CIRCUITS OF RESIDENT IMMUNITY REGULATING TISSUE ADAPTATION AND ORGAN HOMEOSTASIS

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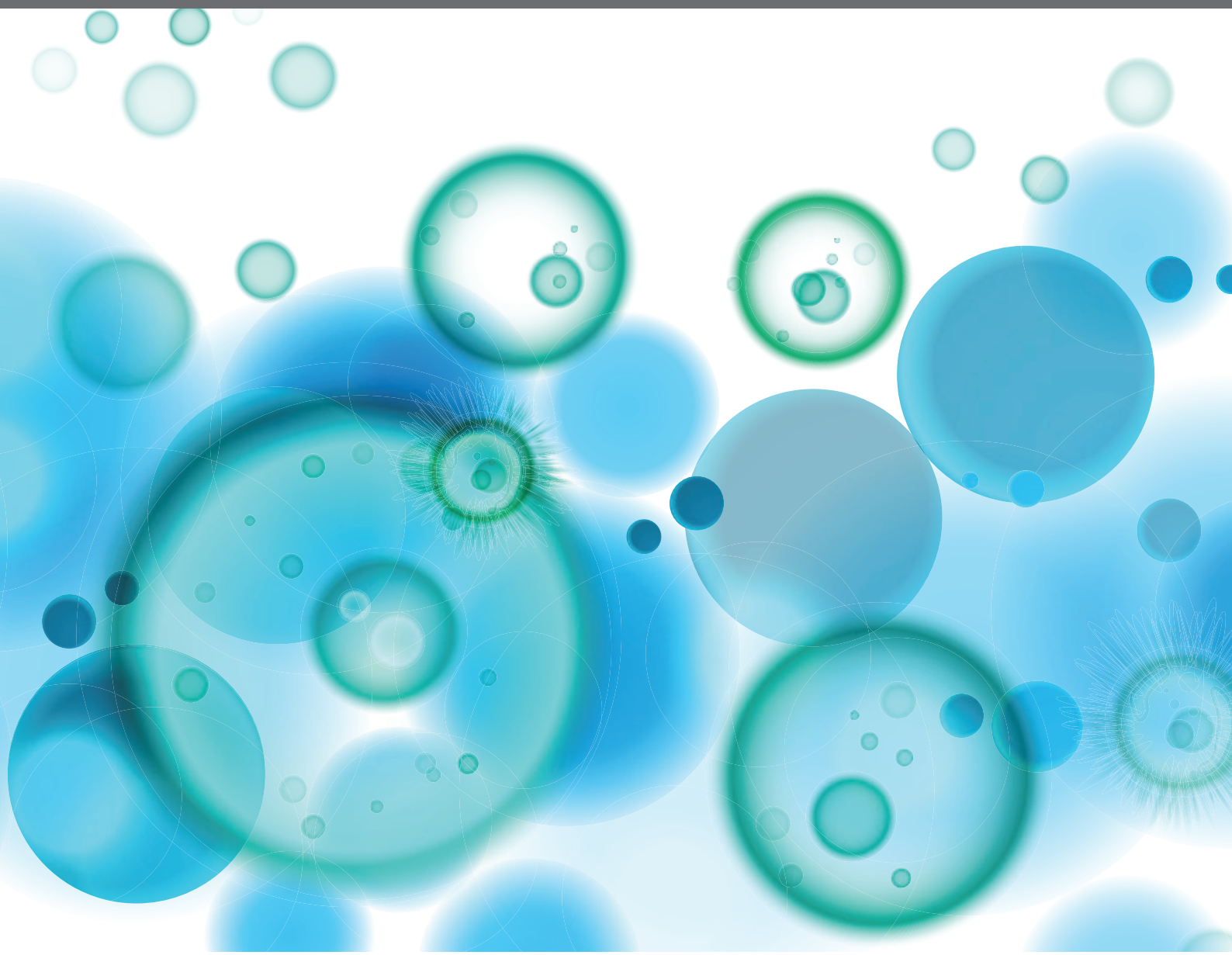
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88976-058-9
DOI 10.3389/978-2-88976-058-9

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CIRCUITS OF RESIDENT IMMUNITY REGULATING TISSUE ADAPTATION AND ORGAN HOMEOSTASIS

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Citation: Klose, C. S. N., Duerr, C. U., Mortha, A., eds. (2022). Circuits of Resident Immunity Regulating Tissue Adaptation and Organ Homeostasis. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-058-9

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frontiers | Frontiers in Immunology

EDITORIAL
published: 11 April 2022
doi: 10.3389/fimmu.2022.901110

Editorial: Circuits of Resident Immunity Regulating Tissue Adaptation and Organ Homeostasis

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Keywords: tissue residency, innate lymphocyte cells (ILCs), T cells, homeostasis, barrier surfaces

Editorial on the Research Topic

Circuits of Resident Immunity Regulating Tissue Adaptation and Organ Homeostasis

OPEN ACCESS

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *21 March 2022*
Accepted: *23 March 2022*
Published: *11 April 2022*

Citation:
Duerr CU, Klose CSN and Mortha A (2022) Editorial: Circuits of Resident Immunity Regulating Tissue Adaptation and Organ Homeostasis. Front. Immunol. 13:901110. doi: 10.3389/fimmu.2022.901110

Each organ in our body serves a determined purpose, follows a distinct development pathway, contains specialized tissue cells and uses unique mechanisms to sustain homeostasis. The disease and pathogens threatening our organs are as diverse as themselves and require distinctly organized responses by the immune system. Patrolling, tissue-resident immune cells populate each organ at defined ratios and support homeostasis, defense and repair. Classical αβ T cells, γδ T cells, invariant T cells, Natural Killer (NK) cells and innate lymphoid cells (ILCs) are effector lymphocytes that aid in this function through local interactions with the microenvironment. Cytokines, growth factors and receptor-ligand interactions play critical roles in this process and are compellingly summarized within this collection of articles centered around the circuits that mediate the adaptation of lymphocytes to their hosting organ and the challenges to ensure defense and homeostasis.

Sheikh and Abraham start discussing the role of the interleukin (IL)-7 receptor alpha chain, a cytokine receptor chain used to identify ILCs but also critical for the survival and development of ILCs. Their review beautifully covers the importance of the IL-7 receptor in ILC biology. In light of cytokines and other pathways of tissue adaptation, Parker and Ciofani discuss the regulatory pathways underlying the earliest event during the effector specification of γδ T cells in the thymus. This review summarizes how the fate of γδ T cell specification in the thymus shapes their later effector profile within the organ. Cong and Wei discuss the role of human and mouse NK cells in the lung and provide a detailed insight into their function during homeostasis, infection, and cancer. Rafei-Shamsabadi et al. review ILCs in allergic skin inflammation. After providing an overview about ILC subsets and plasticity, the authors outline the role of ILCs in atopic dermatitis and contact hypersensitivity. They further discuss the role of group 2 ILCs (ILC2s) in the pathogenesis of allergic skin diseases and close their article with an overarching concept on how these intriguing cells influence the contextual balance of type I and type II immune responses. Centered around skin inflammation, Polese et al. analyze the contribution of T cells, NKT cells and ILCs to the pathogenesis of psoriasis, emphasizing the unique and overlapping contributions of their effector functions at various stages of this disease. The microenvironmental impact on the phenotype and function of heart ILC2s is the topic of an original research article by Deng et al., who define ILC2s as the major innate lymphoid cell (ILC) population in the unique microenvironment of the heart. Using parabiosis, the authors elegantly show that heart ILC2s are readily present at early life, retain tissue-residency during the steady state and increased in a model of myocardial necroptosis

