-DR on human CD5$^-$CD7$^+$ ILCs, CD5$^+$CD7$^+$ ILCs, and NK cells in the lung of HSPC-engrafted MISTRG mice. ILCs were gated as human CD45$^+$Lin$^-$CD3$^-$TCRαβ$^-$CD94$^-$CD127$^+$ cells and NK cells were gated as human CD45$^+$Lin$^-$CD3$^-$TCRαβ$^-$CD94$^+$CD127$^-$ cells as in **Figure 1B**. **(B)** Frequencies of CD45RA$^+$ cells among CD5$^-$CD7$^+$ ILCs, CD5$^+$CD7$^+$ ILCs, and NK cells from the lung of HSPC-engrafted MISTRG mice (n = 7). n.s., not significant; *P < 0.05 by one-way ANOVA, Tukey's post-test. **(C)** Intracellular expression of the proliferation marker Ki67 in CD5$^-$CD7$^+$ ILCs, CD5$^+$CD7$^+$ ILCs, and NK cells from the lung of HSPC-engrafted MISTRG mice and frequencies of Ki67$^+$ cells among each subset (n = 14). **(D)** Intracellular expression of transcription factors (TCF-1, PLZF, E4BP4, GATA3) in human CD5$^-$CD7$^+$ ILCs, CD5$^+$CD7$^+$ ILCs, and NK cells isolated from the spleen of HSPC-engrafted MISTRG mice. ILCs were gated as human CD45$^+$Lin$^-$CD3$^-$TCRαβ$^-$CD127$^+$ cells and NK cells were gated as human CD45$^+$Lin$^-$CD3$^-$TCRαβ$^-$CD127$^-$CD56$^+$ cells. Matched isotype antibodies were used as controls. Data represent mean ± SEM and are representative of at least two independent experiments.

the expression of transcription factors regulating ILC development (54). Human CD5$^+$CD7$^+$ ILCs from the spleen of HSPC-engrafted MISTRG mice expressed intracellular TCF-1 and variable amounts of PLZF and E4BP4 protein, like their CD5$^-$CD7$^+$ counterparts (**Figure 3D**). TCF-1 is associated with "stemness" and known to be expressed by naïve T cells, T memory stem cells, as well as circulating CD117$^+$ ILCPs (24). Furthermore, TCF-1 is expressed by mouse ILCPs (16). This further supports the notion that CD5$^+$CD7$^+$ ILCs have features of immature ILCs. In contrast to the lung (**Figure 2B**), human CD5$^+$CD7$^+$ ILCs, as well as NK cells and CD5$^-$CD7$^+$ ILCs from the spleen of HSPC-engrafted MISTRG mice expressed some GATA3 (**Figure 3D**). While ILC2s have the highest GATA3 expression, GATA3 is also known to be expressed by circulating CD117$^+$ ILCPs (24, 34), consistent with its general role in ILC development (54). Taken together with our results above, we conclude that CD5$^+$CD7$^+$ ILCs contain actively dividing cells with features of immature ILCs.

Human CD5$^+$CD7$^+$ ILCs Respond to Polarizing Cytokines *In Vitro*

To further examine the differentiation potential and effector function of CD5$^+$CD7$^+$ ILCs, we purified both CD5$^+$CD7$^+$ and CD5$^-$CD7$^+$ ILC subsets from human CD127$^+$Lin$^-$CD3$^-$TCRαβ$^-$CD94$^-$ ILCs isolated from adult peripheral blood and co-cultured them with OP9-DL1 cells *in vitro* with various lineage-polarizing

cytokines. CD5-CD7+ ILCs from human blood are known to contain both mature ILCs (mainly ILC2s) and CD117+ ILCPs that are able to differentiate into all types of ILCs (24). Accordingly, purified CD5-CD7+ ILCs produced IL-13 after *in-vitro* culture without any lineage-skewing condition (IL-2+IL-7 only), which was further increased in the presence of ILC2-polarizing cytokines (IL-4+IL-25+IL-33+TSLP) (**Figure 4A** and **Supplementary Figure 3A**). The CD5-CD7+ subsets also gave rise to IFNγ-producing ILC1s (or NK cells) when cultured in ILC1-polarizing cytokines (IL-1β+IL-12) (**Figure 4A**) and, to a lesser extent, to IL-17A-producing ILC3s when ILC3 polarizing cytokines (IL-1β+IL-23) were present (**Figure 4B**). On the other hand, CD5+CD7+ ILCs purified from blood contained some mature ILC1s and ILC2s as demonstrated by spontaneous IFNγ and IL-13 production in neutral culture conditions (IL-2 +IL-7 only) (**Figure 4A**). In lineage-skewing conditions, the frequency of effector cytokine-producing ILCs derived from CD5+CD7+ ILCs increased, with a predominance of IFNγ+ cells (**Figures 4A, B** and **Supplementary Figure 3**). These results indicated that the CD5+CD7+ ILC population either contained pre-existing mature ILCs that expanded during the *in-vitro* culture and/or immature ILCs that acquired the ability to produce effector cytokines in response to polarizing signals. Moreover, we observed that purified CD5+CD7+ ILCs largely lost CD5 surface expression *in vitro*, irrespective of the culture conditions (**Figure 4C**). Further analysis revealed that IFNγ-producing ILC1s (or NK cells) were mainly enriched within purified CD5+CD7+ ILCs that had downregulated CD5 from their cell surface after *in-vitro* culture (**Figure 4C**). In contrast, purified CD5+CD7+ ILCs that had retained CD5 expression consisted of IFNγ+ ILC1s (or NK cells) as well as cells that did not produce IFNγ, even when ILC1-polarizing cytokines were present in the culture (**Figure 4C**). Taken together, we demonstrate that CD5+CD7+ ILCs acquire effector function after downregulating CD5 from their cell surface.

CD5+CD7+ ILCs Mainly Reside in the Lung Vasculature

Our observation that human CD5+CD7+ ILCs were abundant in peripheral blood (**Figure 1E**) suggested that they resided in a different anatomical compartment than conventional CD5-CD7+ ILCs. To investigate whether CD5+ and CD5- ILC subsets occupied different niches in the lung, we performed intravascular cell labeling by intravenously (IV) injecting HSPC-engrafted MISTRG mice with a phycoerythrin (PE)-conjugated antibody against human CD45 (**Figure 5A**). This technique allowed us to distinguish cells within the lung vasculature (stained by IV CD45-PE antibody) from cells within lung tissue, i.e. outside of lung blood vessels, that were not stained by IV CD45-PE antibody (22). As expected, all CD45+ hematopoietic cells in peripheral blood were labeled by the IV CD45-PE antibody, whereas bronchoalveolar lavage (BAL) cells outside of the lung vasculature were not stained (**Supplementary Figure 4**). Flow cytometric analysis of lung tissue after intravascular antibody labeling revealed differences in the intravascular and extravascular distribution of human ILCs in MISTRG mice. Specifically, we found that conventional CD5-CD7+ ILCs resided in both the intravascular and extravascular compartment of the lung (**Figures 5B, C**). In contrast, CD5+CD7+ ILCs mostly had an intravascular localization in the lung, similar to NK cells (**Figures 5B, C**).

To further explore the migratory features of CD5+CD7+ ILCs, we examined the expression of cell surface receptors that regulate ILC trafficking and tissue residency (9, 10). Compared to the other ILC subsets, very few CD5+CD7+ ILCs expressed the tissue residency markers CD69 and CD103 on their cell surface (**Figure 5D**), consistent with their intravascular localization in the lung (**Figures 5B, C**). Instead, CD5+CD7+ ILCs more frequently expressed CD62L surface protein (**Figure 5D**), which is known to mediate the homing of naïve T lymphocytes to secondary lymphoid organs. Overall, these findings demonstrate that CD5+CD7+ ILCs have a unique anatomical location within the lung and that they share an intravascular niche with NK cells.

CD5+CD7+ ILCs Have a Distinct Ontogeny Compared to CD5-CD7+ ILCs

In adult hosts, conventional ILCs develop from a common lymphoid progenitor in the bone marrow and complete their differentiation into mature ILCs after migration into various tissues (54–57). The finding that CD5+CD7+ ILCs inhabited a different anatomical niche than conventional CD5-CD7+ ILCs raised the possibility that CD5+CD7+ ILCs have a distinct developmental origin. Moreover, the expression of CD62L by CD5+CD7+ ILCs (**Figure 5D**) suggested that they home to secondary lymphoid organs, such as the spleen. To determine the origin of CD5+CD7+ ILCs, we examined the reconstitution of the human ILC compartment in bone marrow, spleen, liver, and lung of MISTRG mice at different time points after transplantation with human CD34+ HSPCs. Human T cells and NK cells greatly expanded in all organs between 3 and 5 weeks post-transplantation (**Supplementary Figure 5**). Human ILCs could already be detected in bone marrow, spleen, and liver 3 weeks post-transplantation, whereas there were few ILCs in the lung at this early time point (**Figure 6A** and **Supplementary Figure 5**). As expected, conventional CD5-CD7+ ILCs were present in the bone marrow (**Figures 6A, B**), their known site of development. In contrast, CD5+CD7+ ILCs mainly resided in the liver and spleen already 3 weeks after HSPC transplantation (**Figures 6A, B**), suggesting that they originated from CD34+ HSPCs or local precursors in these organs. We also considered the possibility that human CD5+CD7+ ILCs in MISTRG mice had a thymic origin before migrating to the spleen and liver. Flow cytometry revealed the presence of CD34-Lin-CD5+CD7+ cells lacking CD3 and TCRab surface expression in the thymus of MISTRG mice 3 weeks after transplantation (**Figure 6C**). This result is consistent with previous data showing that CD5+CD7+ ILCs are present in the human post-natal thymus (34) and raised the possibility that CD5+ ILCs originate from precursors in the thymus. The finding that reconstitution of human CD5+CD7+

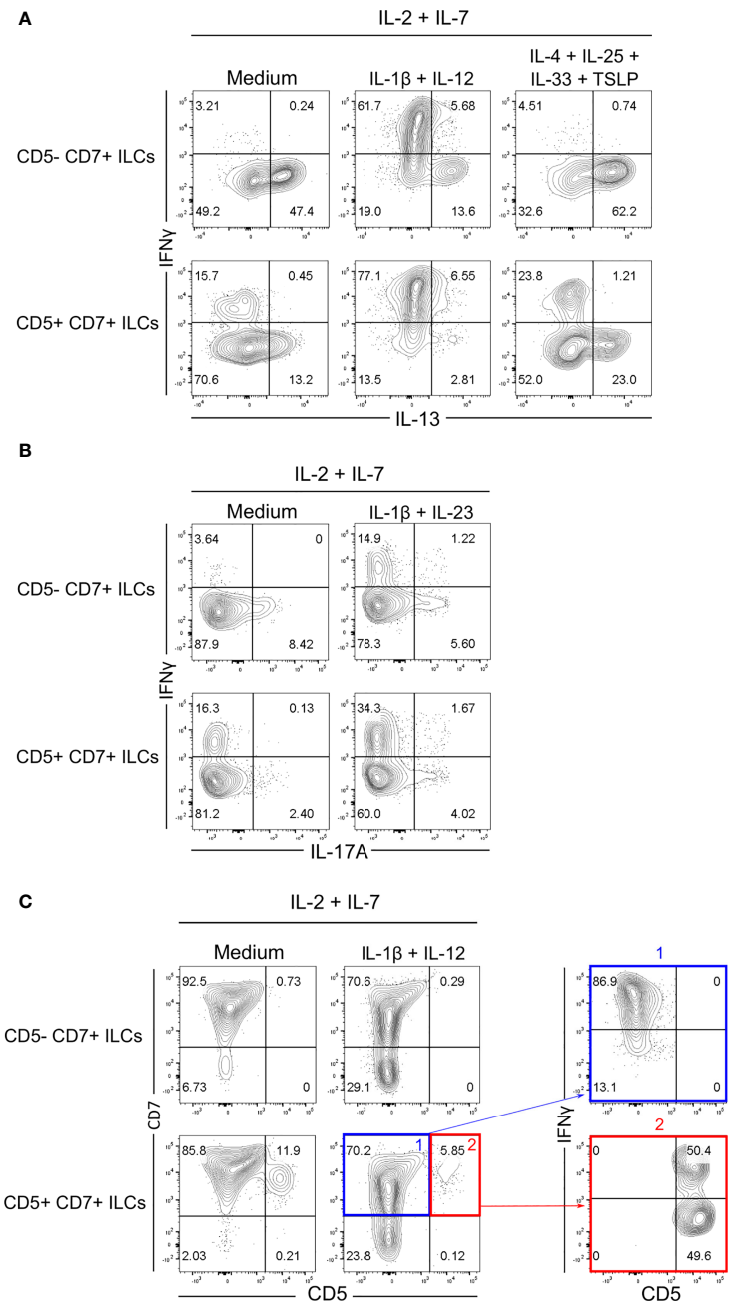

FIGURE 4 | Human CD5⁺CD7⁺ ILCs respond to polarizing cytokines *in vitro*. **(A, B)** Flow cytometry analysis of intracellular IFNγ, IL-13 **(A)**, and IL-17A **(B)** expression by CD5⁻CD7⁺ ILCs (top row) and CD5⁺CD7⁺ ILCs (bottom row) one week after co-culture with OP9-DL1 cells in the presence of the indicated polarizing cytokines. CD5⁻CD7⁺ and CD5⁺CD7⁺ ILCs were purified from human peripheral blood by cell sorting. Cells were gated as in **Supplementary Figure 3A**. Non-stimulated controls are shown in **Supplementary Figure 3B**. **(C)** Cell surface expression of CD5 and CD7 on purified CD5⁻CD7⁺ ILCs (top row) and CD5⁺CD7⁺ ILCs (bottom row) one week after co-culture with OP9-DL1 cells and IL-2+IL-7 only or with OP9-DL1 cells and IL-2+IL-7+IL1β+IL-12. Dot plots on the right show IFNγ production by CD5⁺CD7⁺ ILCs that have down-modulated (blue) or maintained (red) CD5 surface expression. Data are representative of at least two independent experiments.

ILCs in the lung occurred later than in the other organs suggested that lung ILCs were derived from cells from the thymus, liver and/or spleen that had migrated to the lung *via* the blood. In support of this possibility, circulating CD5⁺CD7⁺ ILCs were present in MISTRG mice already at 3 weeks after transplantation with human CD34⁺ HSPCs (**Figure 6D**). Furthermore, human CD5⁺CD7⁺ ILCs were found in both the extravascular and the intravascular compartment of the spleen

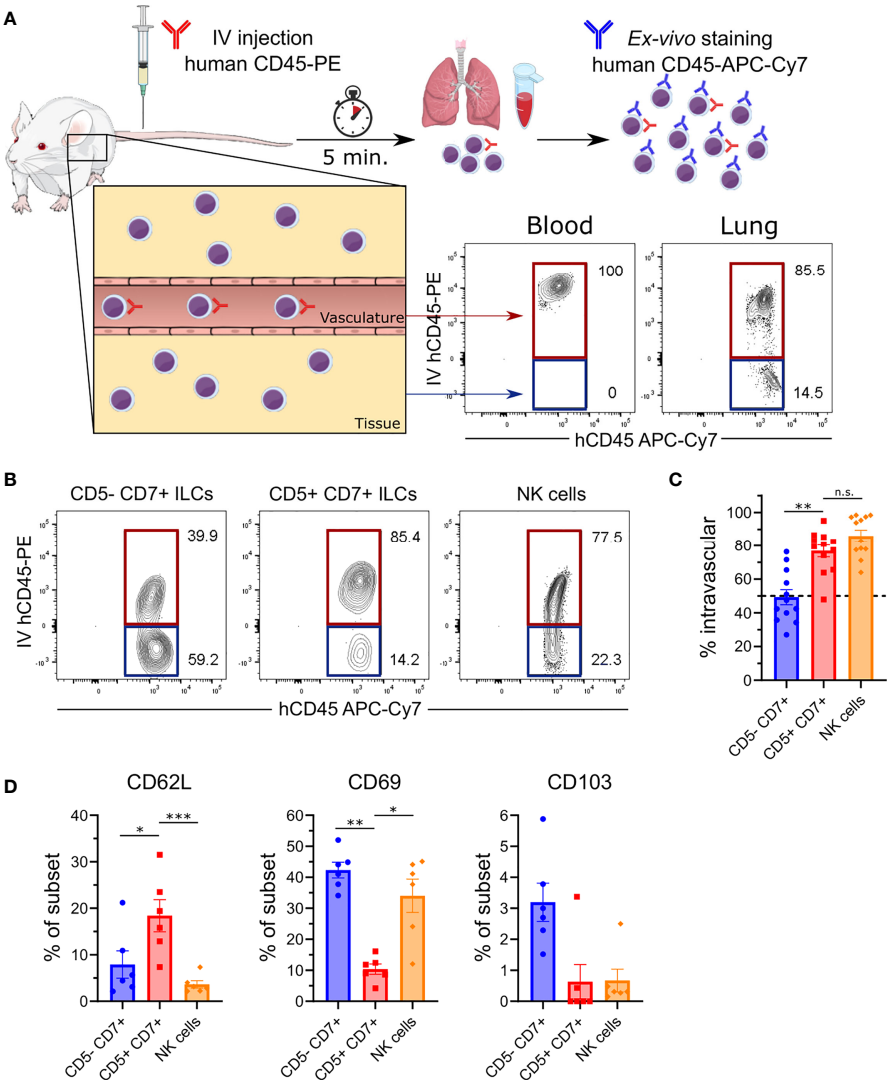

FIGURE 5 | $CD5^+CD7^+$ ILCs mainly reside in the lung vasculature. **(A)** Intravascular labeling of human $CD45^+$ hematopoietic cells in MISTRG mice engrafted with $CD34^+$ HSPCs by intravenous (IV) injection of anti-human CD45-PE antibody. Human cells in the vasculature (highlighted in red) are stained by both the IV-injected anti-CD45 antibody (hCD45-PE) and by the *ex-vivo* anti-CD45 antibody (hCD45-APC-Cy7). Cells residing in the tissue (highlighted in blue) are only stained with the *ex-vivo* hCD45-APC-Cy7 antibody. **(B)** Flow cytometry analysis of intravascular (red) and extravascular (blue) $CD5^-CD7^+$ ILCs, $CD5^+CD7^+$ ILCs, and NK cells in the lung of HSPC-engrafted MISTRG mice. ILCs were gated as human $CD45^+Lin^-CD3^-TCR\alpha\beta^-CD94^-CD127^+$ cells and NK cells were gated as human $CD45^+Lin^-CD3^-TCR\alpha\beta^-CD94^+CD127^-$ cells as in **Figure 1B**. **(C)** Frequencies of intravascular (IV-hCD45-PE^+) human $CD5^-CD7^+$ ILCs, $CD5^-CD7^+$ ILCs, and NK cells in the lung (n = 12) as determined in **(B)**. **(D)** Frequency of CD62L, CD69, and CD103 expression by human lung $CD5^-CD7^+$ ILCs, $CD5^-CD7^+$ ILCs, and NK cells from HSPC-engrafted MISTRG mice (n = 6). n.s., not significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA, Tukey's post-test. Data represent mean ± SEM and are representative of at least two independent experiments. **(A)** was adapted from Mind the Graph.

and liver of HSPC-engrafted MISTRG mice (**Figures 7A, B**). This further supported the notion that $CD5^+CD7^+$ ILCs derived from intrahepatically injected $CD34^+$ HSPCs took up residence in the spleen and liver of MISTRG mice and subsequently gained access to the local vasculature (spleen red pulp, liver sinusoids) and systemic circulation. Importantly, $CD5^+CD7^+$ ILCs were also present in the spleen of human organ donors (**Figure 7C**), confirming the physiological relevance of our findings. Collectively, these results support the notion that $CD5^+CD7^+$ ILCs have a distinct origin and ontogeny than their $CD5^-CD7^+$ counterparts.

DISCUSSION

ILCs are essential for rapid immune responses and organ homeostasis. However, the ontogeny and migration of human ILCs is difficult to study in blood and tissue samples *ex vivo*.

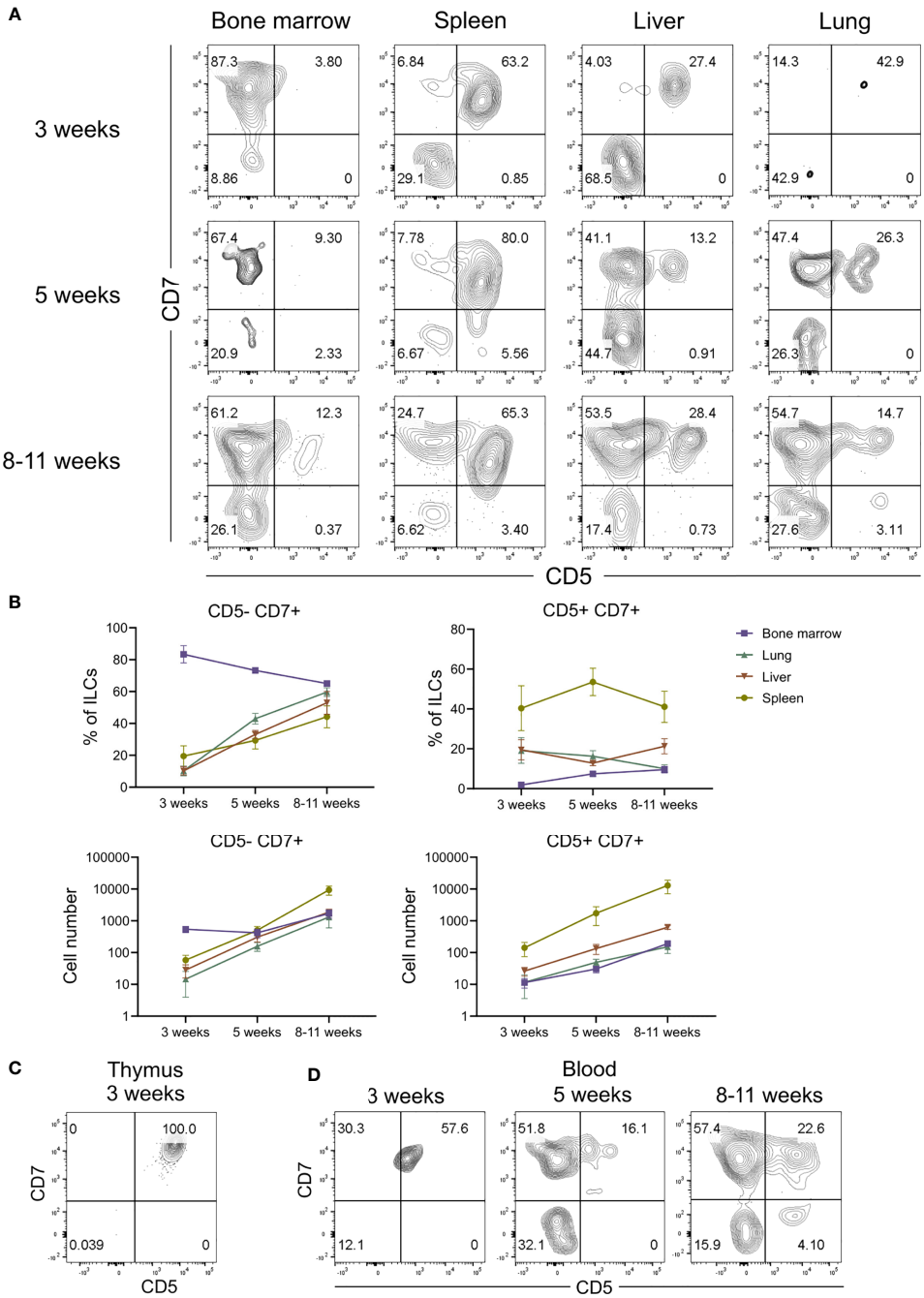

FIGURE 6 | CD5⁺CD7⁺ ILCs have a distinct ontogeny compared to CD5⁻CD7⁺ ILCs. **(A)** Flow cytometry analysis of CD5/CD7-expressing human ILCs in bone marrow, spleen, liver, and lung at 3 weeks, 5 weeks and 8-10 weeks after transplantation of MISTRG mice with human CD34⁺ HSPCs. ILCs were gated as human CD45⁺Lin⁻ CD3⁻TCRαβ⁻CD94⁻CD127⁺ cells. **(B)** Frequency and numbers of CD5⁻CD7⁺ and CD5⁻CD7⁺ ILCs in bone marrow, spleen, liver, and lung of MISTRG mice at 3 weeks, 5 weeks and 8-10 weeks post-engraftment with HSPCs (n = 6-9). **(C)** Flow cytometry analysis of human CD5⁺CD7⁺ ILCs in the thymus of MISTRG mice at 3 weeks after transplantation with human CD34⁺ HSPCs. **(D)** Flow cytometry analysis of human CD5⁺CD7⁺ ILCs in the blood of MISTRG mice at 3 weeks, 5 weeks and 8-10 weeks after transplantation with human CD34⁺ HSPCs. Data represent mean ± SEM and are representative for at least two independent experiments.

Accordingly, the developmental and migratory pathways of human ILCs remain poorly defined. To overcome this limitation, we employed a humanized mouse model to investigate human ILC migration within a surrounding tissue microenvironment *in vivo*. Using this experimental system, we identified a human CD5-expressing ILC population in the lung that has a unique ontogeny and migratory behavior, distinct from that of conventional CD5⁻ ILCs. CD5⁺CD7⁺ ILCs were

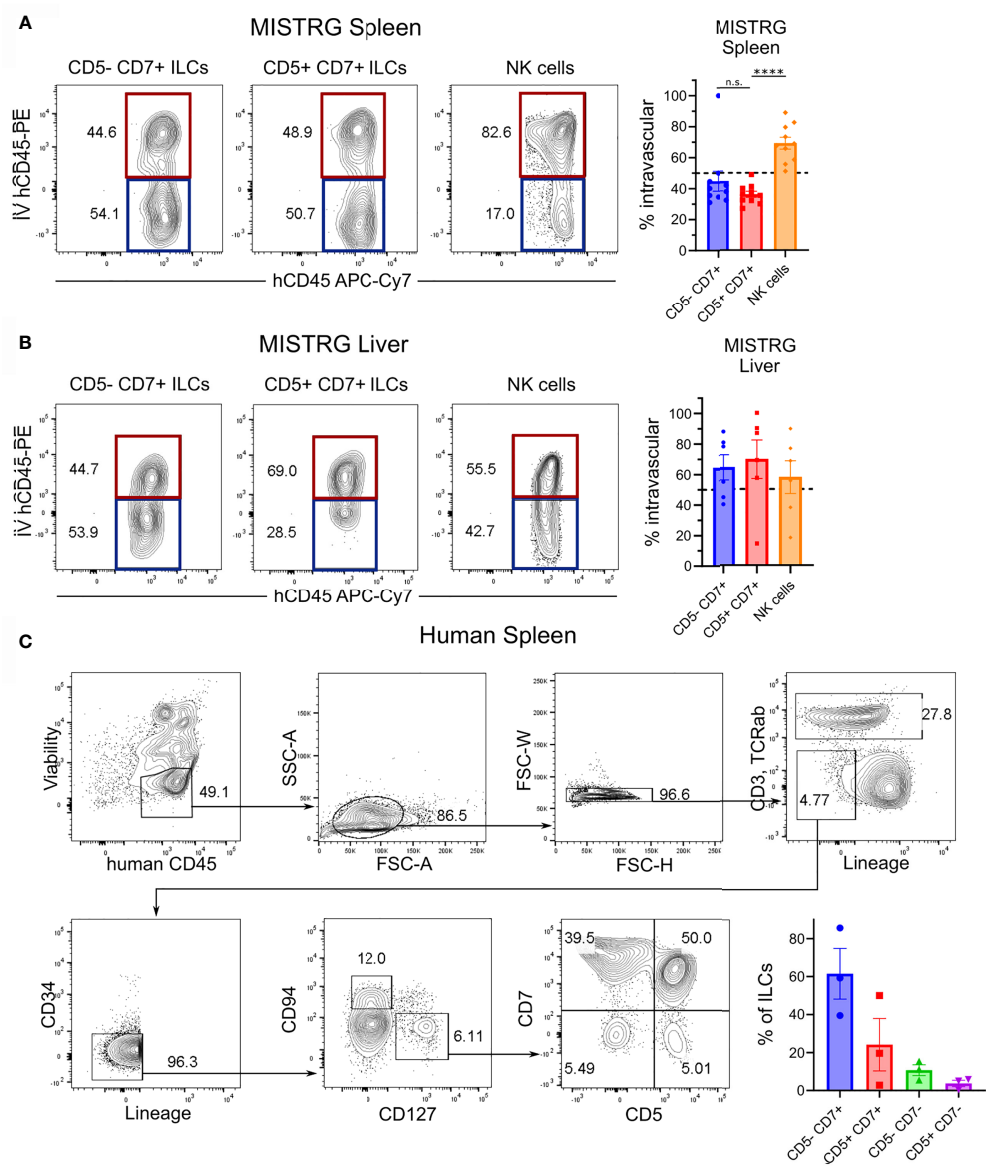

FIGURE 7 | Human CD5$^+$CD7$^+$ ILCs reside in the spleen and liver. **(A, B)** Flow cytometry analysis of intravascular (red) and extravascular (blue) CD5$^-$CD7$^+$ ILCs, CD5$^+$CD7$^+$ ILCs, and NK cells in the spleen **(A)** and liver **(B)** of HSPC-engrafted MISTRG mice. Frequencies of intravascular (IV-hCD45-PE$^+$) spleen and liver ILC subsets and NK cells (n = 6-10) are shown on the right. n.s., not significant; ****$P < 0.0001$ by one-way ANOVA, Tukey's post-test. **(C)** Frequencies of CD5$^-$CD7$^+$ and CD5$^+$CD7$^+$ ILCs in human spleen (n = 3). ILCs were gated as shown as viable human CD45$^+$CD3$^-$TCRαβ$^-$CD94$^-$CD127$^+$ cells that were negative for lineage markers (CD11c, CD14, CD19, CD123, FceRI) and CD34. Data represent mean ± SEM and are representative of three independent experiments.

located within the vasculature and may represent circulating precursors of mature human ILCs. Furthermore, vascular CD7$^+$CD5$^+$ ILCs had a different developmental path than tissue ILCs, first appearing in the spleen rather than in the bone marrow after transplantation of MISTRG mice with human CD34$^+$ HSPCs (**Supplementary Figure 6**). A few reports have studied human CD5$^+$ ILCs (34, 39), but CD5$^+$ ILCs remain poorly characterized and the lack of a relevant *in-vivo* model to study them has been a major limitation. Beyond these previous reports, our study provides new knowledge on the ontogeny, anatomical localization, and migration of human CD5$^+$ ILCs.

The CD5$^+$CD7$^+$ ILC population occupied the intravascular niche in the lung and other highly vascularized organs, such as the liver and spleen, after birth, mostly staying within the circulation in steady state, unlike conventional ILCs that migrate into the lung and become tissue-resident. Furthermore, in contrast to conventional CD5$^-$CD7$^+$ ILCs that develop in the bone marrow, CD5$^+$CD7$^+$ ILCs appeared first in

thymus, liver, and spleen after transplantation of MISTRG mice with $CD34^+$ HSPCs. Therefore, the timing and pattern of tissue reconstitution differed between $CD5^+CD7^+$ ILCs and $CD5^-CD7^+$ ILCs, which suggests that $CD5^+CD7^+$ ILCs may develop along an alternative pathway, distinct from that of bone marrow-derived $CD5^-CD7^+$ ILCs. Specifically, $CD5^+CD7^+$ ILCs could arise locally from intrahepatically injected $CD34^+$ HSPCs before disseminating *via* the circulation. This notion is supported by a recent study in mice demonstrating local differentiation of mouse ILC1s from hematopoietic stem cells in the liver (58). Alternatively, it is possible that $CD5^+CD7^+$ ILCs in MISTRG mice develop from local precursors in the spleen, consistent with CD62L surface expression by $CD5^+CD7^+$ ILCs. Moreover, studies in humanized mice indicate that the spleen environment may favor human ILC1 differentiation (59). The expression of T cell-related molecules suggests that $CD5^-CD7^+$ ILCs may also have a thymic origin, possibly representing ILCs with a failed T cell program. Finally, we cannot exclude the possibility that $CD5^+CD7^+$ ILCs originate from the bone marrow and then migrate to other organs, such as liver and spleen, although this scenario seems less likely based on our data. Overall, our results suggest that there are non-redundant pathways of human ILC development and that distinct developmental paths may relate to specific anatomical niches occupied by ILCs.

As their innate counterparts, ILCs share several features with T cells and they express T cell-related molecules, such as the surface protein CD5, which has important roles in T cell development and regulation (28, 29). Earlier studies demonstrated that ILC1s can express CD5 (33–38). Moreover, ILC populations expressing CD5 have been described as precursors to mature ILC2s and KIR^+NKG2A^- NK cells (34, 39). Consistent with these reports, our study further demonstrates that $CD5^+$ ILCs are heterogeneous and contain ILC populations other than mature ILC1s. We describe $CD5^+CD7^+$ ILCs with a surface phenotype ($CD45RA^+HLA\text{-}DR^-CD62L^+$) that is associated with naïve T cells recirculating between lymphoid organs. Furthermore, these $CD5^+CD7^+$ ILCs were present in highly vascularized organs (lung, spleen, and liver) with a mainly intravascular localization and were able to produce signature cytokines of mature ILC1s, ILC2s, and to a lesser extent ILC3s under polarizing conditions *in vitro*. We observed that these $CD5^+CD7^+$ ILCs downregulated CD5 from the cell surface upon acquiring effector function. A similar finding was reported by the Spits' lab for $CD5^+$ ILC2s that develop into cytokine-secreting $CD5^-$ ILC2s (34). It is therefore possible that CD5 inhibits the effector function of $CD5^+CD7^+$ ILCs.

The exact lineage identity of $CD5^+$ ILCs is still controversial. $CD5^+$ ILCs are related to T cells as shown by the expression of T cell signature genes, intracellular CD3 protein, and the presence of *TCR* gene rearrangements (34, 35, 38, 39, 50, 60, 61). However, these *TCR* gene rearrangements likely result in nonfunctional TCR protein expression. This idea is supported by a recent study, demonstrating non-productive *TCR* gene rearrangements in mouse ILC2s (62). Moreover, in contrast to T cells, ILC1s expressing CD5 are present in the blood of humans with *RAG1* deficiency (37). Overall, this indicates that expression of T cell-associated surface markers, such as CD5, and intracellular CD3 expression is *per se* not sufficient to define T cell identity. $CD5^+$ ILCs may represent immature ILCs with precursor activity or failed T cells with a nonfunctional TCR that underwent reprogramming into ILCs. Therefore, conclusively defining the exact lineage identity of $CD5^+$ ILCs is an important area of future study.

Their intravascular localization within the lung shows that $CD5^+$ ILCs are distinct from conventional $CD5^-$ ILCs that mostly reside outside of the vasculature in the lung. Furthermore, their migratory features are likely linked to their function. Although their residence time within the lung vasculature is unknown, it is possible that $CD5^+CD7^+$ ILCs adhere to the endothelium within lung capillaries and become a marginated pool of cells. Therefore, $CD5^+CD7^+$ ILCs could act as sentinels, patrolling the body *via* the blood vessels, and migrate "on-demand" into tissue during infection and other immune challenges. This might be especially relevant in the lung (63), a highly vascularized organ that is the first to receive the full cardiac output. $CD5^+CD7^+$ ILCs would be well-suited for this task, because they constitute a mixture of mature ILC1s, ready to contribute to type 1 immune responses, and immature cells that could produce effector cytokines when exposed to polarizing cytokines within the inflamed lung. Consistent with this possibility, circulating ILCs are reduced in respiratory infections, such as COVID-19 (64), suggesting their active recruitment into the lung. Therefore, vascular $CD5^+CD7^+$ ILCs may perform similar functions to blood monocytes that patrol blood vessels and gain access to tissue niches during altered organ homeostasis, when they differentiate into tissue-resident macrophages to support host defense against infection (65).

Type 1 immune responses are directed against viruses and intracellular bacteria and put the host in a state of "anticipatory alert", associated with heightened immune surveillance and tissue resistance to infection (66). ILCs involved in type 1 immunity are mainly IFNγ-secreting ILC1s/NK cells and cytotoxic NK cells, which both are mainly intravascular. Cytotoxic NK cells are kept in check by the expression of inhibitory NK cell receptors. We speculate that CD5 performs a similar role to inhibit the activity $CD5^+CD7^+$ ILC1s, in analogy to the inhibitory function of CD5 in T cells. This would allow the host to locally compartmentalize immune responses in order to avoid immunopathology and improper systemic immune activation within the vasculature. Therefore, future studies should investigate the role of $CD5^+$ ILCs in pulmonary infections and various inflammatory lung diseases in humans.

Limitations of the Study

MISTRG mice allow the investigation of the human immune system in a small animal model and are therefore suitable to study human ILCs *in vivo*. Limitations of this and other humanized mouse models include the absence of human fetal hematopoiesis because some ILCs, e.g. in the intestine, develop from fetal progenitors (67, 68). Another limitation is the lack of fully developed lymph nodes, which might affect ILC development and trafficking. Furthermore, human ILCs

interact with mouse epithelial, stromal, and endothelial cells in HSPC-engrafted MISTRG mice as well as in other humanized mouse models (24, 48, 49, 69–71). However, the human cytokines in MISTRG mice are sufficient to support human ILC-poiesis from HSPCs. Moreover, human ILCs migrate into tissues in MISTRG mice, leading to the distribution of the different ILC subsets into distinct vascular and tissue compartments. Overall, despite some shortcomings, our experimental system provides important information on the *in-vivo* biology of human ILCs, complementary to *ex-vivo* studies of ILCs from human blood and tissues.

Previous studies have demonstrated that $CD5^+$ ILCs from human blood contain ILC2 and NK cell precursors (34, 39). We observed that, after bulk culture, purified $CD5^+$ ILCs produced ILC1, ILC2, and, to a lesser extent, ILC3 effector cytokines *in vitro*. However, we cannot exclude the possibility that this is due to the outgrowth of pre-existing mature ILCs. Therefore, future studies, using single-cell ILC differentiation assays, are needed to unequivocally determine whether $CD5^+$ ILCs contain multi-potent ILCPs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Review Boards at Karolinska Institutet (#2006/229-31/3, 2015/1368-31/4, 2015/2122-32, 2016/1415-32, 2019-05016). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Linköping Animal Experimentation Ethics Committee (#29-15, 03127-2020).

AUTHOR CONTRIBUTIONS

AA designed, performed, and analyzed most experiments and wrote the paper. YG and EE designed, performed, and analyzed some experiments. NS helped with mouse experiments. DB, AK, CJ, NM, and JM provided human lung cells. TW conceived and supervised the study, acquired funding, designed, and analyzed experiments, and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.752104/full#supplementary-material

Supplementary Table 1 | List of antibodies used for flow cytometry.

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Myosin 1g and 1f: A Prospective Analysis in NK Cell Functions

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NK cells are contained in the ILC1 group; they are recognized for their antiviral and antitumor cytotoxic capacity; NK cells also participate in other immune response processes through cytokines secretion. However, the mechanisms that regulate these functions are poorly understood since NK cells are not as abundant as other lymphocytes, which has made them difficult to study. Using public databases, we identified that NK cells express mRNA encoding class I myosins, among which Myosin 1g and Myosin 1f are prominent. Therefore, this mini-review aims to generate a model of the probable participation of Myosin 1g and 1f in NK cells, based on information reported about the function of these myosins in other leukocytes.

Keywords: Class I Myosin, NK cell, Myo1g, Myo1f, cytoskeleton

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INTRODUCTION

Innate lymphoid cells (ILCs) neither express T and B lymphocyte receptors but are derived from common lymphoid progenitors (CLPs) (1, 2). There is evidence about the capacity of ILC2 and ILC3 needed to recognize and present antigen to T lymphocytes and, in this way, maintain immune homeostasis (3–6). ILCs have been considered the innate equivalent of T helper lymphocytes (Th), Th1, Th2, and Th17 since ILC releases the same cytokine profile of Th cell (7–10). ILCs can mirror even T regs functions due to their capacity to produce TGF-β and IL-10 (11–13). NK cells belong to the ILC1 group (14). They are crucial in antiviral and antitumor response through their cytotoxic activity (15, 16). NK cells require the optimal function of the actin cytoskeleton and cellular membrane dynamics to perform their functions. In NK cells, the actin cytoskeleton reorganization is achieved by activation signals through several activation receptors, such as; Killer-cell Immunoglobulin-like Receptor (KIR), Natural Cytotoxicity Receptor (NCR), CD16, Signaling Lymphocyte Activation Molecule (SLAM), and others (17, 18). In a reductionist model, CD16, NKp46, NKp30 receptors associate with adapter proteins with Immunoreceptor Tyrosine-based Activation (ITAM) domains such as CD3ζ, FcεRIγ; whereas NKp44 associate with DAP12. Src kinase family members phosphorylate tyrosines in the ITAMs. Phosphorylated ITAMs form a binding site for the Src homology 2 (SH2) domains of the ZAP70 and SYK tyrosine kinase, which induce SLP-76 phosphorylation. Vav1 then recognizes phosphorylated SLP-76 *via* SH2 domain (19, 20). Next, SLAM family receptors transmit activation signals through the SLAM-Associated Protein

(SAP), which recruits tyrosine kinase (Fyn) (21). Then Fyn induces Vav1 phosphorylation (22). ITAM independent signaling through the NKG2D receptor also induces Vav1 recruitment *via* PI3K and Grb2 after DAP10 tyrosine phosphorylation (23–26). In this way, Vav1 has an essential role in NK cell function. Vav1-deficient NK cells show defects in tumor cell killing (27). A synergistic effect is required to achieve ubiquitin ligase c-Cbl inhibition, which controls the availability by Vav1 (28). Consequently, Vav1 regulates actin cytoskeleton polymerization by activating the GTPases Rac, Rho, and Cdc42 since Vav1 has GEF properties (29). In this dynamic process, myosins participate at different levels, either during the polarization or aggregation of integrins, maintaining membrane tension, or interacting directly with other proteins.

NK cells are not abundant as other lymphocytes; this scarcity hinders the analysis of NK functions. Searching in databases and the analysis of the mechanisms reported in other similar cells could help understand NK lymphocytes that eventually will lead to a broader perspective about the function of these cells.

OVERVIEW OF NK CELLS

Natural Killer cells are innate lymphocytes (ILC1s) known primarily for their antiviral and antitumor cytotoxic capacity (16, 30). However, they also have effector functions such as releasing cytokines, such as IFN-γ, TNF-α, IL-10, and others (8, 10, 31). Thus, NK lymphocytes are considered part of the sentinels of the innate immune system. In humans, two populations of NK cells have been described, $CD56^{dim}CD16^{+}$ and $CD56^{bright}$ $CD16^{dim}$ (32, 33). There are differences between both populations; for example, $CD56^{dim}CD16^{+}$ has more cytotoxic capacity than $CD56^{bright}$ $CD16^{dim}$ or $CD16^{-}$.

In contrast, upon monocytes-derived-stimuli, the $CD56^{bright}$ $CD16^{dim/-}$ NK lymphocytes release a high amount of cytokines (7, 32, 33). Thus, $CD56^{-}CD16^{+}$ subpopulation is usually found in HIV-infected individuals presenting the high expression of NK inhibitory receptors, associated with poor cytotoxic activity (34). Regarding their anatomical distribution, the presence of NK cells has been observed in both lymphoid and non-lymphoid tissues (7). The cytotoxic activity of NK lymphocytes depends on their ability to release preformed cytotoxic granules contained in vesicles (35). The exocytosis of lytic granules begins with the contact between NK lymphocyte and target cells, which gives rise to the cytotoxic synapse (36–38).

Furthermore, NK cells express on their surface receptors of the TNF family, such as FasL and TRAIL, which can induce apoptosis by binding to their Fas or TRAIL ligand, respectively (37). Thus, the regulation of NK cell functions depends on the balance between activation and inhibition signals given by receptors present on their membrane (35). Within the group of inhibitory receptors, one can find the KIRs in humans and Ly49 Isoforms (A, B, C, E, G, Q) in mice. These receptors inhibit, inside-out and outside-in, LFA-1 signaling at different levels, preventing polarization and degranulation (39, 40). Therefore, a decrease in MHC-I expression reduces the inhibitory signal and promotes the activation of the NK cell. Additionally, in both humans and mice, the CD94/NKG2A heterodimer recognizes non-classical MHC-I molecules in the context of HLA-E (human) or H2-Qa1 (mouse). The ligands of the activating receptor NKG2D are represented by MICA/B and by ULBP in humans, and Mult1 and Rae1 in mouse (7, 10, 41).

On the other hand, activation signals are given by activation receptors, for example, Ly49 (D, H, L) and KIR isoforms, NKG2D, and natural cytotoxic receptors such as NKp30 and NKp44 in humans and NKp46 in humans and mice (7, 10). Additionally, LFA-1, β1, and β2 integrins can also regulate NK cell function (42), which we will address later. Signaling of activation and inhibition receptors regulate several NK cells functions, for example, degranulation, morphological modifications to increase NK-target cell contacts, cell migration, and cytokine release. Since Myo1g and Myo1f are involved in morphological changes and vesicular traffic, studying these proteins in the NK cell physiology becomes relevant (43). Thus, the functions of NK lymphocytes are dynamic processes that may be regulated by cytoskeletal proteins such as myosins.

Furthermore, there are functional differences among NK cell subpopulations depending on their anatomical distribution (44). For example, IL-12- and IL-18-induced IFN-γ production varies between mouse $CD27^{high}$ spleen-resident and $CD27^{low}$ lung-resident NK cells (32). In humans, NK $CD56^{bright}$ under *in vitro* stimulation of IL-12 and IL-18 induce the release of more IFN-γ and TNF-β than NK $CD56^{dim}$ cell (45). Therefore, a detailed understanding of the intrinsic factors regulating NK cell functions could provide tools to modulate any particular function depending on the type of required response.

CLASS I MYOSINS

Myosins are a family of motor proteins, which are mainly known for their function in cell contractility. However, some members of this family proteins, for example, Myosin V, VI, and Ic participate in moving different cargos along the actin filaments, such as vesicles, mitochondria, and ribonuclear protein particles (46–51). Currently, 35 classes of myosins have been reported in eukaryotic organisms (52). This classification varies depending on the species; for example, 12 classes of myosin's are described in humans (53–55). Class I myosins are non-filamentous myosins, consisting of one heavy chain and a variable number of light chains (56). The heavy chain contains three conserved regions; the ATP-dependent globular or motor domain, which binds to F-actin (57, 58). Adjacent to the motor domain is the neck region, where light chains associate and regulate the globular domain (57). In addition, the neck region has IQ domains, sequences that interact with calmodulin and calmodulin-like chains (57). Finally, the tail variable section bestows different functions depending on the domains present in that region (50, 51, 54, 56, 57). Class I myosins are subdivided into short-tailed and long-tailed myosins; both have a Pleckstrin homology domain inside the TH1 domain, as shown in **Figure 1A**, which allows interaction with several phospholipids

present in the membrane and other compartments in a PH-dependent manner (51, 59). Long-tailed myosins have two additional domains; a proline-rich domain (TH2) and a domain homologous to SRC kinase (SH3) (50, 51, 56, 60). Humans and mice have a total of 8 genes coding for six short-tailed myosins (*Myo1a*, *b*, *c*, *d*, *g*, and *h*) and two long-tailed myosins (*Myo1e*, *f*) (58). Remarkably, only Myo1c, d, e, f, and g have been described in leukocytes (51, 60). Myo1g has a length of 1018 amino acids in humans and 1024 amino acids in mice (https://www.uniprot.org/) and belongs to the group of short-tailed myosins. It has a PH-type domain in the tail region, which allows its binding to lipids in the plasma membrane and microdomains rich in phospholipids and cholesterol, known as lipid rafts (59, 61, 62). The expression of Myo1g has been observed mainly in T and B lymphocytes and mast cells (63–65). Myo1g has been proposed as a bridge that allows the adequate interaction between the membrane and the cytoskeleton in processes such as cytokine secretion, cell migration, mobilization, recycling of membrane molecules, and regulating modifications in the cytoskeleton that favor cell adhesion (63, 65, 66).

On the other hand, Myo1f has a length of 1098 amino acids in humans and 1099 amino acids in mice (https://www.uniprot.org/). Its expression has been confirmed in neutrophils, macrophages, mast cells, and T lymphocytes (67–71). Similar to Myo1g, Myo1f is located adjacent to the plasma membrane, co-localizing with cortical actin and interacting with membrane phosphoinositides (67). As mentioned above, Myo1f has two additional domains, a TH2 and an SH3 that allows the interaction with several proteins. In addition, it has been observed that Myo1f interacts with 3BP2 (69), activating Cdc42 (72) suggesting a Vav1 pathway that potentially activates Rac and RhoA (73), thus regulating the cytoskeletal machinery to favor morphological changes and the generation of membrane protrusions.

As shown in **Figure 1B**, some class I myosins' mRNAs are expressed by NK cells (74). However, it will be necessary to prove this expression at a protein level. The presence of Myosin 1g (Myo1g) and Myosin 1f (Myo1f) mRNA occur from the early stages of NK cell development (https://gexc.riken.jp). Likewise, human peripheral blood NK cells also show high Myo1g and Myo1f mRNA (75–77) (https://www.proteinatlas.org). Interestingly, tumor resident ILC1 and NK cells also express Myo1g and Myo1f (78). These results suggest that both mouse and human NK cells express both myosins and that the expression is maintained in the context of their antitumor effect. Therefore, it would be essential to analyze the role of these myosins in NK-cell differentiation, development, and functions. Class I myosins reported in leukocytes regulate processes requiring the interaction between the plasma membrane and actin cytoskeleton, such as cytokines secretion, cell migration, and mobilization of plasma

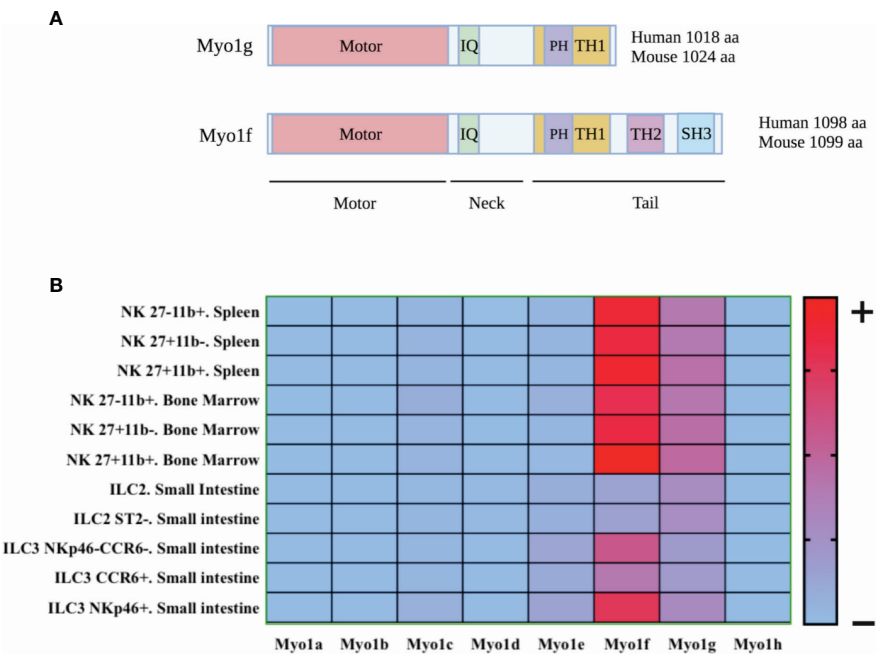

FIGURE 1 | Schematic representation of Myo1g and Myo1f structure and heat map of expression of class I myosin in innate lymphoid cells. **(A)** Myo1g has a sequence of 1018 amino acids (human) and 1024 amino acids (mouse), while Myo1f has a sequence of 1098 amino acids (human) and 1099 amino acids (mouse). In addition, both myosins have a motor domain in the amino-terminal region, following a neck region with an IQ domain and a TH1 domain in the tail region which allows phosphoinositide interaction in a PH-manner dependent. Finally, Myo1f had two additional domains: TH2 (a proline-rich region) and SH3 (a proline-rich-interacting region), allowing protein-proteins interactions. Created with BioRender.com. **(B)** Class I Myosin expression in ILCs was explored with data from: http://www.immgen.org. The heat map was generated using GraphPad Prism version 8.0.0 for Mac OS X, GraphPad Software, San Diego, California USA, www.graphpad.com.

membrane molecules. Therefore, we aim to analyze class I myosins' participation in NKs functions, using information derived from results published in other leukocytes.

MYO1G AND MYO1F COULD REGULATE CYTOTOXIC SYNAPSE THROUGH MORPHOLOGICAL CHANGES

Cytotoxicity is one of the main functions depending on morphological changes regulated by the cytoskeleton. The cytotoxic activity of NK lymphocytes first requires interaction with their target through a cytotoxic synapse and subsequently the release of cytotoxic granules towards the target cell (79). The synapse is dependent on the mobilization of different surface molecules such as adhesion molecules and integrins (80). First, the synapse requires close contact with the target cell by generating projections (filopodia and lamellipodia), this depends on the force generated by the myosins (81). The formation of these protuberances depends on Cdc42 and RhoA (29). Physical properties such as membrane tension allow membrane deformation to generate these projections (81, 82). Myo1g is abundantly expressed in the protuberances generated by B lymphocytes (63).

On the other hand, B lymphocytes show reduced membrane tension in its absence, decreasing their ability to generate filopodia and lamellipodia (83). Besides, *Myo1f* siRNA-treated macrophages decrease their capacity to generate morphological changes (84). In this way, Myo1g and Myo1f could participate in the early stages of the cytotoxic synapse of the NK cell, regulating the formation of membrane protrusions that allow interaction with their target cell.

PROBABLE PARTICIPATION OF MYO1G AND MYO1F IN NK CELL MIGRATION

Cell migration depends on cytoskeleton changes that promotes the interaction of migrating cells with the endothelium. There is evidence showing that class I myosins regulate the expression of the molecules during leukocyte migration (65–67, 85). NK cells are recruited to different tissue compartments, i.e., lymph nodes and inflamed tissues, where they perform different functions such as promoting DC maturation, T cell polarization, and as cytotoxic effector cells (86, 87). NK cells express β1, β2, and β7 integrins, PSGL-1, CD62L, and various chemokine receptors such as CXCR1, CXCR2, CXCR4, CCR5 y CCR7, which allow their interaction with HEV during lymph nodes (86, 88, 89). NK subpopulations in humans and mice show differences in the expression levels of integrins and adhesion molecules (86). Therefore, the mechanisms by which NK cells migrate to different anatomical sites are not yet fully understood. Selectins and integrins regulate the interaction between the cell and the endothelium, so the expression of these molecules and their mobilization is essential during cell migration. Myo1g-deficient B lymphocytes have reduced adhesion to the endothelium due to a lower expression of LFA-1, CD62L and, VLA-4 (65).

Moreover, in the absence of Myo1g, B lymphocytes have a lower capacity for CXCL13-dependent transmigration to the inguinal node, furthermore *in vitro* CXCL12-dependent migration is also reduced (65). Migration defects were attributed to a decrease in the expression of adhesion molecules and a lower capacity to generate morphological changes due to the absence of Myo1g. Myo1f-deficient mice showed a reduction in the recruitment of neutrophils in a lung damage model (68). These neutrophils did not present defects in rolling and adhesion but in extravasation *in vivo*, explained by inefficient nucleus deformation during migration (68). *In vitro* CXCL1-dependent chemotaxis was also affected (68). Although it has not been observed that Myo1f participates directly in cell migration, it has been seen that Myo1f affects the expression of integrins β1 and β7 in mast cells (72).

Additionally, in mast cells, the activation of phosphatidylinositol 3-kinase (PI3K) increases PI(3,4,5)P3, causing the recruitment and association of 3BP2 with Myo1f during KIT activation (69). Although the consequence of the interaction of both proteins in other cell types has not been evaluated, in mast cells, 3BP2 participates in different processes; such as degranulation, by regulating the SYK, LAT, and PLC-γ pathway; in survival, by regulating the KIT, STAT1, Akt and ERK pathway; and during cell migration, by activating the Cdc42 and Rac2 pathway and regulating the expression of integrin β1 (69). Furthermore, 3BP2 is essential for activating Vav1 (73), impacting the activation of GTPases of the Rho family. The absence of Myo1f impacts the activation of Cdc42 (72), then its association with 3BP2 could play a role in the activation of Vav1. Consequently, the activation of the GTPases of the Rho family, essential in the polymerization of the actin cytoskeleton, will be affected. RhoA controls the polymerization of cortical actin through its interaction with ROCK1 and ROCK2, forming stress fibers (90). Rac1 and Rac2 are involved in the polymerization of the actin cytoskeleton *via* the SCAR/WAVE effectors, while Cdc42 controls cell polarity for migration, synapse formation, and cytokine secretion *via* effectors of the WASP family (29, 91). The role played by Myo1f, and 1g could be crucial for the migration of NK lymphocytes since they could participate by independent mechanisms due to their structural differences.

MYO1G AND MYO1F REGULATE ADHESION MOLECULES EXPRESSION IN LEUKOCYTES

Adhesion molecules such as selectins and integrins, in addition to regulating the migration, and increasing the adhesion during cytotoxic synapse, also participate in the activation of NK cells. β1 and β2 integrins regulate the interaction of the NK cell synapsis, while LFA-1 participates in the polarization of cytotoxic granules and increasing adhesion during synapse (92, 93). As an example of its importance in other lymphocytes, Myo1g-deficient B cells have reduced CD62L and LFA-1 (65). It has been speculated that Myo1g participates in the vesicular

trafficking of these molecules (66). However, no significant differences in LFA-1 expression were found in Myo1g-deficient T cells (64). Thus, it is necessary first to evaluate LFA-1 expression and other adhesion molecules in Myo1g-deficient NK cells. LFA-1 has acquired notoriety in NK cells because it participates in the activation, adhesion, and regulation of cytotoxic granules (38).

Similarly, Myo1f is crucial for the expression of integrins in leukocytes. Myo1f-deficient neutrophils increase the expression of β2-integrin, which enhances their adherence to ICAM-1 (67). Additionally, macrophage cell lines such as RAW 264.7 and J774, with stable overexpression of Myo1f-GFP, have an increased expression of integrin αVβ3, leading to increased adhesion vitronectin and promoting an inflammatory phenotype *via* ILK/Akt/mTOR activation (70). Silencing Myo1f in human mast cells negatively impacts the expression of the integrin β1 and β7, affecting exocytosis (72). Therefore, the role of Myo1f may be highly relevant for the cytotoxicity of NK cells because it is plausible to think that it may regulate the expression of integrins or other membrane molecules essential in NK cell activation.

MYO1G AND MYO1F COULD REGULATE NK CELL CYTOTOXICITY

Unlike cytotoxic T lymphocytes, NK cells have performed cytotoxic granules (31). Thus, NK cells have a faster cytotoxic activity, which becomes relevant in viral infections where a quick response is required (94, 95). Once the cell recognizes its target, these granules are mobilized to the synapse site by the mTOC (96). Then, these granules fuse with the cell membrane and are released into the pocket of the synapse (97, 98). Thus, LFA-1 primarily mediates tight maintenance of the synaptic cleft (92). In this regard, Myo1f has been reported to participate during granule mobilization in mast cells through a mechanism dependent on Cdc42 activation (72). However, it has not been evaluated whether Myo1g could have a similar function in activating GTPases of the Rho family (69, 72).

PARTICIPATION OF MYO1G AND MYO1F IN CYTOKINE PRODUCTION AND RELEASE

NK cells produce and secrete IFN-γ and TNF-α (7). It has been reported that IFN-γ production in infections by murine norovirus depends on ISG15 signaling (99–101). The binding of ISG15 to LFA-1 strongly induces the production of IFN-γ and IL-10 (99–101). The absence of Myo1g decreases LFA-1 expression in B lymphocytes (63, 65), suggesting that Myo1g could participate in the LFA-1-dependent IFN-γ production in the context of viral infections. Cytokines release depends on the fusion of secretory vesicles with the plasma membrane, resulting in the content release towards extracellular space (102). Diverse reports have shown the participation of Myo1g and Myo1f in releasing TNF-α, IL-6, IL-1, lactoferrin, IFN-γ, and prolactin in B lymphocytes, neutrophils, and mast cells (63, 67, 70, 72). Whether Myo1g and Myo1f are required for cytokines released by NK cells waits to be determined. However, accumulated evidence with other leukocytes points out in that direction.

MYO1G AND MYO1F COULD REGULATE OTHER ESSENTIAL NK CELL FUNCTIONS

Myo1g, through its PH domain, participates in mobilizing and recycling lipid rafts, indirectly moving molecules, such as CD44 (66, 103). Lipid rafts from NK cells' membrane are mobilized to the contact site of target cells, but they are excluded in cells resistant to lysis (104). It has been suggested that signaling the KIR2DL1 protein in the cytotoxic synapse inhibits the polarization of the lipid rafts, thus preventing the death of the target cell (104). Given the role of Myo1g in mobilizing lipid rafts (66), it is likely that it participates in mobilizing these microdomains during activation and inhibition of NK-cell cytotoxicity. For this reason, it would be interesting to analyze whether Myo1g has a similar function during NK-cell lipid rafts mobilization during synapsis and in other functions, where lipid microdomains mobilization is also required. Besides, it has been reported that in the absence of Myo1g, lymphocytes present a lower membrane tension, which decreases their ability to generate membrane structures (64, 83). In addition to regulating the elasticity and stiffness of the membrane, membrane tension can generate morphological changes through the PLD2-mTORC2 signaling pathway (82, 105). Since, Myo1g and Myo1f are located adjacent to the plasma membrane, co-localizing with cortical actin (63, 67). So then, it would be interesting to know if Myo1g and Myo1f intervene in the mechano-transduction process by NK cells as described in other cell types.

DISCUSSION

To date, there is no information about the role of class I myosins in NK cells functions. However, evidence in other cell lineages suggests that Myo1g and Myo1f could participate by regulating different NK cell functions such as cytokines release, synapse formation, granule mobilization, and migration. The functional defects described by the absence of Myo1g and Myo1f could similarly affect NK cells, causing increased susceptibility to viral infections and tumor development. Due to the tail's structural differences, the mechanism by which Myo1g and Myo1f may regulate these processes will not be the same. Since Myo1f has a TH2 and an SH3 domain, the functions of Myo1f could depend on protein-protein interactions (85, 106), while the function of Myo1g could depend on its interaction with phosphoinositides present in membranes and vesicles (85). The function of class I myosins seems to depend on cell activation but also cell lineage. Therefore, it would be interesting to study these proteins in the context of NK cells and other ILCs subpopulations. To date, there is no information available about myosin mutations in humans that could be associated with NK cell function. However, the use of

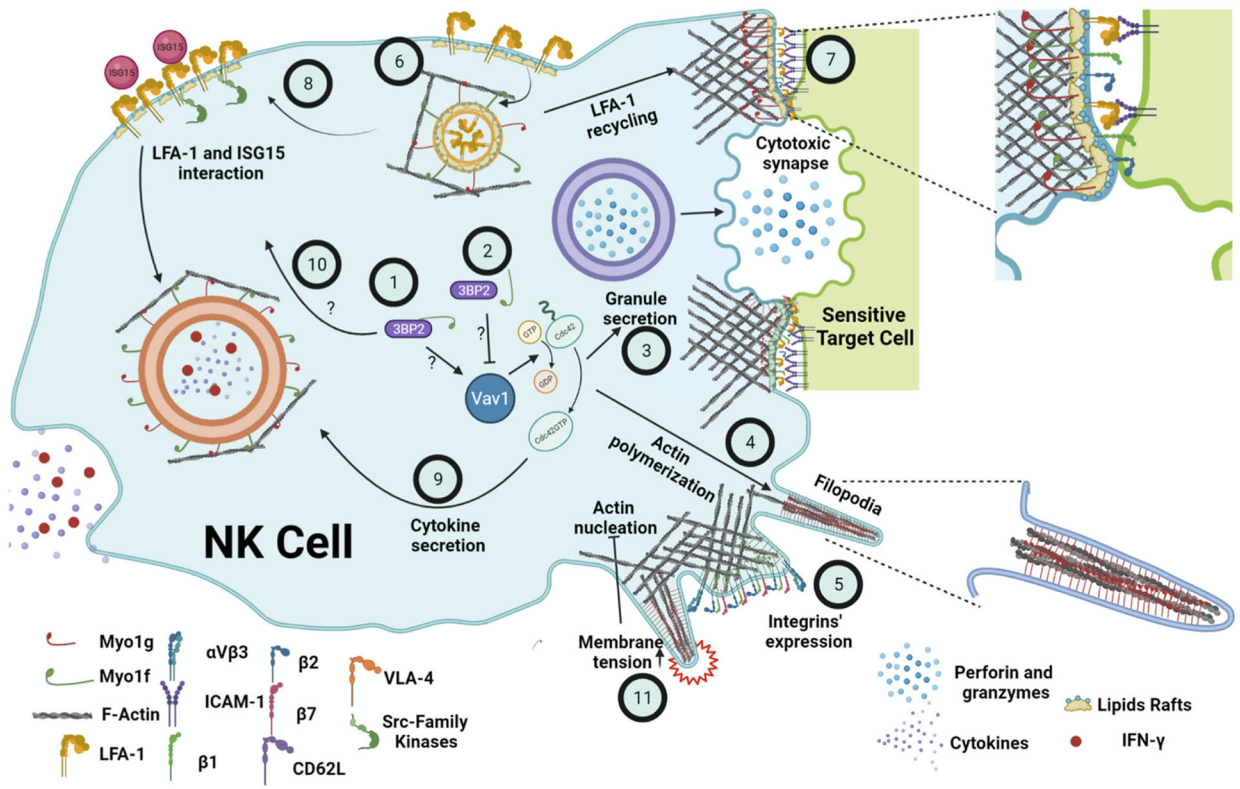

FIGURE 2 | Summary of the possible functions of Myo1g and Myo1f in the NK cell. It is suggested that the interaction of Myo1f with 3BP2 causes the activation of Cdc42 through Vav1 (1). On the other hand, reduced interaction Myo1f and 3BP2 causes a decreased activation of Vav1 and, in turn, lower activation of Cdc42 (2). Consequently, less actin is polymerized during the secretion of cytotoxic granules (3), protrusions formation (4), and cytokine secretion. The participation of Myo1g and Myo1f is also essential, regulating the membrane tension. Furthermore, Myo1f deficiency causes defects in β1, β2, β7 integrins expression (5). Moreover, Myo1g-deficient B cells show lower LFA-1 membrane expression, probably due to defects in recycling (6) similar to the ones present in CD44. A decrease in LFA-1 at the cytotoxic synapse (7) will impair cytotoxic activity. Additionally, a lower amount of LFA-1 in the membrane would cause a lower interaction with ISG15 and defects in Src family kinases signaling (8), which induces the secretion of cytokines and IFN-γ, also mediated by Cdc42 (9). Therefore, it would be possible that the interaction of Myo1f and 3BP2 (10) could regulate Src; however, none of these interactions have been proven in NK cells. Finally, membrane tension negatively regulates actin polymerization through the PLD2-mTORC2 pathway (11). Thus, both Myo1g and Myo1f could regulate NK cell membrane tension and, consequently, mechano-transduction. Created with BioRender.com.

murine models or cell lines deficient or overexpressing Myo1g and Myo1f could reveal the role of these myosins in NK cells and other ILCs subpopulations. **Figure 2** summarizes what we believe may be the participation of Myo1g and Myo1f in the functions of NK cells.

AUTHOR CONTRIBUTIONS

DC-Z, CM-R, IM-V, and LS-A wrote the manuscript with contributions by all authors. DC-Z, CM-R, and IM-V designed at the heat map under LS-A supervision. DC-Z, CM-R, and IM-V designed and drew the illustration under LS-A supervision. All authors contributed to the article and approved the submitted version.

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Efficient *In Vitro* Generation of IL-22-Secreting ILC3 From CD34$^+$ Hematopoietic Progenitors in a Human Mesenchymal Stem Cell Niche

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Innate lymphoid cells (ILCs) and in particular ILC3s have been described to be vital for mucosal barrier functions and homeostasis within the gastrointestinal (GI) tract. Importantly, IL-22-secreting ILC3 have been implicated in the control of inflammatory bowel disease (IBD) and were shown to reduce the incidence of graft-versus-host disease (GvHD) as well as the risk of transplant rejection. Unfortunately, IL-22-secreting ILC3 are primarily located in mucosal tissues and are not found within the circulation, making access to them in humans challenging. On this account, there is a growing desire for clinically applicable protocols for *in vitro* generation of effector ILC3. Here, we present an approach for faithful generation of functionally competent human ILC3s from cord blood-derived CD34$^+$ hematopoietic progenitors on layers of human mesenchymal stem cells (MSCs) generated in good manufacturing practice (GMP) quality. The *in vitro*-generated ILC3s phenotypically, functionally, and transcriptionally resemble *bona fide* tissue ILC3 with high expression of the transcription factors (TF) RorγT, AHR, and ID2, as well as the surface receptors CD117, CD56, and NKp44. Importantly, the majority of ILC3 belonged to the desired effector subtype with high IL-22 and low IL-17 production. The protocol thus combines the advantages of avoiding xenogeneic components, which were necessary in previous protocols, with a high propensity for generation of IL-22-producing ILC3. The present approach is suitable for the generation of large amounts of ILC3 in an all-human system, which could facilitate development of clinical strategies for ILC3-based therapy in inflammatory diseases and cancer.

Keywords: CD34 cells$^+$, mesenchymal stem cells, umbilical cord stem cells (UCSC), innate lymphoid cells (ILCs), ILC3, hematopoietic stem and progenitor cells, GMP—Good Manufacturing Practice

INTRODUCTION

Recently, the therapeutic potential and possibility of translational approaches of human innate lymphoid cells (ILCs) has been highlighted by us (1) and others (2). One subset of ILCs is particularly interesting in this regard: tissue-resident ILC3. ILC3 have been described to express the transcription factor (TF) RORγT and can be divided into two functional subsets: NKp44$^-$ILC3 secreting IL-17A and NKp44$^+$ILC3 secreting IL-22 (3, 4). The latter have been recognized to be essential for promoting tissue integrity, maintaining barrier functions, and promoting homeostasis (3, 5), especially within the gastrointestinal (GI) tract. Secretion of IL-22 seems to play a key role for these functional properties (3). IL-22 secretion by NKp44$^+$ILC3 is activated after food intake (6, 7), sensing danger signals (8, 9) as well as changes in cytokine milieu (3, 10). Furthermore, NKp44$^+$ILC3s express neuroregulatory receptors enabling direct interactions with glial cells (11), but also interact with the microbiome (12) and goblet cells (13). The secreted IL-22 acts upon epithelial cells and intestinal stem cells (ISCs), both expressing the IL-22 receptor (IL-22R) (14), which is absent on cells from the hematopoietic lineage. Within experimental models, IL-22 stimulation had positive effects on ISCs to protect them against tissue damage (15–17) and promoted IFNλ secretion to induce anti-viral activity (18, 19). These studies combined suggest a complex interaction of IL-22-producing NKp44$^+$ILC3 within the GI tract milieu necessary for our wellbeing.

Importantly, recent transplant studies have further strengthened the hypothesis that NKp44$^+$ILC3s are essential for maintaining homeostasis and protection of the GI tract: following human GI transplantation, recipients` ILCs quickly infiltrated into the GI graft (20) and moreover elevated frequencies of human NKp44$^+$ILC3s within the graft have been associated with successful intestinal transplants by reducing the risk of rejection (21). A similar observation has been made in leukemic patients after hematopoietic stem cell transplantation (HSCT). Patients undergoing allogeneic HSCT are in constant risk of graft-versus-host disease (GvHD) by alloreactive cells such as donor T cells attacking dominantly the skin, GI tract, and liver (22, 23). After HSCT, NKp44$^+$ILC3, which are normally not present within the circulation of healthy individuals (24), occurred in peripheral blood and correlated with reduced risk of GvHD (25). The patient`s NKp44$^+$ILC3s expressed chemokine receptors, potentially enabling the migration into the skin or GI tract and therefore possibly promoting protection. On the other hand, NKp44$^-$ILC3 have been described to accumulate and to aggravate inflammation due to their secretion of IFNγ and IL-17A, respectively, especially during inflammatory bowel disease (IBD) (26–28). Therefore, IBD patients, leukemic patients undergoing HSCT, and GI-transplant recipients may benefit from cell-based therapies using human NKp44$^+$ILC3.

Human effector ILC3s are predominantly found in the mucosa of certain organs, such as tonsils and intestine, and are virtually absent from the circulation. Of note, the circulating counterpart in umbilical cord blood (CB) referred to as ILC3-like cells are predominantly immature and lack typical ILC3 effector functions (29). Since the isolation of human ILC3 directly from organs appears not to be a viable option due to ethical issues and low cell recovery, the *in vitro* generation of ILC3 from HSPC appears to be a promising avenue. Indeed, in conditions enabling the generation of NK cell from HSPC *in vitro*, it has been already shown that besides NK cells, an additional population of IL-22-producing ILCs is generated (30, 31). The efficiency of ILC generation was increased by usage of murine stroma cells as stem cell niche (30, 32, 33). In this regard, we recently established a protocol for the generation of NK cells from CD34$^+$ HSPCs in which murine stroma cells were replaced by human mesenchymal stem cells (MSCs) from bone marrow. The MSC-based niche enabled the highly efficient generation of NK cells including expression of KIRs, which are only scarcely generated on murine feeder cell lines (34). In the course of further studies analyzing the effect of human cytomegalovirus infection on NK cell differentiation *in vitro*, a NKp44$^+$CD56$^+$CD94$^-$ cell subset with similarity to tissue ILC3s was identified by us (35). In the present study, we now provide compelling evidence that these cells indeed represent *bona fide* effector IL-22-secreting NKp44$^+$ILC3s and that they can be efficiently generated from umbilical CB-derived HSPC in a human MSC niche. The *in vitro* generated NKp44$^+$RorγT^{high}ILC3 closely resemble tissue-resident ILC3 phenotypically and by global transcriptional analysis. Functionally, the *in vitro* generated NKp44$^+$RorγT^{high}ILC3 secreted high amounts of IL-22 and hardly any IL-17A. The present approach omits xenogeneic components and opens novel avenues for the efficient generation of IL-22-producing ILC3 in GMP-compatible conditions for future human cell-based therapies.

RESULTS

Efficient and Robust Generation of ILC3 from HSPC on Human MSCs

The purpose of this study was to establish a standardized protocol for the GMP-compatible generation of human NKp44$^+$ILC3 employing the HSPC/MSC platform, previously developed for the generation of NK cells (34). As outlined in **Figure 1A**, following isolation of mononuclear cells (MNCs) from CB, CD34$^+$ HSPCs were sorted by flow cytometry, seeded on human MSCs, and differentiated for up to 28 days. Based on our previous study, NK cells and ILC3 could be distinguished on the basis of NKG2A and CD56 expression (35) (see **Supplementary Figure 1** for gating strategy). Thus, three major populations were provisionally identified: ILC3 (NKG2A$^-$CD56$^+$), NK cells (NKG2A$^+$CD56$^+$), and double negative (DN) cells (NKG2A$^-$CD56$^-$) (**Figure 1B**). In terms of cell frequencies, the three populations developed with distinct dynamics between day 14 and day 28: ILC3 frequencies continuously increased over time (ILC3: 14/15 days to 21 days, $p = 0.001$, 14/15 days to 27/28 days, $p = 0.003$), as did NK cells, but the latter with a slightly slower increase in the fourth week of culture (NK cells: 14/15 days to 21 days, $p = 0.015$, 14/15 days to 27/28 days, $p = 0.029$), while DN frequencies continuously decreased (14/15 days to 21 days, $p < 0.0001$; 14/15 days to 27/28 days, $p < 0.0001$) (**Figure 1B**). Thus, the culture conditions enabled a significant expansion of the different innate lymphoid subsets. Total cell counts strongly increased from 2×10^3 CD34$^+$ HSPC at day 0 to $>1 \times 10^6$

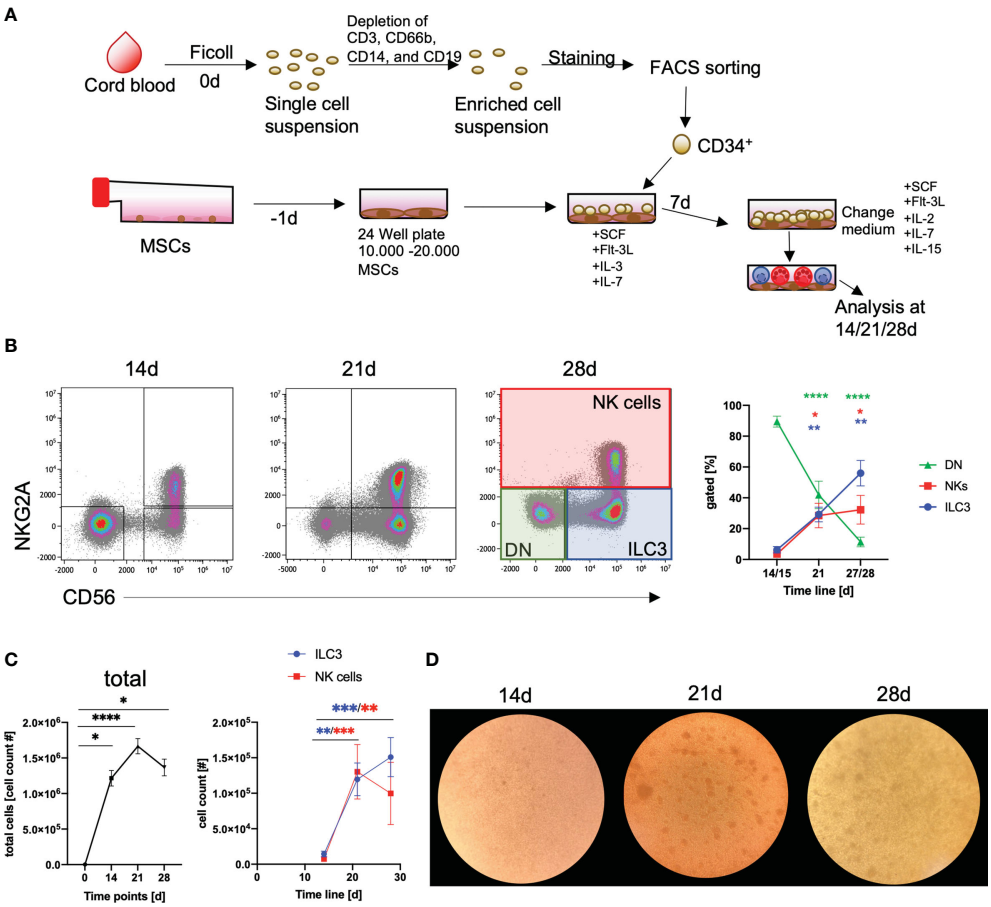

FIGURE 1 | Efficient generation of ILC3 using an MSC-based differentiation platform. $CD34^+$ HSPCs were isolated from fresh cord blood (CB) by cell sorting following depletion of unwanted cell populations (anti-CD3 for T cells, anti-CD66b for granulocytes, anti-CD14 for monocytes, and anti-CD19 for B cells). $CD34^+$ HSPCs (2000/well) were seeded in 24-well plates with MSC, which were plated one day in advance **(A)**. Representative dot plots are shown for NKG2A and CD56 expression and frequency dynamics are shown for DN cells ($NKG2A^-CD56^-$, green box and line), ILC3 ($NKG2A^-CD56^+$, blue box and line), and NK cells ($NKG2A^+CD56^+$, red box and line), for day 0, 14, and 21 ($n = 9$), and day 28 ($n = 7$) (general gating strategy is shown in **Supplementary Figure 1**) **(B)**. Total cell counts (graph, left-hand side) as well as ILC3 (blue line) and NK cell (red line, both in graph, right-hand side) counts are shown for day 0, 14, and 21 ($n = 9$), and day 28 ($n = 7$) **(C)**. Representative microscopic pictures of cultures at day 14, 21, and 28 **(D)**. Error bars represent the mean ± SEM. The data are based on four different experiments with seven to nine individual CB donors and three different MSC lines. Levels of significance were calculated with a nonparametric ANOVA (Kruskal–Wallis with a Dunn`s post-test comparing day 14 to either day 21 or 28) **(B, C)**, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

cells/well until day 21 ($p < 0.0001$). As expected, this was due to the expansion of ILC3 as well as NK cells (**Figure 1C**), while DN cells rather seem to be precursors due to their significant decline from day 14 to 28 and strong CD117 expression (data not shown). In the fourth week of culture, the ILC3 population further increased, whereas NK cell counts slightly dropped. Morphologically, clustering of typical comma-shaped cells, indicating the presence of NK cells and/or ILC3, was microscopically clearly apparent from day 21 of culture (**Figure 1D**). Of note, we used three different MSC lines to validate the stability of our differentiation platform and could not detect systematic differences in the generation of ILC3 between MSC lines, similar to previous observations looking at NK cell differentiation (34). All in all, the MSC-based differentiation platform proved to be a robust system for the *in vitro* generation of human ILC3.

In Vitro Generated ILC3 and NK Cells Can Be Distinguished by Phenotype and Transcriptome

Overall, ILC3 and NK cells follow quite similar kinetics during *in vitro* differentiation with first occurrence of both cell types around day 14 and strong increase until day 21. Furthermore, it is so far not possible to create culture conditions to generate one subset without the other. Notably, both ILC3s and NK cells expressed NKp44 and CD117 on the cell surface (**Figure 2A**); however, *in vitro* ILC3s showed significantly higher mean fluorescence intensity (MFI) values for NKp44 as well as CD117 (**Figure 2B**). To determine if our *in vitro* generated ILC3 and NK cells share transcriptional similarity to their *ex vivo* counterpart, we performed RNAseq analysis of bulk sorted ILC3 (sorted on $NKG2A^-CD56^+$) and NK cells (sorted on $NKG2A^+CD56^+$) to screen for distinguishing

features. As outlined in **Figure 2C**, the transcriptomes of NK cells could be clearly distinguished from ILC3 based on the expression of typical NK cell receptors such as *KLRC1* (NKG2A), *KLRD1* (CD94), several killer cell immunoglobin-like receptors (*KIR2DL1, KIR3DL1, KIR3DL2*, and *KIR3DL2*), and *KLRF1* (NKp80) but also genes encoding granzymes (*GZMM, GZMK*, and *GZMB*) and perforin (*PRF1*) and essential TFs such as *TBX21* (TBET) and *EOMES* (**Figure 2C**). In contrast, *in vitro* ILC3 expressed hallmark genes of tissue-resident ILC3 such as *NCR2* (NKp44), *IL7R* encoding the ILC inclusion "marker" CD127, *CD2*, *KIT* (CD117), *IL1R*, and *IL23R* needed for cytokine stimulation of ILC3, as well as the ILC3 signature TFs *RORC* and *AHR* (**Figure 2C**). The distinct transcriptional signatures of *in vitro* generated ILC3 and NK cells are further clearly illustrated by a heatmap showing the top 100 differentially expressed genes. Notably, these data also illustrate how homogeneous the transcriptomic signatures of the *in vitro* generated ILC3s and NK cell populations are across different donors (**Figure 2D**).

We next looked more closely at previously defined signature TFs of ILC3 (RorγT) and NK cells (EOMES) at the protein levels. With regard to RorγT and EOMES, expression was validated by intranuclear staining: unexpectedly, we detected three different populations based on their RorγT expression: high (RorγT^{high}), intermediate (RorγT^{int}), and no (RorγT^{neg}) expression (**Figure 2E** and **Supplementary Figure 2A**). NK cells were mainly RorγT^{int} with only a small fraction of RorγT^{neg} NK cells of approximately 10% (**Figure 2F**). Importantly, EOMES expression was almost completely restricted to the RorγT^{int} population, indicating that the CD56^{+}NKG2A^{+} RorγT^{int}EOMES^{+} population represents *bona fide* NK cells showing that NK cells also express RorγT but with lower expression levels compared to ILC3. In contrast, as expected, the RorγT^{high} population was lacking EOMES expression leading to a provisional phenotypic definition of *in vitro* ILC3 as CD56^{+}NKG2A^{-}RorγT^{high}EOMES^{-}. By gating on RorγT^{high}EOMES^{-} and RorγT^{int}EOMES^{+} gates, respectively, ILC3 and NK cells could be each defined with high confidence (**Supplementary Figure 2**). Notably, a small ILC3 population of unknown significance was co-expressing RorγT^{int} and EOMES (**Figures 2E, F**).

The Transcriptome of *In Vitro* Generated ILC3 Is Closely Related to Tonsillar ILC3

A key question that we wanted to address in this study pertains to the similarity between the *in vitro* generated ILC3 and *ex vivo* tissue-resident ILC3 in terms of phenotype, transcription, and function. As a first step, we compared *in vitro* generated ILC3 with tonsillar ILC3, representing a well-defined archetypical ILC3 effector cell subset, by bulk RNA-seq analyses. To be most unbiased, we performed a two-dimensional principal component analyses (PCA) based on the top 500 most differentially expressed genes between the *ex vivo* subsets of tonsillar ILC3 and CB ILC3-like cells [published dataset (29)] compared to the new data of the *in vitro* generated ILC3 as well as the DN (NKG2A^{-}CD56^{-}) population. We have recently characterized CB ILC3-like cells in depth and observed that although this subset seems to be phenotypically similar to tissue-resident ILC3, they largely lack typical ILC3 effector function and have a different transcriptomic signature (29). As shown in **Figure 3A**, *in vitro* generated ILC3 closely clustered to tonsillar ILC3, whereas CB ILC3-like cells were located far apart within the first principal component (PC1) representing 68% of the variance. *In vitro* generated DN cells were similar in the PC1 but clearly separated in PC2, representing 16% of variance. We next performed unsupervised clustering of the top 200 most differentially expressed genes between CB ILC3-like cells and tonsillar ILC3 (**Figure 3B**). The resulting heatmap again demonstrated a close similarity between tonsillar and *in vitro*-generated ILC3, clustering apart from CB ILC3-like cells as expected but also showing several divergent gene clusters in comparison to the DN population. Finally, to further test the transcriptional similarity and determine shared gene signatures of *in vitro* ILC3 and tonsillar ILC3, a four-way plot with CB ILC3-like cells as reference sample was calculated. The four-way plot identifies a large number of genes commonly expressed in tonsillar ILC3 that are also expressed in *in vitro* generated ILC3 (upper right quadrant), further validating that *in vitro* generated ILC3 are very similar to *ex vivo* tonsillar ILC3s (**Figure 3C**). Among the shared genes are many, which have been described to be hallmarks for tissue-resident ILC3, such as *IL23R, IL22*, and *NCAM1* encoding CD56, *NCR2* encoding NKp44, and *AHRR* encoding AHR repressor, as well as many other shared genes. Nonetheless, some unique genes distinguishing *in vitro* generated ILC3 and tonsillar ILC3 were identified such as *CCR8* and *NKG7* for the *in vitro* generated ILC3s (upper left quadrant) and *CD200*, *KLF4*, and *EPCAM* for tonsillar ILC3 (lower right quadrant). Finally, the four-way plot also identified a unique set of genes known to be expressed in CB ILC3-like cells, such as the TF *ID3* (29).