implicating their adaptation to environmental stress. Becker et al. summarize recent findings on kidney ILCs during homeostasis and inflammation. Importantly, the authors highlight that kidney ILC2s constitute a permanent immune population in both mouse and human, posing as potential therapeutic target for reinstating. In addition to the innate immune response, adaptive immunity plays an indisputably important role at immunoprivileged locations. During cerebral *Toxoplasma gondii* infections, an intact immunoproteasome (IP) limits the cellular protein stress to ensure an effective T cells response. Deficiency in three key subunits of the IP results in impaired parasitic control as reported in this original research paper by French et al. Domingues et al. focus their review on ILC3s as sentinels and regulators of tissue homeostasis. They discuss the impact of diets, the microbiota, circadian rhythm and neuroimmune interactions on ILC3s biology and function. The interactions with the commensal microbiota, adaptive lymphocytes, and other immune systems are further reviewed. Essential regulators of ILCs comprise neural factors, which are the focus of Jakob et al. Originating from the composition of the peripheral nervous system and, in particular, the enteric nervous system, this article outlines neuro-immune crosstalk, including but not limited to ILCs and the gastrointestinal tract. Therapeutic applications of neuro-immune interactions, such as in inflammatory bowel disease and other chronic inflammatory diseases, are discussed as well. Within the final article of this collection, Murphy et al. provide a comprehensive review of human and mouse ILCs across all tissues, emphasizing their beneficial and detrimental functions during the steady state and organ specific pathologies and infections. The authors highlight the underlying disease mechanisms and each ILC subset's selective role on cause and consequence.

Collectively, these articles excellently summarize current concepts and mechanisms underlying the adaptation of lymphocytes to support organ homeostasis and defense.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

We wish to convey our appreciation to all authors who have participated in this Research Topic and the volunteering efforts of all reviewers providing fair and insightful comments.

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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frontiers in Immunology

REVIEW
published: 25 June 2019
doi: 10.3389/fimmu.2019.01416

Natural Killer Cells in the Lungs

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The lungs, a special site that is frequently challenged by tumors, pathogens and other environmental insults, are populated by large numbers of innate immune cells. Among these, natural killer (NK) cells are gaining increasing attention. Recent studies have revealed that NK cells are heterogeneous populations consisting of distinct subpopulations with diverse characteristics, some of which are determined by their local tissue microenvironment. Most current information about NK cells comes from studies of NK cells from the peripheral blood of humans and NK cells from the spleen and bone marrow of mice. However, the functions and phenotypes of lung NK cells differ from those of NK cells in other tissues. Here, we provide an overview of human and mouse lung NK cells in the context of homeostasis, pathogenic infections, asthma, chronic obstructive pulmonary disease (COPD) and lung cancer, mainly focusing on their phenotype, function, frequency, and their potential role in pathogenesis or immune defense. A comprehensive understanding of the biology of NK cells in the lungs will aid the development of NK cell-based immunotherapies for the treatment of lung diseases.

Keywords: natural killer cells, lung, homeostasis, inflammation, infection, lung cancer

OPEN ACCESS

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *22 March 2019*
Accepted: *04 June 2019*
Published: *25 June 2019*

Citation:
Cong J and Wei H (2019) Natural Killer Cells in the Lungs. Front. Immunol. 10:1416. doi: 10.3389/fimmu.2019.01416

INTRODUCTION

The lungs comprise mucosae that are constantly exposed to environmental and autologous stimuli, and they are sites of high incidence of primary and metastatic tumors (1). Accordingly, a rapid and efficient immune response that prevents tumorigenesis and pathogen invasion without leading to excessive inflammation is needed to maintain pulmonary homeostasis. As a type of innate immune cell, natural killer (NK) cells are regarded as the host's first line of defense against tumors and viral infection (2). Moreover, involvement of NK cells in various lung diseases, such as lung cancer, chronic obstructive pulmonary disease (COPD) and asthma, as well as infections, has been documented (**Table 1**) (34, 35, 37–39).

In humans, NK cells are usually defined as $CD3^{-}CD56^{+}$ cells, and they are divided into two main subsets with different functions and maturation statuses: $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$. The $CD56^{dim}CD16^{+}$ NK cells are known as a highly differentiated subset with killer cell immunoglobulin-like receptor (KIR) expression, potent cytotoxicity and the capacity to induce antibody-dependent cellular cytotoxicity (ADCC), while the less mature $CD56^{bright}CD16^{-}$ NK cells lack KIR expression but are the major producers of cytokines (40–42). In mice, NK cells do not express CD56 and have historically been defined as $CD3^{-}NK1.1^{+}$ cells (and, more recently, $CD3^{-}NKp46^{+}$ cells). However, type 1 innate lymphoid cells (ILC1s) and some subsets of type 3 innate lymphoid cells (ILC3s) also express NK1.1 and NKp46 and are easily confused with NK cells (43, 44). No equivalent subsets to the human NK cell subsets have been established to date among mouse NK cells. Mouse NK cells are divided into four subsets from the most immature to the

most mature, according to the expression of CD27 and CD11b: $CD27^{-}CD11b^{-}$, $CD27^{+}CD11b^{-}$, $CD27^{+}CD11b^{+}$, and $CD27^{-}CD11b^{+}$ (45–47).

NK cell functions are modulated by the balance between activating and inhibitory signals delivered by receptors expressed on the NK cell surface (**Table 2**). Abnormal cells (including cancer cells and infected cells) activate NK cells via lack of ligands of NK cell inhibitory receptors (missing self) or increased expression of ligands of NK cell activating receptors (induced self) (48–50). In addition, cytokines such as interleukin (IL)-12, IL-15, IL-18, and type I interferon (IFN), as well as Toll-like receptor (TLR) ligands, are powerful activators of NK cell functions (51, 52). Activated NK cells then function in various environments mainly through cytotoxicity and cytokine production. Recent findings have revealed that the functions and phenotypes of NK cells vary depending on their local microenvironments (53), mainly due to the distinct cytokines, cellular composition and foreign stimuli of various tissues. In this review, we provide an overview of the current understanding and gaps in knowledge regarding NK cells in the lungs.

LUNG NK CELLS IN HOMEOSTASIS

Lung NK cells are generally thought to originate and develop in the bone marrow, and then migrate to the lungs (54). In human lungs, NK cells, accounting for about 10–20% of the lymphocytes, are located in the parenchyma and are not detected outside the parenchyma (**Figure 1**) (1). In mice, lung NK cells account for about 10% of the lymphocytes, and this percentage is higher than the percentages in other tissues (liver, peripheral blood, spleen, bone marrow, thymus and lymph node) (55, 56). Moreover, the number of mouse lung NK cells is second only to the number of spleen NK cells (55).

Human lung NK cells are mostly composed of the $CD56^{dim}CD16^{+}$ subset. In addition, KIR-expressing NK cells and highly differentiated $CD57^{+}NKG2A^{-}$ NK cells are found at higher frequencies in the lungs than in matched peripheral blood. These findings indicate that human lung NK cells have a well-differentiated phenotype (1). Even as early as the human fetal period, the frequency of KIR-expressing and differentiated NK cells is highest in the lungs compared to other tissues (57). A more mature phenotype is also observed for mouse lung NK cells. The most mature subset, $CD27^{-}CD11b^{+}$ NK cells, is found at a higher frequency among the lung NK cells (>70%) than those in the liver, peripheral blood, spleen, bone marrow and lymph nodes. Moreover, NK cells in the mouse lung express higher levels of the mature markers CD49b, CD122, CD43, Ly49s, and CD11b, but lower levels of the immature marker CD51, than NK cells in other tissues (56, 58).