We next looked at the expression levels of selected ILC3-specific genes (**Figures 3D–F**). Tonsillar and *in vitro* generated ILC3 had comparable read counts for *IL1R1* and *IL23R* encoding the receptors stimulated by IL-1β and IL-23, constituting the presumably most important stimuli for ILC3 function (3) (**Figure 3D**). Whereas expression of IL23R could not be validated on the cell surface due to lack of suitable staining reagents, *in vitro* ILC3 revealed strong expression of the IL1R1 expression (**Supplementary Figure 3**). Both receptors were either low (IL1R1) or negative (IL23R) in CB ILC3-like and also completely negative in DN cells. Recently, a novel ectoenzyme-expressing subset of ILC3 (ecto^{+}ILC3) has been identified within the oral-GI tract and bone marrow (BM) that is reactive to extracellular adenosine triphosphate (eATP), which upon activation have shown immunosuppressive capacities against autologous T cells (8). Given its potential relevance for ILC3-based therapy of GvHD, we checked the respective ectoenzymes *NT5E* encoding CD73 and *ENTPD1* encoding CD39. Indeed, *NT5E* was strongly expressed in tonsillar as well as *in vitro* ILC3, whereas it was very low in CB ILC3-like and *in vitro* DN cells. Flow cytometrically analyses revealed that most *in vitro* ILC3 expressed CD39, but only a fraction expressed CD73 (**Supplementary Figure 3**). Interestingly, the ectoenzyme *ENTPD1*, which was shown to protect from intestinal injury in colitis (36), was found to be strongly expressed

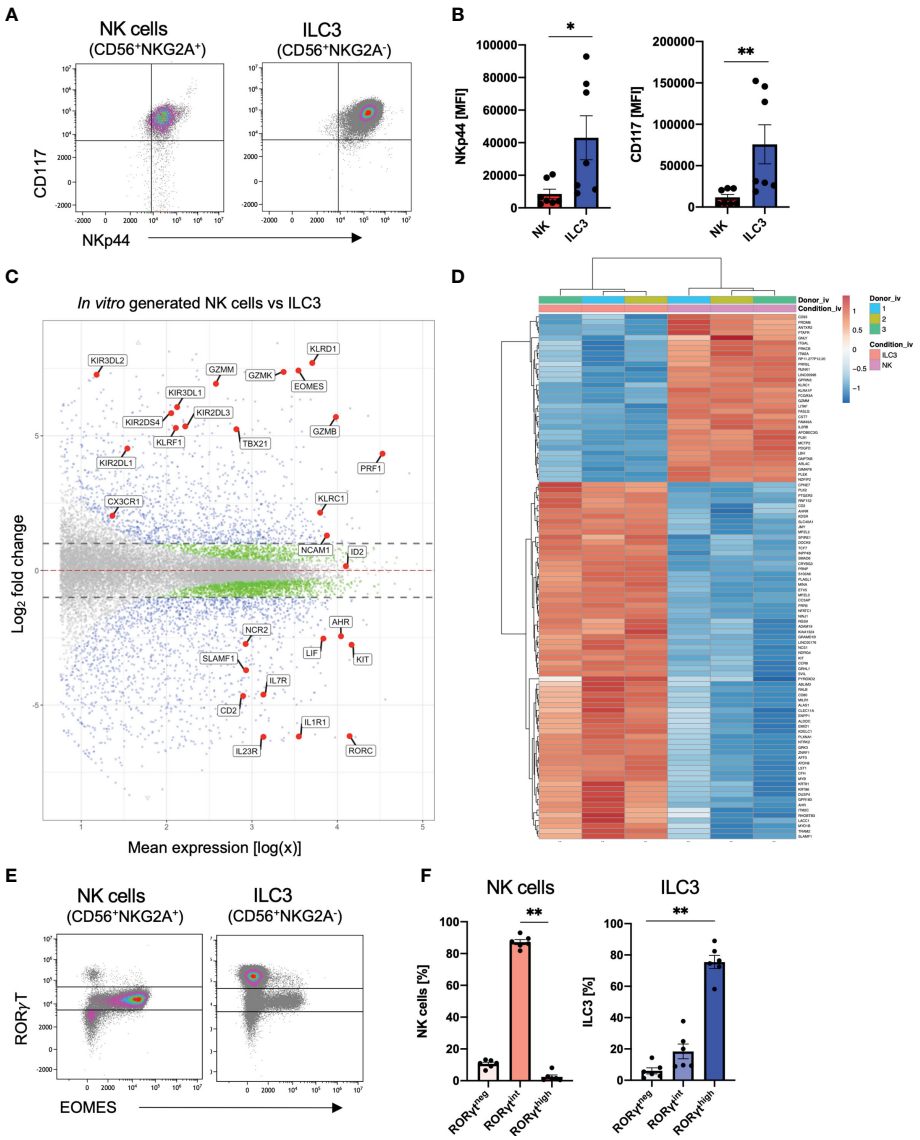

FIGURE 2 | Distinct transcriptional profiles of *in vitro* generated ILC3 and NK cells. Freshly sorted CD34$^+$ HSCs were seeded on MSC. On day 28, cultured cells were flow cytometrically analyzed **(A, B, E, F)** and NKG2A$^+$CD56$^+$ NK cells as well as NKG2A$^-$CD56$^+$ ILC3 were sorted for bulk RNA-seq analyses (n = 3 each). RNA sequencing was done on the Illumina platform **(C, D)**. Representative dot plots for CD117 and NKp44 expression of ILC3 and NK cells on day 28 **(A)**. Quantification of NKp44 (left-hand side) and CD117 (right-hand side) MFI for *in vitro* ILC3 and NK cells, n = 7 **(B)**. MA plot showing all differentially expressed genes between *in vitro* ILC3 and NK cells with dotted lines representing a $\log_2$ fold change cutoff of 1. Blue dots represent a p-value <.05 and a $\log_2$ fold change of >1. Green dots represent a p-value of <.05 and a $\log_2$ fold change of <1. Gray dots represent a p-value > 0.05 and a $\log_2$ fold change of <1. Selected blue genes are highlighted **(C)**. Heatmap showing the top 100 most differentially expressed genes between *in vitro* ILC3 and NK cells **(D)**. Representative dot plots of intranuclear EOMES and RORγT expression of NK cells and ILC3 **(E)**. Frequency distribution of NK cells and ILC3 within the RORγT^{high}, RORγT^{int}, and RORγT^{neg} fraction **(F)**. The data are representative of at least three different experiments **(A, B, E, F)**, and each dot is representative of an individual donor **(B, F)**. The height of the bar graphs represents the mean ± SEM. Level of significance was calculated with an unpaired nonparametric two-tailed t-test (Mann–Whitney test) **(B)** and a nonparametric ANOVA (Kruskal–Wallis with a Friedmann post-test) **(F)**, *$p < 0.05$, **$p < 0.01$.

in *in vitro* ILC3 whereas it was weak in tonsillar ILC3, CB ILC3-like, and DN cells (**Figure 3E**).

In vitro ILC3 also exhibited high expression levels of ILC3-specific transcription factors: we observed a 4.8-fold higher expression for RORC compared to tonsillar ILC3 (**Figure 3F**) and, as previously reported, hardly any expression for CB ILC3-like cells (29). The aryl-hydrocarbon receptor *AHR* exhibited comparable expression within *in vitro* and tonsillar ILC3 with only slightly higher read counts compared to CB ILC3-like cells and *in vitro* DN cells. Finally, the inhibitor of DNA binding factors *ID2* and *ID3* have been described to play major roles within innate lymphoid lineage fate decisions (37, 38), and in particular, ID2 has been described to be vital for ILC development in general (39). We observed highest *ID2* expression in *in vitro* ILC3 with distinctly lower levels in tonsillar ILC3 and only

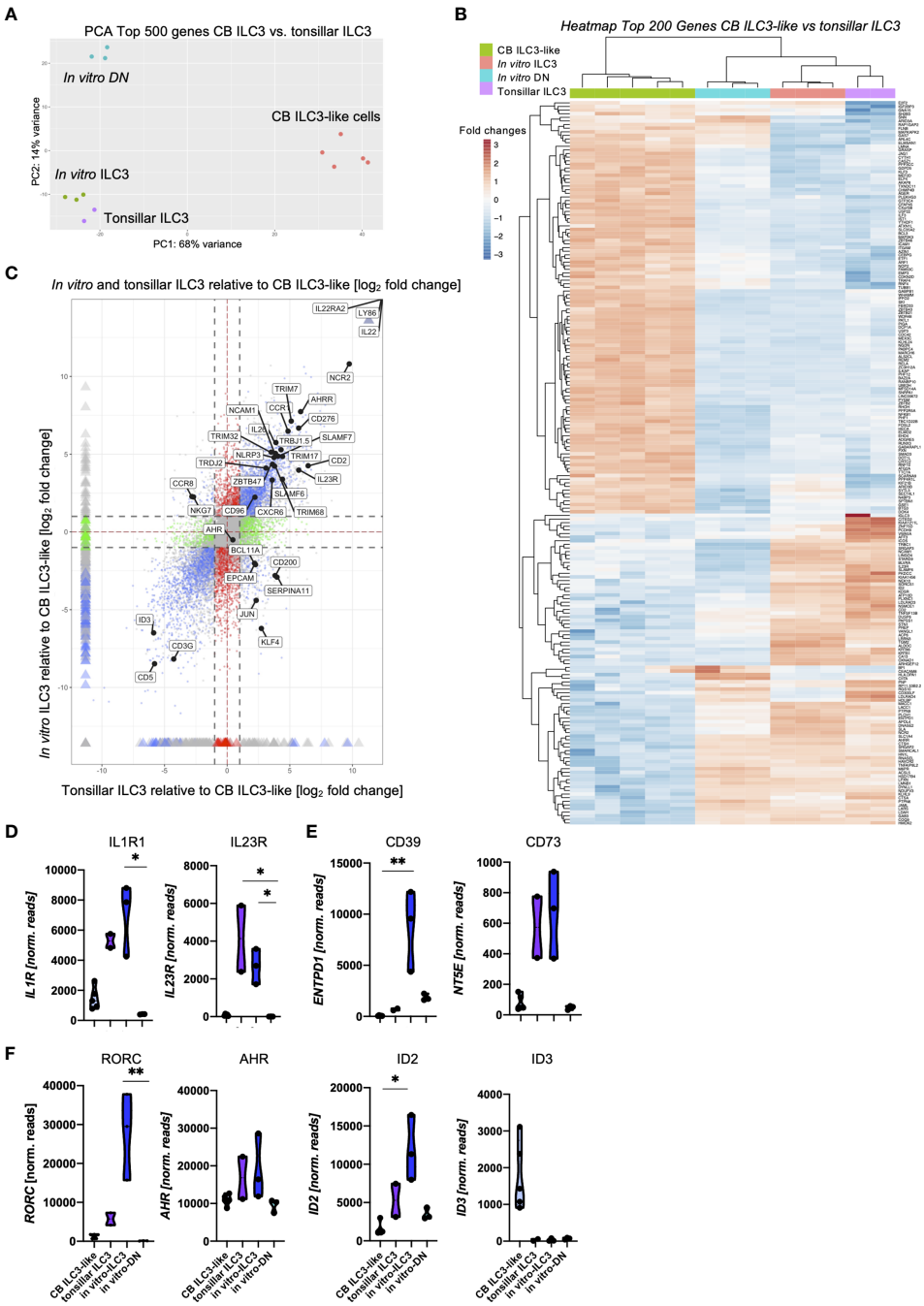

FIGURE 3 | Shared transcriptomic signatures of *in vitro* generated ILC3 and tonsillar ILC3. Freshly sorted CD34$^+$ HSCs were seeded on MSC and NKG2A$^-$CD56$^-$ DN cells and NKG2A$^-$CD56$^+$ ILC3 were sorted at day 14 and 28, respectively, for bulk RNA-seq analyses (n = 3 each). CB ILC3-like cells and tonsillar ILC3 were taken from a published dataset (29) (n = 5 for CB ILC3-like cells, n = 2 for tonsillar ILC3). RNA sequencing was done on the Illumina platform. A two-dimensional principal component analysis based on the top 500 differentially expressed genes of CB ILC3-like cells versus tonsillar ILC3 including the data of *in vitro* generated ILC3 and DN cells is shown **(A)**. A heatmap showing the top 200 differentially expressed genes between CB ILC3-like cells and tonsillar ILC3 with inclusion of *in vitro* ILC3 and DN cells **(B)**. A four-way plot with a cutoff at a $\log_2$ fold change ±1 (dotted lines) and adjusted p-values of.05 showing differently expressed genes of CB ILC3-like cells ("control") compared to tonsillar ILC3 ("x") and *in vitro* ILC3 ("y"). Blue dots represent genes with an adjusted p-value <.05 with a fold change >1. Green dots represent genes with an adjusted p-value <.05 with a fold change between >1 (x-axis) and <1 (y-axis). Gray dots represent genes with an adjusted p-value > 0.05. Red dots represent genes with an adjusted p-value <.05 with fold rates <1 (x-axis) and >1 (y-axis). Selected differentially expressed genes including ILC3 hallmark genes are highlighted **(C)**. Violin plots of normalized RNAseq read counts of known ILC3 functional receptors *IL1R* and *IL23R* **(D)**, ectoenzyme expression *ENTPD1* and *NT5E* **(E)**, and transcription factors *RORC*, *AHR*, *ID2*, and *ID3* **(F)** for CB ILC3-like cells (light blue), tonsillar ILC3 (purple), *in vitro* ILC3 (royal blue), and DN cells (aqua). Level of significance was calculated with a nonparametric ANOVA (Kruskal–Wallis with a Dunn`s post-test) **(B, C)**, *p < 0.05, **p < 0.01.

marginal expression within CB ILC3-like cells and DN cells. In line with our previous observations (29), ID3 was strongly expressed in CB ILC3-like cells, whereas it was not expressed in other ILC populations (**Figure 3F**). Altogether, the transcriptomic signature of *in vitro* ILC3 was shown to be closely related to *ex vivo* tonsillar ILC3.

In Vitro Generated ILC3 Provide a Superior Profile of Effector Functions Including High IL-22 Production and Lack of IL-17A

We next examined cytokine-based effector functions of *in vitro* ILC3. In this regard, the efficient production of IL-22 is highly relevant given its described beneficial function for tissue regeneration and repair within the GI tract (7, 15, 40). To this end, we flow cytometrically sorted *in vitro* generated NKp44$^+$ILC3 and stimulated the cells with medium alone, IL1β/IL-23 (3), or IL1β/IL-23/IL-2 (10) (**Figure 4A**). Surprisingly, the classical ILC3 stimulation protocol using IL1β/IL-23 could be significantly improved by addition of IL-2, leading to substantially increased secretion of IL-22 (**Figure 4B**). ILC3 have also been described to produce IL-17A, which acts inflammatory when produced in larger quantities (41). However, hardly any IL-17A protein secretion was found with any stimulus we used (all means < 5 pg/ml) (**Figure 4C**), which is in line with the hypothesis that NKp44$^+$ILC3 secrete IL-22 whereas NKp44$^-$ILC3 secrete IL-17A (4). Furthermore, in line with published data of *ex vivo* tonsillar ILC3s (10), we detected significantly higher amounts of GM-CSF and LIF when adding IL-2 to the stimulation (**Figure 4D**) (10). Of note, stimulation did not change the overall ILC3 phenotype of CD56$^+$NKG2A$^-$ CD117$^+$ (data not shown). Thus, *in vitro* ILC3 are high producers of IL-22 without accompanying secretion of IL-17A.

We next wanted to compare the efficiency of MSC-based differentiation of *in vitro* ILC3 to previously established protocols (30–32, 42). We first compared our MSC-based protocol to a cytokine-only milieu using the identical media conditions. We observed a similar trend of cell proliferation during both culture conditions with an increase in cell numbers until day 21 with overall stronger proliferation in MSC-based conditions. However, a sharp decline accompanied by massive cell death (data not shown) was observed during the fourth week of culture in the cytokine-only conditions compared to a slight decline using the MSC-based approach (**Figure 4E**). Thus, while *in vitro* ILC3 constantly increased in cell number up to day 28, as already shown in **Figure 1**, a sharp decline was observed for ILC3 in cytokine-only conditions (**Figure 4F**). Hence, compared to the MSC-based approach, the cytokine-only conditions do not appear to be suitable for efficient expansion of ILC3.

Finally, the MSC-based approach was compared to a protocol, using the murine embryonic stroma cell line EL08, which was established by the Verneris group and is widely regarded as the gold standard for generation of ILC3 (30, 32). Indeed, when comparing both methods, we observed significantly higher frequencies of ILC3s on murine EL08 cells at day 14, 21, and 28 of culture (**Figure 4G**). However, when we compared the expression of RorγT, ILC3 generated on murine EL08 mainly exhibited a RorγT^{int} phenotype whereas ILC3 on human MSC largely exhibited a RorγT^{high} phenotype. Although we had shown above that the RorγT^{int} phenotype is largely restricted to NK cells in MSC-based cultures, this was not the case for EL08-supported cells, since they lacked expression of EOMES (**Figure 4H**). Finally, MSC-based generation of ILC3 led to significantly higher IL-22 secretion than ILC3 generated on EL08 (**Figure 4I**). Altogether, MSC-supported differentiation of CB-derived HSPC enables robust and efficient generation of human RorγT^{high}ILC3 secreting high amounts of IL-22 in fully human culture conditions.

DISCUSSION

In this study, we present a detailed protocol for the efficient and GMP-compatible generation of human effector ILC3 from CB-derived hematopoietic progenitors. The utilization of human clinical-grade MSC for the support of hematopoietic differentiation into the ILC3 lineage enables, for the first time, an efficient xenobiotic-free way to generate IL-22-producing ILC3. The protocol is robust and works efficiently with different donors and MSC lines. The *in vitro* generated ILC3s were functionally, phenotypically, and transcriptionally comparable to tissue-resident ILC3s by expressing high levels of NKp44, CD117, RorγT, as well as secreting high amounts of IL-22, but not IL-17A. Transcriptomic analyses demonstrated a close similarity between tonsillar and *in vitro* generated ILC3, which was not necessarily expected, given the fact that this involved comparison of an *ex vivo* with an *in vitro*-generated cell subset. The close relationship between *in vitro* and tonsillar ILC3 was especially evident with respect to genes responsible for ILC3 effector functions, setting both cell types apart from circulating ILC3-like cells that are basically unable to produce IL-22 (24, 29). Of note, when relaxing the analysis parameters of the RNAseq data by accounting for all genes, the effect of cell culturing becomes dominant and *in vitro* ILC3 become more closely related to *in vitro* DN cells taken from the same culture dish compared to the tonsillar ILC3 (data not shown). Furthermore, the study shows that the generation of human ILC3s on MSCs leads to favorable results in terms of IL-22 production compared to previous protocols employing either murine stroma cells (30, 32, 33, 43) or feeder-free conditions (31, 44–46). Notably, bone-marrow-derived MSCs were previously described to promote proliferation and enhanced IL-22 secretion of *ex vivo* isolated tonsillar ILC3 (47), further suggesting that MSC besides providing an efficient stem cell niche, as shown here, are also capable of supporting their effector functions.

Importantly, ILC3 generated on MSC produced high amounts of IL-22 with no co-secretion of IL-17A. This is an essential observation, as IL-17A was previously shown to exacerbate inflammation in IBD, whereas IL-22-secreting ILC3s have been described to be beneficial for IBD patients, leukemic patients after HSCT, and GI tract transplant recipients (15, 21, 25, 27, 48–50). Several issues still have to be addressed concerning the role of ILC3 in inflammatory settings: an increase in IL-17A-producing ILC3s was reported in IBD, correlating

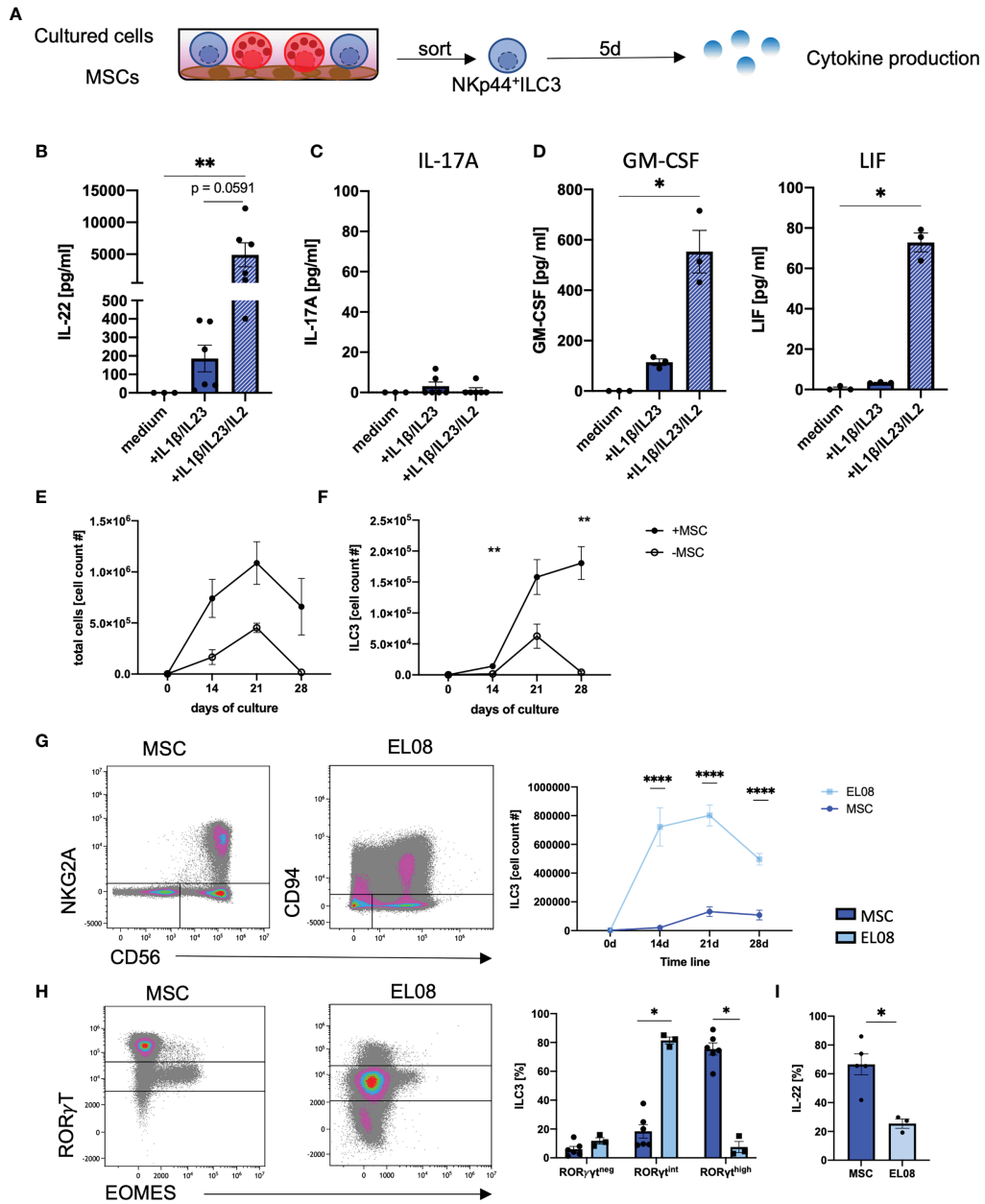

FIGURE 4 | *In vitro* generated ILC3 produce high amounts of IL-22, but not IL17a. At day 28 on MSCs, NKp44$^+$ILC3s were freshly sorted and stimulated either with medium alone or with IL1β/IL-23 (50 ng/ml each) with or without IL-2 (1000 U/ml). Supernatant was taken at day 5 **(A)**. IL-22 **(B)**, IL-17A **(C)**, GM-CSF, and LIF **(D)** levels in the supernatant were measured after a 5-day stimulation with medium alone, IL1β/IL-23 (blue bar), and IL1β/IL-23/IL-2 (blue-white striped bar). CD34$^+$ HSPCs were sorted and 2,000 cells were seeded in parallel in wells with MSC (+MSC) or without MSC (-MSC). Line graphs showing total cell counts **(E)** and ILC3 (NKG2A$^-$CD56$^+$) cell counts **(F)** in cultures with MSC (+MSC, filled dots, n = 5–7) and without MSC (-MSC, open dots, n = 3) from day 0 to day 28. CD34$^+$ HSPCs were cultured on EL08. Representative dot plots of NKG2A and CD56 expression (left panels) and quantification of ILC3 (right panel) cultured on EL08 (light blue, n = 3) or on MSC (dark, blue, n = 7–9) **(G)**. Representative dot plots showing RORγT and EOMES expression (left panels) and quantification of RORγT^{neg}, RORγT^{int}, and RORγT^{high} frequency distribution (right panels, bar graphs) for cells cultured on EL08 (light blue, n = 3) or on MSC (dark blue, n = 7) **(H)**. Cultures derived on MSC or EL08 were re-stimulated with IL1β/IL-23 and stained for intracellular IL-22 secretion. Bar graphs showing IL-22 secretion for MSC-derived ILC3 (dark blue, n = 5) and EL08-derived ILC3 (light blue, n =3). The data are representative of one (EL08 and culture -MSC) and two different experiments (+MSCs) with each dot representative of an individual donor. The height of the bar graphs represents the mean ± SEM. Levels of significance were calculated with a nonparametric ANOVA (Kruskal–Wallis with a Dunn`s post-test) **(B–D)** and Mann–Whitney tests **(E–I)**, $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

with mucosal leakage in ulcerative colitis patients (27, 51). These observations could be due to a switch in effector programs from IL-22- to IL-17-producing ILC3, which would be undesirable for immunotherapy due to IL-17-mediated aggravation of inflammation. We did not experience a switch or a *de novo* expression of IL-17A during *in vitro* generation of ILC3 and also not following prolonged culture and challenge with different stimuli. Nonetheless, the point of a potential plasticity of the *in vitro* generated ILC3 needs to be addressed in the future, employing suitable *in vivo* and *in vitro* models.

The interest in developing protocols for ILC3-based cellular therapy was recently fueled by observations made in clinical GI transplantation. It has been observed that human ILCs quickly infiltrate the transplanted GI tract with >70% of ILCs being recipient-derived (20). This is in line with a recent murine study showing that IL-22-secreting mouse ILC3s were able to infiltrate into transplanted lungs promoting the formation of peripheral nodal addressin-expressing high endothelial venules (52). Importantly, the frequency of human NKp44$^+$ILC3s correlated with successful engraftment of the intestinal transplants. Due to the high correlation and prediction success, human NKp44$^+$ILC3s were suggested to be a biomarker for transplant rejections (21). Both studies combined highlight that human NKp44$^+$ILC3s are beneficial for GI transplantation success, but also that NKp44$^+$ILC3s are able to actively migrate into tissues. These studies provide a paradigm change to the previous notion that ILC3s are largely tissue resident and renew rather locally than by replenishment from external progenitors or migration of mature ILCs into the tissues (53). Hence, although this has to be proven in future studies, it is reasonable to believe that intravenously given NKp44$^+$ILC3s would find their way into GI tissues. Thus, *in vitro* generated IL-22-producing NKp44$^+$ILC3s could exert beneficial effects in the settings of GvHD following allogeneic HSCT and could also provide a promising new approach for treatment of intestinal inflammatory processes in the colon and small intestine of Colitis Ulcerosa and Morbus Crohn patients, respectively. Notably, there is so far no curative treatment available in either setting. The present approach for the efficient generation of clinically applicable ILC3 could facilitate first steps into this translational direction.

It has to be acknowledged that our *in vitro* cultures besides ILC3 predominantly contained NK cells. This is observed in all *in vitro* differentiation approaches reported so far and is probably due to the predominance of common progenitors for NK cells as well as ILC3 within the CD34$^+$ HSPC fraction. On the other hand, the cultures were completely free of critical T-cell contaminations (data not shown) and none of the non-ILC3 cellular components within the culture is expected to constitute a safety issue for clinical application. It has to be stressed that previous experience with NK cell-based therapies did not point towards any GvHD-promoting effect of allogeneic NK cells (54). In fact, contaminating NK cells might rather have immunosuppressive effects due to regulation of inflammatory CD8$^+$ T cells (55). Similarly, MSCs are endowed with immunosuppressive features (56) and any remaining MSC contamination in the culture would not constitute any hazard for the clinical product in contrast to murine feeder cells that would have to be completely removed before clinical use. In any case, it would be feasible to generate highly pure ILC3 products for example by applying a two-step protocol for GMP-compatible enrichment (i.e., magnetic beads) involving depletion of NKG2A$^+$ NK cells and subsequent enrichment of NKp44$^+$ILC3.

Classically, murine stroma cells are employed to support the differentiation of human HSC into mature immune cell types such as NK cells, T cells, and more recently ILCs (30, 32, 33, 57–63). This is based on the premise that the major interactions between hematopoietic stem cells and niche cells are conserved between mouse and humans. However, many important players, such as cytokine receptors, adhesion molecules, and chemokine receptors, are not compatible with the respective murine ligands and might in part explain the superior function of human MSC in this context. It would certainly merit further investigation to define how far these species-specific differences are modulating differentiation programs. Regarding conserved stroma-defined signals, we and others have shown that ectopic expression of human NOTCH ligands such as DLL1 and DLL4 into murine OP9 stroma cells enhanced the efficiency of differentiation into the NK cell lineage (34, 59, 63) and, as recently shown, similarly into the ILC lineage (33). Notably, DLL proteins do not seem to play a major role for MSC-based differentiation of ILC3, since the MSC lines consistently showed either no (DLL4) or only very low (DLL1 and DLL2) expression in RNAseq analyses (data not shown). In fact, Jagged 1 represented the only highly expressed NOTCH ligand in MSC, but its role in this system needs to be explored in the future. In general, it is well established that MSCs constitute one of the key components of the bone marrow stem cell niche (64), and its usage in the present protocol will facilitate future studies of ILC3 development without species-specific receptor/ligand incompatibilities found in xenogeneic settings, mirroring more closely *in vivo* development.

Another advantage of the present approach for generation of human effector ILC3 is the lack of regulatory hurdles attached to MSC in contrast to the use of murine feeder cells. In the latter case, master cell banks have to be established and stringent proof must be provided excluding the risk for transmission of zoonotic pathogens, which precludes clinical phase III studies in many countries. In contrast, human MSCs are used within clinical trials in various disease settings [reviewed here (65)]. The MSC lines used within this study were established from BM of children under GMP conditions and have been subjected to GMP quality controls as described previously (34). These MSC lines are routinely used in the clinic for treatment of GvHD, and thus fully meet the regulatory criteria for the use of clinical products (66). Even though the success of MSC-based therapies is controversial (56), the fact that MSCs are already used in clinical trials shows that it is ethically and regulatory unproblematic to be used within humans. In practical terms, human MSC lines have a high renewal capacity and can be expanded to large quantities if needed. In our experience, the *in vitro* generation of ILC3 as well as NK cells (34) did not

require the usage of particularly early MSC passages but worked equally well with more advanced MSC lines, which facilitated the acquisition of consistent results over time. Adding to this, the present experiments were conducted using three different donor-derived MSC lines, and in fact, all three MSC lines supported a robust generation of human NKp44$^+$ILC3s enabling narrow error margins. Altogether, our protocol enables the robust and GMP-compatible generation of fully functional human IL-22-producing ILC3, hopefully paving the way for their future therapeutic application.

MATERIALS AND METHODS

Human Samples and Ethics Statement

Umbilical CB used within this study was collected by the José Carreras Stem Cell Bank at the ITZ. The protocol was accepted by the institutional review board at the University of Düsseldorf (study number 2019-383) and is in accordance to the Declaration of Helsinki. CBs were processed directly without previous thawing.

Cell Culture

GMP-grade MSCs were obtained as previously described (34). For the experiments, three different MSC lines were used. MSCs were cultured in Dulbecco's high-grade glucose medium (Gibco) with 10% platelet lysate and 1% penicillin/streptomycin (Stock: 10,000 U/ml penicillin; 10,000 µg/ml streptomycin, Gibco). MSCs (10,000–20,000) were plated 1–2 days before the CD34$^+$ isolation in the inner eight wells of a 24-well plate (Gibco Tissue-treated cell culture plates). The murine embryonic liver stroma cell line EL08-1D2 (= EL08) was cultured in Iscove modified Dulbecco medium (Lonza) with 15% fetal calf serum, 5% horse serum, 1% penicillin/streptomycin, and 400 mM 2-mercaptoethanol at 33°C (67, 68). For generation of ILC3, 2,000 freshly sorted CD34$^+$ hematopoietic stem cells (day 0) were seeded on the MSCs, EL08, or without feeder cells in "NK1" medium for 7 days. On day 7, half the NK1 medium was removed and a new "NK2" medium was added. "NK1" and "NK2" medium had been previously described by us (35) except that the "NK1" medium did not contain IL-2 in the present protocol. Half of the medium was changed twice a week. Flow cytometric analyses were done at days 14/15, 21, and 27/28 of culture. The cells are harvested from the wells by careful pipetting and ensuring by visible inspection that all cells have been detached from the well.

Isolation of MNCs From Cord Blood and Sorting of CD34$^+$ Cells

CB was diluted 1:1 with sterile 1×PBS (Gibco by Life Technologies, California, USA) and MNCs were isolated *via* a density gradient centrifugation (Biocoll, 1.077 g/cm^3, Biochrom Merck). For erythrocyte lysis, cells were resuspended in 5 ml of ice-cold ammonium chloride solution (pH = 7.4, University Clinic Düsseldorf) with three consecutive washing steps. MNCs were counted and further prepared for sorting. Monocytes, B cells, and T cells were depleted *via* MojoSort Streptavidin Nanobeads (BioLegend) using the supplier's negative selection protocol as previously described (63). The cells were stained with a lineage panel as previously described (69), as well as CD94, CD127, CD117, and CD34 for sorting of CD34$^+$ HPCs. Cell sorting was performed on a MoFlo XDP (Beckman Coulter).

Flow Cytometric Analyses

Cells were extracellularly stained with the following FITC-conjugated lineage antibodies for sorting as previously described (69): anti-CD3 (UCHT1), anti-CD1a (HI149), anti-CD14 (HCD14), anti-CD19 (HIB19), anti-TCRαβ (IP26), anti-TCRγδ (B1), anti-CD123 (6H6), anti-CD303/BDCA-2 (201A), anti-FceR1a [AER-37(CRA-1)], anti-CD235a (HI264), and anti-CD66b (G10F5), all from BioLegend. These antibodies were further used within the study: anti-CD3-APC/Cy7 (UCHT1), anti-CD14-APC/Cy7 (HCD14), anti-CD19-APC/Cy7 (HIB19), anti-CD56-PE/Dazzle™ 594 or BV650™ (HCD56), anti-CD34-BV510™/-PE or -FITC (581), anti-NKp44-APC (P44-8), anti-CD73-PE/Dazzle™ 594 (AD2), anti-CD39-APC (A1), anti-Thy1-Alexa Flour® 700 (5E10), and anti-CD117-BV421 and -PE/Cy7 (104D2), all from BioLegend. CD127-PE/Cy5 (R34.34) and NKG2A-PE/Cy7 (Z199) were from Beckman Coulter (California, USA). Anti-IL1R1-PE (FAB269P) was purchased from R&D (Minneapolis, Minnesota). Intranuclear staining of anti-Eomes-PE-eFlour® 610 (WD1928, Invitrogen) and anti- RorγT-PE (clone: AFKJS-9, eBioscience) was performed with the Foxp3 staining kit (Thermo Fischer Scientific) and protocol. The initial gating strategy is shown in **Supplementary Figure 1**.

Functional Analyses

For functional analyses, cells were stimulated with human (h) IL-1β (50 ng/ml) and IL-23 (50 ng/ml) with or without IL-2 (1,000 U/ml) overnight with subsequent addition of Brefeldin A solution (1,000×, BioLegend, 1,000-fold dilution) after 1 h of stimulation. The cells were stained extracellularly for detection of ILC3 and NK cells as well as intracellularly for analysis of IL-22 (2G12A41, BioLegend) production using the intracellular staining kit (BioLegend). *In vitro* generated ILC3 were flow cytometrically sorted (see RNA sequencing and analyses for gating) and 50,000 cells were subsequently stimulated for 5 days. Supernatant was collected at day 5. LEGENDplex Human Th Panel and the LEGENDplex Human Hematopoietic Stem Cell Panel from LEGENDplex (Cat: 740722 and 740611, BioLegend, San Diego, California) were used and experiments were done according to the manufacturer's instructions.

RNA Sequencing and Analyses

On day 28, cells were taken from cultures, stained, sorted for ILC3 (Lin$^-$CD34$^-$Thy1$^-$NKG2A$^-$CD56$^+$NKp44$^+$) and NK cells (Lin$^-$CD34$^-$Thy1$^-$NKG2A$^+$CD56$^+$), and stored in TRIzol™ Reagent (Invitrogen) for extraction of total RNA. Reverse transcription and library production were carried out with an Illumina Truseq RNA preparation kit as described in the company's protocol. Sequencing of the libraries was performed with an Illumina HiSeq4000 (single-read 1×50bp). Sequence reads were mapped

to the human genome (hg38) with STAR (version STAR_2.5Oa), and read counts of gene transcripts were determined using gtf file Homo_sapiens.GRCH38.84.gtf and featureCount (v1.5.0-p1). Analysis of differential gene transcription and normalization of read counts and PCA were performed with R package DESeq2 (v.1.22.2) (70), and four-way plots and MA plots were generated with R package vidger (v.1.2.1) (71).

Statistical Analyses

All tests were performed with a nonparametric assumption and significance level of 0.05. All analyses were done using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, California, www.graphpad.com).

DATA AVAILABILITY STATEMENT

RNA sequencing data for ILC3-like cells and in vitro generated ILC3 and NK cells is accessible at NCBI Project ID: PRJNA642003 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA642003) and PRJNA777910 (https://www.ncbi.nlm.nih.gov/sra/PRJNA777910).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission, Medical Faculty, University hospital Düsseldorf (study number 2019-383). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SB: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript. SW: Collection and/or assembly of data, conception and design, and final approval of the manuscript. ÖD: Collection and/or assembly of data, provision of study material, and final approval of the manuscript. RO: Provision of study material and final approval of the manuscript. KR: Collection and/or assembly of data and final approval of the manuscript. GK: Provision of study material, conception and design, and final approval of the manuscript. RM: Collection and/or assembly of data, provision of study material, and final approval of the manuscript. LW: Collection and/or assembly of data, manuscript writing, and final approval of the manuscript. MU: Conception and design, data analysis and interpretation, manuscript writing, financial support, and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.797432/full#supplementary-material

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Innate Lymphoid Cells in Autoimmune Diseases

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Innate lymphoid cells (ILC) are a heterogeneous group of immune cells characterized by lymphoid morphology and cytokine profile similar to T cells but which do not express clonally distributed diverse antigen receptors. These particular cells express transcription factors and cytokines reflecting their similarities to T helper (Th)1, Th2, and Th17 cells and are therefore referred to as ILC1, ILC2, and ILC3. Other members of the ILC subsets include lymphoid tissue inducer (LTi) and regulatory ILC (ILCreg). Natural killer (NK) cells share a common progenitor with ILC and also exhibit a lymphoid phenotype without antigen specificity. ILC are found in low numbers in peripheral blood but are much more abundant at barrier sites such as the skin, liver, airways, lymph nodes, and the gastrointestinal tract. They play an important role in innate immunity due to their capacity to respond rapidly to pathogens through the production of cytokines. Recent evidence has shown that ILC also play a key role in autoimmunity, as alterations in their number or function have been identified in systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis. Here, we review recent advances in the understanding of the role of ILC in the pathogenesis of autoimmune diseases, with particular emphasis on their role as a potential diagnostic biomarker and as therapeutic targets.

Keywords: innate lymphoid cell (ILC), autoimmune diseases, autoimmunity, systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis, ANCA-associated vasculitis, NK cell

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INTRODUCTION

Innate lymphoid cells (ILC) are lymphocytes that lack somatically diversified antigen receptor expression (1). So far, different subtypes of ILC have been described, depending on their specific functional features mirroring $CD4^+$ T helper (Th)1, Th2, and Th17 cells. In 2013, they were classified as group 1 (including NK cells), group 2, and group 3 ILC (1, 2); however, following further research, a new description was proposed in 2018 which classifies ILC into five categories, namely, NK cells, ILC1, ILC2, ILC3, and lymphoid tissue inducer (LTi) cells (**Table 1**) (3).

ILC in humans and mice originate from a common lymphoid precursor (CLP), which is able to give birth to all lymphocyte subsets (37). Studies in murine models have shown that CLP initially differentiates into the common innate lymphoid progenitor (CILP) which serves as a common precursor for both NK cells and ILC. CILP then evolves into the common helper innate lymphoid progenitor (CHILP), which is common to LTi and ILC (6, 38). CHILP finally differentiates into innate lymphoid cell precursors (ILCP) that will give rise to ILC1, ILC2, and ILC3 (3, 12). Of note, in

TABLE 1 | Characteristics of innate lymphoid cell (ILC) populations in humans.

		ILC1	ILC2	ILC3	LTi	ILCreg	NK
Function		Antimicrobial defense (intracellular microbes) (1)	Allergies, parasite elimination (3)	Innate immunity against fungi and extracellular microbes (1, 3)	Mesenchymal organizer for SLN in embryonic development (3)	Resolution of innate intestinal inflammation (4)	Antitumor surveillance, antimicrobial defense (intracellular pathogens and viruses) (5)
Phenotype		Variable depending on tissue residency	$CRTH2^+ST2^+CD161^+$ (6, 7)	Controversial Lin^- ($CD56^{+/-}$) $CD127^+CRTH2^-CD117^+NKp44^{+/-}$ (8, 9)	Controversial NRP1 (10)	$Lin^-CD45^+CD127^+IL10^+$ (11)	$CD56^+CD16^{+/-}$
Transcription factor		T-bet (1, 3, 12)	GATA3, RORα (1, 3, 13)	RORγt (1, 3)	RORγt (3)	–	T-bet, EOMES (3, 5, 12, 14, 15)
Inducing cytokines		IL-12 (16)	IL-33, IL-25, TSLP (1, 7, 17)	IL-7, IL-23, IL-1β (3, 18)	–	–	IL-15 (3, 5, 12, 14, 15)
Produced cytokines		IFN-γ (1, 3, 12)	IL-4, IL-5, IL-13 (1, 7, 17)	IL-22, IL-17A, GM-CSF, IFN-γ, TNF-α (3, 18)	IL-22, IL-17, GM-CSF, TNF-α and TNF-β, IL-8 (10)	IL-10, TGF-β (11)	IFN-γ, perforin granzyme B
Regulated tissues	Main	Tonsils, gut, lung, liver, adipose tissue, skin, LN, spleen (6, 19–21)	Peripheral blood, skin, lungs, adipose tissue (6, 7, 22)	Mucosal tissues (gut) (6, 16)	Lymphoid organs	ILCreg: intestine (11), kidney (23); follicular ILCreg: tonsils, LN (24)	Blood
	Possible	Peripheral/cord blood (6)	Liver, LN, spleen, adenoids (6)	Blood, spleen, LN, tonsils, intestine, skin and lung (6)	–	–	
Association with systemic autoimmune diseases		Increased in Ssc, SLE, active AAV (25–31), Increased or decreased in RA (32, 33)	Increased in SSc and RA (33–35), Decreased in AAV and SLE (27–29, 31)	Increased (29) or decreased (27, 28) in SLE, Increased (36) or decreased (33) in RA Decreased in AAV (31)	Decreased in RA (32)		

IL, interleukin; NRP1, neuropilin1; T-bet, T box expressed in T cells; GATA 3, Trans-acting T-cell-specific transcription factor GATA3; RORγT, retinoid acid-related orphan receptor γ T; RORα, retinoid acid-related orphan receptor α; LN, lymph node; CRTH2, chemoattractant-homologous receptor expressed on Th2 cells; Ssc, systemic sclerosis; SLE, systemic lupus erythematosus; AAV, ANCA-associated vasculitis; RA, rheumatoid arthritis.

mice, lymphoid progenitors (which have the potential to differentiate into all ILC lineages, including NK cells) were identified as inhibitor of DNA binding 2 (ID2) positive (5). ID2 is a transcription factor required for organogenesis of lymphoid tissues, and its loss was shown to disrupt the generation of ILC precursors (14, 15). In humans, the differentiation steps that lead to the development of ILC are less well known even though they are considered to be similar (19). Similarities and differences between human and murine ILC have been excellently reviewed elsewhere (19).

Many phenotypic markers have been used to characterize mature ILC subsets, but no definitive marker universally defining ILC has been identified so far. This is notably due to the fact that their phenotype depends on the tissue they populate and that ILC represent very heterogeneous populations (20, 21). Despite tremendous variability in their definition, ILC can be roughly described as CD3-negative lymphocytes that express IL-7 receptor ($CD45^+CD3^-CD127^+$), although in many tissues ILC1 do not express CD127 (20, 21).

NK cells were initially included in group 1 ILC, together with ILC1, because of important similarities such as the expression of the transcription factor T-bet and the production of interferon γ (IFN-γ) (3, 7). However, subsequent studies indicated that NK cells and ILC1 belong to distinct lineages and represent two separate cell types (6, 13, 17, 21). Indeed, while ILC are mainly tissue-resident cells, NK cells are principally found in blood circulation, constituting 5%–20% of circulating lymphocytes, and are capable of being rapidly recruited to inflammation sites (20, 39, 40). Moreover, NK cells have an important cytotoxic function with high expression levels of perforin and granzyme B, whereas ILC1 are in general noncytotoxic or only weakly cytotoxic. Interestingly, recent evidence in murine models shows that $CD160^-$ILC1 exhibit cytotoxicity against YAC-1 cells (22). In addition, certain populations of splenic ILC1-like NK cells are able to kill cells infected with murine cytomegalovirus (3, 18, 22, 39). NK cells play a special role in antitumor surveillance and in antimicrobial defense against intracellular pathogens and viruses (39). In addition, compared with ILC1, they follow a specific differentiation pathway that requires the expression of the T-box transcription factor Eomes for their development, and the induction of CD122 with subsequent IL-15 responsiveness (8, 13, 16).

In addition, ILC1 also share similarities with Th1 cells, as they react to intracellular pathogens, mainly secrete IFN-γ and depend on the transcription factor T-bet for their differentiation (1, 3, 6). Although they can be detected in peripheral blood or cord blood, they are primarily tissue-resident cells (20). In humans, ILC1 are mainly found in the tonsils, gut, lung, liver, adipose tissue, skin, lymph nodes, and spleen (4, 9, 20, 40). They show significant differences in the

expression of surface markers and transcription factors linked to the microenvironment of the tissue they populate (20).