Despite the well-differentiated phenotype, both human and mouse lung NK cells are hypofunctional in homeostasis. Human lung NK cells are hyporesponsive to stimulation by target cells (irrespective of priming with IFN-α) compared with peripheral blood NK cells (1). This may be caused by suppressive effects of alveolar macrophages and soluble factors in the epithelial lining fluid of the lower respiratory tract (59). Similarly, in mice, lung NK cells exhibit lower cytotoxicity toward targets compared with spleen NK cells when stimulated by IL-2 or IL-2/IL-12/IL-18 (58). In addition, in mice, the expression intensity of molecules associated with activation (NKp46, NKG2D, and CD69) is lower, and the expression of inhibitory receptors (NKG2A and CD94) is higher, on lung NK cells than on NK cells in the spleen and bone marrow. This indicates that lung NK cells are subject to tighter restrictions in the steady state (56). As the lungs comprise mucosal surfaces that are constantly exposed to environmental and autologous antigens, the dominance of hypofunctional NK cells may contribute to pulmonary homeostasis.

CD49a, CD69, and CD103 are regarded as markers of tissue-resident NK cells (53, 60–63). The fact that the vast majority of lung NK cells in mice are non-tissue-resident cells has been demonstrated by a parabiotic mouse model and the very low expression of CD49a and CD69 (35, 64, 65). Although the majority of human lung NK cells are $CD56^{dim}$ with a non-tissue-resident phenotype (1), a small but distinct $CD49a^{+}$ lung NK cell subset (which largely involves $CD56^{bright}$ NK cells) has recently been identified (66). These $CD56^{bright}CD49a^{+}$ lung NK cells strongly co-express CD103 and CD69 and these cells are not found among the $CD56^{bright}$ NK cells in the peripheral blood, implying that $CD56^{bright}CD49a^{+}$ lung NK cells may be tissue-resident cells (66). However, the circulating and tissue-resident characteristics of human lung NK cells still need to be further investigated using humanized mice and "multi-omics" analyses (67).

NK CELLS IN LUNG INFECTIONS

There is increasing evidence that NK cells are involved in lung immune responses to respiratory pathogens. As an important type of innate immune cell, NK cells can respond rapidly to invading pathogens and clear them efficiently. On the other hand, NK cells may cause uncontrolled inflammation and pathological damage in some cases.

Viruses

NK cells are innate immune cells that confer early immunity in acute viral infections and, accordingly, patients with genetic deficiencies that cause the loss of functions of NK cells are subjected to recurrent viral infection (39, 68, 69). However, the rapid immune response mediated by NK cells may sometimes occur at the cost of excessive inflammation. The lungs are continually exposed to various respiratory viruses such as influenza viruses. The evidence of involvement of NK cells in influenza infection dates back to 1982. Ennis et al. (70) demonstrated that individuals infected with influenza viruses exhibit increased peripheral blood NK cell activity in association with interferon (now known as IFN-γ) induction. In mouse models, depletion of systemic or lung NK cells increases the morbidity and mortality of mice during the early course of medium-dose influenza infection (3, 4), indicating a protective role of NK cells. In contrast, depletion of systemic NK cells improves the survival of mice infected with high-dose influenza viruses by alleviating lung immunopathology (5, 6). These findings uncover a dual role for mouse NK cells in influenza

TABLE 1 | Beneficial and/or detrimental roles of NK cells in mouse models of pulmonary disorders.

Pathology	Beneficial role of NK cells	Detrimental role of NK cells	References
VIRUSES			
Influenza virus	Promote host defense via IFN-γ at medium-dose	Induce immunopathology at high-dose	(3–6)
Respiratory syncytial virus	Inhibit type 2 inflammation via IFN-γ; promote host defense via IFN-γ	Exacerbate early acute lung injury via IFN-γ	(7–10)
Herpes simplex virus	Promote host defense via IFN-γ and cytotoxicity		(11, 12)
BACTERIA			
Klebsiella pneumoniae	Promote host defense via IL-22 and IFN-γ		(13, 14)
Streptococcus pneumoniae	Promote early clearance of bacteria in WT mice (3 h post infection)	Amplify pulmonary and systemic inflammation in scid mice; impair clearance of bacteria in scid mice (24 h post infection)	(15, 16)
Pseudomonas aeruginosa	Promote host defense via NKG2D and IFN-γ		(17, 18)
Mycobacterium tuberculosis	Promote host defense via IFN-γ in T cell-deficient mice		(19)
Bordetella pertussis	Promote host defense via IFN-γ		(20)
Staphylococcus aureus	Promote host defense via IFN-γ and TNF		(21, 22)
Haemophilus influenzae	Promote host defense via IFN-γ		(23)
Chlamydia trachomatis	Promote host defense via regulation of Th1/Treg and Th17/Treg balances		(24)
FUNGI			
Aspergillus fumigatus	Promote host defense via IFN-γ		(25, 26)
Cryptococcus neoformans	Promote host defense via IFN-γ		(27, 28)
Asthma	Promote inflammation resolution via clearance of eosinophils and $CD4^+$ T cells in OVA-induced asthma	Promote allergic sensitization via initiation of type 2 response in OVA-induced asthma; promote pathogenesis via NKG2D and granzyme B in HDM-induced asthma?	(29–33)
COPD		Kill autologous lung epithelial cells	(34)
Lung cancer	Inhibit tumorigenesis in *Kras*-driven cancer; inhibit lung metastasis of cancer cells		(35, 36)

TABLE 2 | Main surface markers of the lung NK cells discussed in this review.

Relevance and function	NK cell-surface molecules
Activating receptors	NKG2D, $DNAM1^h$, $NKp30^h$, $NKp44^h$, NKp46, $NKp80^h$, CD16
Inhibitory receptors	CD94/NKG2A, $ILT2^h$, $KIR2DL^h$, $KIR2DL2^h$
Activation marker	CD69
Mature and differentiation markers	KIR^h, $CD57^h$,CD11b, CD43, CD49b, CD122, $Ly49s^m$
Tissue-resident markers	CD49a, CD69, CD103
Adhesion molecules	CD11b, CD49a, CD49b, $CD57^h$, CD103,

^{h}*Expressed only by human NK cells.*
^{m}*Expressed only by mouse NK cells.*

infection, providing protection or contributing to pathogenesis, depending on the virus dose.

During influenza infections, NK cells are activated by infected cells via contact-dependent mechanisms (71), and by cytokines such as IL-12, IL-2 and type I IFN, which are derived from infected cells and possibly from other cell types (71–76). In addition to these conventional recognition modes during viral infection, the NK cell receptors NKp44 (which is only expressed on human NK cells) and NKp46 can bind to influenza haemagglutinins (HAs). This allows NK cells to directly recognize influenza viruses and lyse influenza virus-infected cells (77–80). Recent studies have found that influenza vaccines induce the immune memory of human NK cells (81). Similarly, in mice, influenza infection also induces memory-like NK cells, which protect the mice against secondary influenza infection. Intriguingly, these memory-like NK cells reside in the liver rather than in the lungs (82–84), and NK cell-mediated recall responses are not dependent on the NKp46-HA interaction (85).