ILC2, like Th2 cells, produce high levels of interleukin (IL)-4, IL-5, and IL-13 in response to epithelial cell-derived IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) (1, 10, 41). They express high levels of the transcription factors GATA3 and RORα (3, 11). Phenotypically, they are characterized, in mice, by the expression of suppression of tumorigenicity (ST2, also known as IL-1RL1) (23, 24), CD161, and inducible T-cell COStimulator (ICOS), whereas in human peripheral blood, they are described as chemoattractant-homologous receptor expressed on Th2 cells ($CRTH2^+$), $ST2^+$, and/or $CD161^+$ (20, 41). ILC2 are involved in allergies and parasite elimination (3).

In mice, they are abundant in the airways, lungs, skin, and gut, especially in models of asthma (1, 41, 42). In humans, ILC2 represent the main population of ILC that inhabit peripheral blood, skin, lungs, and adipose tissue, but they are little or not present in adult gut (20, 41, 43). Furthermore, the presence of ILC2 has also been described in liver, lymph nodes, spleen, and adenoids (20).

ILC3 are the innate counterpart of Th17 cells. They play a role in innate immunity against fungi and extracellular microbes and depend on the transcription factor RORγt (1, 3). Like other ILCs, they require IL-7 for their development. More specifically, they secrete IL-22 and certain subsets can produce IL-17A, in response to IL-23 and IL-1β (3, 44). In mouse models, ILC3 participate in the secondary antibody response by promoting the survival of $CD4^+$ T cells through the expression of OX40 ligand and CD30 ligand (44). They are also able to express antigen-presenting molecule major histocompatibility complex-II (MHC-II) and present processed antigens to $CD4^+$ T cells (44).

In humans, ILC3 and notably $NKp44^+$ ILC3 are particularly found in mucosal tissues such as the gut (20, 45). However, they may also be found in blood, spleen, lymph nodes, tonsils, skin, and lung (20). Phenotypically, in humans, they were notably described as $Lin^-CD56^{+/-}CD127^+CRTH2^-CD117^+NKp44^{+/-}$ (46) or $Lin^-CD45^+CD127^+cKit^+CRTH2^-NKp44^-$ or $NKp44^{+/-}$ (47).

LTi were previously included in the group 3 ILC because of their capacity to produce IL-17 and IL-22. They undergo differentiation from a specific progenitor, the lymphoid tissue inducer progenitor (LTiP) and depend on RORγt for their differentiation (3). However, now considered a specific population, LTi have a specific role as mesenchymal organizer cells in the formation of secondary lymphoid structures during embryonic development (3). According to data from studies in mice, the crosstalk between LTi and lymphoid tissue stromal cells continues postnatally, as it has been demonstrated that LTi cells contribute to the restoration of lymphoid tissue architecture following infection with LCMV (48). In humans, LTi express neuropilin-1, produce IL-17, IL-22, GM-CSF, TNF-α, TNF-β, and IL-8, and play possibly a role in the Th1 and Th17 immune response (49, 50).

Lately, another ILC subpopulation was described, which harbors a regulatory phenotype, and hence named regulatory ILC (ILCreg) (51). These cells, phenotypically defined as $Lin^-CD45^+CD127^+IL\text{-}10^+$, were initially described in mouse and human intestine secrete high amounts of IL-10 and TGF-β and are devoid of CD4 and Foxp3 expression (51). They show a distinct gene expression profile compared with other ILC and play an important role in the resolution of innate intestinal inflammation through the suppression of ILC1 and ILC3 *via* IL-10 secretion, in a mouse model of colitis (51). In addition, the secretion of TGF-β acts in an autocrine way to support the expansion of ILCreg during gut inflammation (51). Of note, the existence of IL-10-producing ILCreg as a distinct population of ILC remains controversial. From this point of view, in various mice models, the main source of IL-10 in the gastrointestinal tract comes from activated populations of ILC2, which expresses KLRG1, IL-25R, and the transcription factor GATA-3 (52).

In another context, ILCreg were also described in mouse and human kidney, where they play a protective role in ischemia-reperfusion injury (53). Another regulatory population of ILC, named follicular regulatory ILC, has been described in human tonsils and lymph nodes and secretes high amounts of TGF-β (54).

Interestingly, ILC have been recently shown to exhibit plasticity, similarly to T cells. They have the ability to coexpress lineage-determining transcription factors in response to signal from their microenvironment (23). This is especially true for $CD127^+CD117^+$ ILC precursors, a cell subset which expresses CD45RA and CD62L and shows similarities to naive $CD4^+$ T cells (23). Their differentiation depends on cytokines present in the tissue they populate (23). Balance between ILC1 and ILC3 changes in the presence of inflammatory stimulations, with ILC1 numbers increasing and ILC3 decreasing in the intestine in pathological conditions such as Crohn's disease (45). This process occurs through a differentiation of ILC3 to ILC1, which depends on exposure to IL-12. In addition, this differentiation has been shown to be reversible, as the presence of IL-23 and IL-1β favors the differentiation of ILC1 to ILC3 (45). Recently, ILC3–ILC1 intermediate subsets were identified in human tonsils and intestinal mucosa, describing ILC3 and ILC1 as the ends of a spectrum, with the cells closest to ILC1 having the maximal ability to produce IFN-γ *in vitro* (4). Another study showed that human ILC3 that are transferred to humanized mice acquire ILC1-like features in the spleen more than in the liver (5). These results support the hypothesis that tissue specific triggers cause local transdifferentiation of ILC (23).

Since their discovery, numerous studies suggest that ILC play a key role in the pathogenesis of systemic autoimmune conditions such as systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and antineutrophil cytoplasm antibody (ANCA)-associated vasculitides. In this review, we discuss the latest advances on the role of ILC in the pathogenesis of human autoimmune diseases and their potential use as diagnostic biomarkers and/or therapeutic targets.

ILC IN AUTOIMMUNE CONDITIONS

Systemic Sclerosis

Systemic sclerosis (Ssc) is an autoimmune connective tissue disease characterized by vasculopathy and fibrosis in multiple

organs. Ssc prototypically causes Raynaud phenomenon, arthralgias, fingertip lesions, skin thickening, hypertensive renal crisis, lung fibrosis, and pulmonary arterial hypertension (55–57). The pathophysiology of Ssc is unclear but involves genetic and environmental factors (i.e., silica solvents, epoxy resins, breast implants, skin microbiota), leading to chronic inflammation, endothelial injury, vascular dysfunction, fibroblast activation, and tissue fibrosis (58, 59). Numerous immune cells, antibodies, and cellular pathways contribute to the processes that lead to tissue fibrosis. In particular, dysregulation of interferon α (IFN-α) is an important alteration in patients with antitopoisomerase I antibodies (58, 60). This dysregulation is characterized by an IFN-α overproduction by plasmacytoid dendritic cells in response to the activation of toll-like receptors (TLR) 7 and 9 by immune complexes generated by endothelial cell death (60). Constitutive fibroblast activation driven by mediators such as tumor growth factor β (TGF-β) also represents a key process, which leads to tissue fibrosis (58).

Few studies have examined the role of ILCs in the pathophysiology of human Ssc (**Figure 1**). A study published in 2015 by Wohlfahrt et al., including 69 Ssc patients, showed that ILC2 number is elevated in the skin and peripheral blood of patients with Ssc compared with healthy controls (34). There was also a positive correlation between the number of ILC2 in the skin and the modified Rodnan Skin Score. In addition, patients with extensive pulmonary fibrosis showed the highest numbers of circulating ILC2 (34). Of note, ILC2 were defined using two different marker panels, both including ST2 ($ICOS^+$ $ST2^+CD3^-CD11b^-$ or $ST2^+IL\text{-}17RB^+KLRG1^+$), with consistent results (34). These data suggest a potential pathogenic role of ILC2 in Ssc, although the mechanism is still unclear. As it was shown that type 2 cytokines such as IL-4 and IL-13 can increase TGF-β production in bronchial epithelial cells in diseases such as asthma (61), one could hypothesize that ILC2, which secrete such cytokines, could thus induce TGF-β secretion from fibroblasts or other epithelial cells such as keratinocytes, and therefore, increase fibrosis (62). Moreover, in murine models, TGF-β is required for the development of ILC2, suggesting a potential crosstalk between fibroblasts and ILC2 (63). However, data are still missing in Ssc, and this hypothesis needs to be investigated.

On the other hand, a study published in 2016 by Roan et al. showed that a subset of ILC1, defined as $CD4^+$ ILC1, and $NKp44^+$ ILC3 were increased in the peripheral blood of Ssc patients compared with healthy subjects (25, 26). An interesting point is that the $CD4^+$ ILC1 expressing IL-6Rα were decreased in SSc, suggesting that these cells are overactivated and contribute to the amplification of the inflammatory response that characterizes SSc (25, 26). In another study, the authors showed that $KLRG1^{low}$ ILC2 are increased in the fibrotic skin from SSc patients. This population is activated by TGF-β and produces lower levels of IL-10 compared with $KLRG1^{high}$ ILC2. These $KLRG1^{low}$ ILC2 cells fail to negatively regulate collagen production by dermal fibroblast, a process which is physiologically IL-10 dependent, thus enhancing skin fibrosis (35). Despite these interesting findings on the role of ILC1 and ILC2 in Ssc pathogenesis and fibrosis development, data are still missing to fully understand the importance of ILC in the pathogenesis of Ssc.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease affecting mainly young women of childbearing age. Its pathophysiology is complex, involving loss of self-tolerance with an imbalance between apoptotic cell abundance, extracellular exposition of nuclear antigens, and disposal of this apoptotic material. The free nuclear antigens will activate TLR notably on plasmacytoid dendritic cells (pDC),

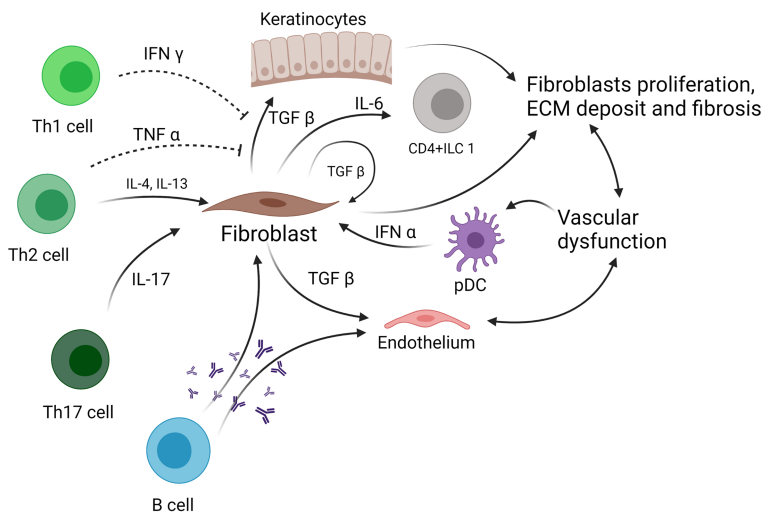

FIGURE 1 | Role of ILC in systemic sclerosis. ILC, innate lymphoid cell; TNF-α, tumor necrosis factor α; TGF-β, tumor growth factor β; pDC, plasmacytoid dendritic cell; IL, interleukin; ECM, extracellular matrix.

with secretion of type I IFN (known as the "interferon signature") and other cytokines that drive B-cell differentiation, and the production of autoantibodies (64). These antibodies directed against self-antigens then form immune complexes that deposit in the tissues, leading to chronic inflammation and tissue damage (27, 64).

The role of ILC in SLE pathogenesis is poorly understood, particularly in humans (**Figure 2**). In 2019, a study by Guo et al. examined circulating ILC in the peripheral blood of 49 SLE patients and showed an increase in ILC1 (defined as $Lin^{-}CD127^{+}CRTH2^{-}CD117^{-}$) compared with healthy controls, while ILC2 ($Lin^{-}CD127^{+}CRTH2^{+}$) and ILC3 (including 2 subpopulations, defined as $Lin^{-}CD127^{+}CRTH2^{-}CD117^{+}NKp44^{+}$ or $NKp44^{-}$) were decreased (28). Moreover, the greatest increase in ILC1 and decrease in ILC2 and ILC3 were observed in patients with moderate and severe disease activity, with a positive correlation of ILC1 numbers to systemic lupus erythematosus disease activity index (SLEDAI) (28). This altered distribution of ILC in active SLE with lupus nephritis was reversed after initiation of treatment (steroids and cyclophosphamide), suggesting that ILC1 may represent a biomarker of disease activity (28). Recently, a study by Jiang et al. examined the number of ILC in the peripheral blood of SLE patients (29). They also found an increase in ILC1 and a decrease in ILC2 in patients with active SLE, but, in contrast to Guo et al., they found an increase of ILC3 in the blood of patients with active SLE compared with inactive (SLEDAI<5). Interestingly, there was a positive correlation between ILC3 absolute numbers in the peripheral blood and the SLEDAI score. This discrepancy between the two studies might be due to differences in gating used to define ILC subsets, as the markers used to distinguish between ILC1 and ILC3 were similar. Heterogeneity of SLE patients might also contribute to such differences. An interesting point in the research by Jiang et al. is a positive correlation between ILC3 and serum anti-dsDNA titers, and a decrease in ILC1/ILC3 and ILC2/ILC3 ratio in SLE patients with arthritis compared with patients without arthritis (29).

In a study by Blokland et al., which also included patients with primary Sjögren's syndrome (pSS) patients, ILC1 were increased in the peripheral blood of SLE patients (27). In pSS, the abundance of total ILC did not differ from healthy donors but was associated with disease activity as measured by the EULAR Sjögren's syndrome disease activity index (ESSDAI). The

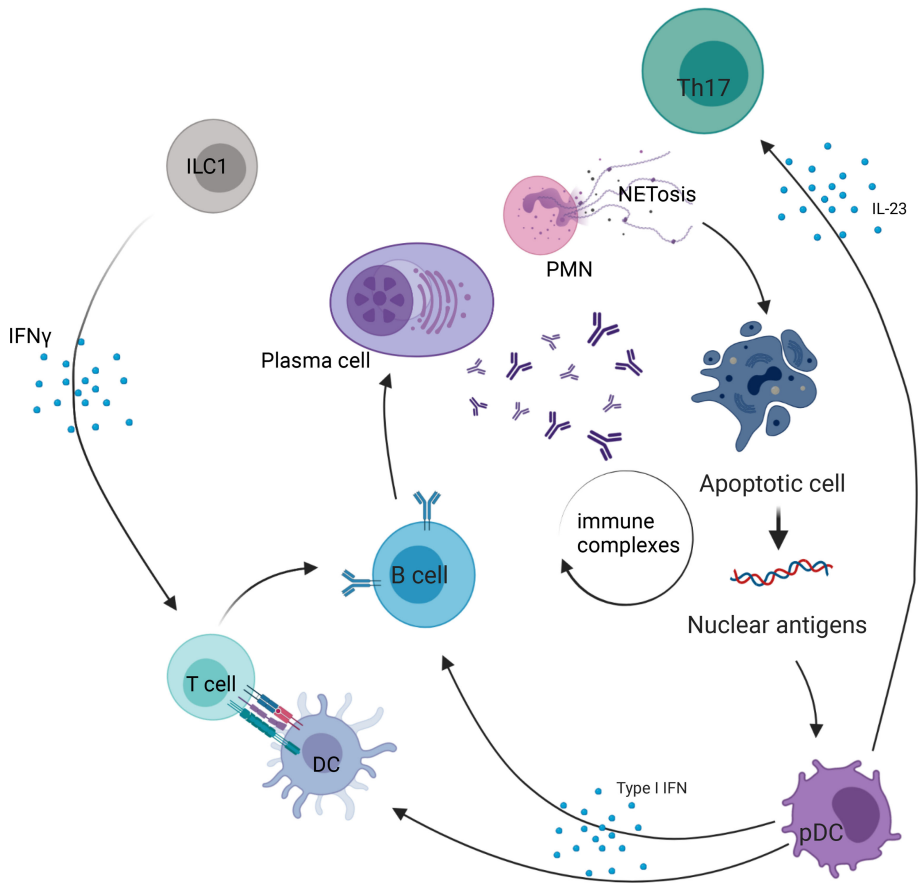

FIGURE 2 | Role of ILC in systemic lupus erythematosus. PMN, polymorphonuclear cell; ILC, innate lymphoid cell; NETosis, process of neutrophil extracellular traps; IL, interleukin; DC, dendritic cell; pDC, plasmacytoid dendritic cell; Th17, T helper 17 cell; IFN-γ, interferon γ.

patients (SLE and pSS) showing an interferon (IFN) signature (defined by an elevated IFN score) had an increased FAS expression, with a decrease in ILC2 and ILC3 frequency (27).

Finally, a study including 51 SLE patients also showed an increase in ILC1 in the peripheral blood. They also identified a positive correlation between increased ILC1/ILC3 count and disease activity (30). These data suggest that ILC1 may participate in/constitute a response to the inflammatory process, while ILC3 may play a role in the development of the autoantibody response in SLE. However, further studies are warranted to explore these hypotheses and understand the role of the altered abundance of ILC in the peripheral blood of SLE patients. Moreover, in humans, ILC1 definition is still controversial (65), and no data are currently available on ILC phenotype in the organs and tissue from SLE patients. This would be of paramount importance to shed light on the role of these cells at the epithelial barrier sites in SLE.

Antineutrophil Cytoplasm Antibody-Associated Vasculitis

ANCA-associated vasculitis (AAV) encompasses three distinct entities: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (66).

These inflammatory diseases are all characterized by small- and medium-vessel inflammation, but with relatively distinct clinical presentations, specific biologic features, and ANCA serotype. Anti-PR3 are mainly associated with GPA, while anti-MPO are more frequently associated with EGPA and MPA (67). GPA often manifests as granulomatous inflammation of the upper and lower airways and ear/nose granulomatous inflammation and kidney damage. MPA is characterized by necrotizing glomerulonephritis and pulmonary capillaritis. EGPA is prototypically associated to eosinophilia, pulmonary infiltrates, and asthma (68).

The pathogenesis of AAV relies on the production of antibodies that target myeloperoxydase and proteinase 3 (67). These two proteins are abnormally overexpressed on the surface of neutrophils, and, subsequently to antibody binding, neutrophils are activated and produce cytokines, reactive oxygen species, and neutrophil extracellular traps (NETosis) (67). Overactivation of B and T cells is also involved in the pathogenesis of AAV and leads to the production of ANCA (67).

As ILC have been shown to play particularly a role in tissue homeostasis at mucosal sites, and especially at the level of airways epithelia, examination of their role in AAV is of particular interest (**Figure 3**). From this point of view, one study examined the frequencies of ILCs in the peripheral blood of AAV patients (26 GPA and 15 MPA subjects) compared with healthy controls (31). Samples were collected during acute phase, defined by Birmingham vasculitis activity (BVAS) score >3, before any treatment, or during remission phase, defined as BVAS 0. Total ILCs, defined as $Lin^{-}CD127^{+}$, were decreased during acute phase in AAV patients compared with controls. More precisely, ILC2 and ILC3 were decreased while ILC1 were increased when compared with healthy controls or AAV patients

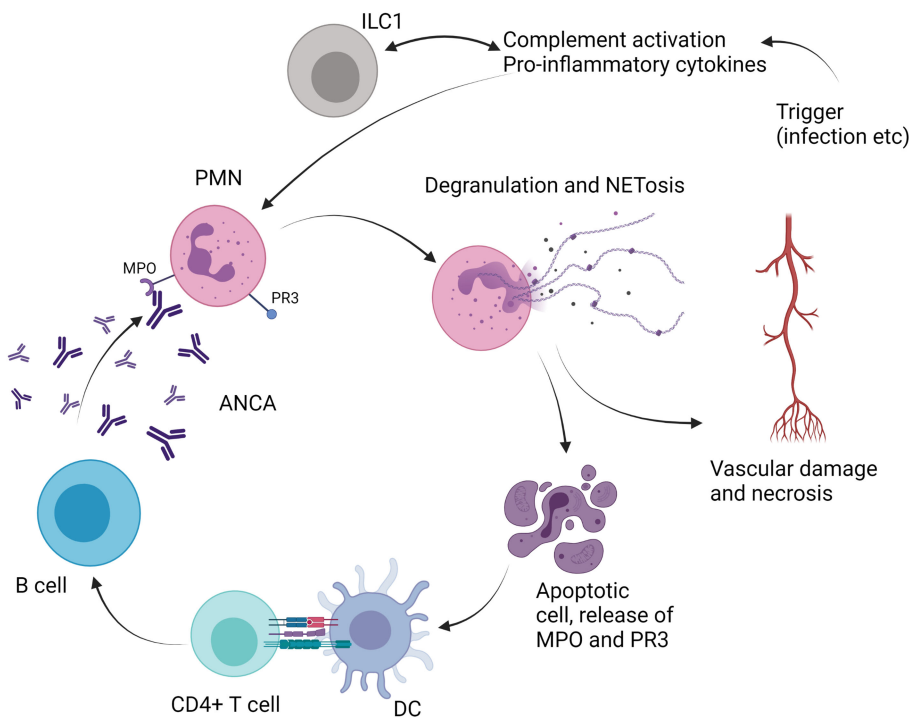

FIGURE 3 | Role of ILC in antineutrophil cytoplasm antibodies (ANCA)-associated vasculitis. PMN, polymorphonuclear cell; ILC, innate lymphoid cell; DC, dendritic cell; MPO, myeloperoxidase; PR3, proteinase 3; NETosis, neutrophil extracellular traps; IL, interleukin.

in remission (31). Even if these data are of interest, it remains difficult to draw any definitive conclusion on the role of ILC in the pathogenesis of AAV. Further studies are warranted to address this point.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease affecting the joints with synovial inflammation and cartilage/bone destruction (69). The pathogenesis is complex and involves the development of auto-antibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), which can be detected years before the onset of clinical disease (70). Development of ACPA and/or RF is triggered by a complex interplay between genetic, epigenetic, and environmental factors (smoking, pathogens, obesity, dysbiosis, toxic substances) (69, 71, 72). Innate immunity is central to the pathogenesis of RA, with the presence of macrophages, mast cells, and NK cells in the synovial membrane, and neutrophils in the synovial fluid (69). NK cells are also increased in the synovial fluid of RA patients (73).

The humoral immune response plays also an essential role in the pathogenesis of RA, and B cells, plasmablasts, and plasma cells are very abundant in the inflamed synovium (69). ACPA promote the production of TNF-α by macrophages, a cytokine that is the cornerstone of RA joint damage, by activating fibroblasts and chondrocytes (69). Additionally, cytokines involved in the Th17 response, including IL-6, IL-21, IL-17, IL-23, and IL-1β, are also elevated in the peripheral blood and synovial fluid of patients with RA (71, 74).

Some recent studies evaluated the role of ILC in RA patients (**Figure 4**). In 2017, Rodriguez-Carrio et al. showed that ILC distribution differed in lymph nodes (LN) of RA patients compared with at-risk patients (defined as patients with RF and/or ACPA positivity, and arthralgia without arthritis) or healthy controls (32). LTi cells were shown to be decreased in RA patients, while ILC1 were increased in RA and at-risk patients. ILC3 were increased in RA patients compared with healthy controls and at-risk patients. A positive association of LTi frequency with VCAM expression on LN endothelial cells was also shown, suggesting a potential crosstalk between ILC and the stromal cell compartment (32). In 2019, Takaki-Kuwara et al. found that a subset of CCR6$^+$ ILC3 was increased in the synovial fluid of RA patients compared with osteoarthritis controls, and positively correlated with RA clinical activity (36). Moreover, a positive correlation was established between the number of CCR6$^+$ ILC3 cells and CCL20 concentration in synovial fluid of RA patients, suggesting that CCR6$^+$ILC3 may play a role in RA pathogenesis through the production of Th17 cytokines such as IL-17 and IL-22 (36). Finally, Yang et al. described that RA patients with stable disease depicted decreased ILC1 and

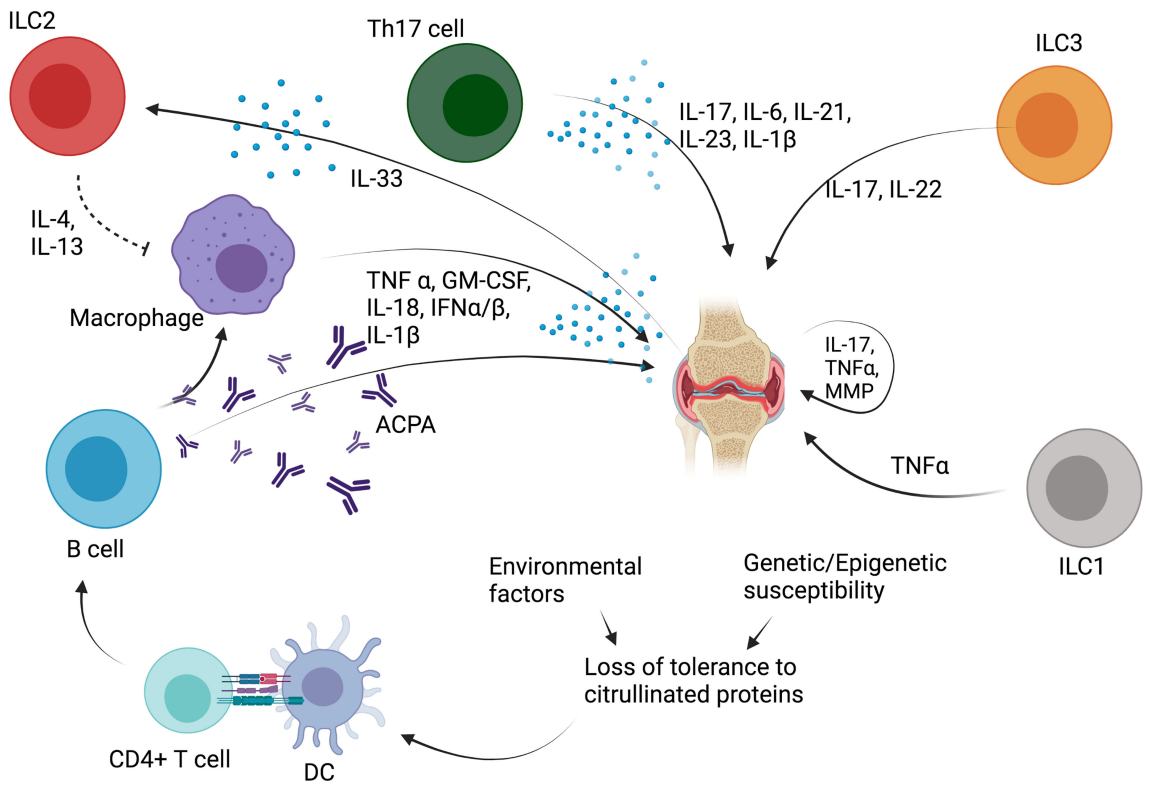

FIGURE 4 | Role of ILC in rheumatoid arthritis (RA). ACPA, anticitrullinated protein antibodies; TNF-α, tumor necrosis factor α; IL, interleukin; DC, dendritic cell; MMP, metalloproteinase; ILC, innate lymphoid cell; IL, interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; Th, T helper CD4$^+$ T cell.

increased ILC2 proportion in the peripheral blood compared with healthy controls and with patients with active disease, while both active and stable RA patients had a decreased percentage of ILC3 (33). A positive correlation between disease activity and ILC1 proportion was also found, while there was a negative correlation between ILC2 percentage and disease activity (33). This suggests that ILC2 may counterbalance the proinflammatory effect of ILC1 through the production of IL-13 (75, 76), which has been shown to have anti-inflammatory effect on synovitis in rheumatoid arthritis (77).

DISCUSSION

Since their initial description 10 years ago, ILC have been increasingly recognized as important players in the immune response, but their role in human autoimmune diseases remains controversial. Currently available data suggest that they could be useful as biomarkers of disease severity or response to treatment.

A major limitation to identify the role of ILC in human diseases is related to the fact that ILC are tissue-resident cells. Access to barrier site requests invasive biopsies, which are not easy to be routinely performed. Therefore, most studies are limited to the examination of cells from the peripheral blood, where ILC are only present at low abundance and where they display a phenotype that might differ from their tissue-resident counterpart. Technically, examination of restricted subpopulations of ILC may be difficult due to the important number of markers needed to identify these populations. Recent advances in single cell mass cytometry and flow cytometry that allow the examination of high amount of parameters in limited biological samples should facilitate future studies on the subject.

Therapeutic approaches targeting ILC are challenging, because ILC are highly heterogeneous. Moreover, no specific markers for ILC have been identified to date, making it difficult to develop a drug that specifically targets ILC or ILC subsets. Accordingly, the border between pathogenic versus beneficial role of ILC is not always obvious. ILC2, for example, seem to be pathogenic in atopic dermatitis, but in a mouse model of RA, they foster the resolution of inflammation (1, 78). Since ILC exhibit an important plasticity that depends on their microenvironment, targeting cytokines or soluble factors involved in their differentiation and maintenance would likely affect ILC subpopulation distribution and alter diseases course. Recent evidence, for example, showed that patients with inflammatory bowel disease exhibit an altered distribution of ILC subsets in the gut and blood during active disease. This anomaly is partly restored after treatment with the anti-IL12/23 monoclonal antibody ustekinumab (79). Overall, future studies are warranted to explore the role of ILC in human diseases, because currently available data remain largely descriptive and functional data are lacking.

AUTHOR CONTRIBUTIONS

AC, MH, NF, MK, and DC researched the data for the article. AC, MK, and DC wrote the manuscript. AC, MH, NF, MK, and DC reviewed the manuscript. All authors accepted the finale version of the manuscript

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REVIEW
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A NK Cell Odyssey: From Bench to Therapeutics Against Hematological Malignancies

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In 1975 two independent groups noticed the presence of immune cells with a unique ability to recognize and eliminate transformed hematopoietic cells without any prior sensitization or expansion of specific clones. Since then, NK cells have been the axis of thousands of studies that have resulted until June 2021, in more than 70 000 publications indexed in PubMed. As result of this work, which include approaches *in vitro*, *in vivo*, and *in natura*, it has been possible to appreciate the role played by the NK cells, not only as effectors against specific pathogens, but also as regulators of the immune response. Recent advances have revealed previous unidentified attributes of NK cells including the ability to adapt to new conditions under the context of chronic infections, or their ability to develop some memory-like characteristics. In this review, we will discuss significant findings that have rule our understanding of the NK cell biology, the developing of these findings into new concepts in immunology, and how these conceptual platforms are being used in the design of strategies for cancer immunotherapy.

Keywords: NK cell, cell signaling, cancer, hematological malignancies, immuno-therapy

INTRODUCTION

The natural cytotoxicity of NK cells is based on the directed release of the content of lytic granule rich in granzymes, delivered into the cytosol of the target cell through perforin pores assembled at the target cell membrane. This response is mediated and regulated by different types of cell surface receptors. When this cytotoxicity is triggered by NK cell Fc receptors that recognize IgG antibodies bound to a target cell, it is named antibody-dependent cellular cytotoxicity (ADCC). NK cell lytic function is a complex process that involves a series of coordinated events regulated by different receptors and signaling proteins (1, 2). Cell adhesion plays a central role by securing stable contacts between NK cells and target cells and guiding further cellular events. This adhesion step is followed by a stepwise movement of lytic granules to the microtubule-organizing center (MTOC), which then guides lytic granules towards the contact site of the target cell. The highly-organized movement of lytic granules along cytoskeleton elements has been described as granule polarization. Once lytic granules have positioned beneath the plasma membrane, their content is released into a well-defined

secretory cleft formed at interface with the target cell, a cellular event defined as degranulation or exocytosis of lytic granules. NK cells can also kill stressed cells through engagement of CD95L and TNF-related apoptosis-induced ligand (TRAIL), which are ligands for the death receptors CD95 and TRAILR respectively (3, 4). Death receptor activation induces the formation of the death-inducing signaling complex that leads to activation of caspase-8 and -10, and ultimately to apoptosis.

In humans, NK cell development initiates with the appearance of the NK-cell precursor (NKP) which arises from a multipotent hematopoietic precursors and early lymphoid precursors (ELP). The NKP give rise to immature NK cells, which in turn are the precursors of more mature NK cells. During maturation, NK cells acquire the expression of various NK cell receptors including those for self MHC class I molecules. The bone marrow represents the main tissue where NK cell development occurs, both in human and mice. By analyzing the phenotype of immature and mature NK cells, different models have proposed the distinction of several stages for developing NK cells (5). Immature NK cells exist in the bone marrow but they have been also found in other tissues including the peri-natal liver in mice and lymph nodes in human, favoring the idea that multiple sites can sustain NK-cell differentiation. An alternative hypothesis sustains that once generated in bone marrow, NKP and immature NK cells access to circulation and populate different peripheral tissues. Once NK cells have gain functional competence, they can be resident cells in various peripheral tissues. However, it is still poorly understood how NK cell maturation is regulated. In mice, mature NK cells can be found in spleen, liver, lung and blood and, to a lesser extent in the bone, marrow, thymus and lymph nodes. In human, mature NK cells represent a substantial fraction of circulating lymphocytes (up to 20%) but they are less frequent in the spleen and bone marrow (5-10%) (6). In contrast, other circulating innate lymphoid cells (ILCs) subsets are found at very low frequencies (less than 0.2%) as they are predominantly found in tissues. In circulating lymphocytes, two major human NK cell subsets are defined by the differential cell-surface expression of CD16 and CD56. $CD16^{+}CD56^{dim}$ is the most abundant subset, with high expression of perforin and enhanced cytotoxicity, whereas the $CD16^{-}CD56^{high}$ subset is the less abundant and produce greater amounts of IFN-γ and TNF-α (7, 8). The phenotype and function of both NK cell subsets can be modified, as for example, cytokine stimulation downregulates CD16 expression and upregulates CD56 and CCR7 expression. Moreover, cytokines can greatly impact on cytotoxicity and cytokine secretion in $CD16^{-}CD56^{high}$ and $CD16^{+}CD56^{dim}$ respectively (7). Whereas the phenotype of circulating NK cells is frequently described in terms on CD56 density, NK cell phenotype and function can be shaped through life in response to genetic and environmental factors (8, 9).

NK cells can adapt their behavior to environmental "clues", such as cytokines present in the milieu or those produced by chronic virus infections. This adaptation may result in enhanced NK functionality and, due to the acquisition of new phenotypic and functional attributes, these NK cells are referred as "memory like" cells. In humans, infections with HCMV are associated with an increased in the percentage of NK cells expressing high levels of the NKG2C receptor (10). In addition, CD16 engagement by anti-HCMV antibodies favors the preferential expansion of adaptive NK cells that upregulated the expression for NKG2C (8, 11, 12). Moreover, other NK cell subsets lacking key B-cell and myeloid-cell signaling proteins, such as FcεRIγ, SYK, and EAT-2, have also been identified in HCMV-infected individuals (8, 9). Importantly, most of these phenotypic changes are due to epigenetic modifications, providing a mechanism for altered signaling and function in adaptive NK cells compared to conventional NK cells. Whether adaptive NK cells emerge in other scenarios either in health or disease remains to be determined. All these studies clearly indicate that NK cells, as other immune cells, including macrophages and innate lymphoid cells, show high plasticity in response to diverse environmental stimuli such as antigens, acute viral infections, and cytokines (13, 14).

SPECIFICITY FOR NK RECEPTORS

In contrast to T lymphocytes and macrophages, NK cell cytotoxicity does not need prior sensitization or require proliferation of specific clones. When discover, NK cell cytotoxic activity seemed to be independent of antigen receptors and complement receptors. A first clue of NK cell specificity came from the "hybrid resistance" phenomenon, where a F_1 hybrid mice host rejects a graft derived from either inbred parent but not from an F_1 hybrid, a rejection that was not predicted to occur according to the laws of transplantation settled at that time (15). This graft rejection was shown to be thymus-independent and mediated by NK cells (16–18). The "hybrid resistance" implies that the absence of at least one major histocompatibility complex (MHC) class I (MHC-I) allele, is sufficient for NK cells from the recipient to eliminate cells "missing" such given MHC-I molecules. Thus, it was proposed that NK cells were able to sense the presence of a whole set of MHC-I molecules (haplotype) in the graft and that their absence would somehow stimulate NK cells. This idea was in line with an earlier suggestion by Snell that rejections of hematopoietic transplants occurs when donor and host differ genetically at the major histocompatibility complex (19). A second hint came from the study on the lifestyle of colonial tunicates, where individual cells are allowed to fuse in order to form colonies. In these invertebrates, the rejection or acceptance between colonies is controlled by a single gene locus with multiple alleles (20). Although the presence of MHC-like molecules is not documented in tunicates, it was possible to stablish an analogy based on the presence of molecules that determine histocompatibility. Despite these clues, there were still some contradictory observations about the relation between MHC-I molecules and NK cell specificity. The most notorious was that the *in vitro* killing of autologous cancer cells by NK cells, which appeared to have no relation to the MHC I molecules expressed on targets cells. Comparing the susceptibility of various cancer

cell lines to NK-mediated, allowed to conclude that high levels of MHC class I expression on target cells was sufficient to explain the resistance of targets to be lysed by NK cells. Thus, altering the expression of MHC class I molecules, by inducing cellular stress (neoplastic transformation) or a mismatch for MHC class I recognition, it was possible to tune the susceptibility of target cells to NK cell-mediated lysis. All these observations contributed to build up a model that allowed to explain why NK cells were able to kill "spontaneously" certain target cells (**Figure 1**). Such model was postulated as the "missing-self" hypothesis (21). According to the missing-self hypothesis, NK cells recognize and eliminate cells that have downregulated MHC class I expression, as consequence of viral infections or malignant transformation. At the same time, it was possible to interpretate the fact that metastatic tumor cells could escape from NK cell surveillance by re-expressing MHC class I molecules, lost by the primary tumor (22). All these advances paved the way to the next fundamental breakthrough: the discovery and characterization of inhibitory receptors for self-MHC-I.

NK CELL-BASED INHIBITION IN THE IMMUNE SYSTEM

The ability of the FcγRIIB receptor to shut down signaling through B cell receptor (BCR) provided the first model to understand how inhibition operates in immune system. The immunoreceptor tyrosine-based activation motif (ITIM) is a 13 amino acid sequence located in the cytoplasmic tail of the FcγRIIB, and is responsible for BCR signaling inhibition (23–25). This sequence resulted to be a specific binding site for the inositol phosphatase SHIP1. This ITIM sequence was reminiscent of a half-ITAM (Immunoreceptor Tyrosine-based Activation Motif), which allows activating signaling through various immune receptors (26). Further studies demonstrated that FcγRIIB was able to inhibit not only BCR-dependent signals but also those emanating from the T cell receptor (TCR) and the high-affinity IgE receptor or FcεRI, suggesting that the inhibitory properties of the FcγRIIB were not limited to any specific cell and could be exerted to any ITAM-containing receptor (25, 27).

Receptors for MCH class I molecules in human NK cells also contained an ITIM sequence (28, 29). Further studies demonstrated that these receptors were able to inhibit NK cell activation upon binding to different MHC class I alleles (30). Unlike ITIMs found in the cytoplasmic tail of FcγRIIB, two YxxL motifs separated by 26 amino acids were present in the cytoplasmic domain of inhibitory receptors for MHC-I molecules (31). A deeper characterization of the two ITIMs in the cytoplasmic tail of the NK cell ITIM-containing receptors (ITIM-Rs) revealed a selective binding motif for SH2 domains of the tyrosine phosphatases, SHP-1 and SHP-2. The selectivity of such inhibitory NK cell receptors for binding SHP-1 and SHP-2 was demonstrated for human and mice. Even thought the recruitment of SH2-containing phosphatases to phosphorylated ITIMs provided a mechanism to explain the inhibitory role of NK cell receptors for MHC-I, this was not sufficient to explain how NK

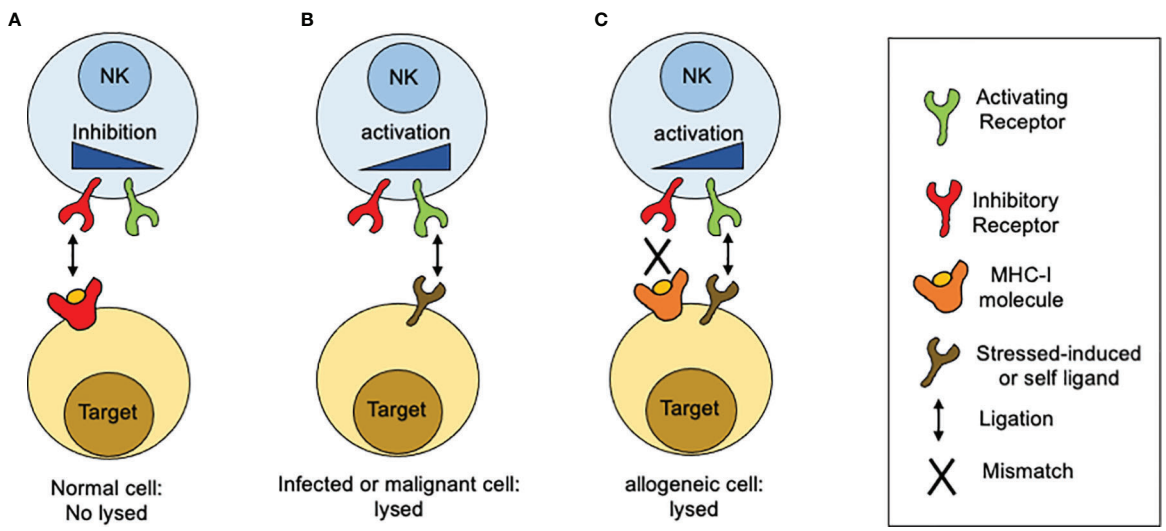

FIGURE 1 | How MHC-I molecules regulate NK cells reactivity. The idea of the missing-self was developed from different observations related with sensing MHC-class I molecules. **(A)** Normal or unstressed cells express MHC-I molecules which are recognized by inhibitory receptors expressed on the NK cell surface. When such receptors are engaged by MHC-I molecules, inhibitory signals are displayed. In the absence of ligation for activating receptors, the inhibitory signals predominate and target cells are not lysed. **(B)** When targets cells NK cells downregulate MHC class I expression, as consequence of viral infections or malignant transformation, inhibitory receptors are no longer engaged. In contrast, target cells start to upregulate the expression of surface molecules that are recognized by activating receptors. As consequence, activation signals are displayed unrestricted and target cells are lysed. **(C)** In the setting of allogeneic recognition, MHC class I molecules are expressed on target cells but are not recognized by NK cells as result of a mismatch. In consequence, activation signals proceed unrestricted and target cells are lysed.

cell ITIMs-R dampen signaling through activating receptors. Two possible mechanisms for phosphatases activity could be envisioned: either tyrosine phosphatases would mediate dephosphorylation of a large number of substrates, which are recruited and positioned through activating receptors, or their phosphatase activity displays a more sophisticated mechanism that targets only key signaling elements that control activation signals. Further studies suggested that inhibitory receptors in NK cells are more suited to block specific signaling events (32). The adaptor protein linker for activation of T cells (LAT) and PLC-γ are SHP-1 substrates, and it has been proposed that NK cell inhibitory receptors focus on regulating the function of adapter LAT rather than directly inhibiting PLC-γ tyrosine phosphorylation (33). Thus, by targeting adapter proteins rather than signaling enzymes, NK cell inhibitory receptors are able to influence multiple stimulatory pathways simultaneously instead of specific signaling pathways. Inhibitory signaling involves the dephosphorylation of Vav1, the only signaling protein associated with the catalytic site of SHP-1 upon recognition of target cells expressing HLA molecules (34). Moreover, the recruitment of Vav to the receptor is independent of actin polymerization, suggesting that inhibitory receptors can dampen NK cell-mediated cytotoxicity through an actin polymerization-independent mechanism (34). Inhibitory receptors can also promote the tyrosine phosphorylation of the adaptor protein Crk and its association with the tyrosine kinase c-Abl, concomitant with its dissociation from c-Cbl and p130 Cas (35, 36). These findings suggested that inhibitory receptors do not only control NK cell activation by dampening tyrosine phosphorylation of signaling proteins but also by promoting tyrosine phosphorylation of downstream signaling elements. How inhibitory receptors promote tyrosine phosphorylation of Crk, and how the association of Crk with c-Abl contribute to inhibit NK cell activation remains to be determined.