In mice, NK cells quickly accumulate in the lungs within the first few days of influenza infection (56, 86). These activated lung NK cells then contribute to viral clearance through IFN-γ production, activation of adaptive immune cells, ADCC and cytotoxic lysis. More recently, Kumar et al. (87) reported that conventional $NKp46^+NK1.1^+CD127^-ROR\gamma t^-$ NK cells in the bronchoalveolar lavage fluid (BALF), trachea and lung tissue produce IL-22 during influenza infection, which facilitates tissue regeneration and prevents excessive lung inflammation. These findings indicate the multiple roles of NK cells in response to influenza viruses.

Due to the difficulties of obtaining lung tissues from humans infected with influenza viruses, most studies exploring the responses of human NK cells to influenza viruses use peripheral blood NK cells (73, 88–90), and less is known about human

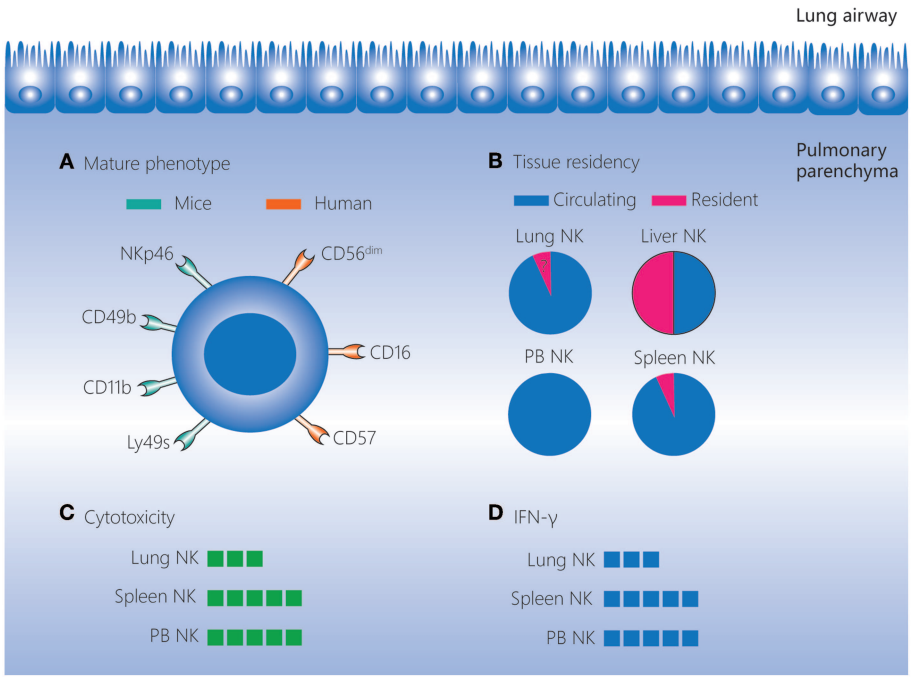

FIGURE 1 | Lung NK cells in homeostasis. NK cells account for 10–20% of lymphocytes in the human and mouse lungs, and these cells are located in the lung parenchyma. **(A)** NK cells in the lungs have a more mature phenotype compared to those in other tissues. In mice, lung NK cells express high levels of the mature markers NKp46, CD49b, CD11b, and Ly49s. In humans, lung NK cells are mostly composed of the $CD56^{dim}CD16^{+}$ subset, and highly differentiated $CD57^{+}NKG2A^{-}$ NK cells are present at a higher frequency in the lungs than in matched peripheral blood. **(B)** The vast majority of lung NK cells are circulating, and the existence of a small percentage of seemingly tissue-resident NK cells in the lungs remains to be confirmed. **(C,D)** Lung NK cells are hypofunctional in homeostasis, and their cytotoxicity and IFN-γ production levels are lower than those of NK cells in the spleen and peripheral blood. IFN, interferon; NK, natural killer; PB, peripheral blood.

lung NK cells in influenza infections. Recently, Cooper et al. (66) utilized a lung explant model to characterize human lung NK cells during the early course of influenza infection. The lung NK cells responded quickly upon *ex vivo* influenza infection of lung explants, with upregulation of CD107a by 24 h after infection. Compared with $CD56^{bright}CD49a^{-}$ NK cells, $CD56^{bright}CD49a^{+}$ lung NK cells, which possibly represent a tissue-resident and trained NK cell subset, express higher levels of CD107a. Recent studies have shown that some activated $CD56^{dim}CD16^{+}$ NK cells lose CD16 expression through ADAM17-mediated shedding and become $CD56^{dim}CD16^{-}$ NK cells (91). However, the expression of CD107a on $CD56^{bright}$ and $CD56^{dim}$ NK cells is comparable, and there is no difference in expression between $CD56^{dim}CD16^{-}CD49a^{+}$ and $CD56^{dim}CD16^{-}CD49a^{-}$ NK cells (66). Although granzyme B and IFN-γ are induced in lung explants after influenza infection, and enhanced IFN-γ responses are detected in peripheral blood NK cells following influenza vaccination (66, 73, 88, 90), there is no direct evidence that granzyme B and IFN-γ are released by lung NK cells. Thus, the immune responses of human lung NK cells in influenza infection remain to be further explored.

Despite the potent antiviral function of NK cells, recurrent influenza infections are common, suggesting that influenza viruses employ complex strategies to evade NK cell-mediated immunosurveillance (92). First, influenza viruses replicate rapidly before NK cells accumulate robustly in the lungs, providing sufficient time for virus dissemination (93). Second, mutation of influenza HA may impair the capacity of NK cells to recognize and lyse infected cells (94). Third, activation of NK cells can be inhibited by influenza HA in a dose-dependent manner (95, 96). On the other hand, when the levels of HA are too low for NK cell recognition, NK cells may not be activated sufficiently to clear viruses (93, 97). Fourth, influenza viruses can directly infect NK cells and induce apoptosis, leading to decreased NK cell cytotoxicity (98).

Bacteria

NK cells are generally regarded as important contributors to the host defense against tumors and viruses, but recent studies have shown that NK cells also play a role in resisting bacterial infections.

Mycobacterium Tuberculosis

Tuberculosis is a leading cause of bacterial infections worldwide. *M. tuberculosis* (MTb) maintains a latent state in most infected individuals, and active disease usually progresses slowly, manifesting later in life (99). *In vitro* studies demonstrate that human peripheral blood NK cells can be activated by MTb-infected monocytes, and this is mediated by NKG2D recognition of ULBP1 and by NKp46 recognition of vimentin (100, 101). Moreover, human NK cells can directly recognize MTb by the binding of TLR2 and NKp44 to peptidoglycan and unknown

components of MTb cell walls, respectively, and then become activated (102–104).