In contrast to FcγRIIB, which binds a single-SH2 domain-containing phosphatidylinositol 5-phosphate (SHIP), inhibitory NK cell receptors bind a two-SH2 domain containing tyrosine phosphatase (SHP1/2) (31, 37, 38). Moreover, ITIM-containing NK cell receptors are constitutively associated with Src family kinases and as consequence they can signal independently of activating receptors ligation (39). However, co-aggregation of an inhibitory NK cell receptor with activating ITAM-containing receptors is necessary to inhibit cell activation **(Figure 2)**. Therefore, NK cells represent a unique model not only to understand the mechanisms of cell regulation by inhibition, but also to appreciate how inhibitory receptors maintain homeostasis and contribute to pathology.

INHIBITORY NK CELL RECEPTORS FOR MHC-CLASS I MOLECULES: STRUCTURE AND SPECIFICITY

In human, MHC-I genes are also designed as human leukocyte antigen (HLA). The cloning of human NK cells showed that NK cells clones displayed a spectrum of different cytotoxic activities towards allogeneic target cells (40). These studies suggested that NK cells were able to recognize different isoforms of HLA class I molecules and the existence of a NK cell repertoire with a given reactivity towards such allogeneic cells (41, 42). The immunization of mice with different NK cell clones allowed the generation of monoclonal antibodies that led to the identification and characterization of NK cell receptors specific for HLA-I molecules. Several studies using such monoclonal antibodies demonstrated that: (i) the expression of NK cell receptors for self HLA-I is maintained unaltered upon cell activation, proliferation or cloning; (ii) the repertoire of NK cell receptors is compound of different members belonging to the same molecular species; and (iii) receptors for HLA-I alleles display different frequencies of expression not only among NK cells from the same individual, but also among different donors (43).

Most of the NK cell receptors for MHC class I molecules, both in human and mice, belong to two major families, which major difference lies in whether the extracellular domain possess a carbohydrate-recognition domain of C-type lectins, or an immunoglobulin-like domains. Gene families that encode for C-type lectins-like receptors are members of the NK complex (NKC) (44), whereas those that encode for immunoglobulin-like receptors belong to the leukocyte receptor complex (LRC) (45). Beyond these differences in structure and genetic organization, both families of receptors have provided two structurally unrelated but functionally complementary systems of inhibitory receptors for MHC-I molecules that drive NK cell development and function.

In humans the LRC includes members of the family of killer cell immunoglobulin-like receptors (KIR), leukocyte Ig-like receptors (LILRs), and leukocyte-associated Ig-like receptors (LAIRs). In humans, the *KIR* gene family contains 15 genes and 2 pseudogenes that are cluster in the chromosome 19q13.4. All members of this family differ for the number of extracellular Ig-like domain and the length of their cytoplasmic tail. In consequence KIR are characterized by the presence of two (KIR2D) or three (KIR3D) Ig-like extracellular domains and by long (KIR2DL and KIR3DL) or short (KIR2DS and KIR3DS) cytoplasmic tails (46). KIRs also contain activating forms that lack ITIMs in their cytoplasmic tails (47, 48). Compared to others NK cell receptor families, KIRs display unique features such as the number of KIR genes present in the genome of any given individual varies within the population (haplotype), the existence of a high polymorphism for each KIR gene, and the stochastic and variegated expression of the KIR repertoire among NK cell subsets. The major consequence of having a variable gene content and allelic polymorphisms for KIR receptors is that unrelated individuals rarely share identical KIR genotypes (49) and that all ethnic populations have their own distribution of KIR-genotypes frequencies (50). Moreover, the number of KIR genes varies between primates, ranging from polymorphic single-copy genes to complex multigene families that result in high levels of haplotypic complexity. In human, there are more than 130 KIR haplotypes that can be divided in at least two distinct groups designated as A and B according to the genes they contain (51).

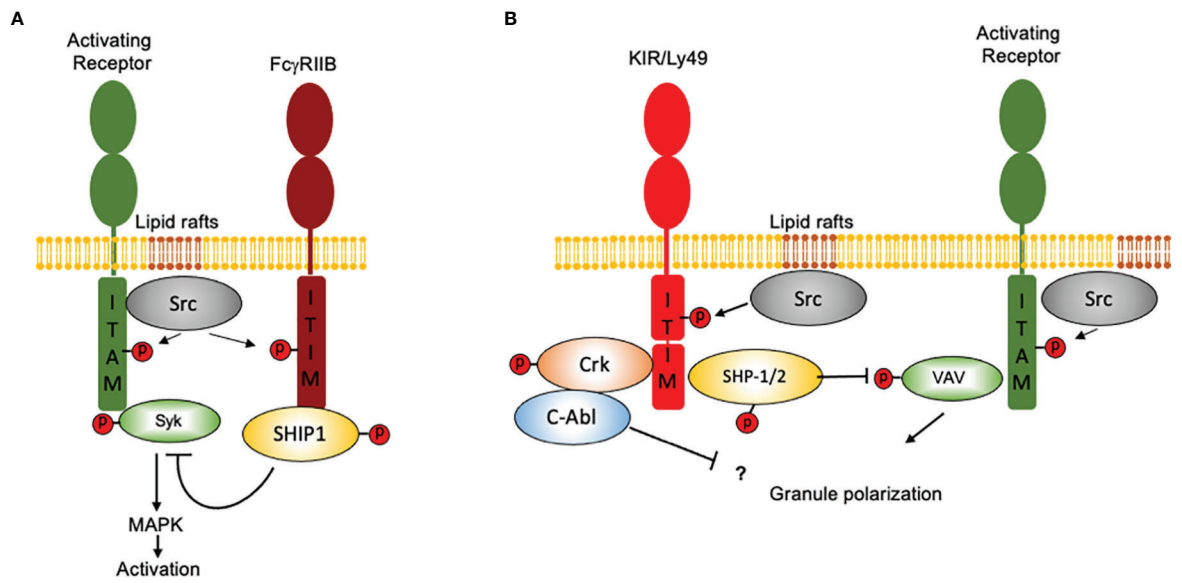

FIGURE 2 | How NK cells contributed to our idea of "inhibition" in the immune system. Inhibition in immune cells is based on the presence of ITIM-containing molecules and in the recruitment of SH2 domain-containing protein phosphatases. Although the presence of an ITIM sequence may represent a basic operation mode for inhibition, there are important differences in how signals are displayed downstream ITIM phosphorylation. For simplicity, only two major systems of inhibition are depicted. **(A)** The FcγRIIB receptor binds a single-SH2 domain-containing phosphatidylinositol 5-phosphate (SHIP1). Activation of SHIP-1 leads to dephosphorylate various substrates downstream ITAM-containing receptors. FcγRIIB coaggregation with activating receptors is mandatory to allow Src family kinases to phosphorylate both, the ITAMs and the ITIMs. **(B)** Inhibitory NK cell receptors bind a two-SH2 domain containing tyrosine phosphatase (SHP1/2). ITIM-containing NK cell receptors are constitutively associated with Src family kinases and as consequence they can signal independently of activating receptors ligation However, coaggregation of an inhibitory NK cell receptor with activating ITAM-containing receptors is necessary to inhibit cell activation. For ITIM-containing NK cell receptors, Vav1 seems to be the only signaling protein associated with the catalytic site of SHP-1 upon recognition of target cells expressing HLA molecules. Moreover, NK cell inhibitory receptors seem to operate no only by promoting tyrosine dephosphorylation, since they are also able to promote the tyrosine phosphorylation of the adapter protein Crk and to promote its association with the tyrosine kinase c-Abl.

Whereas the former has a common organization of seven genes and two pseudogenes, the latter contains more variable gene content. In general, B haplotype contains more genes that encode for activating KIRs than A haplotype. In contrast to humans, mice and rats encode a single or no KIR gene at the LRC, but they encode for multiple Ly49 genes at the NKC, which provide a similar NK cell receptor system for recognizing missing-self of MHC-I molecules. Despite their differences in protein structure and phylogenies, the KIR and Ly49 gene families arose from gene duplication and diversified by gene conversion, both families are highly polymorphic, expressed in NK cells and some T cell subsets, and their expression in NK cells is influenced by the host MHC class I haplotype.

In humans, KIR genes are expressed in variable combination by NK cells and some subsets of effector/memory αβ T cells, γδ T cells, but absent in thymocytes or naïve T cells (52, 53). Members of the KIR family include receptors for of HLA-A, HLA-B, and HLA-C proteins, which are also the products of highly polymorphic genes that are cluster on chromosome 6. However, the expression of KIRs does not depend on the expression of their HLA class I ligands and as a result, a given individual may express KIRs with no specificity for self-HLA alleles (51). The inhibitory receptors KIR2DL1, KIR2DL2, and KIR2DL3 recognize a polymorphism in HLA-C molecules which are distinguished by the presence of Ans77 and Lys80 or Ser77 and Asn80 on the α1 domain of the HLA-A heavy chain. In addition, the inhibitory receptors KIR3DL1 and KIR3DL2 recognize HLA-B and HLA-A molecules respectively. Variation at position 80 of HLA-C defines two groups of KIR ligands, the MHC-C1 allotypes (Asn80), and MHC-C2 allotypes (Lys80). The KIR2DL2 and KIR2DL3 receptors are specific for group C1, whereas KIR2DL1 is specific for group C2. The consequence of this natural variation at position 80 of HLA-C is an alteration in the specificity and strength of the HLA-C binding by KIR members. The interaction of C2 with KIR2DL1 is stronger and more specific than that of C1 with KIR2DL2 or KIR2DL3. However, both C allotypes are well represented in all human populations suggesting that both allotypes may have complementary functions providing variation in the HLA-C-mediated inhibition. As for HLA-C, HLA-B also presents a dimorphism in the C-terminal region of the α1-helix that determines the specificity for binding of inhibitory KIRs. As a result, two epitopes defined as Bw4 and Bw6 have been identified (54), but only Bw4 function as a KIR ligand which is recognized by KIR3DL1 (55, 56). Both epitopes are also present in all human populations suggesting complementary functions.

Besides members of the LCR family, the NKC also encodes for receptors that bind MHC class I molecules. These lectin-type

receptors are type II transmembrane proteins and the inhibitory and activating CD94/NKG2 receptors. In contrast to the evolutionary dynamic KIR system, the CD94:NKG2 is an older and more conserved system. CD94 associates with various NKG2 receptors such as NKG2A, NKG2C and NKG2E. In contrast, NKG2D is a homodimer. In contrast to human, which only encodes a single Ly49L pseudogene, the NKC of mouse includes the Ly49 family of lectin-type NKRs (44, 57). The expansion of Ly49 genes is only observed in rodents and horse. Of the three heterodimers, only NKG2A contains and ITIM motif and as consequence, is able to trigger inhibitory signals that tune NK cell activation (58). In contrast, NKG2C and NKG2E, by mean of a charged residue in their cytoplasmic tail, are able to associate with DAP-12 and to transmit activating signals (59, 60). The binding partner for NKG2A and NKG2C is the non-classical MHC class I molecule HLA-E (61, 62). HLA-E bind peptides derived from the leader peptide of HLA class I sequences (61, 63), which contain polymorphisms that either enhance or diminish peptide binding to HLA-E. In addition, HLA-E can bind heat-shock-protein derived peptides (64), viral peptides (65), and other ligands that also influence NK cell sensitivity (66). Because a proper folding and cell surface expression of HLA-E is dependent on such peptide binding, the surface abundance of HLA-E detected by CD94/NKG2A correlates with the amounts of HLA-A, HLA-B, and HLA-C being produced by any given cell. Therefore, polymorphism of HLA class I has important implications in NK cell development and function. A particular dimorphism in the leader peptides of HLA-B modulates its binding to HLA-E. Binding of this peptide to HLA-E is stronger when a methionine is at position -21 of the HLA-B leader sequence that when a threonine is at the same position (67). Based on this HLA-B dimorphism, humans are divided in three groups (M/M, M/T, and T/T). Those groups containing the M/M and M/T haplotypes have CD94-NKG2A+ NK cells that also express a repertoire of cell surface receptors more diverse and NK cell effector function are more potent than those NK cells form T/T haplotype (68). Thus, a simple dimorphism in HLA-B has had in important influence in NK cell function and diversity (68). Despite KIR and CD94/NKG2A evolved independently, they complement each other to direct NK cell development and function during immune response.

ACTIVATING NK CELL RECEPTORS

Receptors can favor NK cell activation through any of the following mechanisms: adhesion, granule polarization, and degranulation. Members of the natural cytotoxic receptor (NCR) family are among these receptors, including Nkp30 (69), Nkp44 (70, 71), (only in humans), and Nkp46 (72, 73). Members of this family are associated with ITAM-containing adapter transmembrane proteins such as CD3-ζ, FcεRIγ, and DAP-12. Many ligands have been identified for these receptors including viral and bacterial proteins, as well as cell surface endogenous molecules, although the physiologic relevance of these interactions remains uncertain. Other important activating receptors include NKG2D (74) and DNAX accessory molecule (DNAM)-1 (75). NKG2D is a C-type-lectin receptor, which recognizes several MHC class I-related ligands that are induced in cells undergoing viral infection or malignant transformation. DNAM is an activating receptor that binds to the poliovirus receptor CD155 and to the nectin adhesion molecule CD112, both of which are up-regulated in cancer cells. In consequence, DNAM has an essential role in preventing spontaneous tumor formation and in controlling tumor growth (76, 77) 4. DNAM promotes NK cell activation *via* an immunoreceptor tyrosine tail (ITT)-like motif that couplesDNAM-1 to Grb2 and to downstream effectors such as Vav-1 (78). Even though all these receptors contribute with positive signaling, none of them activate a full-cytotoxic function by itself.

Therefore, all these receptors can interplay and cooperate to overcome a critical threshold that counterbalances the effect of inhibitory receptors. In contrast, CD16, the low-affinity receptor for IgG (FcγRIII) and the mediator of antibody-dependent cellular cytotoxicity (ADCC), is the only receptor that seems to fulfill the necessary requirements to promote strong effector functions without requiring synergy through costimulatory receptors, although the molecular mechanism underlying this function remains unknown (79, 80) 47,48. CD16 is found to be associated with FcεRIγ chains or CD3ζ chains either as homodimers or heterodimers, each one containing ITAM motifs.

Beside receptors with a well-defined function of activating or inhibiting, NK cells also express receptors with the dual capability to activate or inhibit NK cell-mediated functions. The prototypic receptors with this "dual" function are those grouped in the SLAM family. This family belongs to the Ig superfamily and is composed by 6 members, named SLAM, CD244, Ly9, NTBA, CD84, andCD319 (81). Apart from CD244 (2B4), which recognizesCD48 (other member of the SLAM family) as a ligand, all members of this family are self-ligands and participate in the context of heterotypic or homotypic cell interactions. The expression of these receptors is restricted to the hematopoietic compartment. All members of the SLAM family harbor at least one tyrosine-based motif named immunoreceptor tyrosine-based switch motif (ITSM). This motif plays an essential role in determining the signals that are delivered downstream of the cytoplasmic tail of SLAM receptors. The tyrosine residue present in the ITSM motif is a bona fide site for interaction with members of the SAP family (82). In addition to the tyrosine residue of the ITSM motif, the cytoplasmic tail of SLAM receptors also harbors tyrosine residues that are potential sites for phosphorylation by Src family kinases. As a consequence, these tyrosine residues become sites for recruitment of SH2 domains-containing phosphatases. When these receptors are engaged, the SLAM receptors deliver signals that favor NK cell activation, in part by regulating inside-out signaling through LFA-1 or by enhancing granule polarization (83). In contrast, when SLAM receptors are engaged in NK cells lacking expression of SAP adapters, the signals emanating downstream lead to the recruitment and activation of SHIP1, which in turns favors Vav-1 dephosphorylation, a key element required to mediate NK cell activation. Therefore, SAP adapters are critical elements that

regulate NK-mediated functions in part by uncoupling SLAM receptors from recruiting phosphatases such as SHP-1 and SHIP1.

NK CELL REACTIVITY AND SELF-TOLERANCE

The missing-self hypothesis explained the close relation between inhibitory receptors for MHC-I molecules and NK cell reactivity and further studies allowed the following conclusions: (i) NK cells simultaneously express multiple inhibitory receptors in a random manner and some of these are specific for self-MHC class I, (ii) a unique NK cell repertoire is influenced by the extension of the number of MHC class I alleles in the host, and (iii) not all NK cells display the same reactivity towards allogeneic target cells (84, 85). Nevertheless, various studies also demonstrated the existence of NK cells lacking expression of inhibitory receptors with specificity for self-MHC class I (86), and according to the missing self-hypothesis, NK cells with no expression of inhibitory receptors for self-MHC class I, loss tolerance in an MHC class I-deficient host. However, observations made in humans who has loss the expression of MHC I molecules due to genetic errors, indicated that NK cells do not spontaneously overreact against the self (87). Same observations were obtained from studies in mice deficient for B2-microglobulin (β_2m) or transporters associated with antigen presentation (TAPs) (88–92). In contrast to expected, NK cells from MHC class I-deficient mice displayed a poor cytotoxicity activity towards MHC class I-deficient targets, despite they were normal in numbers, tissue distribution and expression of activating receptors. Moreover, NK cells from a host lacking MHC-class I expression, were unable to reject MHC class I-deficient bone marrow grafts compared to NK cells from MHC class I-sufficient mice. More detailed studies assessing NK cell responses on a per cell basis, found that NK cells expressing an inhibitory receptor for self-MHC class I displayed efficient effector functions in response to stimulation through activating receptors, but NK cells lacking such inhibitory receptors failed to mount effector responses (93). Moreover, the presence of MHC-I ligands increased the frequency of human NK cells expressing KIR family members, suggesting that frequencies of NK cell subsets expressing inhibitory receptors for MHC-I molecules is shaped by the self-MHC-I environment (94). Therefore, the capacity of a given individual NK cell to become functionally competent was correlated with the expression of an inhibitory receptor with specificity for self-MHC class I. This idea was further supported by studies in transgenic mice for a single-chain trimer specific for the inhibitory receptor Ly49, in which only NK cell expressing Ly49 inhibitory receptor showed a normal production of IFN-γ as compared to NK cells lacking the expression of such receptor. The explanation for this phenomenon was forged under different names including NK cell "education", "licensing", "arming", "disarming", among others. The basic idea is that mammals have evolved a mechanism by which NK cells, are positively selected during development for the expression of inhibitory receptors that recognize self-class I alleles. If such recognition takes place, then NK cells are allowed to undergo further maturation to become functionally competent. In contrast, when NK cells fail to recognize such self-class I molecules, further differentiation is arrested. This model allows explaining how NK cells become tolerant to self-MHC class I molecules while ensuring reactivity to allogeneic-class I molecules.

Three major models have been proposed to explain how NK cells become functionally competent. The first model is based on a "disarming" mechanism and propose that activating signals in immature NK cells are by default in "ON" mode. If such NK cells acquire inhibitory receptors, then interactions with MHC class I molecules prevent such default "ON" mode, NK cell tolerance is set-up and NK cells mature normally (**Figure 3A**). Under this scenario, functional activation signals ensure proper NK cell responses towards cells that have loss or downregulated MHC-I expression. However, if such NK cells fail to express inhibitory receptors for self-MHC-I, then prolonged or unopposed activation signals would lead eventually to a state of NK cell hypo-responsiveness. Conversely, the second model, based on a "licensing" or "arming" mechanism, sustains that activating signals are by default in "OFF" mode. When NK cells acquire the expression of inhibitory receptors for self-MHC-I, then the signaling through such inhibitory receptors capacitate activating receptors to become functionally competent (**Figure 3B**). Once stablished, same inhibitory receptors mediating capacitance for activating receptors would restrain activating signals, avoiding self-reactivity. If NK cells fail to express inhibitory receptors, then the activating receptors would remain functionally incompetent.

An essential difference between these two models is the nature of the signals that are triggered by inhibitory receptors for self-MHC-I. In immature NK cells, signaling through inhibitory receptors is dependent on ITIMs present in their cytoplasmic domains. When NK cells express an inhibitory receptor with a mutated ITIM, signals through activating receptors are dysfunctional rendering NK cells functionally incompetent. This dependence of acquiring NK cell reactivity for an ITIM domain is consistent with the two models. However, a qualitative difference for signals through inhibitory receptors is assumed. The default in "ON" mode implies a conventional inhibitory role, whereas the default in "OFF" mode connotes a new instructive role for inhibitory receptors. However, only the default in "ON" model assumes that a continuous and unrestrained signaling through activating receptors would induce an anergy-like responsiveness. A similar scenario was described in NK cells exposed to NKG2D ligand-expressing tumor cells *in vitro* (95), or enforced expression of NKG2D ligand *in vivo* (96), where continuous engagement by such ligands render NKG2D signaling dysfunctional. Same outcomes were found in mice expressing a viral protein from MCMV, where continuous engagement of a self-specific activating receptor during development induces NK cell tolerance (97, 98). Interestingly, the continuous NKG2D engagement not only affected NKG2D signaling but also those triggered by other activating receptors. For example, the signals through NK1.1 and CD16 also resulted

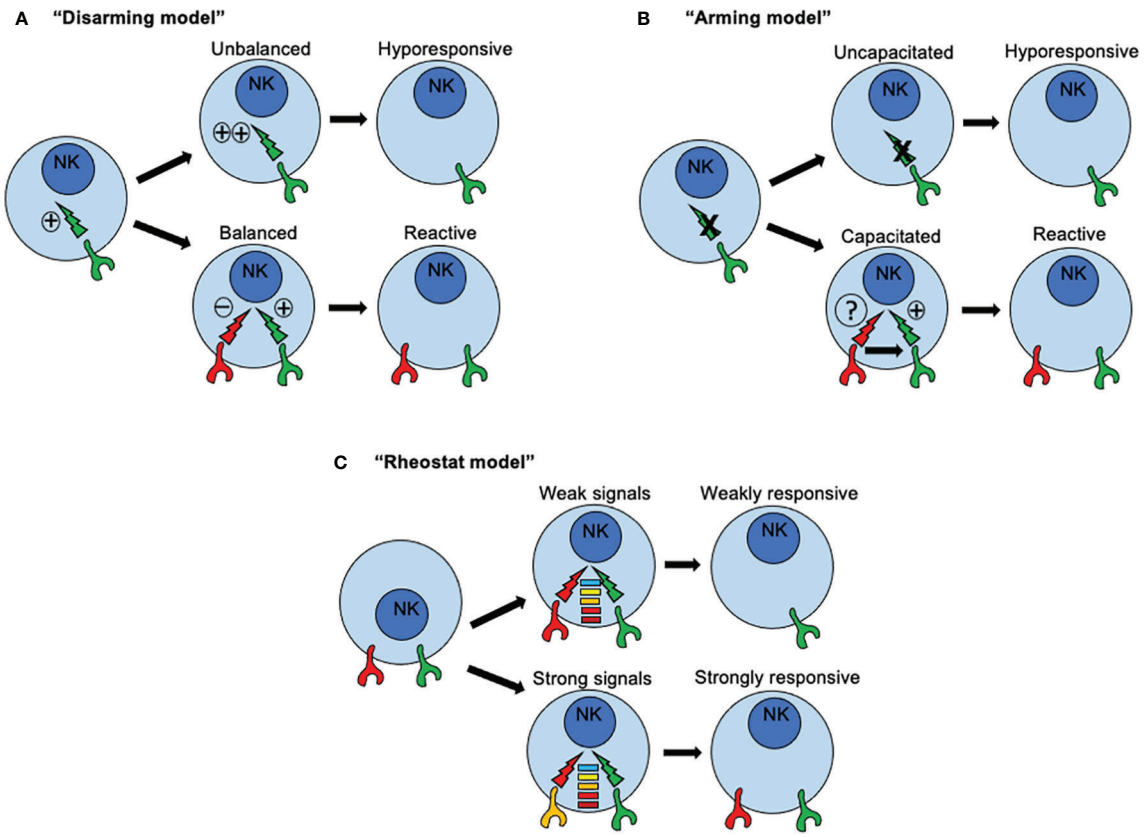

FIGURE 3 | How recognition of MHC class I molecules drive NK cell reactivity and self-tolerance. Three major models have been proposed to explain how NK cells become functionally competent. **(A)** The first model is based on a "disarming" mechanism and propose that activating signals in immature NK cells are by default in "ON" mode. If such NK cells acquire inhibitory receptors, then interactions with MHC class I molecules prevent such default "ON" mode, NK cell tolerance is set-up and NK cells mature normally. However, if such NK cells fail to express inhibitory receptors for self-MHC-I, then prolonged or unopposed activation signals would lead eventually to a state of NK cell hypo-responsiveness. **(B)** The second model, based on a "licensing" or "arming" mechanism, sustains that activating signals are by default in "OFF" mode. When NK cells acquire the expression of inhibitory receptors for self-MHC-I, then the signaling through such inhibitory receptors capacitate activating receptors to become functionally competent. If NK cells fail to express inhibitory receptors, then the activating receptors would remain functionally incompetent. **(C)** A third model known as the "rheostat" model has been proposed to explain how NK cell responsiveness in regulated during NK cell development According to this model, NK signals are not displayed as a binary system ("on" or "off"), but as an analogue system. The latter means that NK cell responsiveness can be "tune up" or "tune off" in a quantitative rather than in a binary manner depending on the inhibitory signal triggered by any particular inhibitory receptor for self-MHC class I ligand.

impaired due to sustained NKG2D signaling. These studies suggest that a cross-tolerogenic effect can be induced in NK cells (95). Further studies have observed that a single MHC class I allele may impact on different subsets of NK cells according to the repertoire of inhibitory receptors, and that a given subset of NK cells may can be influenced by several different MHC class I alleles. All these observations point to that idea that NK cell education is not an all-or-none phenomenon but this it can be described in quantitative terms (99, 100). As consequence, a third model known as the "rheostat" model has been proposed to explain how NK cell responsiveness in regulated during NK cell development (101). According to this model, NK signals are not displayed as a binary system ("on" or "off"), but as an analogue system (**Figure 3C**). The latter means that NK cell responsiveness can be "tune up" or "tune off" in a quantitative rather than in a binary manner depending on the inhibitory signal triggered by any particular inhibitory receptor for self-MHC class I ligand. This model does not exclude the previous mechanisms proposed.

Although we have learned that self-MHC-I molecules play a central role shaping the NK cell repertoire and regulating reactivity and tolerance, several issues remain to be addressed. For example, are NK cells educated only at certain time window during development? Or mature NK cells can be "reeducated"? A clue for this question may come from a recent study where the SLAMF6 receptor was found to be critical to control NK cell responsiveness. NK cells lacking the adapter protein SAP displayed enhanced responsiveness towards nonhematopoietic cells, this effect was also observed in response to the engagement of various activating receptors including DNAM, NKp46 and CD16, a phenomenon also observed during NK cell education mediated by KIRs and Ly49. This effect was no longer observed

in the absence of SLAMF6, indicating that this receptor was largely responsible to mediate these effects. Interestingly, the loss of SLAMF6 in the NK cell line YT-S also resulted in a diminished responsiveness towards nonhematopoietic cells. Therefore, this study suggest that education of NK cells may not be restricted to only during NK cell maturation but also may occur in mature NK cells, and that NK responsiveness may not be equally regulated for hematopoietic and nonhematopoietic target cells.

NK CELL-BASED THERAPY IN HEMATOLOGICAL MALIGNANCIES

Since their discovery in the 1970s, NK cells have been highlighted by their unique ability to recognize and eliminate cells undergoing neoplastic transformation. NK cells can not only directly kill tumor cells, but also can enhance antibody and T cell responses, therefore NK cell- immunotherapy is emerging as a promising therapeutic approach in cancer. Below we will discuss the major advances in NK cell-based immunotherapy against hematological malignancies.

Early clinical evidence in hematopoietic stem cell transplants (HSCTs) suggested that NK cells in the donor graft recognize and eliminate residual malignant cells (102). These findings encouraged to investigate the NK cells as effectors in immunotherapies against hematological malignancies. However, the clinical use of NK cells faces big challenges such as to obtain sufficient cells for adoptive transfer, the persistence of transferred cells and the low activity of naïve NK cells. In initial studies, NK cells for autologous therapy were expanded and activated *ex vivo* using interleukin-2 (IL-2), and even though the infusion of activated NK cells proved to be safe, it did not improve patient outcome (103). In a different approach, haplotype-mismatched HSCTs showed that NK cells exert potent antileukemic effects and did not cause graft-versus-host disease (GvHD), preventing leukemia relapse in acute myeloid leukemia (AML) patients (104, 105). This graft versus leukemia (GvL) effect is associated with a mismatch between host HLA groups and donor NK cell KIR ligands, in which the lack of "self" MHC class-I molecules on leukemia cells is crucial to unrestraint NK cell cytotoxicity (104–106). In addition, NK cells can suppress GvH disease by lysing alloreactive T cells and antigen-presenting cells (APCs), or either by inhibiting T cell proliferation (107–109).

In adoptive NK cell therapy, to avoid side effects such as GvHD or B-cell lymphoproliferative disease, is recommended to purify NK cells with minimal contamination by B or T cells (110, 111). A pioneering study of allogeneic NK cell transfer showed that haploidentical NK cells purified from related donors can expand *in vivo* after subcutaneous IL-2 administration and lymphodepleting chemotherapy. Although 30% of poor prognosis AML patients reached complete remission, the high intensive immune suppressive regimens and the high doses of IL-2 resulted in significant toxicities (112). In a posterior study, milder conditioning regimen and purified NK cells without *in vitro* exposure to cytokines allowed the engraftment of haploidentical NK cells and reduced the risk of relapse in pediatric patients with AML (113).

Cytokines such as IL-2, IL-15, IL 18 and IL-21 promote the proliferation, activation and cytotoxicity of NK cells against leukemia cells (112, 114–118). Feeder cells also support the expansion and activation of NK cells, including irradiated PBMCs (116, 119), EBV-LCL Epstein–Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) (120, 121) or gene-modified K562 cells expressing membrane-bound (mb)IL-15 or mbIL-21 (86, 122, 123). IL-15 has a preponderant role in NK cell function, differentiation, proliferation and survival (124, 125). Preclinical studies showed that IL-15 was able to expand and activate T cells and NK cells *in vivo* (126). IL-15 immunomodulatory properties are being explored alone or in combination with other agents to potentiate anti-tumor responses. IL-15 is frequently found associated to the receptor IL-15Rα on dendritic cells (127). It has been shown that soluble IL-15/recombinant IL-15Rα complexes elicit a rapid and strong expansion and activation of CD8+ T cells and NK cells, improving immune activation *in vivo* (128). The safety and efficacy of superagonist IL-15-IL-15Rα (ALT-803) was investigated in a clinical trial in hematological malignancies relapsed after HSCT. This study demonstrated that ALT-803 selectively expanded and

TABLE 1 | CAR-NK-cell therapy for hematological malignacies registered in clinicaltrials.gov.

Clinical trial identifier	Phase	Disease	Target	NK-cell source	Location
NCT02892695	I/II	Relapsed/Refractory Leukemia and Lymphoma	CD19	NK-92 cell line	China
NCT03056339	I/II	Relapsed/Refractory B-Lymphoid Malignancies	CD19	CB-NK cells	USA
NCT04555811	I	Diffuse large or high-grade B cell lymphoma.	CD19	hiPSC-derived NK cells (FT596)	USA
NCT04245722	I	Relapsed/Refractory B-cell Lymphoma and CLL	CD19	hiPSC-derived NK cells (FT596)	USA
NCT04887012	I	Refractory/Relapsed B-cell NHL	CD19	HLA haploidentical-NK cells	China
NCT04796675	I	Relapsed/Refractory B Lymphoid Malignancies	CD19	CB-NK cells	China
NCT00995137	I	Relapsed/Refractory B-lineage ALL	CD19	HLA haploidentical-NK cells	USA
NCT03690310	Early Phase I	Relapsed/Refractory B Cell Lymphoma	CD19	N/A	China
NCT04639739	Early Phase I	Refractory/Relapsed B-cell NHL	CD19	N/A	China
NCT03824964	Early Phase I	Relapsed/Refractory B Cell Lymphoma	CD19/CD22	N/A	China
NCT03692767	Early Phase I	Relapsed/Refractory B Cell Lymphoma	CD22	N/A	China
NCT02944162	I/II	Relapsed/Refractory AML	CD33	NK-92 cell line	China
NCT04623944	I	Relapsed/Refractory AML or MDS	NKG2D	N/A	USA
NCT03940833	I/II	Relapse/Refractory MM	BCMA	NK-92 cell line	China
NCT02742727	I/II	Relapsed/Refractory Leukemia and Lymphoma	CD7	NK-92 cell line	China

activated CD8+ T cells and NK cells, producing favorable responses in almost 20% of the patients (including 1 complete remission) (129). Currently, some clinical trials in hematological malignancies are investigating safety and efficacy of ALT-803 in combination with NK cell adoptive therapy or antibodies that elicit antibody-dependent cell cytotoxicity (ADCC) (**Table 1**) (clinicaltrials.gov: NCT02384954, NCT01898793 and NCT02782546). In a different study, the recombinant human IL-15 (rhIL-15) receptor agonist NKTR-255 was designed to obtain a sustainable activation of the IL-15 pathway. A phase I study of NKTR-255 as monotherapy and in combination with antibodies in relapsed/refractory hematologic malignancies is ongoing (clinicaltrials.gov: NCT04136756) (130).

The first in-human trial with activated NK cells using the K562-membrane-bound IL-15 (K562-mb15-41BBL) feeder cell line, derived from HLA-matched donors and infused into patients with high-risk solid tumors, resulted in an unexpected high incidence of acute GvHD, even after T-cell depletion, suggesting that allogenic IL-15/4-1BBL activated NK cells can contribute to GvHD (131). In contrast, adoptive transfer of donor-derived NK cells activated and expanded (NKAE) using the K562-mbIL21 line in patients with hematological malignancies, did not increase the risk of GvHD (132). Furthermore, autologous transfer of NKAE cells using the K562-mIb15-41BBL cell line was well tolerated in patients with a bad prognosis multiple myeloma (MM) and showed clinical efficacy in two of five subjects (133). Currently, some clinical trials in pediatric acute leukemia (clinicaltrials.gov: NCT01944982, NCT02074657 and NCT02763475) are addressing the clinical efficacy of haploidentical NKAE cells in combination with chemotherapy to eliminate chemotherapy-resistant leukemic cells (118, 134). Human NK cells have the ability to retain an intrinsic memory of prior activation after stimulation with cytokines, exhibiting enhanced IFN-γ and TNF production and cytotoxicity upon reactivation (135, 136). In a phase I study of adoptive transfer in poor-prognosis AML patients, memory-like NK (ML NK) cells were generated by pre-activation of haploidentical NK cells with IL-12, IL-15, and IL-18. ML NK cells efficiently expanded *in vivo* and displayed enhanced cytotoxicity against leukemia, and favorable outcomes were observed in five of nine patients, including four complete remissions (117). Preliminary results of other phase I study in patients with relapsed myeloid malignancies after haploidentical donor transplant (**Table 1**) (clinicaltrials.gov: NCT040247761), showed that ML NK cells expanded massively and persisted for several months after infusion (/doi.org/10.1182/blood-2020-133933).

Sources of NK Cells for Therapy

Early studies in adoptive cell therapy used NK cells derived from peripheral blood (PB) since they are easy to obtain and have a mature phenotype. However, NK cells represent a small and heterogeneous fraction of PB cells with a reduced proliferative capacity, and it is difficult to implement large-scale expansion methods for clinical uses (112, 137, 138). In addition, autologous PB-NK cells have not given the expected results in the clinic since the NK cells from cancer patients are often functionally impaired (103, 137). Therefore, alternative allogenic sources of NK cells are being explored for cancer immunotherapy.

Higher percentage of NK cells can be isolated from Umbilical Cord Blood (UCB), which are easy to collect and cryopreserve, offering an attractive source of NK cells for therapy (139). In one study NK cells derived from cord blood (CB) were expanded and activated *ex vivo* using K562 cells expressing IL-2, and transferred into multiple myeloma patient in combination with chemotherapy and autologous HSCT. This study resulted in 10 of 12 patients achieving a good partial response and revealed that CB-NK cells maintain an active phenotype *in vivo* but did not persist for long-term after infusion (130). Another clinical trial used CD34+ progenitor cells isolated from partly HLA-matched UCB units to generate NK cells. The hematopoietic stem and progenitor NK cells (HSPC-NK) generated *ex vivo* were safely infused in AML patients after immunosuppressive chemotherapy and exerted a transient antileukemic effect. This study demonstrated that HSPC-NK cells engraft and undergo *in vivo* maturation (140).

The NK-92 cell line, derived from a lymphoma patient, is a homogeneous source of NK cells that can be easily genetically manipulated, cryopreserved and expanded with a high purity and function (141). Nevertheless, the NK-92 cell line needs to be irradiated before infusion due to its tumorigenic potential, which limits the *in vivo* survival and expansion of these cells, and very high doses of NK-92 cells are required for allogenic administration (142). A phase I trial for refractory hematological malignancies relapsing after autologous HSCT, demonstrated that irradiated NK-92 cells infusions are safe even at high doses (142). Two more clinical trials are addressing the safety and efficacy of NK-92 cells in hematological malignancies (**Table 1**) (clinicaltrials. gov: NCT00900809 and NCT00990717).

NK cells also can be derived from human pluripotent stem cells (hPSCs) (143). Human PSC-derived NK cells (hPSC-NK) have a potent anti-tumour activity and proliferative capacity, and are easy to genetically modify (143). One major advantage of hPSC-NK cells and NK-92 cells is the possibility to obtain enough NK cells to allow multiple infusions into patients, in a cost effective and standardized manner. However, hPSC-NK cells have the potential for malignant transformation and safety should be careful addressed in clinical trials. Since NK cells have a lifespan of about 2 weeks (144), stimulation with IL-15 has been used to improve the persistence of NK cells after infusion *in vivo* (129). The Cytokine-inducible SH2-containing protein (CIS), encoded by the CISH gene, is a negative regulator of IL-15 signaling in NK cells (145). To improve survival and anti-tumor activity of NK cells derived from human induced pluripotent stem cells (iPSC), the CISH gene was deleted in iPS cells using the CRISPR/Cas9 technology. CISH−/− iPSC-NK cells resulted in increased IL-15 signaling, increased expansion and enhanced cytotoxicity. In an AML xenograft model, CISH −/− iPSC-NK cells had an improved *in vivo* persistence and superior anti-tumor activity in comparison with WT iPSC-NK cells and (146).

However, iNK cells are characterized by a lower CD16 expression compared to PB-NK cells (147). The Fc receptor CD16a is cleaved by the ADAM17 metalloprotease upon stimulation (148). To stabilize the expression of CD16a, iPSCs were genetically engineered to express a high-affinity variant of CD16a with a mutation that confers resistance to ADAM17-mediated cleavage (149) (hnCD16). NK cells derived from hnCD16-iPSC (hnCD16-iNK) were functionally mature,

maintained high levels of stable CD16a expression and exhibited enhanced ADCC against multiple cancer cell lines *in vitro*. Preclinical xenograft studies with hnCD16-iNK cells and the anti-CD20 antibody led to improved regression of B-cell lymphoma tumors compared with PB-NK cells or unmodified iNK cells (149). A phase I study of hnCD16-iNK cells (FT516) as monotherapy in AML and in combination with an anti-CD20 antibody in B-cell lymphoma is undergoing (clinicaltrials. gov: NCT04023071). Preliminary results in patients with relapsed/refractory B-cell lymphoma showed that two of four patients achieved a complete response and another patient achieved a partial response, with no associated toxicities (Fate-Therapeutics-Reports-Positive-Interim-Data-from-its-Phase-1-Study-of-FT516-in-Combination-with-Rituximab-for-B-cell-Lymphoma.html. Accessed 12 August 2021).

Monoclonal Antibodies Enhance NK Cell-Mediated ADCC in Hematological Cancers

NK cells are the main lymphocyte subset responsible for the ADCC. In cancer immunotherapy, antibody-dependent tumor killing through NK cells relies in the development of monoclonal antibodies (mAB) directed against highly expressed antigens in tumor cells. The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved several monoclonal antibodies to mediate ADCC in hematological cancers, used as single agent or in combined regimens. Rituximab was the first mAb approved by FDA for the treatment of Non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) (150). Rituximab is a chimeric mouse/human anti-CD20 mAb, which mainly acts by inducing ADCC of NK cells and complement-dependent cytotoxicity (CDC) (150). Since approximately half of all patients become refractory to rituximab treatment, new anti-CD20 mAbs with an increased ADCC have been developed, such as Ocrelizumab (151), Obinutuzumab (152) and Ublituximab (153). Alemtuzumab is another mAB approved for the treatment of patients with relapsed/refractory CLL (154). Alemtuzumab targets CD52 and its mechanisms of action mainly rely on NK-mediated ADCC (155). Daratumumab is a human mAB that targets CD38, approved as monotherapy and in combination with standards of care for MM. Preclinical studies have shown that daratumumab induces cell death through mechanisms such as ADCC and CDC (156). Elotuzumab is a humanized mAB that targets the glycoprotein SLAM Family Member 7 (SLAMF7), also known as CS1, approved for the treatment of relapsed and/or refractory MM in combination with other agents. Elotuzumab induces NK cell cytotoxicity against myeloma cells directly thought ADCC or indirectly by facilitating SLAMF7-SLAMF7 interactions between NK cells and myeloma cells to enhance natural cytotoxicity (157).

Furthermore, antibodies directed to immune checkpoint receptors, capable to regulate NK cells response, are being tested in cancer therapy. Lirilumab is a mAB that binds to KIR2DL1 and enhance NK antitumor effect by increasing NK-cell-mediated killing of HLA-C expressing tumor cells (158). Phase 1 clinical trials of lirilumab in hematological malignancies have demonstrated that prolonged KIR blockade is safe and well tolerated by patients (158, 159), and additional studies as single agent or in association with other immunomodulatory drugs are underway (**Table 1**) (clinicaltrials.gov: NCT02252263, NCT01687387). Monalizumab is an anti NKG2A-mAb that block NKG2A-HLA-E binding and restore the function of NK cells by blocking the inhibitory signals given by tumor cells expressing HLA-E (160). Preclinical studies in CLL demonstrated that blockade of NKG2A though monalizumab restores cytotoxicity of NK-cells against HLA-E+ CLL cells (161). An ongoing phase I trial is investigating the safety of monalizumab in hematologic malignancies after HLA matched allogenic HSTC (clinicaltrials.gov: NCT02921685).

Bispecific and Trispecific Killer Cell Engagers

New NK cell-based therapeutic approaches involve the use of bispecific and trispecific killer engagers, BiKEs and TriKEs respectively, which target CD16 and tumor antigens to induce the formation of immune synapses between NK cells and tumor cells, triggering cytotoxic responses (162). The group of Dr. Jeffrey Miller has developed several BiKEs and TriKEs as potential agents for leukemias and lymphomas immunotherapy. They have demonstrated that the CD16/CD19 BiKE and the CD16/CD19/CD22 TriKE enhanced NK cell cytotoxicity against B-cell tumor cell lines and primary leukemia cells (163). Also a BiKEs that targets CD16 along with CD33 (CD16/CD33) facilitated effective NK cell elimination of primary CD33+ AML and CD33+ MDS patient cells (164, 165). However, they found a direct correlation between NK cell activation and the downregulation of CD16 expression after exposure to the CD16/33 BiKE (164). The same group designed the CD16/IL-15/CD33 TriKE by integrating IL-15 into the CD16/CD33 BiKE, to deliver a proliferation/survival signal to NK cells and enhance the *in vivo* persistence. The CD16/IL-15/CD33 TriKE not only was able to enhance NK function against CD33+ cell lines and AML blasts, but also to expand and sustain human NK cells *in vivo*, reducing tumor burden in a xenograft model (166). This TriKE (GTB-3550) is currently in Phase I/II clinical trial for high-risk hematological malignancies (clinicaltrials.gov: NCT03214666).

Chimeric Antigen Receptor-Engineered NK Cells

The chimeric antigen receptor (CAR) -T cell therapy has achieved great success treating patients with hematological malignancies (167). The approval of CAR-T cell therapy in the United States, Europe, Canada, Australia and Japan represent a major milestone of genetically modified cell immunotherapy (168). The FDA in the US and the EMA in Europe had already approved three CAR-T products for B-cell malignancies that target the CD-19 antigen: (1) Tisagenlecleucel (KYMRIAH), Novartis Pharmaceuticals Corporation, for relapsed B-cell Acute Lymphoblastic Leukemia (ALL) and relapsed or refractory large B cell lymphoma, (2) Axicabtagene Ciloleucel (YESCARTA), Kite Pharma Inc., for relapsed or refractory large B cell lymphoma, and brexucabtagene autoleucel (TECARTUS), Kite Pharma Inc., for the treatment of relapsed or refractory mantle cell lymphoma.