A study in immunocompetent mice showed that activated NK cells with upregulated CD69, IFN-γ, and perforin accumulated in the lungs in the early stage after aerosol infection with MTb, but depletion of NK cells did not influence the course of infection (105). Nevertheless, another study in T cell-deficient mice demonstrated that NK cells mediated early defense against MTb infections via IFN-γ (19, 106). Given that mice infected with MTb progress directly to active disease without experiencing latency, these reports indicate the redundant role of NK cells in the active stages of MTb infection. In humans, NK cells in the peripheral blood stimulated with MTb or live *M. bovis* Bacillus Calmette-Guerin (BCG) upregulate IFN-γ expression (107, 108). More recently, Chowdhury et al. (109) conducted a long-term study on a cohort of South African adolescents and found that the frequency of NK cells in the peripheral blood can inform disease progression, therapeutic responses and lung inflammation of patients with active tuberculosis. Pleural fluid, which is the excess fluid that collects around the lungs of pulmonary tuberculosis patients, may be closer to the pulmonary milieus than peripheral blood. The pleural fluid is enriched with IFN-γ-producing $CD56^{bright}$ NK cells due to selective apoptosis of cytotoxic $CD56^{dim}$ NK cells induced by soluble factors present in tuberculous effusions (110). Together, these findings in mice and humans suggest that NK cells may function at the site of active MTb infections mainly through IFN-γ production rather than cytotoxic lysis. Although Chowdhury et al. (109) showed that peripheral blood NK cells from individuals with latent tuberculosis infection display elevated cytotoxicity and increased frequency, whether cytotoxic lysis is employed by NK cells in the defense against MTb, especially latent MTb, remains to be further researched.

Klebsiella Pneumoniae

K. pneumoniae is an important cause of nosocomial pneumonia and is infamous for multidrug resistance. *In vitro* studies have shown that human NK cells can be activated by TLR2 recognition of recombinant protein A, the pathogen-associated molecular pattern expressed by *K. pneumoniae* (111). However, whether NK cells can recognize live *K. pneumoniae* and lyse *K. pneumoniae* after this direct recognition is unclear. In mice, lung NK cells promote host defense against *K. pneumoniae* by IL-22 and IFN-γ production (13, 14). On the other hand, Wang et al. (112) demonstrated that *K. pneumoniae* pre-infection alleviated influenza virus-induced death and acute lung injury by inhibiting lung NK cell expansion. These findings suggest a complex role of NK cells in response to various pathogens. Thus, accurate and in-depth research into NK cells in different infection conditions is needed and this will contribute to the development of effective interventions for lung infections.

NK CELLS IN LUNG INFLAMMATION

Asthma and COPD are very common and serious chronic inflammatory diseases of the lungs that may lead to pulmonary fibrosis (113, 114), and NK cells are implicated in both diseases.

Asthma

Asthma is a chronic airway inflammatory disease, and the majority of cases involve allergic asthma which is typically characterized by type 2 immune responses (114). Asthma can be induced and exacerbated by many factors, such as environmental pollutants, allergens, obesity and viral infections.

Current reports on NK cells in asthmatic patients seem to be somewhat contradictory, both regarding the numbers and functions of NK cells. An early study showed that the percentage of peripheral blood NK cells increases in asthmatic children during acute exacerbations relative to asthmatic children who are in a stable state after prednisolone therapy (115). Nevertheless, Barning et al. (29) and Duvall et al. (116) demonstrated that asthmatic patients have fewer NK cells in both the peripheral blood and BALF than healthy individuals, and the loss of NK cells is increased in patients with severe asthma. The $CD56^{dim}$ NK cell subset (but not the $CD56^{bright}$ NK cell subset) is selectively lost in the peripheral blood of asthmatic patients, whereas BALF NK cells are skewed toward a $CD56^{dim}$ phenotype in asthmatic patients. With regard to the functions of NK cells, there is evidence that NK cells can facilitate inflammation resolution by inducing eosinophil apoptosis (29). In healthy individuals and patients with mild asthma, NK cells in the peripheral blood can induce the apoptosis of eosinophils efficiently. In contrast, despite displaying a more activated phenotype, the cytotoxicity of peripheral blood NK cells from patients with severe asthma is impaired, and the decreased cytotoxicity can be exacerbated by corticosteroids (29, 116). These results indicate the attenuated capacity of NK cells to resolve inflammation in severe asthma. In contrast, earlier studies reported that NK cell cytotoxicity is elevated in the peripheral blood of patients with asthma compared to healthy individuals, and it declines immediately after acute antigen challenge (117, 118). On the other hand, Wei et al. (37) showed that there were increased $IL\text{-}4^{+}$ NK cells in the peripheral blood of asthmatic patients compared to healthy individuals, and $IL\text{-}4^{+}$ NK cells decreased when the patients recovered owing to erythromycin treatment. This implies a role for NK cells in promoting IgE-mediated ongoing allergic inflammation.

The contradictory results have also been observed in mouse models. The ovalbumin (OVA)-induced asthmatic mouse model is widely used in the study of allergic asthma, and many studies have demonstrated an important role for NK cells at all stages of asthma using this model. OVA sensitization and challenge does not change the total number of NK cells in the lungs, but it selectively increases the number of immature NK cells in the lung draining lymph nodes, as well as upregulating the expression of CD86 on NK cells in both the lungs and lung draining lymph nodes (119). Lack of NK cells either throughout life or just prior to sensitization leads to decreased type 2 cytokine secretion, decreased OVA-specific IgE production, and decreased pulmonary eosinophil infiltration (30, 31). Furthermore, adoptive transfer of OVA-specific T cells from sensitized wild-type (WT) mice, but not mice lacking NK cells, can induce the development of asthma in allergen-challenged $RAG^{-/-}$ mice (31). These results indicate that NK cells are essential for allergic sensitization, and that NK cell-mediated

initiation of the type 2 response is probably involved in this process. However, once mice have been sensitized, NK cells may not regulate the established type 2 response but instead they may promote pulmonary eosinophilia, as evidenced by the fact that NK cell depletion during allergen challenge significantly reduces BALF eosinophilia without altering airway hyperresponsiveness or serum OVA-specific IgE levels (119). Nevertheless, Haworth et al. (32) found that depletion of NK cells at the peak of inflammation delays the clearance of airway $CD4^+$ T cells and eosinophils. Taken together, these findings suggest that besides the pathologic role of NK cells in allergic sensitization and inflammation promotion, NK cells also provide protection by contributing to the resolution of allergic lung inflammation in mice with OVA-induced asthma. In house dust mite (HDM)-induced asthma mouse models, HDM exposure leads to the accumulation of NK cells in the BALF and lung draining lymph nodes, as well as the activation of NK cells in the lungs (120). Farhadi et al. (33) have shown that NK cells play a critical role in the pathogenesis of HDM-induced asthma via NKG2D and granzyme B. However, a more recent study demonstrated that NK cells are not required for the development of HDM-induced asthmatic disease (120).

There is evidence that viral infection is associated with the development of asthma, and NK cells have been shown to play an important regulatory role in this setting. In mice with pre-existing allergic inflammation and asthma, the induction of asthma-activated NK cells confers more potent protection against influenza infection (121). Nevertheless, NK cells activated by the viral mimic polyinosinic:polycytidylic acid (poly(I:C)) exacerbate OVA-induced asthma via IL-17a production (122). However, when mice are infected with respiratory syncytial viruses and then subjected to allergic sensitization, NK cells inhibit viral- and bystander allergen-specific type 2 responses, possibly through IFN-γ production (7). Recent studies have reported the presence of an altered microbial composition in patients with asthma, and airway dysbiosis is relevant to the clinical features in these individuals (123, 124). Airway colonization by *Haemophilus influenzae* and *Streptococcus pneumonia* at 1 month of age was associated with an increased odds ratio of childhood asthma (125). Although NK cells produced higher levels of IFN-γ during *H. influenzae* and *S. pneumonia* infections (15, 23), colonization by *H. influenzae* and *S. pneumonia* did not inhibit asthma, in contrast to the anti-asthma role of NK cells during respiratory syncytial virus infections. This may be because *H. influenzae* and *S. pneumonia* activate other cell types and pathways involved in asthma occurrence and exacerbations. Moreover, the children were also colonized by many other bacteria, and the integrated effect on NK cells caused by diverse bacteria may lead to variable consequences. Thus, the exact associations between NK cells, airway bacteria and asthma need further study.