The CAR is an engineered protein based on the T cell receptor (TCR) that is composed of an extracellular antigen binding domain, followed by a transmembrane region and an

intracellular signaling transduction domain (167). The CAR protein engages the immune cell with the cancer cell antigen and then transform the binding signal into a signaling cascade that ultimately induce tumor killing (167). The expression of a CAR in NK cells is a promising strategy to improve anti-tumor functions by enhancing NK cells cytotoxicity and overcoming immune evasion. In contrast to CAR-T cells, CAR-NK cells transfer do no mediate GvHD, cytokine release syndrome or immune effector cell-associated neurotoxicity syndrome (169). In addition, CAR-NK cells are capable of killing malignant cells either by their intrinsic cytotoxic capacity or through the CAR-dependent mechanism (170). Predominantly CAR-NK cells have been engineered using the same constructs designed for CAR-T cells. The first generation of CARs consisted in a synthetic protein that fused an antibody-like domain to the CD3ζ chain of a TCR. Second generation CARs coupled additional co-stimulatory signaling domains, like CD28, CD134 (OX40) or CD137 (4-1BB), to improve the activation and enhance survival and expansion of the modified cells. Third-generation CARs combine two co-stimulatory domains. Fourth-generation CARs are being designed to improve the *in vivo* proliferation and persistence of immune effectors or to control potentially side effects, by for example the insertion of cytokines or suicide genes (167, 171). Few CAR constructs have been designed specifically for NK cells by incorporating DAP10 as co-stimulatory domain and NKG2D as transmembrane domain (172, 173). The therapeutic potential of CAR-NK cells in hematological malignancies is being explored in several preclinical studies and some clinical trials.

CAR-NK Cells Pre-Clinical Development

The initial CAR-NK cells studies focused on B cell malignancies (174–176). As an example, allogenic PB NK cells were expanded and nucleofected with the anti-CD20-4-1BB-CD3ζ construct to generate anti-CD20 CAR-NK cells that improved cytotoxicity against NK cell resistant B-cell lymphoma cells *in vitro*, and also reduced tumor burden and enhanced survival in a xenograft mouse model (177). In another study, memory like NK cells were generated after stimulation of PB NK cells with IL-12, IL-15, and IL-18, and then genetically modified to express the CD19-4-1BB-CD3ζ construct. The 19-CAR-ML NK cells effectively expanded and persisted *in vivo*, and also controlled tumor growth and prolonged survival in a human xenograft model. Importantly, lymphoma patient 19-CAR-ML NK cells displayed an improved antitumor activity against autologous lymphoma cells (178). The group of Dr. Rezvani expanded and modified CB NK cells with a fourth generation CAR vector (iCasp9-CAR19-CD28-CD3ζ-IL-15) that incorporated the IL-15, to enhance CAR-NK cells proliferation and persistence, and the inducible caspase-9 (iC9) suicide gene. The iC9/CAR.19/IL-15-NK cells efficiently killed a lymphoma cell line and primary CLL leukemia cells *in vitro*, and showed long-term persistence and improved antitumor activity *in vivo* (179). Deletion of the CIS negative regulator of IL-15 signaling (CISH gene knock down) improves the metabolic fitness and antitumor efficacy of CB-NK cells (180). The CISH gene was deleted in iC9/CAR.19/IL-15-NK cells to generate CISH-/- iC9/CAR19/IL-15 CAR-NK cells. *In vivo* studies revealed that CISH-/- iC9/CAR19/IL-15 CB-NK cells persisted twice as long as control CAR19/IL-15 NK cells and significantly improved anti-tumor responses since animal treated with CISH KO iC9/CAR19/IL-15 CB-NK cells were tumor free (181).

Human iPSC-derived CAR19 NK cells (FT596) were generated by modifying the hnCD16-iNK cells (149) with the CD19-NKG2D-2B4-CD3ζ CAR construct and an IL-15 receptor fusion (IL-15RF) gene. FT596 in combination with rituximab promoted synergistic anti-tumor responses against CD19+ CD20+ B lymphoblast cells *in vitro*. Also, FT596 in combination with rituximab showed enhanced killing of tumor cells in a lymphoma mouse model, as compared to CAR19 T cells (doi.org/10.1182/blood-2019-129319). Despite the good results obtained in CD19-directed therapies in B-cell malignancies, some patients still relapse due to the loss of the CD19 antigen (182) (183). To overcome altered CD19 expression or loss of the antigen in the treatment of pre-B cell acute lymphoblastic leukemia (B-ALL), FMS-like tyrosine kinase 3 (FLT-3)-specific CAR-NK-92 cells (NK-92/4G8.28.z) were generated by transduction of NK-92 cells with an FLT3-CD28-CD3ζ vector. NK-92/4G8.28.z cells were specifically activated by FLT3+ leukemia cells *in vitro* and significantly delayed disease development in an aggressive leukemia xenograft model (184). To improve the safety of this system, the FLT3-specific CAR 4G8.28.z was co-expressed with iCasp9 an inducible caspase-9 (iCasp9) suicide gene in NK-92 cells, which resulted in the elimination of NK-92/iCasp9_2A_4G8.28.z cells upon iCasp9 activation (184).

Multiple myeloma is a heterogenous hematologic malignancy that urgently needs novel therapies as most of the patients relapse with current treatments. Preclinical data using an anti-CD138 CAR, generated after transduction of the NK-92MI cell line (which express human IL-2) (185), with the CD138-CD3ζ vector, showed enhanced cytotoxicity on CD138+ MM cells *in vitro* and *in vivo* (186). Other study used the NK-92 cell line to express the CS1-CD28-CD3 ζ CAR construct. CS1-CAR NK cells efficiently eradicated CS1-expressing MM cells, both *in vitro* and *in vivo* (187). NKG2D is an activating receptor with broad target specificity and NKG2D-based CAR therapy could potentially target a large variety of human tumors. The efficacy of a NKG2D-CAR, generated by genetic engineering of activated and expanded NK cells (NKAE) with the NKG2D-4-1BB-CD3z-CAR construct, was analyzed in a preclinical study for MM. NKG2D-CAR-NKAE cells demonstrated to be safe and efficiently eradicated MM cells *in vitro* and displayed potent anti-tumor efficacy *in vivo* (188).

One major obstacle to targeting AML with immunotherapy is that many myeloid antigens are expressed at similar levels on normal and malignant cells. The interleukin-3 receptor alpha chain (CD123) is highly expressed on the many primary AML cells (189). The NK-92 cell line and PB NK cells were genetically modified to express the CAR construct CD123- CD28, 4-1BB-CD3ζ to generate CD123-CAR-NK. Both, CD123-CAR-NK-92 cells and CD123-CAR-NK cells, showed anti leukemic function, however CD123-CAR-NK-92 cells were more stable and had stronger cytotoxic activity against leukemia cells lines and primary AML cells (190). CD4 is an antigen expressed in M4 and M5 subtypes of AML. NK-92 cell were transduced with the CD4-CD28,

4-1BB-CD3ζ CAR vector to obtain CD4CAR NK-92 cells, which successfully eradicated CD4+ AML cells *in vitro* and demonstrated robust anti leukemic activity in a CD4+ AML xenograft mouse model (191). Other strategy to target AML cells utilized CAR NK cells expressing the activating receptor NKG2D (NKX109), generated with the CAR vector NKG2-OX40-CD3ζ, and the mbIL-15. Pre-clinical data in AML xenografts mouse models showed that NKX101 had enhanced antitumor function as compared with WT NK cells (doi.org/10.1182/blood-2020-134625)

T-cell based malignancies remain a challenge for CAR T-cell therapy since most target antigens are shared between malignant cells and the therapeutic agent, leading to CAR-T cell fratricide, therefore CAR-NK therapy is a promising alternative for T cell malignancies. CD4 is also expressed in Peripheral T-cell lymphomas (PTCLs) and CD4CAR NK-92 cells could also eliminated CD4+ T-cell leukemia and lymphoma cells *in vitro* and reduced tumor burden and prolonged survival in a T cell lymphoma xenograft model (192). CD3 is an antigen expressed in PTCLs and a small subset of T-cell acute lymphoblastic leukemia (T-ALL). CD3 CAR NK-92 cells, generated by genetic engineering of NK-92 cells using the C3-4-1BB-CD28-CD3ζ construction, specifically eliminated CD3+ lymphoma/leukemic cells, suppressed tumor growth and significantly prolonged survival in a CD3+ lymphoma xenograft model (193). Other preclinical study targeted the CD5 antigen, which is expressed in a majority of T-cell malignancies. Anti CD5-specific CAR NK cells were generated by modifying NK-92 cells with the CD5-4-1BB-CD28-CD3ζ vector. CD5 CAR-NK-92 cells displayed specific cytotoxic responses against CD5+ cell lines and CD5+ primary tumor cells *in vitro* and significantly inhibited disease progression in xenograft mouse models (194).

CAR-NK Cell Clinical Studies

Currently, there are 15 clinical trials registered in ClinicalTrials.gov (August 2021), evaluating the safety and efficacy of CAR-NK cells in hematological malignancies, with two published studies (NCT02944162 and NCT03056339).

This fist clinical trial (NCT02944162) included only 3 patients with relapsed or refractory AML and used anti-CD33 CAR-NK cells as therapeutic agent, generated by transduction of NK-92-MI cells with the CD33-CD28-4-1BB-CD3ζ CAR construct. CD33-CAR NK-92 cell infusions were safely administrated to patients in three increasing doses; however, this study did not show durable responses (195). Dr. Rezvani's group reported the result of a dose escalation study of HLA-mismatched CB-derived CAR-NK-CD19 cells [iC9/CAR.19/IL-15-NK cells (179)] in combination with lymphodepleting chemotherapy in 11 patients with relapsed or refractory CD19- B-cell lymphoid malignancies (NCT03056339). After infusion CAR-NK-CD19 cells expanded and persisted at low levels for at least 12 months, in the absence of neurotoxicity, CRS or GvHD. 8 out of 11 patients showed a clinical response, with 7 patients going into complete remission (169).

For the rest of the trials clinical results are currently pending. Most of the clinical are focused in B-cell malignancies and target lineage marker CD19 (9) or CD22 (2). Two studies are focused in AML/Myelodysplastic Syndromes and target CD33 or utilized NKG2D CAR-NK cells, while one study targets BCMA in MM and other CD7 in T-cell malignancies. Trials utilize NK-92 cells, allogeneic NK cells, two studies use IPSC-derived NK cells (FT596) and one CB NK cells.

CONCLUSIONS

Since the first description, more than 50 years ago, important advances in the field of immunology has been made from studying and understanding the NK cell function. The rising of the missing self-hypothesis led us to appreciate how immune system, by mean of NK cells, is able to recognize the self and the non-self through a system that differs from other immune cells (pattern recognition receptors). This system relies on an array of receptors specific for self-MHC class I molecules, which expression is often perturbed in infected or malignant cells. These arrays of receptors are coupled to a module of inhibition that prevents unsolicited NK cell effector functions. When inhibition signals are in an Off-mode, as consequence of absence of self-MHC class I molecules, or due to a miss-match between inhibitory receptors and MHC-class I molecules, then NK cells can mobilize the cytotoxic machinery. This last scenario, is observed in the graft versus leukemia effect, and has become in an important immunotherapeutic resource for the treatment of hematological malignancies. Moreover, blocking the ligation of inhibitory receptors through monoclonal antibodies has been shown to be useful to unleash T cell responses and this option needs to be more explored in NK cells. However, a deeper understanding of the signaling mechanism, both positive and negative, that control NK cell reactivity and tolerance may allow to identify novel therapeutic targets. In addition, a better understanding of the mechanisms that regulate NK cell education may represent another important opportunity field to re-educate NK cells. In this sense, the ability of NK cells to undergo "adaptation" to new environmental clues indicates that NK cells display inherent cellular plasticity, and as consequence, this plasticity may be tuned-up to improve NK cell responsiveness. Therefore, NK cells represent a successful example of how basic research still represent our best way to generate new knowledge that can be interpretated to design and develop new therapeutic approaches against hematological malignancies and perhaps other cancers.

AUTHORS CONTRIBUTIONS

VR-M write the manuscript; JG write the manuscript; JA write the manuscript; MC-M conceive and write review. All authors contributed to the article and approved the submitted version.

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IL-33 and the PKA Pathway Regulate ILC2 Populations Expressing IL-9 and ST2

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Type 2 Innate lymphoid cells (ILC2s) are tissue-resident immune cells activated by epithelial-derived alarmins upon tissue damage. They regulate immunity against helminth parasites and allergies by expressing type 2 immune response cytokines including IL-9, known to be critical for inducing and potentiating the immune response in such context. Although ILC2s are reported to be the main source of IL-9 in mice during *N. brasiliensis* infection, the mechanisms that regulate the expression of IL-9 in these cells are yet to be described. Recent studies have shown that in addition to cytokines, multiple molecules can differentially modulate the functions of ILC2s in various contexts both *in vitro* and *in vivo*. Among these stimuli are lipid mediators and neuropeptides, which activate the PKA pathway and have been associated with the regulation of type 2 immune cytokines. In this work we found that ILC2s in mice infected with *N. brasiliensis* can be classified into different groups based on the expression of IL-9 and ST2. These distinct populations were distributed in the lung and the small intestine. Through the development of an *in vitro* culture system, we sought to determine the stimuli that regulate the expression of these markers in ILC2s. We identified the alarmin IL-33 as being a key player for increased IL-9 expression. Additionally, we found the PKA pathway to be a dual regulator of ILC2 cells, working synergistically with IL-33 to enhance IL-9 production and capable of modulating proliferation and the expression of ILC2 markers. These data provide further evidence of a high heterogeneity between ILC2 subsets in a context dependent manner and calls for careful consideration when choosing the markers to identify these cells *in vivo*. Distinguishing ILC2 subsets and dissecting their mechanisms of activation is critical for a deeper understanding of the biology of these cells, allowing their manipulation for therapeutic purposes.

Keywords: IL-9, ILC2, ST2, IL-33, PKA, pathway, regulation

INTRODUCTION

Innate lymphoid cells (ILC) are tissue-resident immune cells lacking receptors to recognize specific antigens; hence their activation depends on cytokines present in the microenvironment (1). Type 2 ILCs or ILC2s are mainly found in the lung, intestine and skin, where they are activated by the epithelial cell-derived alarmins IL-25, TSLP, and IL-33 upon tissue damage (2), although they can also respond to cytokines derived from the immune compartment such as IL-2 and IL-7 (3).

ILC2s are mainly tissue resident cells; however, their precursors reside in the bone marrow, where they maturate after going through different stages of differentiation. Migration of ILC2 precursors from the bone marrow to different peripheral tissues such as skin and lung has been shown to occur in response to intravenous IL-33 administration or IL-33-dependent pulmonary fungal allergen challenge (4). In addition, intranasally administered IL-33 can have a direct effect on bone marrow derived ILC2s, inducing their production of IL-5, demonstrating an important role for these cells in the induction of eosinophilia derived from inflammation in the upper airways (5).

Recent studies have shown that multiple molecules besides cytokines can regulate the functions of ILC2s in different contexts both *in vitro* and *in vivo*. These stimuli include the lipid mediators leukotrienes and prostaglandins, neuropeptides, hormones and nutrients (6), thereby demonstrating that the regulation of ILC2 function is tightly controlled by a variety of signals within the cellular microenvironment. Several of these stimuli act through the cAMP/PKA pathway.

Cyclic adenosine monophosphate (cAMP) has been established as a universal regulator of metabolism and gene expression in all living organisms (7). The levels of this messenger are regulated by different enzymes such as adenylate cyclase and phosphodiesterases (8). Although cAMP acts in multiple downstream pathways, its most prominent role is the activation of the protein kinase A (PKA). Upon binding of cAMP, PKA dissociates into its regulatory and catalytic subunits. The catalytic subunits then phosphorylate specific Ser and Thr residues on numerous downstream target proteins involved in different signaling pathways. For example, cAMP-activated PKA binds and phosphorylates cAMP-responsive transcription factors, including cAMP-response element binding protein (CREB), activating transcription factor-1 (ATF-1), Nuclear Factor κB (NFκB), and nuclear receptors (8).

Studies exploring the effects of cAMP, PKA or CREB activation on ILCs are limited. However, on helper T cells, ILCs adaptive cellular counterparts, they regulate cytokine expression, proliferation and apoptosis (8–12). Altogether these reports suggest that activation of the cAMP/PKA pathway has a negative effect on T cell survival by inhibiting cell proliferation and increasing apoptosis (10–12). The cAMP pathway was recently shown to suppress ILC2 function through mediators such as PGE2 and PGI2 (13, 14). Contrastingly, cAMP signaling mediated by neuropeptides activates ILC2s (6), demonstrating a dual regulation of these cells that is context dependent.

ILC2s are characterized by the expression of cytokines associated with the type 2 immune response, including IL-4, IL-5, IL-13 and IL-9, all important in the regulation of immunity against helminth parasites and allergies (15). The expression of IL-9 derived from ILCs was first confirmed in 2011 using a fate reporter in models of sterile inflammation of upper airways (16). A couple of years later, the first evidence of IL-9 expression specifically in ILC2s *in vivo* was reported, when ILC2s were found to be the main source of IL-9 expression in mice during *Nippostrongylus brasiliensis (N. brasiliensis)* infection (17). ILC2 cells were confirmed to express high levels of the IL-9 receptor (18) and this cytokine was found to act in an autocrine manner, promoting IL-5, IL-13 and amphiregulin expression as well as ILC2 survival (19). Despite these findings, the mechanisms controlling the expression of IL-9 in ILC2s remain unclear.

Most studies on the regulation of ILC2 cytokines by cAMP-associated pathways have been focused on IL-5 and IL-13 in different inflammation models (13, 20). However, studies exploring how the cAMP pathway could potentially regulate IL-9, and cellular processes such as proliferation and survival, are lacking.

Suppressor of Tumorigenicity 2 (ST2), a component of the IL-33 receptor, is commonly used as a marker for the identification of ILC2s. In mice, ST2 is expressed on ILC2s in the lung, and can also be found in bone marrow and adipose tissue, while its basal expression is limited in tissues such as the gut and skin (21, 22). IL-33 signals through ST2 to activate ILC2s, which produce IL-5 and IL-13 in models of upper airway inflammation and helminth infection (23–25).

In this work, we explored the signals that control IL-9 expression in ILC2s and how the regulation of PKA directs the function of these cells. We found that during *N. brasiliensis* infection, different populations of ILC2s can be distinguished based on their IL-9 and ST2 expression. These populations are distributed in the different anatomical compartments analyzed. *In vitro*, IL-33 and the cAMP/PKA pathway modulated the proliferation of ILC2s and regulated their expression of IL-9 and other markers, establishing the stimuli that could be regulating the heterogeneity and function of these cells in the different compartments during infection with *N. brasiliensis in vivo*.

EXPERIMENTAL PROCEDURES

Mice

All mice used were on C57BL/6 background at 8–12 weeks of age. INFER and KN2 mice were generated and genotyped as previously described, used in heterozygosity (17, 26) and maintained according to the bioethics and biosecurity norms from the Instituto de Fisiología Celular in the National University of Mexico.

N. brasiliensis Infection

Mice were infected subcutaneously with 200 viable third stage *N. brasiliensis* larvae. Animals were sacrificed at different time points post-infection, and tissues harvested and processed for flow cytometry staining as described below.

Tissue Processing

The mediastinal and mesenteric lymph nodes were isolated, homogenized, filtered and resuspended in RPMI media

supplemented with 10% fetal bovine serum (FBS) for subsequent analysis by flow cytometry. The lung was isolated and digested with 1 mg/mL collagenase D (Sigma) and 20 μg/mL DNAse (Sigma). The samples were subsequently homogenized and subjected to an Optiprep gradient followed by resuspension in medium for analysis by flow cytometry. The small intestine was processed according to the protocol of Ferrer-Font et al. (27) with some modifications for the recovery of intraepithelial cells. Cells isolated from the small intestine are extremely sensitive and viability is compromised with prolonged processing times; hence, we recommend working as fast as possible with no more than 3 mice simultaneously. In short, the small intestine was isolated and the Peyer patches were extracted. The intestine was then opened longitudinally, cut into small pieces, and washed 3 times with PBS. The small intestine pieces were incubated with EDTA solution (2mM) in HBSS, and the supernatants were recovered for the analysis of intraepithelial cells. These supernatants were washed once with PBS and resuspended in RPMI media supplemented with 10% FBS and 20 μg/mL DNAse (Sigma) for subsequent staining and analysis by flow cytometry. For lamina propria cell isolation, the remaining tissue was digested with 1 mg/mL collagenase D (Sigma) and 50 μg/mL DNAse (Sigma) for 30 minutes shaking vigorously every 5 minutes. The samples were then passed through a cell strainer, homogenized, washed once with PBS and resuspended in RPMI media supplemented with 10% FBS and 20 μg/mL DNAse (Sigma) for subsequent staining and analysis by flow cytometry.

In Vitro Bone Marrow Cultures

Bone marrow was extracted from femur, tibia and humerus of mice. Cell suspensions were prepared after ACK erythrocyte lysis and resuspended in RPMI medium supplemented with 10% FBS, penicillin (100 Units/mL), streptomycin (100 μg/mL), glutamine (292 μg/mL) and 2-mercaptoethanol (50 μM). Bone marrow cultures were performed in the presence of 10 ng/mL of either IL-2 (Peprotech), IL-7 (Peprotech), TSLP (Peprotech), or IL-25 (eBioscience) with or without 10 ng/mL IL-33 (Peprotech). 100 μM 8-Br-cAMP (Sigma) was added to the cultures where indicated. The cells were incubated at an initial density of 1 x 10^6 cells/mL in 48-well plates at 37°C in 5% CO_2. Media was refreshed every 4 days where appropriate.

In Vitro ILC2 Cultures (BM-ILC2 Cultures)

Whole bone marrow cells were cultured for 3 days in the presence of IL-2, IL-7, TSLP and IL-25 at an initial density of 2 x 10^6 cells/mL under previously specified culture conditions. Sorted ILC2 (CD45+ lineage- $CD90^+$ CD25+) were recovered in fetal bovine serum and resuspended in RPMI medium supplemented with 10% FBS, penicillin (100 Units/mL), streptomycin (100 μg/mL), glutamine (292 μg/mL) and 2-mercaptoethanol (50 μM) and containing 10 ng/mL of either IL-2 (Peprotech), IL-7 (Peprotech), TSLP (Peprotech), or IL-25 (eBioscience). 10 ng/mL IL-33 and 100 μM 8-Br-cAMP (Sigma) was added to the cultures where indicated. The cells were incubated at an initial density of 4 x 10^4 cells/mL in 96-well plates at 37°C in 5% CO_2 for 4 days.

Flow Cytometry

For the lineage cocktail, biotin-coupled antibodies against B220, CD4, CD8, CD11b, CD11c, CD19, FcεRI, Gr-1, NK1.1, TCRβ, TCRγδ, Ter119 and SiglecF (eBioscience) were used. Fluorescent coupled streptavidin or antibodies against CD45, ST2, $CD90^+$, human CD2, CD25, KLRG1 and CD127 (Biolegend) were used.

For intracellular staining antibodies against IL-5 (BD Pharmingen), IL-13 (eBioscience) and GATA-3 (eBioscience) were used. To determine the cell viability, we stained the cells using Zombie aqua reagent (Biolegend). The ILC2s were sorted using a Facs ARIA II sorter in the Instituto Nacional de Enfermedades Respiratorias (INER) and the Instituto de Investigaciones Biomédicas, UNAM. The samples were analyzed using an Attune Nxt cytometer (Thermofisher) located in the Laboratorio Nacional de Citometría de Flujo of Instituto de Investigaciones Biomédicas, UNAM and a BD FACSMelody Cell Sorter located in the Instituto de Fisiología Celular.

Proliferation Assays

Cultures were stained on day 3 of whole bone marrow culture before sorting for ILC2s with the Tag-it Violet reagent according to the manufacturer's instructions (Biolegend). Proliferation assays were performed by following the dilution of the violet dye by flow cytometry and the division index was calculated using the FlowJo software.

Apoptosis Determination

Cells were incubated with viability dye Zombie (Biolegend), stained for surface markers and incubated with annexin V (eBioscience) after staining according to the manufacturer instructions. The frequencies of living, apoptotic and necrotic cells were determined by flow cytometry.

Statistical Analysis

Statistical analyzes were calculated in Prism 6.0 (Graphpad Software) using a two-tailed Student's test. Experiments with more than two groups, were analyzed using one way and two-way ANOVA tests with *post hoc* Tukey and Bonferroni tests. $p \leq 0.05$ was considered significant.

RESULTS

Helminth Infection Results in the Generation of Distinct ILC2 Populations

ST2 is one of the main markers used to specifically identify ILC2s within the ILC group. The presence of this marker on ILC2s has been reported in different organs and study models *in vivo* (23, 28, 29). Along with ST2, expression of type 2 immune response cytokines is also a unique feature of this population. Within these type 2 cytokines, IL-9 plays an essential role in the immune system defense against helminth infections (17). However, this cytokine has only been reported to be present under inflammatory conditions (16, 17). In order to assess the expression of ST2 and IL-9 in ILC2s, we infected IL-9 reporter mice (INFER) with *N. brasiliensis* larvae and analyzed lung and

small intestine tissue at 0, 4, 7 and 10 days post-infection. The IL-9 INFER reporter allows tracing of cells that express this cytokine without altering expression levels when used in heterozygosity as previously reported (17). Evaluation of the simultaneous expression of IL-9 and ST2 revealed the presence of separate ILC2 populations specific to the analyzed compartment (**Figure 1**; **Supplementary Figure 1A**).

In the lung at steady state conditions, we found most of the ILC2s to be $ST2^+$ and $IL\text{-}9^-$, with only a small percentage of ILC2s expressing IL-9 (**Figures 1A, B**). At 7 days post-infection, we observed an increase in this predominant $ST2^+$ $IL\text{-}9^-$ population along with trending increases in the double positive ILC2 population, and a slight but significant decrease in the double negative ILC population (**Figures 1A, B**; **Supplementary Figure 1B**).

In the small intestine within the intraepithelial cell compartment, most ILCs are double negative under basal conditions (**Figure 1C**). At day 7 and 10 post-infection, there were trending increases in the $ST2^+IL\text{-}9^+$ and $ST2^-IL\text{-}9^+$ populations (**Figure 1C**), however these differences were not statistically significant (**Figure 1D**). In the lamina propria, a low expression of ST2 was observed, along with a small percentage of $ST2^+$ $IL\text{-}9^-$ cells that remained unchanged with infection. In contrast, we observed an increase trend in the double positive population at day 4 post infection, and a significant increase in the $ST2^-$ $IL\text{-}9^+$ population on day 10 post infection (**Figures 1E, F**).

Analyses of lung and small intestine draining lymph nodes revealed increased frequencies and numbers of these ILC2 populations on day 10 post infection, mostly in the mesenteric lymph nodes (**Supplementary Figures 1C–F**).

Overall, we observed a clear distribution of ILC2 populations based on the expression of ST2 and IL-9 in the infected tissues. Importantly, the expression of these markers is not conserved among tissues and seems to be regulated in a microenvironment-specific manner.

Development of a Novel Bone Marrow-Derived ILC2 Culture Model

To determine which signals contribute to the generation of different ILC2 populations in tissues, we developed an *in vitro* differentiation model. First, cells extracted from bone marrow were cultured with different combinations of cytokines known to be important for the differentiation and proliferation of ILC2s including IL-2, IL-7, IL-25, TSLP and IL-33 and selected the combinations that resulted in the highest number of ST2 or IL-9 positive ILC2s (**Figure 2A**). We observed that the combination of IL-2, IL-7, TSLP and IL-25 induce the highest numbers of $ST2^+$ ILC2s with low expression of IL-9 (**Figure 2A**). IL-33 is known to induce bone marrow ILC2s to produce IL-5 locally and to stimulate their exit towards the periphery (4, 5). Interestingly, adding IL-33 to our culture resulted in increased frequency of $IL\text{-}9^+$ cells within the $lineage^-$ $CD90^+$ population (**Figure 2A**).

Based on this information, we designed a protocol to expand ILC2s termed BM-ILC2 culture (**Figure 2B**) in which we

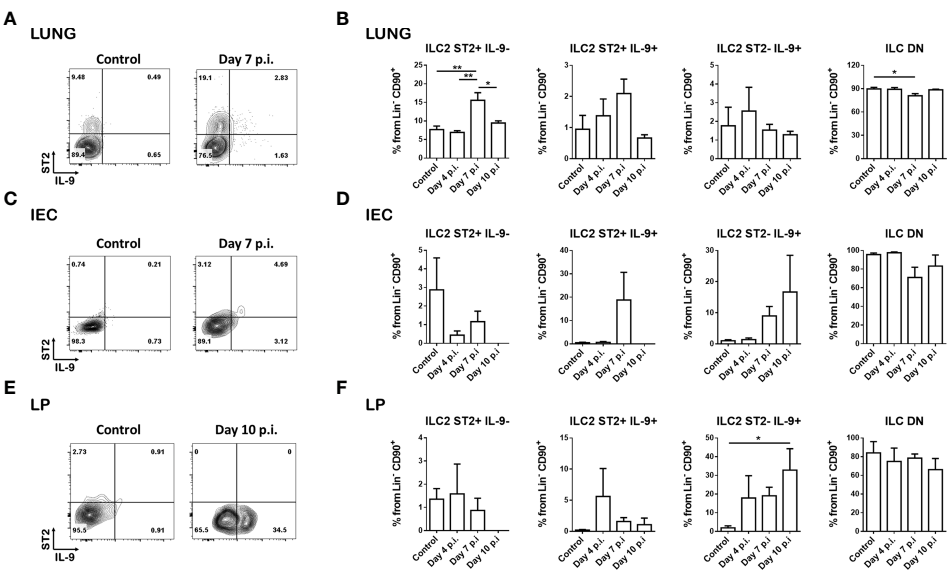

FIGURE 1 | Helminth infection results in distinct ILC2 populations. INFER mice were subcutaneously infected with 200 N. *brasiliensis* larvae and ILC2s from lung and small intestine were analyzed at different times post-infection. **(A)** Representative dot plots of the ILC2 populations generated in the lung on day 7 post-infection compared to day 0 (control) (gated on live $CD45^+$ $lineage^-$ $CD90^+$ cells). **(B)** Frequencies of the ILC2 subsets in the lung on day 0 (control), 4, 7 and 10 post-infection. **(C)** Representative dot plots of the ILC2 populations generated in the intraepithelial cells (IEC) compartment on day 7 post-infection compared to day 0 (control) (gated on live $CD45^+$ $lineage^-$ $CD90^+$ cells). **(D)** Frequencies of the ILC2 subsets in the intraepithelial cell compartment on day 0 (control), 4, 7 and 10 post-infection. **(E)** Representative dot plots of the ILC2 populations generated in the lamina propria (LP) on day 10 post-infection compared to day 0 (control) (gated on live $CD45^+$ $lineage^-$ $CD90^+$ cells). **(F)** Frequencies of the ILC2 subsets in the lamina propria on day 0 (control), 4, 7 and 10 post-infection. Data shown are mean ± SEM. n = 2 for day 4 and day 10 experiments; n = 4 for day 7 experiments; 1-2 mice were analyzed per experiment. *p < 0.05, **p < 0.01.

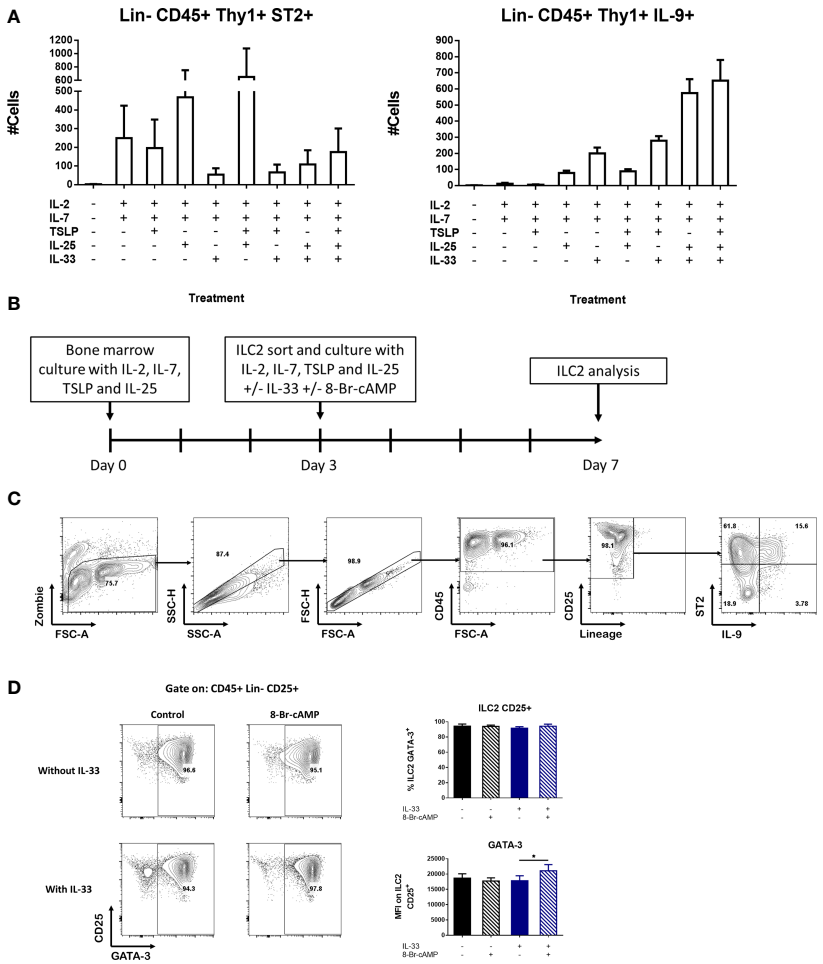

FIGURE 2 | Novel bone marrow-derived ILC2 culture model. **(A)** Induction of ILC2 numbers expressing ST2 or IL-9 in the presence of different combinations of cytokines in whole bone marrow cultures. The medium was changed at day 4 and the cells were analyzed at day 7. Data represent the mean ± SEM of three mice per group. **(B)** Schematic of the protocol used for the BM-ILC2 culture. **(C)** Gating strategy to identify populations in the BM-ILC2 culture. **(D)** Representative dot plots of ILC2 and graphs of GATA-3 expression in BM-ILC2 at day 7. Data represent the mean ± SEM of four mice per group analyzed in four independent experiments. *$p < 0.05$.

performed a 3-day bone marrow culture in the presence of cytokines (IL-2, IL-7, IL-25 and TSLP) that induce ST2$^+$ ILC2 cells. After this incubation period, we sorted the ILC2s identified as CD45$^+$ lineage$^-$ CD90$^+$ CD25$^+$ (**Supplementary Figure 2A**), and cultured them an additional 4 days with the same cocktail of cytokines in the presence or absence of IL-33. ILC2 cells cultured without IL-33 express ST2 and therefore are capable of responding to this alarmin, hence the addition of IL-33 post-sort (**Figure 2A**). Additionally, the cAMP analog 8-Br-cAMP was also added to the sorted cultures to see the effects of PKA activation on ILC2 cells. Culturing our cells in the described conditions resulted in a greater than 90% ILC2 population identified as CD45$^+$ lineage$^-$CD25$^+$ by the end of the incubation period (**Figure 2C**). In addition, the frequency of GATA-3 expressing cells was above 90% regardless the culture conditions tested (**Figure 2D**), confirming their identity as ILC2 cells.

Finally, we assessed the expression of CD127, another marker associated with the ILC family, in our cultures at different stages of our model. We found that throughout the protocol, the expression of this receptor is lost. In the newly isolated bone marrow all the CD25$^+$ cells express CD127; however, after the initial 3-day incubation only 35% of the CD25$^+$ ILC2 cells maintained the expression of this marker. By day 7 and depending on the treatment, only between 3 to 30% of CD25$^+$ ILC2 cells expressed CD127 (**Supplementary Figure 2B**). For this reason, we decided to exclude CD127 as a selective marker for isolation of ILC2s in our model.

IL-33 and cAMP Enhance IL-9 Expression and Regulate Specific Markers in Bone Marrow Derived ILC2 Cells

The presence or absence of IL-33 in our BM-ILC2 cultures allowed us to identify three distinct populations based on the expression of ST2 and IL-9 (**Figure 3A**). No expression of IL-9 is observed in the

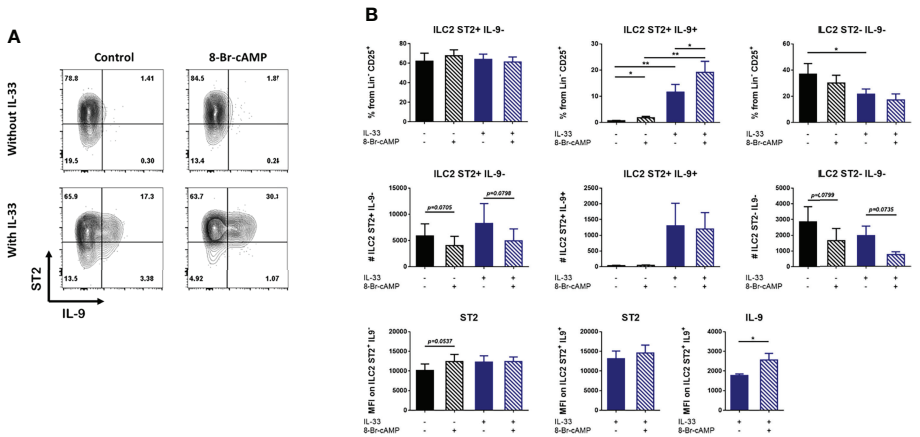

FIGURE 3 | IL-33 and cAMP enhance IL-9 expression in BM-ILC2 cultures. **(A)** Representative dot plots of ILC2 populations generated with IL-2, IL-7, IL-25 and TSLP in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture (gated on live CD45$^+$ lineage$^-$ CD25$^+$ cells). **(B)** Frequency, absolute numbers and MFI of ST2 and IL9 in ILC2 populations generated with IL-2, IL-7, IL-25 and TSLP in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. Data represent the mean ± SEM of seven mice analyzed per group in 6 independent experiments. *p < 0.05, **p < 0.01.

absence of IL-33, therefore we were able to identify the ST2$^+$ IL-9$^-$ and ST2$^-$IL-9$^-$ ILC2 populations, while the addition of IL-33 resulted in the generation of an additional ILC2 population that expresses ST2 and IL-9 (**Figure 3A**); demonstrating that IL-33 is an important signal for IL-9 induction in our system.

While IL-33 decreased the number of ST2$^+$ cells in whole bone marrow cultures (**Figure 2A**), no regulation of ST2 induced by IL-33 or PKA was observed in isolated ILC2s (**Figure 3A**). However, IL-33 treatment did increase the frequency and absolute numbers of IL-9-expressing ILC2 cells (**Figure 3B**). Treatment with 8-Br-cAMP also resulted in increased expression of IL-9 and the analog further synergized with IL-33 in the induction of ST2$^+$ IL-9$^+$ cells, increasing the MFI of IL-9 on these cells (**Figure 3B** lower panel). The positive regulation of IL-9 by IL-33 has been described in lung resident ILC2s (30), indicating that ILC2s induced by our model share this regulation with other tissue resident ILC2s.

We also evaluated the expression of CD25, KLRG1 and CD127, markers associated with ILC2s (**Supplementary Figure 3**). Our results indicate that IL-33 increases the expression of CD25 while we did not detect any type of PKA-mediated regulation of this marker (**Supplementary 3A, 3B**). In addition, the percentage of ILC2 expressing KLRG1 is increased by IL-33 but this increase is inhibited by PKA (**Supplementary Figures 3C, D**). Finally, IL-33 does not seem to induce changes in the expression of CD127; however, in the presence of this alarmin, activation of PKA leads to increased frequencies of ST2$^+$ IL-9$^-$ ILC2 cells expressing CD127 (**Supplementary Figures 3E, F**).

In conclusion, IL-33 and PKA activation synergize to induce IL-9 expression but differentially regulate other markers of ILC2 *in vitro*.

cAMP Inhibits ILC2 Proliferation *In Vitro*

Despite the fact that we did not observe significant differences in the numbers of ILC2 cells associated with PKA activation, there is a trend indicating a possible decrease in the numbers of these cells in the presence of the cAMP analog (**Figure 3B**). With this data in mind and based on reports about PKA activity in T cells (11, 12), we decided to analyze whether PKA is capable of regulating cellular processes such as proliferation and apoptosis that could impact ILC2 numbers. To address this, we performed a dye dilution assay. ILC2s from bone marrow cultures were stained with the Tag-it Violet dye at day 3 of our protocol, previous to the ILC2 cell sorting. The cells were then cultured under the different conditions and dye dilution was monitored at day 7. The division index was calculated in order to quantify cell proliferation.

Due to limited numbers of some ILC2 populations in our purified cultures, we could not evaluate the effect of 8-Br-cAMP on each different ILC2 subset; therefore, proliferation was assessed on the total CD45$^+$ lineage$^-$ CD25$^+$ cells. The division index of cells with no IL-33 treatment was lower compared to the IL-33-treated culture, which is consistent with the capacity of IL-33 as a proliferative stimuli for ILC2 (**Figures 4A, B**). The cAMP analog induced an IL-33-independent proliferation arrest, as shown by the histograms and division index graphs in **Figures 4A, B**. These trending decreases are consistent in the 3 independent experiments performed. In conclusion, 8-Br-cAMP treatment can inhibit proliferation of our cells in an IL-33-independent manner, recapitulating one of the effects of the PKA activation previously described on T cells (11, 12).

cAMP Does Not Regulate ILC2 Survival *In Vitro*

PKA can induce apoptosis of T lymphocytes (10), therefore we tested whether this signaling pathway works similarly on ILC2s by determining the percentages of living, apoptotic and necrotic cells in our ILC2 cultures in the presence or absence of IL-33 and 8-Br-cAMP (**Figure 4C**). We stained the ILC2s with a viability dye (Zombie) and Annexin V, which allows for the discrimination

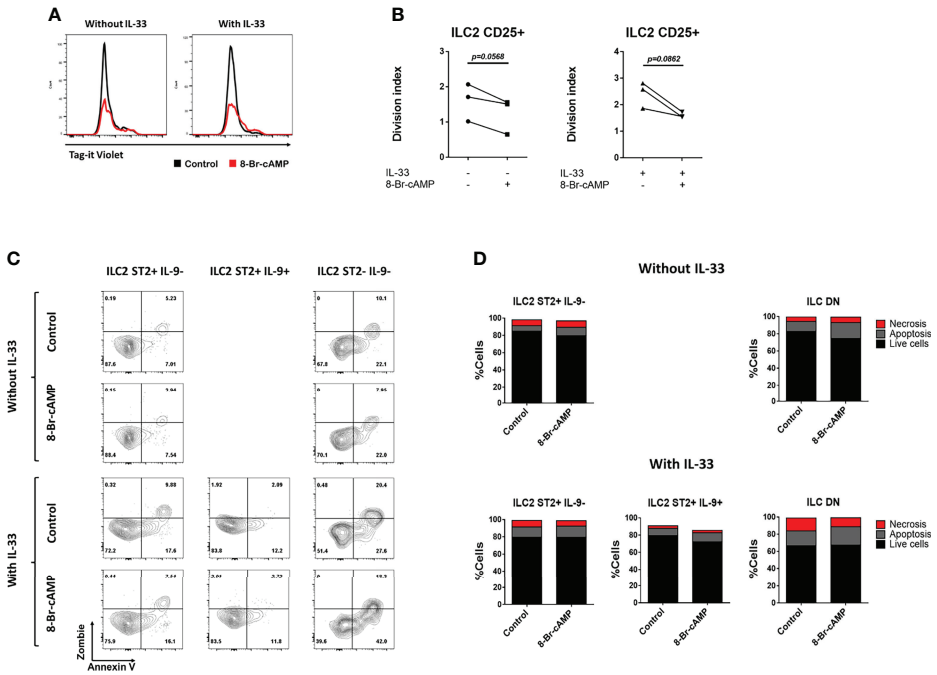

FIGURE 4 | 8-Br-cAMP regulates proliferation but not cell death of ILC2s. **(A)** Representative histograms of dye dilution in total ILC2s treated with or without 8-Br-cAMP in the presence or absence of IL-33 at day 7 of the BM-ILC2 culture (gated on live CD45$^+$ lineage$^-$ CD25$^+$ cells). **(B)** Division index on total ILC2s treated with or without 8-Br-cAMP in the presence or absence of IL-33 at day 7 of the BM-ILC2 culture. Data represent three mice analyzed per group in 3 independent experiments. **(C)** Representative dot plots for the annexin V and viability staining (zombie) of the different ILC2 populations treated with or without 8-Br-cAMP in the presence or absence of IL-33 at day 7 of the BM-ILC2 culture. **(D)** Frequencies of living, apoptotic and necrotic cells of ILC2 populations generated in the presence (lower panel) or absence (upper panel) of IL-33 at day 7 of the BM-ILC2 culture. Data represent the mean of seven mice analyzed per group in 6 independent experiments.

between viable cells (Annexin V$^-$ Zombie$^-$), cells in early apoptosis (Annexin V$^+$ Zombie$^-$) and cells in late apoptosis or necrosis (Annexin V$^+$ Zombie$^+$). The cellular status in our cultures was measured at day 7. No significant difference in the frequencies of live, apoptotic and dead cells was observed in any of the culture conditions analyzed (**Figures 4C, D**), leading us to conclude that under this model the PKA pathway does not regulate apoptosis of any of the ILC2 populations. However, our data suggest that expression of ST2 or IL-9 improves viability on ILC2 cells, as percentages of live cells are higher in ST2$^+$IL-9$^-$ and ST2$^+$IL-9$^+$ ILC2 cells compared with DN ILC2s cultured in the presence of IL-33, further supporting a positive signal promoted by IL-33 and IL-9 on ILC2s (18, 19).

Characterization of the Cytokine Expression Profile in ILC2s in the Presence of cAMP *In Vitro*

ILC2s are an important source of type 2 cytokines in different inflammatory models (31–33). Several reports suggest that ligands like neuropeptides and lipid mediators that activate ILC2s and regulate the expression of IL-5 and IL-13, could also activate the PKA pathway (14, 20, 34). Hence, we analyzed the expression of type 2 cytokines and the effect of PKA activation on the different ILC2 populations obtained from our *in vitro* model. For this purpose, sorted ILC2s were cultured in the presence or absence of IL-33 with or without the cAMP analog to determine the effect of PKA activation on the induction of other type 2 cytokines. The different ILC2 populations were stained for IL-5 and IL-13 and analyzed by flow cytometry.

The percentages and numbers of cells expressing IL-5 significantly increased only in the presence of IL-33 (**Figures 5A, B**). In contrast, PKA activation resulted in decreased numbers of IL-5$^+$ ILC2 cells; however, this inhibitory effect was restricted to ILC2s that do not express IL-9 (**Figures 5A, B**). Hence, IL-5 expression appears to be dependent on IL-33 while PKA activation negatively regulates it.

IL-33 was not required for IL-13 expression. Even in the absence of this alarmin, 50% of ILC2s are positive for IL-13, nevertheless we do observe significantly higher expression on IL-13 induced by IL-33 (**Figures 5C, D**). Similarly to the observed effects on IL-5, PKA activation decreases the numbers of IL-33-induced IL-13$^+$ ILC2 cells that do not express IL-9 (**Figures 5C, D**). We also observed a particular effect of cAMP decreasing the numbers of ST2$^-$IL-9$^-$ ILC2 cells expressing IL-13 that was independent of IL-33 (**Figure 5D**).The MFI data in our ILC2 model shows a modest yet significant PKA-associated difference in IL-13 (**Supplementary Figure 4C**). Although the MFI of IL-13 increases significantly with IL-33, a decrease in IL-13 associated with PKA activation is observed in ST2$^-$ IL-9$^-$ ILC2 cells in the

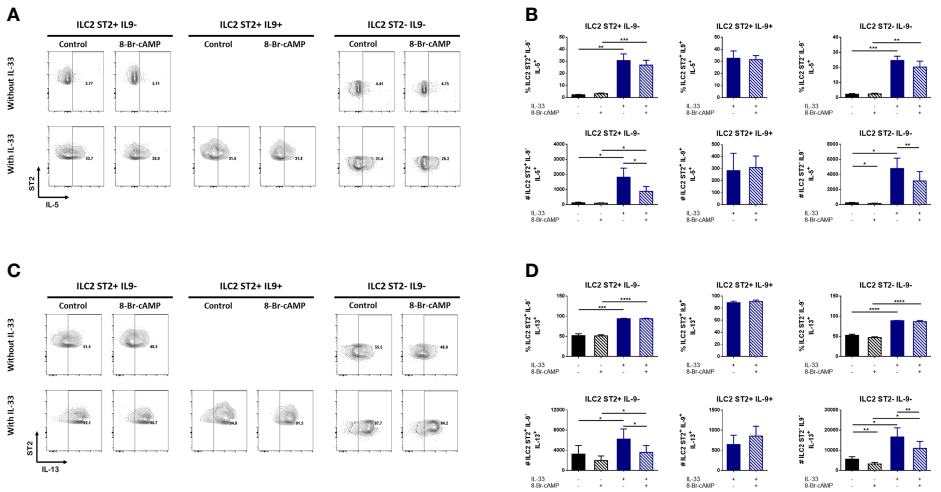

FIGURE 5 | cAMP regulates IL-5 and IL-13 expression in IL-9 negative bone marrow derived ILC2s. **(A)** Representative dot plots of IL-5 expression in different ILC2 populations in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. **(B)** Frequency and absolute numbers of IL-5 expression in different ILC2 populations shown in **(A)**. **(C)** Representative dot plots of IL-13 expression in different ILC2 populations in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. **(D)** Frequency and absolute numbers of IL-13 expression in different ILC2 populations shown in **(C)**. Data represent the mean ± SEM of seven mice analyzed per group in 6 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

absence of IL-33 (**Supplementary Figure 4C**). This indicates that PKA can regulate certain ILC2 markers in the absence of this alarmin.

Since the discovery of Th9 cells as a specialized population different from Th2 cells, the expression of IL-4 and IL-9 in T lymphocytes was found to be mutually exclusive (17, 35). To determine if this occurs in a similar way in ILC2s, we used a KN2 reporter mouse in heterozygosis which expresses the human CD2 protein under the endogenous IL-4 promoter (26) to monitor the expression of IL-4. Different stimuli have been reported to induce the expression of IL-4 in ILC2 cells. However, we were unable to detect the presence of IL-4 in any of the culture conditions tested in this work (**Supplementary Figure 4D**). Thus, we conclude that IL-33 and cAMP are not sufficient stimuli to induce IL-4 in ILC2s *in vitro*.

Together, these results suggest that in the presence of IL-33, PKA activation have opposite effects regulating the numbers of IL-5 and IL-13 expressing cells but only in IL-9 negative ILC2s. A similar effect was observed in the MFI of IL-13 in $ST2^+IL\text{-}9^-$ and $ST2^-IL\text{-}9^-$ ILC2 cells. Finally we believe that other stimuli different from IL-33 and cAMP are necessary to induce IL-4 in ILC2 cells in our culture.

DISCUSSION

In this work we report the presence of distinct ILC2 populations based on ST2 and IL-9 expression in a helminth infection model. These populations, which include $ST2^+$ $IL\text{-}9^-$, $ST2^+$ $IL\text{-}9^+$, $ST2^-$ $IL\text{-}9^+$ and $ST2^-$ $IL\text{-}9^-$ had not been previously reported *in vivo*. Here, we provide the first evidence that they are found in tissues and to a lesser extent in lymphoid organs. The $ST2^+$ $IL\text{-}9^-$ cells predominate in the lung, and along with $ST2^+$ $IL\text{-}9^+$ cells, increase in the *N. brasiliensis* infection model. Infection with the parasite seems to induce the expression of ST2 and IL-9 in the intraepithelial compartment of the small intestine, however this induction is very discrete and did not reach the statistical significance. In the lamina propria, both $IL\text{-}9^+$ populations are increased, however the $ST2^-$ $IL\text{-}9^+$ is the most abundant subset induced by the infection. Two different populations of ILC2 cells have been described: natural (nILC2), characterized by the expression of KLRG1 and ST2; and inflammatory (iILC2), which are induced by *N. brasilienis* infection and express KLRG1 but apparently lack ST2 (36, 37). This absence of ST2 expression on iILC2s might be explained by downregulation of the receptor driven by its ligand IL-33 in the small intestine, as recently demonstrated (38). IL-33 is constitutively expressed in this tissue and, together with IL-25, is further induced by *N. brasiliensis* infection to promote the generation of iILC2 cells. Hence, the presence of the $ST2^-$ $IL\text{-}9^+$ ILC2 population in the small intestine described here might be explained by this regulatory loop, where IL-33 and IL-25 are induced upon infection, promoting iILC2s that lack ST2 and express effector cytokines *in vivo*. Altogether, our results demonstrate a dynamic transformation of the ILC2 subsets dependent on the tissue and time of infection that can be modulated by the inflammatory context.

An important aspect to consider is that isolating cells from *N. brasiliensis* infected small intestine is extremely challenging and results in low frequencies of live cells, potentially masking real absolute numbers of cells present in the tissue *in vivo*. However, analyses of cell frequencies are feasible and reproducible using our protocol. To our knowledge, this is the first report showing hematopoietic cell isolation from *N. brasiliensis* infected small intestine, followed by analysis of ILC2 cells by flow cytometry,

and hence represents a useful tool to evaluate these and other immune cells from this particular complicated tissue.

In order to dissect the signals that originate these ILC2 populations, we developed an *in vitro* culture model from bone marrow cells in which different combinations of cytokines were tested to determine which ones are important for the induction of ST2 and IL-9. We found that IL-33 is not necessary for the induction of its receptor since the combination of IL-2, IL-7, IL-25 and TSLP is sufficient to generate $ST2^+$ ILC2s. On the contrary, IL-33 decreased ST2 expression, which could be due to internalization of the receptor upon ligand binding as described above. Additionally, our studies confirmed IL-33 as a key stimulus to increase IL-9 expression in our cells. This data agrees with previous reports by Mohapatra et al., where lung ILC2s expressed IL-9 in response to IL-33 and this expression was increased when the treatment was performed in combination with other cytokines such as IL-2, IL-7 and TSLP (30). Therefore, we can conclude that the ILC2s in the whole bone marrow culture model resemble the behavior of ILC2s derived from other tissues such as small intestine and lung.

In order to determine the effects of IL-33 and PKA activation directly on ILC2s, we developed another culture model termed BM-ILC2, in which we expanded bone marrow ILC2s in the presence of IL-2, IL-7, TSLP and IL-25 for 3 days. ILC2s where then sorted and cultured 4 additional days in the same cocktail with the addition of IL-33 and/or a cAMP analog. We further demonstrated that with our novel experimental strategy, we were able to generate cultures highly enriched on ILC2 cells expressing GATA-3 and CD25, as expected.

In this model, IL-33 did not have a negative effect on the expression of ST2, differing from the results observed in the whole bone marrow culture. We believe that the effect observed in total bone marrow cultures may depend on further signals provided by other cells, in addition to the presence of IL-33. On the other hand, the IL-33-dependent induction of IL-9 on our BM-ILC2 cells was independent of those potential signals included in the total bone marrow culture, since purified cultures of ILC2s exposed to IL-33 still showed a strong induction of IL-9.

The addition of IL-33 to our sorted ILC2 cultures increased the expression of other markers associated with the identification of these cells such as CD25 and KLRG1. In addition, the differential expression of IL-9 generated three different subpopulations of ILC2 cells, all identified as $CD45^+$ $lineage^-$ $CD25^+$ $GATA\text{-}3^+$. These subsets are $ST2^+$ $IL\text{-}9^-$, $ST2^+$ $IL\text{-}9^+$ and $ST2^-$ $IL\text{-}9^-$. The first 2 populations correspond to those previously found in the lung and in the intraepithelial compartment of the small intestine. The $ST2^-$ $IL\text{-}9^+$ ILC2 population identified in lamina propria of *N. brasiliensis* infected mice, could not be induced in our *in vitro* model; we found the frequencies of these cells to be less than 3 percent of the total population. As previously mentioned this could be due to the requirement of other signals in combination with IL-33 to induce the negative regulation of ST2 while maintaining the expression of IL-9.

The PKA pathway is known to regulate the expression of cytokines in T lymphocytes and ILC2s. Here, we confirmed that 8-Br-cAMP-mediated PKA activation in the presence of IL-33 increased IL-9 expression, which does not modify the distribution of the ILC2 populations but could enhance the function of $ST2^+$ $IL\text{-}9^+$ ILC2 cells. To our knowledge, there are no previous reports associating PKA activation with an increase in IL-9 in innate lymphoid cells, however this effect has been observed in Th9 lymphocytes where the CGRP/cAMP/PKA pathway promotes IL-9 production *via* NFATc2 activation (39). This establishes a possible common regulatory pathway between ILC2 and T lymphocytes.

The synergic effect of PKA and IL-33 on IL-9 expression described here is contrasting with studies that report PKA-mediated inhibition of type 2 cytokines in ILC2s (14, 20, 34). This suggests that although our *in vitro* ILC2s have characteristics in common with lung ILC2s, there are also specific differences associated with the microenvironment of these cells. Along with the increase in IL-9, PKA activation induced CD127 and decreased KLRG1 expression. Previous reports have identified KLRG1 not only as a marker of ILC2 maturation but also as a negative regulator of the function of these cells. The interaction of activated ILC2s with the KLRG1 ligand E-cadherin results in a down regulation of GATA-3, IL-5, IL-13, amphiregulin and reduced ILC2 proliferation (29). In other tissues such as the stomach, ILC2s can be activated by IL-7, inducing proliferation and expression of cytokines (40). We propose that PKA activation could prime these cells for a better activated phenotype, perhaps as a mechanism to ensure proliferation and proper function. However, future experiments are necessary to verify this.

A striking finding is that when PKA is activated, ILC2s seem to decrease in numbers except for the IL-9-producing population. Our results suggest that this can be attributed to the inhibition of proliferation since we did not find PKA activation to be associated with apoptosis. However, the induction of IL-9 by PKA could be compensating for these proliferation effects, which is why we do not see differences in absolute numbers of $IL\text{-}9^+$ ILC2 cells when we activate PKA. Our results are in accordance with previous studies that report IL-9 to be a potent inducer of proliferation and survival for ILC2 cells (19).

The inhibitory effects on proliferation described here for ILC2s have already been reported in T cells (11, 12, 41). In this model, inhibition of proliferation by the cAMP analog is observed independently of IL-33. cAMP has been reported to inhibit proliferation in T cells through different mechanisms. For example, it can generate cell cycle arrest through the inhibition of cyclins D and E (12) and PKA activation can block IL-2 receptor signaling (11). Further research is necessary to determine the regulatory mechanism carried out by PKA to inhibit proliferation of the ILC2s in our model. In addition, although we did not observed an effect on apoptosis induced by IL-33 and the PKA pathway in our cultures, we did see a lower frequency of apoptotic and death cells on the ILC2 subset expressing IL-9, further supporting the role of IL-9 as a positive signal acting in an autocrine way on these cells (18, 19).

Additional data derived from our BM-ILC2 cultures validate this protocol as a means to generate bona fide ILC2 cells, since our ILC2 populations express both IL-5 and IL-13. In agreement with other

works, our data indicate that IL-5 induction is dependent on IL-33 as has been observed in lung-derived ILC2s (42). Contrastingly, IL-13 expression does not require IL-33, however it is significantly enhanced by the presence of the alarmin. Our data is in agreement with previous reports of ILC2s in the intestine, where IL-25 produced by Tuft cells further activated these cells to produce IL-13 in a parasitic infection context (43).

Lung ILC2s have been reported to express IL-5 and IL-13, which can be blocked by cAMP analogs and ligands that activate PKA (13). The effects of PKA on these two cytokines are similar in our model. We established that cAMP has regulatory effects on the numbers of ILC2 cells expressing IL-5 and/or IL-13. These effects were observed only in the presence of IL-33 and were restricted to ILC2s that do not express IL-9. These data suggest that while there is a decrease in the induction of IL-5 and IL-13 in ILC2 cells, the increase in IL-9-expressing cells compensates for these defects, resulting in no change in the absolute numbers of ILC2 that co-express IL-5 or IL-13 with IL-9.

Finally, the induction of IL-4 has been reported in ILC2s through activation with other mediators such as leukotriene D4, but not with IL-33 (44, 45); further supporting the observation in our model that IL-33 alone is unable to promote IL-4 expression *in vitro*. This could be because in addition to the cytokines used here and in previous reports, other signals are required to generate IL-4$^+$ ILC2 cells; however, this has yet to be demonstrated.

In general, the ILC2s differentiated from bone marrow precursors obtained with our *in vitro* model share various characteristics with tissue-derived ILC2s. It is tempting to speculate that the different ILC2 subpopulations characterized in this work are found in different anatomical locations and that their modulation depends on the conditions and time of stimuli presentation. *N. brasiliensis* infection for example, results in tissue damage and IL-33 release (46). Worm-derived factors can modulate this IL-33 response (25) and consequently generate the different ILC2 populations. Therefore, it would be interesting to determine if these ILC2 populations are induced in response to IL-33 *in vivo* as well. Here, we found that stimulation with IL-33 and PKA activation results in an enrichment of IL-9$^+$ ILC2 cells that are competent regarding the expression of IL-5 and IL-13. Therefore, expansion of this population could contribute to immunity against helminth infections.

Based in our results, the PKA pathway antagonizes IL-33 in several processes such as proliferation and production of IL-5 and IL-13, while inducing a phenotype that favors its ability to respond to other stimuli in the microenvironment (IL-9$^+$, CD127$^+$, KLRG1$^-$). Whether changes in PKA activation are generated during helminth infection with *N. brasiliensis* is a matter of interest and remains to be determined along with the identification of the potential ligands promoting such activation *in vivo*.

To conclude, the data presented here provide further evidence of the high heterogeneity between ILC2 subsets in a helminth infection context and calls for careful consideration when choosing the markers to identify these cells. In addition, by providing a protocol for differentiation and expansion of bona fide ILC2 cells, we contribute with a useful tool for studies focused on ILC2 biology. Finally, the findings that IL-33 and PKA induce IL-9 and regulate the different ILC2 population dynamic, could help us better understand the physiology of these cells, allowing their manipulation for therapeutic purposes in specific anatomical locations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by CICUAL Instituto de Fisiología Celular, UNAM.

AUTHOR CONTRIBUTIONS

EO-M conceived, design and performed the experiments, analyze the data and wrote the manuscript, OM-P performed experiments and discussed the data, BER-M revised the manuscript, JLR-B performed experiments, acquired the data and provided reagents, IL-L discussed and thoroughly revised the results and conclusions of the manuscript, PL-L conceived the idea, design the experiments, discussed and revised the manuscript, supervised the project and acquired the funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.787713/full#supplementary-material

Supplementary Figure 1 | INFER mice were subcutaneously infected with 200 N. *brasiliensis* larvae subcutaneously and ILC2s from lung and small intestine

draining lymph nodes were analyzed at different times post-infection. **(A)** Gating strategy to identify the different ILC2 populations in tissues. **(B)** Absolute numbers of the different ILC2 subsets in the lung on day 0 (control), 4, 7 and 10 post-infection. **(C)** Representative dot plots of different ILC2 populations generated in the mediastinal lymph nodes on day 0 (control) and 10 post-infection (gated on live $CD45^+$ $lineage^-$ $CD90^+$ cells). **(D)** Frequencies and absolute numbers of the different ILC2 subsets in the mediastinal lymph nodes on day 0 (control), 4, 7 and 10 post-infection. **(E)** Representative dot plots of different ILC2 populations generated in the mesenteric lymph nodes on day 0 (control), and 10 post-infection (gated on live $CD45^+$ $lineage^-$ $CD90^+$ cells). **(F)** Frequencies and absolute numbers of the different ILC2 subsets in the mesenteric lymph nodes on day 0 (control), 4, 7 and 10 post-infection. Data represent the results of two (days 4 and 10) or four independent experiments (day 7) with one or two mice analyzed per dot. *p<0.05, **p<0.01, ***p<0.001, , ****p<0.0001

Supplementary Figure 2 | (A) Gating strategy used for sorting ILC2s at day 3 of the BM-ILC2 culture and purity post-sort of those cells. **(B)** Representative dot plots of CD127 expression on different days of BM-ILC2 culture (gated on $CD45^+$ lineage- $CD90^+$ for the days 0 and 3 and $CD45^+$ lineage- for the day 7). **(C)** Representative fluorescence minus one (FMO) dot plots for the markers used in the sort and analysis of ILC2s, controls were performed in total bone marrow cultures to ensure positive and negative populations.

Supplementary Figure 3 | (A) Representative histograms of CD25 expression in different ILC2 populations in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. **(B)** MFI of CD25 expression in different ILC2 populations shown in **(A)**. **(C)** Representative dot plots of KLRG1 expression in different ILC2 populations in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. **(D)** Frequency of KLRG1 expression in different ILC2 populations shown in **(A)**. **(E)** Representative dot plots of CD127 expression in different ILC2 populations in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. **(F)** Frequency of CD127 expression in different ILC2 populations shown in **(A)**. Data represent the mean ± SEM of 7, 6 or 5 mice analyzed per group for CD25, KLRG1 and CD127 respectively. *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure 4 | (A) Representative FMO dot plots for IL-5 and IL-13 staining. **(B)** MFI of IL-5 expression in different ILC2 populations with or without 8-Br-cAMP in the presence of IL-33 at day 7 of the BM-ILC2 culture. **(C)** MFI of IL-13 expression in different ILC2 populations in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. **(D)** Representative dot plots of IL-4 expression on total ILC2s from a KN2 mice, in the presence or absence of IL-33 and 8-Br-cAMP at day 7 of the BM-ILC2 culture. Data represent the mean ± SEM of seven mice analyzed per group in 6 independent experiments. *p<0.05, **p<0.01, ****p<0.0001.

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FOXO1 and FOXO3 Cooperatively Regulate Innate Lymphoid Cell Development

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Natural killer (NK) cells play roles in viral clearance and early surveillance against malignant transformation, yet our knowledge of the underlying mechanisms controlling their development and functions remain incomplete. To reveal cell fate-determining pathways in NK cell progenitors (NKP), we utilized an unbiased approach and generated comprehensive gene expression profiles of NK cell progenitors. We found that the NK cell program was gradually established in the CLP to preNKP and preNKP to rNKP transitions. In line with FOXO1 and FOXO3 being co-expressed through the NK developmental trajectory, the loss of both perturbed the establishment of the NK cell program and caused stalling in both NK cell development and maturation. In addition, we found that the combined loss of FOXO1 and FOXO3 caused specific changes to the composition of the non-cytotoxic innate lymphoid cell (ILC) subsets in bone marrow, spleen, and thymus. By combining transcriptome and chromatin profiling, we revealed that FOXO TFs ensure proper NK cell development at various lineage-commitment stages through orchestrating distinct molecular mechanisms. Combined FOXO1 and FOXO3 deficiency in common and innate lymphoid cell progenitors resulted in reduced expression of genes associated with NK cell development including ETS-1 and their downstream target genes. Lastly, we found that FOXO1 and FOXO3 controlled the survival of committed NK cells *via* gene regulation of IL-15Rβ (CD122) on rNKPs and bone marrow NK cells. Overall, we revealed that FOXO1 and FOXO3 function in a coordinated manner to regulate essential developmental genes at multiple stages during murine NK cell and ILC lineage commitment.

Keywords: innate lymphocyte cells (ILCs), development, FOXO, natural killer cells, IL-15

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INTRODUCTION

The evolutionarily conserved forkhead box transcription factors of the O class (FOXO) are critical regulators of metabolism, lifespan, fertility, proliferation, and cellular differentiation (1, 2). In mammals, the FOXO family is comprised of four members (FOXO1, FOXO3, FOXO4, and FOXO6) that, apart from FOXO6, are widely co-expressed throughout the immune system. Growth factor and survival signals activate the phosphoinositide 3-kinase-Akt signaling pathway, which leads to phosphorylation of the FOXOs and their subsequent nuclear exclusion and degradation (3). This counteracts the FOXO family's role in promoting apoptosis (4, 5) and cell cycle arrest (6, 7). In the adaptive immune system, FOXOs control a wide range of functions including homing and survival of naïve T cells (1, 8, 9), expansion of $CD8^+$ memory T cells (10, 11), differentiation of regulatory T cells (12–15), as well as B cell lineage commitment, homing, and germinal center proliferation (16–18).

NK cells are innate immune cells important for controlling viral infection and cancer (19–21). The IL-15-dependent NK cell lineage (22) is similar to B and T cells – derived from common lymphoid progenitors (CLP) (23). Downstream of the CLP, NK cells develop *via* two hierarchically related NK progenitor (NKP) stages originally defined by the loss of FMS tyrosine kinase 3 (FLT3) on preNKPs and the subsequent acquisition of IL-15Rβ (CD122) on refined NKPs (rNKP) (23). Recent studies have refined this developmental scheme by demonstrating that the preNKP compartment represents a heterogeneous population of innate lymphoid cell progenitors that give rise not only to NK cells but also to the non-cytotoxic ILC subsets (24–27).

Despite the identification of these intermediate NK cell progenitors and committed ILC progenitors within the preNKP compartment (24, 26), the precise stages where the ILC lineage-specific developmental programs are activated and the underlying mechanisms that lead to NK lineage restriction remain to be understood in detail. However, on the gene regulatory level, several transcription factors, including ETS1, NFIL3, and TCF7, have been shown to impact the development of NK cells at the preNKP and rNKP progenitor level (28–31).

Little is known about the role of the FOXOs in the development of non-cytotoxic ILCs. In contrast, their role in NK cell maturation has been investigated, but this has provided contradictory results. Relying on the specific Cre mediated deletion in $Ncr1^+$ (NKp46) cells, Deng et al. observed a more mature phenotype in NK cells lacking FOXO1 or FOXO1 and FOXO3 (32). As this was not observed in NK cells lacking FOXO3 alone, this lead to the conclusion that FOXO1 is dispensable for NK cell development but negatively regulates NK cell maturation (32). Using a similar model, Wang et al., in contrast, observed that NK cell development was abrogated by the loss of FOXO1 (33). Noteworthy, Ncr1 expression is acquired only after commitment to the NK cell lineage and therefore, these studies did not address a potential role of the FOXOs in early NK cell development. In line with this, deletion of FOXO1 in the hematopoietic stem cell has been reported to result in increased frequencies of NK cell progenitors and committed NK cells (34). Together, this underlines the need for further studying the role of the FOXOs in NK cell progenitors and NK cell maturation.

Using ablation of FOXO1 and/or FOXO3 throughout the hematopoietic system, we show that the FOXOs are critical for NK progenitor development, establishment of the early NK gene regulatory network, and NK maturation. In addition, we show that the loss of FOXO perturbs the development of the non-cytotoxic ILC lineages. These findings provide novel insights into NK and ILC development and the gene regulatory program that underpins this process.

RESULTS

The NK Gene Expression Program Is Initiated in preNKPs and rNKPs

To characterize gene regulation in early ILC and NK cell development, we performed RNA sequencing (RNA-seq) on FACS sorted $LY6D^{neg}$ CLPs (35, 36), preNKPs and rNKPs (23–26) (**Figure 1A** and **Table S1**). In agreement with preNKPs representing a developmental stage between CLPs and rNKPs (23), principal component analysis (PCA) revealed three distinct groups with the first component (PC1 60%) positioning the related progenitor subsets in the expected hierarchical order (**Figure 1B**). Further in line with this, preNKPs expressed genes otherwise only expressed (≥0.3 TPM in all replicas) in CLPs or rNKPs (**Figure 1C**).

Next, we investigated the expression pattern of genes known to be crucial for NK and ILC development (37, 38) (**Figure 1D**). Many of these genes, including *Bcl11b, Tox2, Zbtb16, Tcf7, Rorc, Id2*, and *IL2rb*, were found to be upregulated at the preNKP stage (**Figure 1D**). Looking directly at genes with significant expression changes in the CLP to preNKP (**Figure 1E** left) and preNKP to rNKP (**Figure 1E** right) transitions, revealed an overall pattern where preNKPs displayed down-regulation of genes expressed at the CLP stage (**Figure 1F**, cluster II, and in part I) and up-regulate genes expressed at the rNKP stage (**Figure 1F**, cluster III, and in part IV). When annotated (using Metascape), we as expected found that cluster I and II were enriched for B cell lineage associated genes (35, 36). In contrast, genes in cluster III and IV were enriched for genes associated with the NK cell lineage (**Figure S1A**). Hence, suggesting that the B-lineage associated gene program observed in CLPs (35, 36) is shut down in preNKPs. The preNKPs instead adopt a general ILC gene program before a more refined NK gene program is established in the NK-lineage committed rNKPs (23).

To characterize changes in cell-signaling pathways occurring at the developmental transitions, we performed KEGG pathway analysis (**Figure 1G**) on the differentially expressed genes (**Figures 1E, F**). This revealed a significant enrichment of genes involved in cytokine-cytokine receptor interactions as well as in the PI3K-Akt, MAPK, and Rap1 signaling pathways (**Figure 1G**). This is in line with prior observations of the critical involvement of cytokines and downstream signaling for early NK cell development and maturation (22, 39–41). Interestingly, the

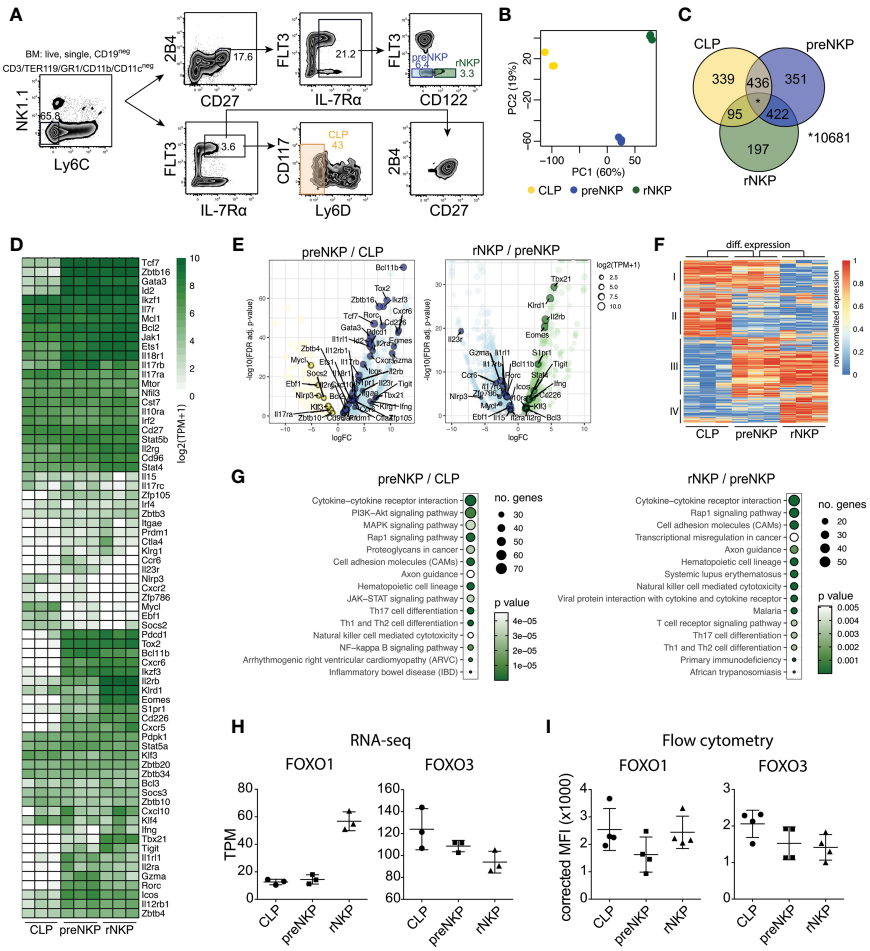

FIGURE 1 | The rNKP gene expression program is established gradually in the CLP to preNKP and preNKP to rNKP developmental transitions. **(A)** Gating strategy for FACS sorting of BM NK cell progenitors (LY6D^{neg} CLP, preNKP and rNKP). **(B)** Principal component (PC) analysis of RNAseq data from indicated cell populations FACS sorted from WT (FOXO1$^{flox/flox}$ FOXO3$^{flox/flox}$) mice (n = 3 per population). The variation explained by each PC is displayed in parenthesis. **(C)** Venn diagram showing the overlap between expressed protein-coding genes in indicated populations. Genes with ≥0.3 transcript per million (TPM) in all three replicas were considered expressed. **(D)** Hierarchically clustered heatmaps showing expression of protein-coding genes important for NK cell or ILC development. **(E)** Volcano plots showing differentially expressed genes for the comparisons between preNKP versus CLP (left panel) and rNKP versus preNKP (right panel). Differentially expressed genes (regulated by ≥2-fold at an FDR<0.05) are highlighted in color. Circle sizes indicate expression values in log2(TPM+1). **(F)** Hierarchically clustered heatmap showing row normalized expression of differential expressed genes (identified in **E**). Clusters I-IV are indicated. **(G)** KEGG pathway analysis of differentially expressed genes comparing preNKP versus CLP (left) and rNKP versus preNKP (right). Genes regulated by ≥2-fold at an FDR<0.05 were considered differentially expressed and used in the analysis. The size and color of the circles indicate the number of genes in each category and significance of enrichment respectively. **(H–I)** Expression levels of FOXO1 and FOXO3 from indicated progenitor populations, obtained by **(H)** RNA-seq or **(I)** flow cytometry. Dots represent individual analysed animals (*n* = 2-4). Bars indicate mean and SD. Data shown in **(I)** is from one representative experiment out of two independent experiments.

cytokine, PI3K-Akt, MAPK, and Rap1 pathways all coalesced on the FOXO family. This by either modulating FOXO localization and activity or by altering expression of genes that are direct transcriptional targets of the FOXO family (42–45). We found that FOXO1 and FOXO3 were co-expressed in LY6D^{neg} CLP, preNKP, and rNKP on the mRNA level (**Figure 1H**). To further validate this observation, we confirmed that FOXO1 and FOXO3 were expressed at the protein level at all three progenitor stags (**Figure 1I**) as well as in committed NK cells from spleen and BM (**Figures S1B, C**). This prompted us to further explore the role of the FOXO family in NK cell development (32, 34).

NK Cell Development and Maturation Are Dependent on the FOXO Proteins

To understand the role of FOXO1 and FOXO3 in NK cell development, we utilized Vav-iCre (46) to conditionally ablate FOXO1 (FOXO1$^{\Delta Vav}$) (47) and FOXO3 (FOXO3$^{\Delta Vav}$) (48) individually or in combination (FOXO1,3$^{\Delta Vav}$) throughout the hematopoietic system. Littermates lacking Vav-iCre (mainly FOXO1$^{flox/flox}$FOXO3$^{flox/flox}$ animals) were utilized as controls (WT). While residual FOXO1 and FOXO3 proteins were detected in the conditional mice (**Figure S2A**), the floxed DNA binding domains of both genes were found to be very efficiently

deleted by Vav-iCre when investigated at the mRNA level (**Figure S2B**).

Neither the loss of FOXO1 or FOXO3 alone resulted in a significant alteration to the number of NK cells in spleen and BM (**Figures 2A, B**). In sharp contrast, NK cell numbers were severely reduced in the spleen (5-10-fold reduced) and clearly decreased in the BM of FOXO1,3$^{\Delta Vav}$ animals (2-5-fold reduced) (**Figures 2A, B**). Altogether, this implies that FOXO1 and FOXO3 display functional redundancy in NK cell development and together are critical for the generation or maintenance of normal NK cell numbers.

We next assessed NK cell maturation in the three FOXO-deficient mouse strains based on CD11b and CD27 expression (49, 50). In this scheme, the first stage of NK cell maturation is characterized by low expression of both CD27 and CD11b (CD27^{low}CD11b^{low}). CD27 is then increased (CD27^{high}CD11b^{low}) before the subsequent upregulation of CD11b (CD27^{high}CD11b^{high}) and finally CD27 being down-regulated again (CD27^{low}CD11b^{high})

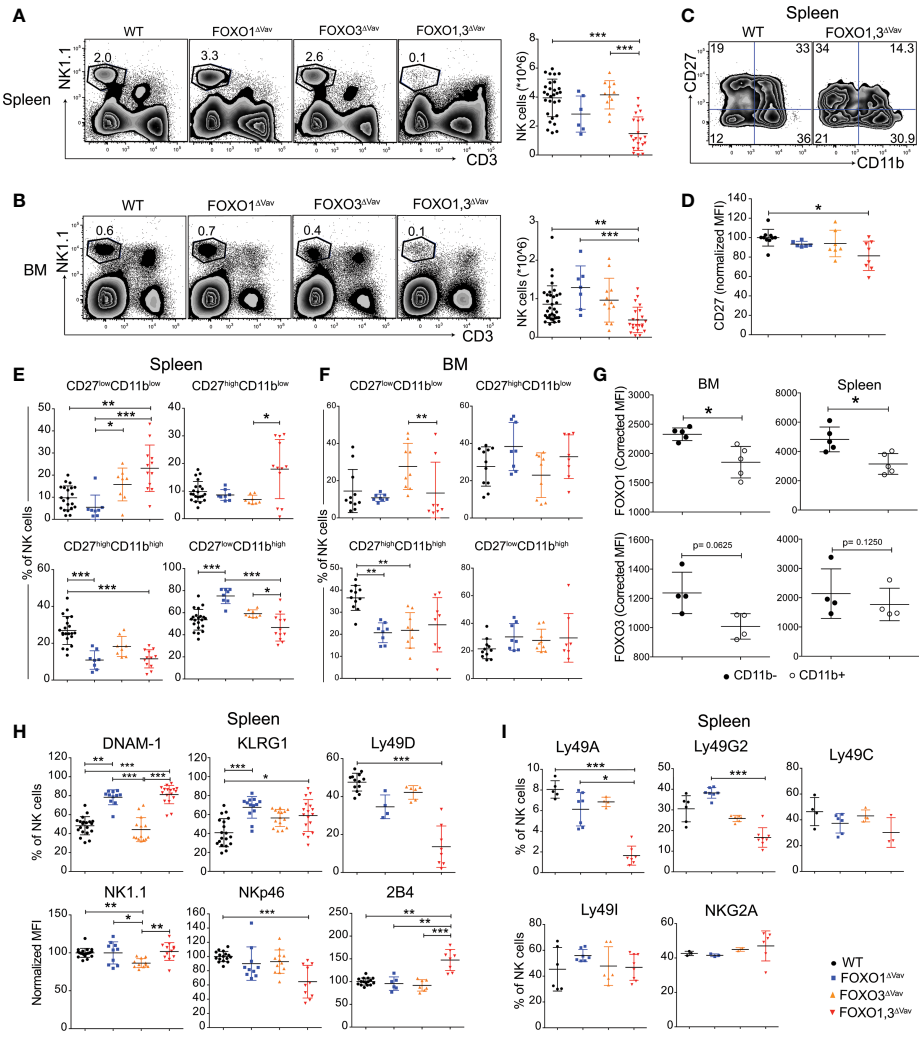

FIGURE 2 | NK cell development is dependent on FOXO1 and FOXO3. **(A)** Representative flow cytometry profiles (left panel) and total number of NK cells (single, live NK1.1^{+}CD3^{-} cells) (right panel) in spleen from animals with the indicated genotypes (n = 6-14). **(B)** Representative flow cytometry profiles (left panel) and total number of NK cells (single, live NK1.1^{+}CD3^{-} cells) in bone marrow (BM) (right panel) from animals with the indicated genotypes (n=7-17). **(C)** Representative flow cytometry profiles showing splenic NK cell maturation stages in animals with the indicated genotypes. **(D)** CD27 MFI of CD27^{+} NK cells from spleens (n = 6-12). **(E)** Frequency (%) of splenic NK cells from each indicated maturation stage (*n* = 7-17). **(F)** Frequency (%) of BM NK cells from each indicated maturation stage (*n* = 8-11). **(G)** FOXO1 and FOXO3 protein expression (MFI) in BM and splenic NK cells (n = 4-5) from WT mice. Data is from one representative experiment out of two independent experiments. **(H)** Frequency (%) or normalized MFI of indicated activating receptors on splenic NK cells (*n* = 14-19). **(I)** Frequency (%) of splenic NK cells with indicated inhibitory receptors (*n* = 3-8). In panels **(A, B, D–I)**: dots represent individual analyzed animals; bars indicate mean and SD; *, ** and *** indicates p-values <0.05, <0.01 and <0.001 respectively. P-values were calculated using: Kruskal Wallis tests with Dunn's multiple comparisons test (panels **A, B, D–F, H–I**) or the paired non-parametric Wilcoxon T test (panel G). Symbols utilized to indicate the genotype of analyzed mice throughout the panels are shown in the bottom right corner of the figure.

at the last step of maturation. The expression of CD27 on $CD27^{high}$ NK cells was significantly reduced in FOXO1,$3^{\Delta Vav}$ as compared to WT (**Figures 2C, D**) but regardless, all four maturation stages could be distinguished (**Figure 2C**). Analysis of the maturation subsets in spleen revealed that FOXO1,$3^{\Delta Vav}$ NK cells were less mature compared to NK cells from WT and single knockouts, with an accumulation of the $CD27^{low}CD11b^{low}$ population and a significant increase of the $CD27^{high}CD11b^{low}$ population (**Figure 2E**). Interestingly, there was an accumulation of the terminally differentiated mature $CD27^{low}CD11b^{high}$ NK cells in FOXO$1^{\Delta Vav}$ mice, that was not observed in FOXO$3^{\Delta Vav}$ and FOXO1,$3^{\Delta Vav}$ mice (**Figure 2E**). This suggests that in the periphery FOXO1 might act as a brake for FOXO3-driven maturation as its absence only has an effect when FOXO3 is present. In contrast to what was found in the spleen, we observed no accumulation of the immature $CD27^{low}CD11b^{low}$ subset and only a trend towards mature $CD27^{high}CD11b^{high}$ NK cells being reduced in the BM of FOXO1,$3^{\Delta Vav}$ mice (**Figure 2F**).

To investigate whether the observed phenotype had any relation to FOXO expression, we quantified FOXO protein expression in early ($CD11b^{low}$) and late ($CD11b^{high}$) NK maturation (**Figure 2G**) in WT mice. In line with the reduced generation of later $CD11b^{high}$ NK cells in FOXO1,$3^{\Delta Vav}$ mice, we found that both FOXO1 and FOXO3 generally displayed higher expression in the more immature $CD11b^{low}$ NK fraction (**Figure 2G**). Together this suggests that the FOXO proteins to a higher extent influence developmental progression of $CD11b^{low}$ NK cells and that peripheral NK cell development is more dependent on the FOXO proteins than BM NK cell development. The latter in line with the higher FOXO protein expression observed in splenic NK cells (**Figures S1B, C**).

Loss of FOXO Results in Perturbed NK Receptor Expression

We next investigated the impact of the FOXO genes on the expression of activating and inhibitory receptors that control signaling and functionality in NK cells (21, 51). Loss of FOXO3 caused no significant changes in the NK receptor repertoire except for a reduction in NK1.1 expression (**Figures 2H, I** and **Figure S3A, B**). In contrast, the loss of FOXO1 alone was enough to cause significant changes in DNAM-1 and KLRG1 expression (**Figure 2H**). As FOXO1,$3^{\Delta Vav}$ mice displayed a similar phenotype as FOXO$1^{\Delta Vav}$ mice (**Figure 2H**), this suggests that the perturbation of DNAM-1 and KLRG1 is caused solely by the loss of FOXO1. In addition, FOXO1,$3^{\Delta Vav}$ mice uniquely displayed significant changes in the expression of the activating receptors Ly49D, NKp46 and 2B4 (**Figure 2H** and **Figure S5A**) as well as the inhibitory receptors Ly49A and Ly49G2 (**Figure 2I** and **Figure S5B**). Hence, the FOXO transcription factors individually or cooperatively influence the expression of specific activating and inhibitory receptors.

CD122 Expression on NK Cells Is Reduced in the Absence of FOXO1 and FOXO3

As IL-15 is an important cytokine for NK cell development and survival (52), we investigated if the reduced expression of CD122 - a central component of the IL-15 receptor on NK cells (38) - could be a contributing factor to the observed reduction in NK cell numbers in FOXO1,$3^{\Delta Vav}$ mice. We found a significant reduction in CD122 expression on both BM and splenic NK cells from FOXO1,$3^{\Delta Vav}$ mice (**Figure 3A**) and that there was a clear correlation between NK cell numbers and CD122 expression in peripheral NK cells (**Figure 3B**). Interestingly, dependence on the FOXO proteins differed between BM and splenic NK cells with the CD122 expression on BM NK cells seemingly only relying on FOXO3 (**Figure 3A**). In contrast, the combined deletion was required to affect CD122 expression in splenic NK cells (**Figure 3A**). This, potentially due to the higher expression of FOXO proteins in splenic NK cells as compared to BM NK cells (**Figures S1B, C**).