The abovementioned contradictory results may be influenced by the fact that asthma-associated factors (such as viral and bacterial infections, obesity, allergens, other environmental insults and corticosteroids) may directly affect NK cell functions and the fact that NK cell-depleting antibodies may impair natural killer T cells (antibodies against NK1.1) or some granulocytes and subsets of T cells in certain conditions (antibodies against asialo-GM1) (126–128). In the future, investigations of the exact roles of NK cells in asthma could be enhanced by using improved tools to specifically deplete lung NK cells, generating transgenic mice that temporarily lack lung NK cells, establishing novel humanized asthmatic mouse models, and carrying out large-scale univariate analyses in asthmatic patients.

COPD

COPD, caused mainly by cigarette smoking (CS) and biomass fuel, is a common worldwide healthcare issue (129). Chronic inflammation drives the irreversible airway obstruction in COPD, eventually resulting in a decline in lung function (130). Unlike asthma, COPD typically involves the infiltration of neutrophils, Th1 cells and $CD8^+$ T cells (130). NK cells are also thought to be responsible for the progression of COPD. Although the number of NK cells in the peripheral blood, BALF and lung parenchyma of COPD patients are the same as in smokers without COPD (34, 131), $CD57^+$ cells in pulmonary lymphoid follicles have been reported to be significantly increased in COPD patients compared to in smokers without COPD (132). CS enhances the IL-15 trans-presentation of dendritic cells to induce NK cell priming (133). NK cells exhibit hyperresponsiveness in COPD, as evidenced by the findings that $CD16^+$ NK cells kill autologous lung $CD326^+$ epithelial cells and that NK cells from CS-exposed mice produce higher levels of IFN-γ upon stimulation with cytokines or TLR ligands (poly(I:C), ssRNA40, or ODN1826) (34, 134, 135). An imbalance of activating and inhibitory signaling contributes to NK cell hyperresponsiveness. NK cells from CS-exposed mice show greater cytotoxic activity in response to the NKG2D ligand RAE-1 (134). Moreover, CS-induced lung inflammation is impaired in NKG2D-deficient mice, revealing the critical role of NKG2D in COPD development (134). In addition, the inhibitory receptor CD94 has been found to be decreased on NK cells of COPD patients, which may be related to increased granzyme B production (131). The state of NK cells in the CS-induced COPD mouse model cannot completely recapitulate that in patients with COPD. BALF NK cells displayed comparable cytotoxic potential between current smokers with COPD and ex-smokers with COPD, suggesting that the alterations of NK cells are not solely caused by CS and that other factors such as genetics and infections may contribute. In contrast, the hyperresponsiveness of NK cells was lost following smoking cessation in the CS-induced COPD mouse model, which indicated the limitation of using this model to study COPD and that a better mouse model is urgently needed.

Bronchial colonization by potentially pathogenic microorganisms is frequently found in COPD, and COPD exacerbations are closely associated with viral and bacterial infections (136–138). An NK cell-related mechanism may contribute to enhanced lung inflammation during influenza-induced COPD exacerbations. In a CS-induced COPD mouse model, after influenza infection, NK cells were found to produce more IFN-γ (135). Microbiome analyses of sputum samples from patients with COPD exacerbations demonstrated an alteration in bacterial diversity, with an overrepresentation

of the *Proteobacteria phylum*, which includes most of the bacteria considered to be pathogenic (139). Although chronic colonization by *Pseudomonas aeruginosa* has been found in COPD patients suffering from exacerbations, in most cases, the exacerbations are due to other pathogenic microorganisms (140, 141). Given that lung NK cells in mice infected with *P. aeruginosa* produced increased IFN-γ through NKG2D-mediated activation (17), a phenotype similar to influenza-induced exacerbations, it would be fascinating to investigate the precise effect of *P. aeruginosa*-only infections on COPD exacerbations utilizing murine experiments and to explore the role of NK cell-related NKG2D and IFN-γ in this situation. In addition, colonization by *H. influenzae*, which can lead to NK cell activation (23), has also been reported in COPD patients (142), but the exact interaction between NK cells and *H. influenzae* in COPD is yet to be determined. Collectively, local hyperresponsive NK cells are responsible for smoke-induced lung inflammation, leading to accelerated progression of COPD. Therefore, targeting NK cells may represent a new strategy for treating COPD.

NK CELLS IN LUNG CANCER

Lung cancer is the leading cause of death related to cancer worldwide (143). It is classified into non-small-cell lung cancer (NSCLC; ∼80%) and small-cell lung cancer (SCLC; ∼20%). NK cells are cytotoxic lymphocytes that were originally identified based on their ability to kill cancer cells, and their potent antitumor effects have been confirmed in numerous tumor types including lung cancer (144, 145). The localization of NK cells in lung cancer is similar in humans and mice; NK cells are located mostly in the invasive margin surrounding the tumor lesions, with rare direct contact with cancer cells (35, 146, 147).

The most direct evidence of an anti-lung cancer role for NK cells comes from *Kras*-driven spontaneous lung cancer and cancer cell-injection experiments in mice, in which mice lacking NK cells were generated by *Nfil3* knockout or administration of antibodies against NK1.1 or asialo-GM1. The lung tumor burden was found to be significantly increased in the mice lacking NK cells (35, 36). However, the robust protective role of NK cells against tumors is limited to the early stage of lung cancer, at least in *Kras*-driven lung cancer in mice, because NK cells become dysfunctional during the late stage. In mice, NK cell dysfunction in the lung cancer microenvironment mainly manifests as attenuated cytotoxicity, diminished responsiveness and impaired viability (**Figure 2**) (35).

Similar phenomena have been observed in NK cells in tumor tissues of patients with NSCLC. NK cells isolated from tumors in these patients have a decreased cell number, a distinctive receptor expression pattern (downregulated expression of NKp30, NKp80, CD16, DNAM1, ILT2, KIR2DL1, and KIR2DL2, but upregulated expression of NKp44, NKG2A, CD69, and HLA-DR), impaired IFN-γ production and CD107a degranulation, lower cytotoxicity, and a proangiogenic phenotype compared with non-tumoral NK cells (146–149). Moreover, NK cells infiltrating NSCLC are enriched with the $CD56^{bright}CD16^{-}$ subset (146), which is a minor subset among non-tumoral lung NK cells. The enrichment of $CD56^{bright}CD16^{-}$ NK cells is probably due to the exclusion of $CD16^{+}$ NK cells from lung tumor lesions, because the frequency of $CD16^{-}$ NK cells among leukocytes in lung tissues is comparable in tumor and non-tumor tissues (149). However, whether the loss of $CD16^{+}$ NK cells is caused by the impaired viability or failure to infiltrate tumor lesions, and how $CD56^{bright}CD16^{-}$ NK cells, which express high levels of the tissue-resident marker CD69 (53), maintain their survival and residency in the lung cancer environment, remain to be determined.

Little is known about NK cells in patients with SCLC. Limited information has shown that, compared to the peripheral blood NK cells of healthy individuals, the peripheral blood NK cells of patients with SCLC are present at the same frequency, exhibit weakened cytotoxicity, and have downregulated NKp46 and perforin expression (150). So far, no studies have reported on the status of NK cells in the tumor microenvironment in patients with SCLC, and this needs to be further investigated.

Platonova et al. (147) found that NK cell infiltration is not correlated with clinical outcomes in NSCLC, similar to the finding by Platonova et al. (147), we recently observed that NK cell depletion in late-stage Kras-driven mouse lung cancer does not influence tumor development (35). The limited prognostic significance of NK cells in NSCLC may be caused by intratumoral NK cell dysfunction in patients who mainly have intermediate- or advanced-stage tumors. Thus, both intratumoral NK cell functions and cell densities may be critical to clinical outcomes in NSCLC. A deeper understanding of the mechanisms associated with NK cell dysfunction in the lung cancer microenvironment will contribute to the development of NK cell-based lung cancer immunotherapy.

NK cell dysfunction in tumor microenvironments can generally be caused by tumor cells, myeloid-derived suppressor cells, macrophages, Tregs and platelets in a contact-dependent manner or via secretion of soluble factors such as transforming growth factor (TGF)-β, IL-10, indoleamine-2,3-dioxygenase, prostaglandin E_2, and adenosine (145, 151). However, NK cell characteristics may be affected by their tissue localization, and each tumor type has a unique microenvironment composed of diverse immune cells (53). Whether the abovementioned mechanisms are applicable to NK cell dysfunction in lung cancer is yet to be fully investigated. Among these mechanisms, in *Kras*-driven lung cancer in mice, TGF-β may be involved in FBP1 upregulation in NK cells, and FBP1-mediated glycolysis inhibition and FBP1-mediated impaired viability have been confirmed to induce NK cell dysfunction (35). Additionally, Donatelli et al. (152) demonstrated that TGF-β-inducible microRNA-183 silenced human NK cells via DNAX-activating protein of 12 kDa (DAP12) depletion. Moreover, higher levels of TGF-β in the human lung cancer microenvironment and reduced DAP12 expression in tumor-associated NK cells were observed simultaneously, further indicating another TGF-β-involved mechanism associated with NK cell dysfunction.

NK cell dysfunction favors tumor immunoevasion, so focusing on restoring NK cell functions represents important

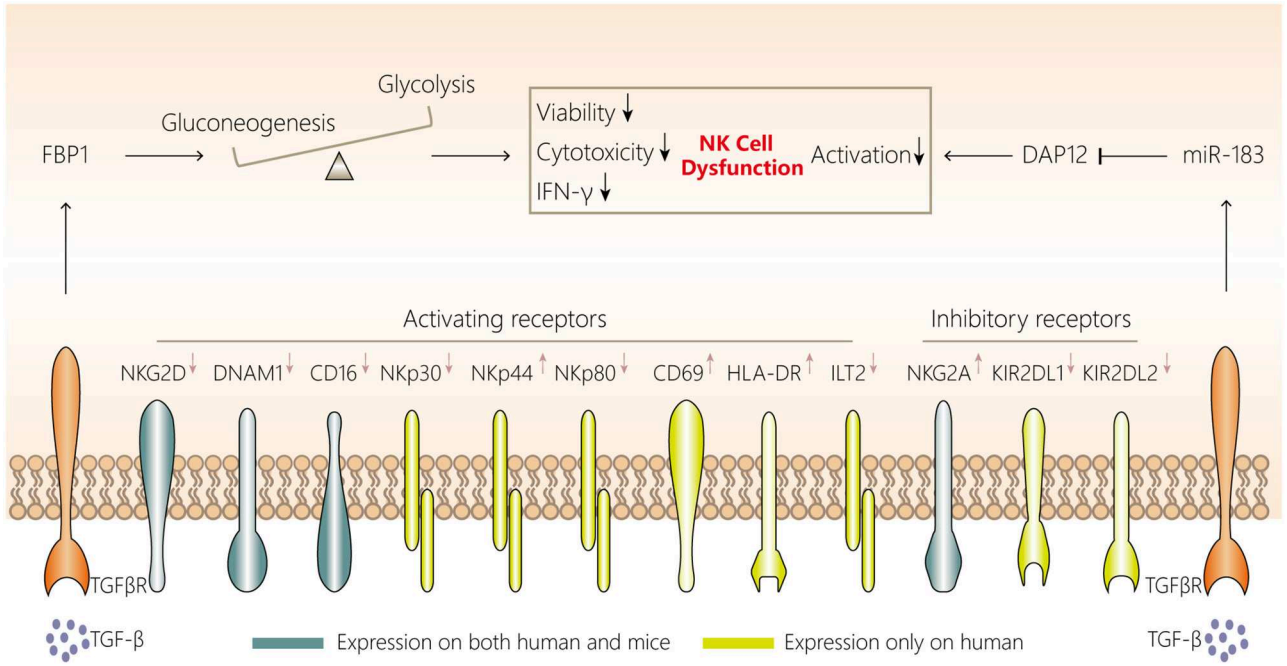

FIGURE 2 | NK cell dysfunction in lung cancer. NK cells in the lung cancer microenvironment display attenuated cytotoxicity, impaired viability and a distinct phenotype, with downregulated expression of NKG2D, DNAM1, CD16, CD27, NKp30, NKp44, and NKp80 and upregulated expression of NKG2A, KIR2DL1, and KIR2DL2. Two mechanisms are involved in NK cell dysfunction in the lung cancer microenvironment. First, aberrant FBP1 expression in NK cells leads to dysfunction by inhibiting their viability and glycolysis. Second, increased microRNA-183 reduces DPA12 expression in NK cells and thus suppresses NK cells. The initiation of both mechanisms may be associated with tumor microenvironment-derived TGF-β. TGF-β, transforming growth factor-β; FBP1, fructose-1,6-bisphosphatase; IFN, interferon; NK, natural killer; DAP12, DNAX activating protein of12 kDa; TGFβR, transforming growth factor β receptor.

potential strategies for inhibiting lung cancer. These strategies include activating NK cells using IL-2/IL-12/IL-15/IL-18, blocking inhibitory receptors on NK cells by targeting NKG2A, KIR2DL1, and KIR2DL2, enhancing NK cell glycolysis by inhibiting FBP1 and altering the immunosuppressive microenvironment by neutralizing TGF-β.

CONCLUDING REMARKS

Although the biology of NK cells has been well-documented, most studies have focused on peripheral blood NK cells in humans, and bone marrow and spleen NK cells in mice, and less is known about NK cells in the lungs. Recently, the concepts of tissue-resident NK cells and tissue microenvironments have attracted investigators' attention. This has raised the issue that NK cells may be profoundly affected by their local tissue microenvironment, and the characteristics of NK cells in distinct tissues have gradually been uncovered. Our current knowledge about NK cells in the lungs is from studies on WT and transgenic mice and studies comparing healthy individual samples and patient samples (predominately lung tumor resection samples and, less frequently, BALF and sputum samples). The present review shows that NK cells in the lungs appear to be conserved between humans and mice regarding several aspects, including the high degree of differentiation, hypofunction and tissue localization during homeostasis, and responses to tumors and influenza infections. Thus, elaborate mouse models that closely mimic human disease have helped to understand the biology of NK cells in the lungs, such as the *Kras*-driven lung cancer mouse model and influenza viral infection mouse model. However, for some common lung diseases such as tuberculosis, the lack of a good mouse model and the difficulty in obtaining lung tissue from patients had led to a lack of understanding of NK cells in these conditions. Moreover, as mouse NK cells do not express CD56 and KIRs, which are important for human NK cells, the characteristics and functions of different human lung NK cell subsets subdivided by these molecules remain unclear.