It has previously been shown that the expression of the activating receptor NKG2D on NK cells is dependent on IL-15 signaling (53–55) and hence it can be utilized as a surrogate marker for IL-15 signaling. Much resembling the expression pattern of CD122 (**Figure 3A**), we found that NKG2D expression was significantly reduced on both BM and splenic NK ells from FOXO1,$3^{\Delta Vav}$ mice but that the dependence on the FOXO proteins varied between BM and spleen (**Figure 3C**). Hence, we concluded that the loss of FOXO results in reduced surface expression of CD122 and decreased IL-15 signaling. This strongly suggests that the reduced NK cell numbers in part can be directly contributed to a diminished IL-15 response in FOXO1,$3^{\Delta Vav}$ NK cells.

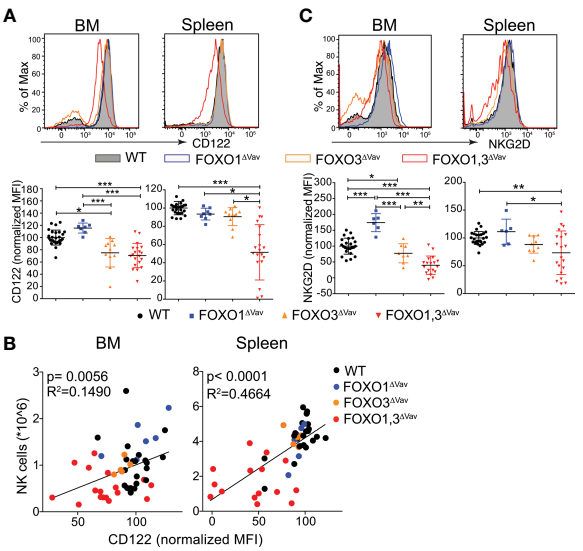

FIGURE 3 | FOXO regulates CD122 expression on NK cells. **(A)** Representative flow cytometry profiles (top) and normalized MFI (bottom panel) showing CD122 expression on BM and splenic NK cells (n = 8-28). **(B)** Correlation between CD122 expression and NK cell numbers (n = 50). **(C)** Representative flow cytometry profiles (top) and normalized MFI (bottom panel) showing NKG2D expression on BM and splenic NK cells (n = 6-26). In panels **(A–C)**: dots represent individual analyzed animals; bars indicate mean and SD; *, ** and *** indicates p-values <0.05, <0.01 and <0.001 respectively. P-values were calculated using: Kruskal Wallis tests with Dunn's multiple comparisons test (panels **A, C**) and linear regression (panel **B**).

FOXO Deficiency Results in a Developmental Block at the preNKP to rNKP Transition

The significant decrease in NK cell numbers (**Figure 2B**) coupled with the mild phenotype in BM NK cell maturation (**Figure 2F**) in FOXO1,3$^{\Delta Vav}$ mice, hinted at a defect in NK cell progenitors. To investigate this, we performed phenotypic analysis of the CLP, preNKP, and rNKP compartments (**Figure 4A**). This showed a significant decrease in the numbers of CLPs in FOXO1,3$^{\Delta Vav}$ mice but no overt changes to the number of preNKPs (**Figures 4A, B**). In contrast, the downstream CD122^{+} (IL15Rβ) rNKP population was significantly decreased in the FOXO1,3$^{\Delta Vav}$ mice (**Figures 4A, B**). No significant changes were observed in preNKP and rNKP numbers in mice lacking only FOXO1 or FOXO3 alone (**Figure S4**). Hence, the combined loss of FOXO1 and FOXO3 results in seemingly increased generation of preNKPs from CLPs and developmental block at the preNKP to rNKP transition. The latter argues for a significant part of the observed reduction in NK cell numbers – particularly in BM – being due to decreased generation of early NK cell progenitors.

The Perturbation in NK Development Is Intrinsic to the Hematopoietic Cells

To verify that the observed phenotype was intrinsic to hematopoiesis, we utilized the CD45.1/2 system and adoptively transfered FOXO1,3$^{\Delta Vav}$ BM (CD45.2) to irradiated WT (CD45.1) hosts. The number of NK cells generated 12 weeks post transplantation from adoptively transferred FOXO1,3$^{\Delta Vav}$ BM cells were markedly reduced in spleen (**Figures S5A, B** left), BM (**Figure S5B** middle), and blood (**Figure S5B** right). The perturbation in NK maturation was also recapitulated with reconstituted FOXO1,3$^{\Delta Vav}$ NK cells displaying accumulation of immature (CD27^{low}CD11b^{low}) NK cells and reduced number of more mature (CD27^{high}CD11b^{high}) NK cells (**Figure S5C**). Further, we observed a significant reduction in CLP numbers and block at the preNKP to rNKP transition in the progeny of FOXO1,3$^{\Delta Vav}$ BM cells (**Figures S5D, E**). Hence, the presence of normal cells in the transplantation setting does not rescue NK cell development from FOXO1,3$^{\Delta Vav}$ donor BM cells, supporting the notion of a cell autonomous FOXO requirement in the regulation of early NK progenitors and NK cell maturation.

Loss of FOXO Impacts Expression of NK Associated Genes Already at the CLP Stage

With the loss of FOXO perturbing the NK developmental pathway already at the level of the CLP (**Figures 5A, B**), we next sought to investigate if we could identify NK lineage related changes at this step of development. To this end, we performed RNA-seq on LY6D^{neg} CLP (GR1/MAC1/NK1.1/TER119/CD3ε^{-} CD11C^{-}LY6C^{-}IL-7Rα^{+}FLT3^{+}KIT^{low}LY6D^{-}) from WT, FOXO1$^{\Delta Vav}$ mice, FOXO3$^{\Delta Vav}$ mice, and FOXO1,3$^{\Delta Vav}$ mice. We found 469 genes that displayed significant differential expression between LY6D^{neg} CLPs from FOXO1,3$^{\Delta Vav}$ and WT mice (**Figures 5A, B** and **Table S2**). The observed perturbation in gene expression became progressively more distinct with the loss of both FOXO1 and FOXO3 function (**Figure 5A**). This suggests that the FOXO proteins have mainly synergistic functions at this step of development.

In agreement with earlier reports, we found that the *Il7ra* gene was significantly down-regulated (**Figures 5A–C**) confirming the known positive regulatory role of FOXO in controlling IL-7R*a* expression (9, 16). Further, looking specifically at genes previously described to be NK cell signature genes (28), we found that a significant number of these genes displayed altered expression at the LY6D^{neg} CLP stage (**Figures 5B, C**). Of note, we found that *Tcf7, Id2, Il18r1*, *Il12rβ1, Cxcl10*, and *Cxcl9* – all genes encoding proteins important for NK cell development, migration, and functions (31, 56–59) - were up-regulated in the FOXO1,3$^{\Delta Vav}$ cells. Conversely, looking at the down-regulated genes, we interestingly found that *Ets1* – a gene known to be important for the development of NK progenitors and NK

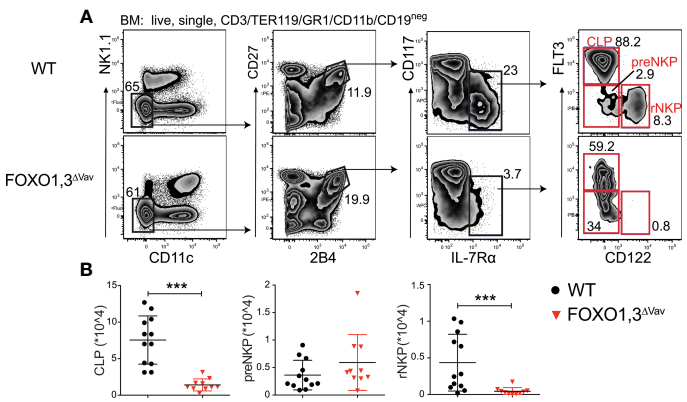

FIGURE 4 | FOXO deficiency results in a block at the preNKP to rNKP transition. **(A)** Representative flow cytometry profiles showing the identification of CLP, preNKP and rNKP in animals with the indicated genotype. **(B)** Total number of CLP, preNKP and rNKP in BM of animals with the indicated genotype (n = 10-12). In panel **(B)**: dots represent individual analyzed animals; p-values were calculated using Mann-Whitney; bars indicate mean and SD; *** indicates p-values <0.001

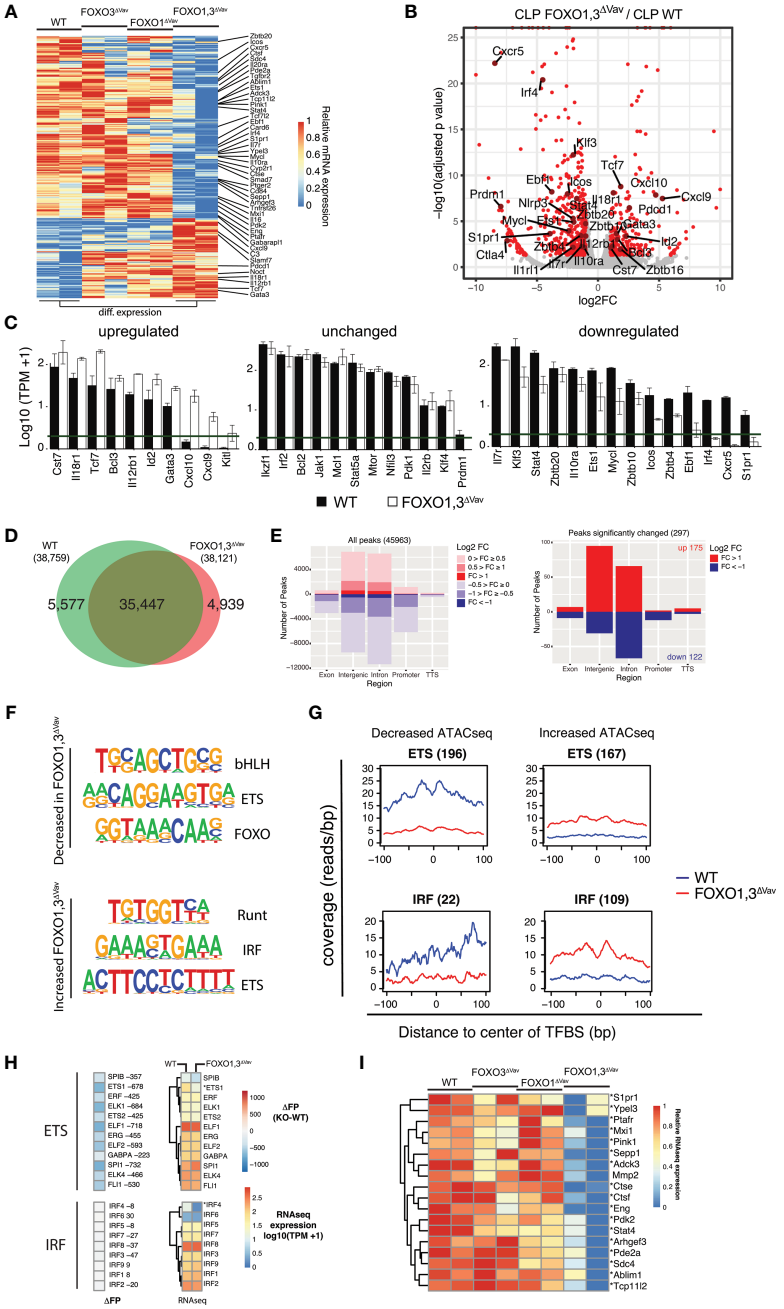

FIGURE 5 | Removal of FOXO results in NK associated gene regulatory changes at the CLP stage. **(A)** Heatmap showing row normalized expression of genes with differential expression (adjusted p-value ≤0.01, ≥2-fold change in expression and ≥1 TPM in 2+ samples) between WT and FOXO1,3$^{\Delta Vav}$ LY6D^{neg} CLPs. **(B)** Volcano plot showing log2 fold change and adjusted p-value for the comparison of WT to FOXO1,3$^{\Delta Vav}$ LY6D^{neg} CLPs. Red dots indicate genes with >2-fold change in expression and adjusted p-value < 0.05. **(C)** Bar graphs showing expression (TPM) of select NK associated genes. Genes with adjusted p-value ≤ 0.01, ≥2-fold change in expression and ≥1 TPM in 2+ samples were considered to have the decreased or increased expression. The green line indicates 1 TPM. **(D)** Venn diagram showing the overlap between ATACseq peaks identified in LY6D^{neg} CLPs from WT and FOXO1,3$^{\Delta Vav}$ mice. Only peaks identified in ≥2 replicas each with >30 reads were considered. **(E)** Annotation of all ATAC-seq peaks identified (left) and peaks with significantly altered chromatin accessibility (adjusted p-value < 0.01 and ≥2-fold change in signal) when comparing LY6D^{neg} CLPs from WT and FOXO1,3$^{\Delta Vav}$ mice (right). Number of regions and log2 fold change (FOXO1,3$^{\Delta Vav}$/WT) in ATAC-seq signal is indicated. **(F)** Motifs enriched in differential ATAC-seq peaks. Top three most significantly enriched motifs existing in >10% of regions are displayed. **(G)** Cut-profiles for differential ATAC-seq peaks with ETS- and IRF-family transcription factor binding sites (TFBS). Number of regions with each TFBS is indicated in parenthesis. **(H)** Genome-wide difference in the number of footprints (identified in WT and FOXO1,3$^{\Delta Vav}$ LY6D^{neg} CLPs) (left) and expression (right) of indicated genes from the ETS- and IRF-families. * indicates significant differences in gene expression between LY6D^{neg} CLPs from WT and FOXO1,3$^{\Delta Vav}$ mice.
(I) Expression of known ETS1 targets in LY6D^{neg} CLPs from mice with indicated genotypes.

maturation (30, 60) - was significantly down-regulated in CLPs (**Figures 5A–C**). The down-regulation of ETS1 was also confirmed at the protein level in CLPs (**Figure S6A).**

The NK Associated Gene Regulatory Network in CLPs Is Perturbed by the Loss of FOXO

We next investigated whether the changes in the expression of known NK-cell-development genes could be related also to changes in the gene regulatory landscape. Using the assay for transposase-accessible chromatin followed by sequencing (ATAC-seq), we identified close to 46,000 open chromatin regions across analyzed LY6D^{neg} CLP with the vast majority existing both in WT and FOXO1,3$^{\Delta Vav}$ cells (**Figure 5D**). Overall, most of the identified open chromatin regions were localized in intergenic and intronic region (**Figure 5E**, left). Next, we identified regions with significant differences (adjusted p-value <0.01 and ≥2-fold change in signal amongst peaks identified in ≥2 samples and having >30 reads) in chromatin accessibility between WT and FOXO1,3$^{\Delta Vav}$. This revealed 297 regions that were mainly localized in intergenic and intronic regions (**Figure 5E**, right). Hence, this suggests that loss of FOXO in LY6D^{neg} CLPs results in relatively few but distinct changes to overall chromatin accessibility and that the major effect on gene regulation is *via* distal elements while promoters remain largely unaffected.

To identify transcription factors where altered binding could cause the alterations in chromatin accessibility, we performed *de novo* motif enrichment analysis on the peaks with altered chromatin accessibility. This revealed that peaks with decreased accessibility in the FOXO1,3$^{\Delta Vav}$ LY6D^{neg} CLP were enriched for transcription factor binding (TFBS) sites related to the bHLH-, ETS- and, as expected, the FOXO family (**Figure 5F** top). Correspondingly, RUNT, IRF, and ETS motifs were found in regions that gained chromatin accessibility (**Figure 5F** bottom). To further corroborate that TF binding was altered, we analyzed the Tn5 integration sites (cut-profiles) around the putative TFBS for each of these TF-families. Out of the motifs identified (**Figure 5F**), ETS and IRF produced clear cut-profiles (**Figures 5G** and **S6B).** This supports that altered ETS and IRF binding directly contribute to the changes in chromatin accessibility while suggesting that the other TFBS are either not used or that TF binding does not produce distinct cut-profiles on these analyzed regions.

Altered binding of a single transcription factor might not cause significant changes to the overall chromatin accessibility at the level of a whole chromatin region as defined by the ATAC-seq peaks. A complementary approach is footprinting analysis, which instead attempts to localize sudden decreases in the number of reads within an open chromatin region to identify individual TF bound regions (61). By means of footprint analysis, we assessed the changes in genome-wide binding of ETS and IRF. We found no major changes in the overall number of IRF associated footprints (**Figure 5H**). This suggests that increased IRF binding is specifically associated with peaks displaying increased chromatin accessibility in FOXO1,3$^{\Delta Vav}$ CLPs (**Figures 5F, G**), while the observed decrease *Irf4* expression (**Figures 5C, H**) has no major impact on overall IRF-binding.

In contrast, we found a significant decrease in footprints associated with ETS motifs also at the genome-wide level (**Figure 5H**). The fact that Ets1 is the only identified ETS-family member displaying significant changes in expression (**Figures 5C, H**), suggests that the decreased number of ETS-bound regions reflects reduced binding of ETS1. This conclusion is further supported by putative ETS1 target genes (62, 63) including *Stat4, Pdk2, Adck3* showing significantly lower expression in FOXO1,3$^{\Delta Vav}$ CLPs (**Figure 5I**). Taken together with the reduced expression of ETS1 (**Figures 5B, C** and **S6A**) and clear ETS cut-profile (**Figure 5G**), this suggests that loss of ETS1 binding in FOXO1,3$^{\Delta Vav}$ CLPs contribute to the altered gene regulatory landscape and potentially the reduced capacity to generate NK cells downstream of the CLP (30).

We further looked specifically at TCF7 (31) and NFIL3 (29), as both are known to be critical for BM NK progenitor development. TCF7 expression was increased in CLPs from FOXO1,3$^{\Delta Vav}$ mice (**Figures 5B, C**) but no significant change in binding as assessed by footprinting analysis (46 less footprints in the FOXO1,3$^{\Delta Vav}$ CLPs) was observed. Hence, TCF7 is potentially controlled by FOXO at the transcriptional level but only has a minor impact in terms of chromatin accessibility at the CLP stage. NFIL3 showed no significant change in expression or overall binding (seven less footprints in the FOXO1,3$^{\Delta Vav}$ CLPs). Hence, we find no indication that perturbed TCF7 and NFIL3 activity contribute to the changes observed in FOXO1,3$^{\Delta Vav}$ CLPs.

preNKPs Lacking FOXO Fail to Up-Regulate NK-Lineage Related Genes

With FOXO1,3$^{\Delta Vav}$ mice displaying a block at the preNKP to rNKP transition, we next sought to characterize the transcriptional changes caused by the loss of FOXO in preNKPs. Based on PCA, the FOXO1,3$^{\Delta Vav}$ preNKPs overall maintained a preNKP transcriptional profile as compared to its WT counterparts (PC2) with the top loadings of PC2 being CLP and NK-lineage related (**Figures 6A** and **S7A**). In line with this, the expression of Id2 – which marks the formation of preNKP from CLP (24) – was not altered and generally the expression of ILC related transcription factors (37) was also found to be similar (**Figure 6B**). However, the combined loss of FOXO1 and FOXO3 did cause distinct gene expression changes as observed both by PCA (PC1) (**Figure 6A**) and direct comparison of expression profiles (**Figure 6C** and **Table S1**). In relation to normal development, the FOXO1,3$^{\Delta Vav}$ preNKPs displayed lower expression of genes commonly expressed throughout the early NK progenitor hierarchy (**Figure 6D**, cluster I) and, to a lesser extent, maintained expression of CLP associated genes (**Figure 6D**, cluster IV). We additionally found that the FOXO1,3$^{\Delta Vav}$ preNKPs failed to properly express genes normally upregulated in the CLP to preNKP transition and further increased in expression in the subsequent preNKP to rNKP transition (**Figure 6D**, cluster II). The later gene cluster

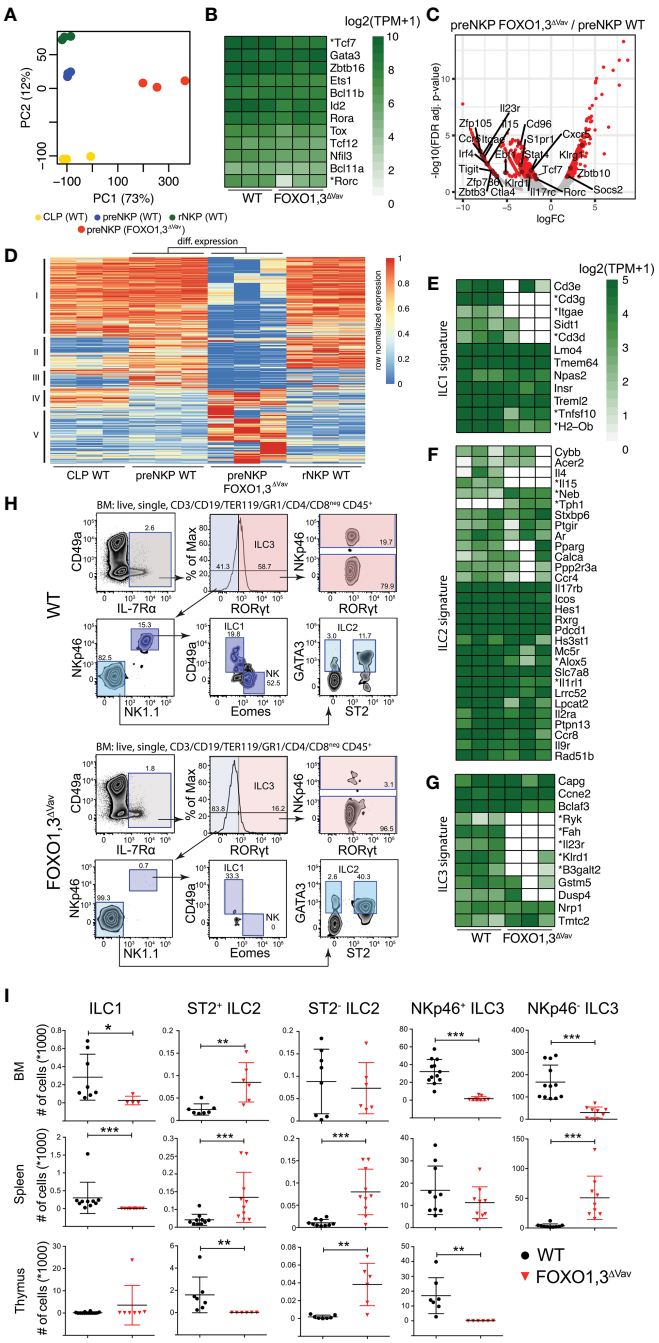

FIGURE 6 | Loss of FOXO results in a perturbation of the preNKP transcriptional program and the development of non-cytotoxic ILCs. **(A)** Principal component (PC) analysis of RNAseq data from indicated cell populations (for gating strategy see **Figure 1A**) from wildtype and FOXO1,3$^{\Delta Vav}$ mice (n = 3). The variation explained by each PC is displayed in parenthesis. **(B)** Hierarchically clustered heatmaps showing gene expression for selected transcription factors critical for NK/ILC development. * indicates significant differences in expression between WT and FOXO1,3$^{\Delta Vav}$ preNKPs (FDR < 0.05, >2-fold change). **(C)** Volcano plot showing log2 fold change and adjusted p-value for the comparison of WT to FOXO1,3$^{\Delta Vav}$ preNKPs. Red dots indicate genes with >2-fold change in expression and adjusted p-value < 0.05. **(D)** Hierarchically clustered heatmap showing row normalized expression of differential expressed genes (identified in C). Clusters I-V are indicated. **(E–G)** Hierarchically clustered heatmaps showing gene expression of **(E)** ILC1, **(F)** ILC2 and **(G)** ILC3 signature genes defined by Robinette et al. (64), * indicates significant differences in expression between WT and FOXO1,3$^{\Delta Vav}$ preNKP (FDR < 0.05, >2-fold change). **(H)** Representative flow cytometry profiles showing the identification of ILC subsets in BM from WT and FOXO1,3$^{\Delta Vav}$ mice. **(I)** Total number of indicated ILC subsets in BM, spleen and thymus from WT and FOXO1,3$^{\Delta Vav}$ mice (n = 6-12). NKp46- ILC3 population had less than 50 cells per thymus in all mice from both mouse strains so was not shown. Dots represent individual analyzed animals. Bars indicate mean and SD. P-values were calculated using Mann-Whitney tests with *, ** and *** indicates p-values <0.05, <0.01 and <0.001 respectively.

was as expected enriched for NK lineage associated genes (**Figure S7B**) including amongst others *Klrd1* (encoding for CD94) and *Tigit* shown to function as NK immune checkpoint inhibitors (65). The reduced generation of rNKPs from FOXO1,3$^{\Delta Vav}$ preNKPs is hence associated with a failure to properly express an early NK gene program.

Looking specifically at individual genes impacting NK development, we found that TCF7 - in contrast to what was observed in CLPs (**Figures 5B, C**) - was downregulated in FOXO1,3$^{\Delta Vav}$ preNKPs while NFIL3 expression remained unaffected (**Figures 6B, C**). Hence, the lower TCF7 expression in preNKPs could potentially contribute to the impaired generation of rNKPs (31). Further, in line with data from the CLP stage, we found that ETS1 displayed a clear trend toward being down-regulated in FOXO1,3$^{\Delta Vav}$ preNKPs (**Figures 6B** and **S7C**). In concordance with the CLP expression and epigenetic data, this argues for the loss of FOXO causing ETS1 downregulation which in turn contributes to the observed impairment generation of NK cells. Further in line with perturbed ETS1 expression contributing to the observed NK phenotype, we found that *Ets1* expression was significantly down-regulated also in splenic NK cells (**Figure S7D).**

Interestingly, we also found a significant decrease in expression of zinc finger protein 105 (*Zfp105)* (**Figure 5B**) – a transcription factor previously shown to be regulated by FOXO1 (15, 66), to augment differentiation toward NK cell lineage (67) and to be a direct target of FOXO1 in CD8 T cells – which might suggest that FOXO-mediated regulation of Zfp105 expression plays a role in NK cell development.

Loss of FOXO1 and FOXO3 Disrupts Development of Non-Cytotoxic ILCs

The preNKP represents a heterogenous population which gives rise not only to NK cells but also to the other non-cytotoxic ILC subsets (24, 25). In agreement with this, we observed expression of genes encoding transcription factors linked to development of the non-cytotoxic ILCs, including *Tcf7, Tox, Bcl11b, Zbtb16, Rorc, Ets1, Nfil3* (37) in both WT and FOXO1,3$^{\Delta Vav}$ preNKP (**Figure 6B**).

We next sought to investigate if we could observe transcriptional changes in the FOXO1,3$^{\Delta Vav}$ preNKP indicative of disruptions potentially influencing also the non-cytotoxic ILC subsets. To this end, we utilized the ILC1-3 gene signature published by Robinette et al. (64). Interestingly, we found down regulation of expression across all the three gene signatures (**Figures 6E–G**) but most notably within the ILC1 (**Figure 6E**) and ILC3 (**Figure 6G**) gene signatures.

These transcriptional changes made us speculate that the development of the non-cytotoxic ILC subsets in addition to NK cells could be perturbed in FOXO1,3$^{\Delta Vav}$ mice. To investigate this, we performed phenotypic analysis of ILC subsets from BM, spleen, and thymus from WT and FOXO1,3$^{\Delta Vav}$ mice (**Figure 6H**). Indeed, we found that the ILC1 population was reduced in BM and spleen (**Figures 6H, I**). ILC3 numbers were similarly reduced in BM and thymus while, in contrast, NKp46^{-} ILC3s were increased in spleen (**Figures 6H, I**). In contrast, ILC2 subsets were generally increased in the analyzed organs (**Figures 6H, I**). Hence, we concluded that the loss of FOXO causes broad perturbation of the non-cytotoxic ILC subsets. Potentially these changes are due to gene regulatory changes already at the level of the preNKP, meaning that FOXO determine lineage specification of ILC.

DISCUSSION

In this study, we show that FOXO1 and FOXO3 are expressed in the early progenitors of the innate lymphoid lineages and cooperatively regulate the generation of NK cell progenitors and NK cells. In addition, we discovered a hitherto undescribed role of the FOXO family in establishing the NK/ILC gene expression program in progenitor cells and in the development of the ILC1, 2, and 3 subsets. Hence, the loss of FOXO1 and FOXO3 disrupts development of both the cytotoxic and non-cytotoxic ILC lineages.

Using a combination of RNA-seq and ATAC-seq data to study the underlying gene regulatory mechanisms, we found that the loss of FOXO proteins disrupted the regulation of NK and ILC associated genes already at the CLP stage and more markedly so at the preNKP stage. Likely the failure to establish the NK/ILC gene program in preNKPs directly results in the observed reduction in rNKPs. Interestingly, we found a decrease in ETS1 at the CLP stage onwards in FOXO1,3$^{\Delta Vav}$ mice. In line with ETS1 being a critical downstream target of FOXO, the NK cell phenotypes of the ETS1 knockout very much resemble the FOXO1,3$^{\Delta Vav}$ phenotype with reduced splenic NK cells and rNKPs while preNKPs seemingly remain unaffected (30, 60). Further, altered NKp46 and Ly49D expression were also observed in ETS1 deficient animals (30). With the activity of ETS1 being modulated *via* interaction with FOXO1 (68), the loss of FOXO could mimic the ETS1 knockout by both lowering ETS1 expression and ETS1 activity throughout NK cell development.

The preNKP compartment is heterogeneous and contains several progenitor populations out of which only a subset is involved in the generation of the NK lineage (24, 25). While these subsets are yet to be readily identifiable without the use of reporter genes, we observed distinct changes both in the activation of the overall preNKP gene expression program and in genes associated with the ILC1-3 lineages. These changes were associated with decreased numbers of ILC1 and ILC3 as well as increased numbers of ILC2. Hence, this argues that the altered gene expression caused by the loss of FOXO is directly reflected in the ILC lineages though it is unclear if these alterations reflect changes at the progenitor composition of the preNKP compartment or in the overall transcriptional program. Addressing this point will require further studies using reporter mice to distinguish the different progenitor populations within the preNKP compartment.

The loss of FOXO could also directly influence the development of ILCs (i.e., by acting downstream of important preNKP transcription factors). Indeed, the expression of BCL11B

in preNKP, especially in combination with ZBTB16, marks the development of ILC2 (24). On the other hand, the absence of both markers in preNKP allows for a balanced development of NK cells and all ILC lineages (24). FOXO proteins have been suggested to repress cell cycle progression downstream of BCL11B, which is a critical regulator of basal cell quiescence in the mammary gland (69). ZBTB16 overexpression leads to reduced FOXO phosphorylation (70) while FOXO1 expression is induced in ZBTB16 heterozygosity as compared to homozygosity (71). Altogether, this makes it tempting to speculate that FOXO serve as crucial transcriptional regulator acting downstream of ZBTB16 and/or BCL11B to control ILC development in a cell type-specific manner.

Prior studies using Ncr1-iCre and Vav-iCre have reported contradictory data on the role of the FOXO1 in NK cell development (32–34). Using the VAV-iCre model, we in agreement with Deng et al., found that the deletion of FOXO1 did not significantly alter total NK cell numbers while $CD27^{low}CD11b^{high}$ NK cells were accumulated in line with FOXO1 suppressing Tbx21 expression needed for maturation (32). However, in contrast to what was observed following the combined deletion of FOXO1 and FOXO3 in the Ncr1-Cre model, we found that NK cell numbers declined significantly in both the BM and spleen of $FOXO1,3^{\Delta Vav}$ animals. The splenic NK cells that developed displayed an accumulation of immature $CD11b^{low}$ NK cells but lacked the accumulation of $CD27^{low}CD11b^{high}$ NK cells observed after the deletion of FOXO1 alone. The discrepancy in maturation status between $FOXO1,3^{\Delta Ncr1}$ and $FOXO1,3^{\Delta Vav}$ might be attributed to their differential roles in hematopoietic progenitors and committed NK cells.

In addition, FOXO1 single deficient mice exhibit an increase in terminally mature splenic NK cells, suggesting that FOXO1 exerts negative regulation on NK cell maturation. These data support the model where FOXO1 inhibit NK cell maturation by repressing Tbx21 (32). The additional deletion of FOXO3 reversed this phenotype leading to accumulation of immature subsets potentially suggesting that in the periphery FOXO1 might act as a brake for FOXO3 driven maturation. Thus, both FOXO1 and FOXO3 expression is key rheostat of NK cell maturation. Such a reciprocal regulation was recently suggested for Tbx21 and Eomes in NK cell maturation (72). FOXO1 acted also as a negative regulator for NK cell receptors DNAM-1 and KLRG1. At least for the DNAM-1 receptor, this might be independent of the maturation status, as we found that immature and mature NK cell subsets were similarly represented in $DNAM\text{-}1^{-}$ and $DNAM\text{-}1^{+}$ NK cells (73).

Somewhat surprisingly, BM NK cell maturation in the $FOXO1,3^{\Delta Vav}$ animals was left rather unperturbed. Potentially, this argues for the lower NK cell numbers in BM being a consequence of the reduced numbers of rNKPs rather than major issues with NK cell maturation. Speculatively, this would in addition suggest that NK maturation in spleen and BM to some extent have different requirements and that the environment causes different reliance on the FOXO proteins.

IL-15 signaling is critical for NK cell development and survival (74–76). We found that the $FOXO1,3^{\Delta Vav}$ NK cells displayed markedly reduced CD122 (IL15Rβ) and that expression was quantitatively correlated with NK cell numbers. Supporting the notion that the $FOXO1,3^{\Delta Vav}$ NK cells have reduced IL-15-signaling, we also found that the IL-15 dependent expression of NKG2D (53–55) was significantly lower on BM and splenic NK. Hence, this argues that the reduced NK cell numbers in $FOXO1,3^{\Delta Vav}$ mice result from defects in the generation of NK cells *via* rNKPs in combination with decreased ability to respond to IL-15 signaling critical for maintaining the normal NK population. Furthermore, the reduction of the ILC1 population in the absence of FOXO1,3 could be explained to occur similarly to the reduction of the NK cell population through their indispensable requirement of IL-15 needed for cell development (38, 77).

It is appealing to speculate that the changes in progenitor development as well as the reduced CD122 expression can be attributed to the reduction in ETS1 expression. Indeed, chromatin immune precipitation experiments revealed that ETS1 binds to the promoter of the CD122 gene and CD122 expression was reduced in mature ETS1 KO NK cells (30). FOXO1 and FOXO3 hence might tune CD122 expression indirectly through regulating a network of factors including ETS1 to gradually modulate IL-15 responsiveness, rather than causing an "on/off" situation in CD122 expression. Given that once NK cells acquire NKp46 expression (and hence gene deletion occurs in the Ncr1-Cre model), CD122 expression is not perturbed by the loss of FOXO1 and FOXO3 activity (32), this suggests that the loss of FOXO activity in $FOXO1,3^{\Delta Vav}$ NK progenitors or very early NK cells (prior to Ncr1-Cre mediated deletion) cause a defect that cannot be corrected in later stages of NK cell development. This hypothesis is in line with recent studies showing that IL-15 signaling creates a positive regulatory loop to modulate expression of its receptors and several components of the IL-15 signaling pathway (40). Hence, FOXO1 and FOXO3 would then serve as crucial early regulator of NK cell fate by establishing proper IL-15 receptor expression.

In conclusion, the co-expression and regulatory function of FOXO1 and FOXO3 is critical throughout the NK cell development and maturation. Mechanistically, we propose that FOXO1 and FOXO3 – amongst other genes - control the expression of ETS1 and CD122 that both are integral for NK cell development. In addition, FOXO proteins selectively promote the development ILC1 and ILC3 but not ILC2. The very well controlled intrinsic modes of NK cell development, differentiation, and maturation by FOXO1 and FOXO3 revealed in our study can drive future efforts to develop anti-tumor and anti-viral immunotherapies targeting FOXO proteins.

MATERIALS AND METHODS

Mice

To generate animals conditionally lacking FOXO1 and/or FOXO3 throughout the hematopoietic system, we crossed $FOXO1^{flox/flox}$ (47) and/or $FOXO3^{flox/flox}$ (48) with Vav^{-}iCre

(46) mice. All alleles were maintained on a C57BL/6 background and mice were predominantly analyzed at 8 to 14 weeks of age. Congenic CD45.1 WT C57BL/6 mice were used as recipients in transplantation experiments. All animal experiments were approved by the local animal ethics committee.

Flow Cytometry

Single-cell suspension of bone marrow, spleen, blood, or thymus were incubated with Fc block (anti-FcγRIII, clone 2.4G2) and subsequently stained with fluorescent antibodies (**Table S3**) and viability markers (LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit or propidium iodide, both from Invitrogen). Staining was done at 4°C in PBS with 2% FBS for 20 min. Results were acquired mainly using the BD LSRFortessa™ or BD FACSymphony™ Flow Cytometers (BD Biosciences).

For FACS sorting of progenitor cells, mature cells were depleted using antibodies against TER119, CD3, CD19, GR1, and MAC1 together with sheep anti-rat IgG Dynabeads (Invitrogen) prior to staining with fluorescent antibodies. Cell sorting was performed mainly on a FACSAriaIIu or FACSAria Fusion (BD Biosciences).

Further analysis of FACS data was performed using Flowjo v9.9.6 (TreeStar, Ashland, OR). Corrected mean fluorescence intensities (MFI) were calculated by subtracting the MFI of control sample (stained only with secondary antibody) from the MFI of the FOXO stained sample. Normalized MFIs were calculated by dividing MFIs with the average MFI observed for WT samples in each independent experiment.

Transplantation Assay

To determine *in vivo* NK lineage output from FOXO1,3$^{\Delta Vav}$ hematopoietic stem- and progenitor cells, unfractionated bone marrow (0.2 x 10^6 WT or 3 x 10^6 FOXO1,3$^{\Delta Vav}$ CD45.2 donor cells), was injected intravenously into irradiated (950cGy) CD45.1 recipients. The number of unfractionated BM cells transplanted were proportional to the frequency of phenotypic hematopoietic stem cells in WT and FOXO1,3$^{\Delta Vav}$ mice (data not shown). Part of the FOXO1,3$^{\Delta Vav}$ transplanted animals were in addition given 0.2 x 10^6 unfractionated WT BM cells as support. Reconstitution was analyzed at 12 weeks post-transplantation using flow cytometry.

TotalScript Based RNA-Seq and Analysis

RNA-seq was done using TotalScript (Epicenter) on RNA prepared from approximately 5000 FACS sorted cells using RNeasy micro (Qiagen) as previously described (78). Libraries were sequenced pair end (2x50 cycles) on the Illumina platform. Reads were mapped to the mouse reference genome (mm10) using STAR v2.3.2b (https://github.com/alexdobin/STAR). Strand-specific reads in exons were quantified using HOMER and assessment of differential gene expression analysis was done using EdgeR (79) on raw read count. Data visualizations were mainly done using ggplot2, pheatmaps, and R base graphics.

ATAC Sequencing and Analysis

ATAC sequencing was performed (using 3000-5000 FACS sorted cells) as previously described (80). Libraries were sequenced pair-end (2x50 cycles) on the Illumina platform (Illumina). Reads were trimmed (using Trim Galore v0.4.1), mapped to the mouse reference genome (mm10) (using Bowtie2 v2.3.3.1) and PCR duplicates removed when making HOMER tag directories (using makeTagDirectory with -tbp 1). Peaks were subsequently identified in sub-nucleosomal reads (read-pairs within 100bp) using HOMER's findPeaks.pl. Peaks with differential chromatin accessibility were identified using EdgeR on raw read counts in identified peaks. Peaks displaying an adjusted p-value ≤0.01 and ≥2-fold change in read count were considered to have differential chromatin accessibility. Only peaks identified in ≥2 replicas each with >30 reads were considered in the analysis. Annotation and motif enrichment analysis of differential peaks were done using the HOMER's annotatePeaks.pl and findMotifsGenome.pl with -size given respectively.

To make cut-profiles, the localization of known HOMER transcription factor binding sites (TFBS) belonging to members of the enriched TF family (identified by the motif enrichment in differential ATAC-seq peaks) were localized in the genome using HOMER's findMotifsGenome.pl. To take into account the position of the Tn5 integration into the genome, custom HOMER tag directories were made off-setting reads on the plus and minus strand with +4 and -5 bases respectively. Read depth centered around TFBS from a specific family were subsequently plotted using HOMER's annotatePeaks.pl with a -fragLength of 9 (corresponding to the bp covered by Tn5) and -hist 1 (1 bp bins).

Genome-wide footprinting to identify TF binding sites was performed using DNase2TF. In brief, Bowtie2 mapped reads were deduplicated (using Picacard tools' MarkDuplicates), data from the same population/genotype merged (using SamTools' merge) and down-sampled to 39 million read-pairs per sample (using Picard Tool's DownsampleSam.jar). Localization of reads were off set in the.bam file to take into account the Tn5 integration (as described above) using custom scripts. HOMER tag directories and peak finding were done as described above. Peak files and downsampled.bam files were subsequently used as input for DNase2TF (81). Identified footprints with a p-value ≤0.05 were overlapped with the TFBS identified in the mouse reference genome (mm10) using the transfac catalogue as previously described (82). Footprints were associated with TFBS when the center of the TFBS fell within the footprint.

Data visualizations were mainly done using ggplot2, pheatmaps and R base graphics.

RTqPCR

NK cells were FACS sorted using BD AriaIII (BD Biosciences). CD117+ BM cells were enriched using CD117 MicroBeads, mouse (Miltenyi). RNA was purified using RNeasy micro kit (Qiagen) and cDNA prepared using MultiScribe Reverse Transcriptase (Life Technologies) or SuperScript II (Life Technologies) in combination with random hexamer priming. qPCR was performed using TaqMan™ Universal PCR Master

Mix (Life Technologies) and TaqMan probes against: FOXO1 (Mm00490672_m1), FOXO3 (Mm00490673_m1), hprt (Mm01545399_m1 or Mm00446968_m1) and Ets1 (Mm0 1175819_m1).

SMART-Seq Based RNA-Seq and Analysis

Two hundred to 500 progenitor cells were FACS sorted into lysis solution with DNase I from the Single Cell Lysis Kit (Invitrogen) and samples prepared according to manufacturer's instructions. RNA-seq libraries were subsequently prepared using the SMART-Seq Stranded Kit (Takara) according to the manufacturer's instruction. Quality of cDNA library was determined using an Agilent Bioanalyzer according to the manufacturer's protocol. Libraries were quantified by using the KAPA-SYBR FAST qPCR kit (Roche) and sequenced pair-end (2x75 cycles) on the Illumina NextSeq 500.

Adaptor sequences were trimmed, and low-quality reads removed using Trimmomatic (v.0.36). All sequencing reads aligning (HiSAT2, v.2.1.0) to annotated mouse ribosomal RNA genes were discarded. High-quality and ribosomal RNA depleted sequencing reads were aligned to the genome GRCm38.p6/mm10 genome using HiSAT2. Using sorted bam files (Samtools v.1.10), the number of aligned reads were counted (featurecount in subread package v. 2.0.0). After normalization (TMM: trimmed mean of M-values), a differential gene expression analysis (edgeR v. 3.28.1) was performed. Significant differentially expressed genes were distinguished by a false discovery rate (FDR) <0.05. Data were plotted using ggplot2 (v.2.3.3) in R (v. 3.6.1). Gene ontology analysis was conducted using clusterprofiler (v.3.14.3) with the database org.Mm.eg.db (v.3.10.0) in R. All scripts used for processing of SMART-seq data are deposited on Github: https://github.com/jonasns/NK_FOXO.

Statistics

Statistical analysis of FACS data was performed using Graphpad Prism version 6 for Mac OSX (Graphpad 83) or R version 3.3.3 (R Development Core 84). Statistics pertaining to RNA-seq and ATAC-seq data were performed as described above.

DATA AVAILABILITY STATEMENT

SMART-seq RNAseq data are deposited at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9560/. RNA-seq and ATAC-seq data are available from the European Nucleotide Archive (www.ebi.ac.uk/en) under accession numbers PRJEB20316, PRJEB41018 and PRJEB40258.

ETHICS STATEMENT

The animal study was reviewed and approved by Stockholms Södra Djurförsöksetiska Nämnd and Linköpings Djurförsöksetiska Nämnd.

AUTHOR CONTRIBUTIONS

TL performed experiments, analyzed data, and contributed to writing the manuscript. JS performed RNA-seq experiments, analyzed RNA-seq data and contributed to writing the manuscript. LP-P analyzed RNA-seq data, analyzed ATAC-seq data and contributed to writing the manuscript. SK FACS sorted cells and assisted with animal experiments. AK and CG performed RNA-seq and ATAC-seq experiments. SM and LS assisted with flow cytometry staining, *in vitro* experiments, and manuscript discussion. NF, YH, and TB assisted with animal experiments. MK provided critical input on the ATAC-seq analysis. AW and BC helped with functional assays and manuscript discussion. AA and CK assisted with RNA-seq experiments, bioinformatic analysis and discussion of the manuscript. PH, RM, and NK supervised the study. RM and NK designed the study, performed experiments, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.854312/full#supplementary-material

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