Over the past decade, ILCs, which include NK cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue-inducer cells, have emerged as an important cell population with potent roles in host defense in mucosal tissues including lung tissues (153, 154). Generally, ILC1s respond to tumors and intracellular pathogens such as viruses, ILC2s react to extracellular helminths and allergens and ILC3s resist extracellular microbes such as fungi and bacteria; some of these effects have been demonstrated in the lungs (154–158). Although other ILCs in the lungs are far less abundant than NK cells, ILC1s and parts of ILC3s are easily confused with NK cells (43, 44), so previous conclusions about lung NK cells may be influenced by the effects of other ILCs. Thus, it is necessary to exclude other

ILCs to further investigate the exact characteristics of lung NK cells.

With regard to lung NK cell research, several interesting issues remain to be solved: (i) whether lung-resident NK cells are present in the lungs in the context of homeostasis and/or disorders; (ii) if so, how lung-resident NK cells function in certain conditions; (iii) the differences and connections between NK cells and ILCs in the lungs; (iv) why memory-like NK cells induced by influenza virus infection are present in the liver rather than in the lungs; (v) whether memory NK cells can form and be maintained in the lungs (as the lungs are frequently challenged by tumors and pathogens); and (vi) how to establish immunocompetent mouse models that can closely mimic human lung diseases. In the future, advanced technologies and tools, such as humanized mice, omics analyses and living microscopy imaging, may be needed to further study lung NK cells. With deeper knowledge of the biology of lung NK cells, effective therapeutic strategies based on NK cells are expected to be applied to treat lung diseases.

AUTHOR CONTRIBUTIONS

JC wrote the manuscript. HW designed the review and revised the manuscript.

FUNDING

This work was supported by the key project of the National Natural Science Foundation of China (81330071, 81788101, 91442202, and 81602491), The Strategic Priority Research Program of the Chinese Academy of Sciences (CAS grants XDA12020217, XDPB10) and Postdoctoral Innovative Talent Support Program.

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Conflict of Interest Statement: 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.

frontiers in Immunology

REVIEW
published: 06 November 2019
doi: 10.3389/fimmu.2019.02591

Context Dependent Role of Type 2 Innate Lymphoid Cells in Allergic Skin Inflammation

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The discovery of innate lymphoid cells (ILC) has profoundly influenced the understanding of innate and adaptive immune crosstalk in health and disease. ILC and T cells share developmental and functional characteristics such as the lineage-specifying transcription factors and effector cytokines, but importantly ILC do not display rearranged antigen-specific receptors. Similar to T cells ILC are subdivided into 3 different helper-like subtypes, namely ILC1-3, and a killer-like subtype comprising natural killer (NK) cells. Increasing evidence supports the physiological relevance of ILC, e.g., in wound healing and defense against parasites, as well as their pathogenic role in allergy, inflammatory bowel diseases or psoriasis. Group 2 ILC have been attributed to the pathogenesis of allergic diseases like asthma and atopic dermatitis. Other inflammatory skin diseases such as allergic contact dermatitis are profoundly shaped by inflammatory NK cells. This article reviews the role of ILC in allergic skin diseases with a major focus on ILC2. While group 2 ILC are suggested to contribute to the pathogenesis of type 2 dominated inflammation as seen in atopic dermatitis, we have shown that lack of ILC2 in type 1 dominated contact hypersensitivity results in enhanced inflammation, suggesting a regulatory role of ILC2 in this context. We provide a concept of how ILC2 may influence context dependent the mutual counterbalance between type I and type II immune responses in allergic skin diseases.

Keywords: innate lymphoid cells, allergic contact dermatitis, atopic dermatitis, counter regulation, immune crosstalk

OPEN ACCESS

Edited by:
Marina Cella, Washington University School of Medicine in St. Louis, United States

Reviewed by:
Stanley Ching-Cheng Huang, Case Western Reserve University, United States
Christoph Wilhelm, University of Bonn, Germany

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *12 May 2019*
Accepted: *18 October 2019*
Published: *06 November 2019*

Citation:
Rafei-Shamsabadi DA, Klose CSN, Halim TYF, Tanriver Y and Jakob T (2019) Context Dependent Role of Type 2 Innate Lymphoid Cells in Allergic Skin Inflammation. Front. Immunol. 10:2591. doi: 10.3389/fimmu.2019.02591

INTRODUCTION

Innate lymphoid cells (ILC) are innate immune cells of the lymphoid lineage, which have a similar functional diversity as T cell subsets based on the developmental dependency on lineage-specifying transcription factors and effector functions. Like T and B lymphocytes, all ILC derive from a hematopoietic stem cell-derived common lymphoid precursor (CLP) cell in the bone marrow (**Figure 1**). The CLP gives rise to an early innate lymphoid precursor (EILP) that expresses the transcription factor (TF) T-cell factor 1 (Tcf-1). From this branching point natural killer (NK)

cells develop via a NK precursor (NKp) and by upregulating the TFs eomesodermin (EOMES) and T-box transcription factor TBX21 (T-bet). The other branch develops into an Id2 expressing common helper-like ILC progenitor (CHILP). C-C chemokine receptor type 6 positive ($CCR6^+$) ILC3 can directly evolve form the CHILP depending on the expression of RAR-related orphan receptor (ROR)γt. All the remaining helper-like ILC subtypes, namely ILC1, ILC2, and ILC3, evolve from an innate lymphoid cell precursor (ILCP) which expresses the TF promyelocytic leukemia zinc finger (PLZF). $CCR6^-$ ILC3 can adapt a more ILC1-like phenotype by downregulating RORγt and upregulating T-bet. These cells are called ex ILC3. Production of their marker cytokines attributes certain physiological and pathological roles to the particular ILC subtype (**Figure 1**). Effector ILC can be classified into three interleukin-7 receptor positive ($IL\text{-}7R^+$) helper-like ILC groups (ILC1-3) and one $IL\text{-}7R^-$ cytotoxic ILC group (NK cells) (1–3). More recently, several groups have also identified IL-10 secreting ILC with proposed regulatory functions (4–6). Helper-like ILC and NK cells are mainly populated at barrier surfaces like the skin, gut, and the respiratory tract, although significant numbers can be detected in secondary and tertiary lymphoid organs in homeostasis and disease (7). Besides the bone-marrow, alternative sites of development exist, such as secondary lymphoid organs or even non-hematopoietic organs such as the gut (8–10). While ILC development continues throughout life, it is known that some ILC lineages are long-lived, and seed their designated tissues early in embryogenesis as demonstrated by parabiosis experiments in mice that show only little replenishment of helper-like ILC from the bone marrow in later life (11–13). Although some helper-like ILC express homing receptors for certain tissues these cells are mainly thought to proliferate on site under proinflammatory conditions (7, 14). Given their localization at barrier surfaces ILC perfectly serve as sensors for danger signals but also allergens and subsequently mount early immune responses by rapid cytokine production. They can act as initiators of the adaptive immune response by crosstalk with dendritic cells and T cells finally shaping full blown type 1, 2, or 3 immune responses [reviewed in (15)]. This review

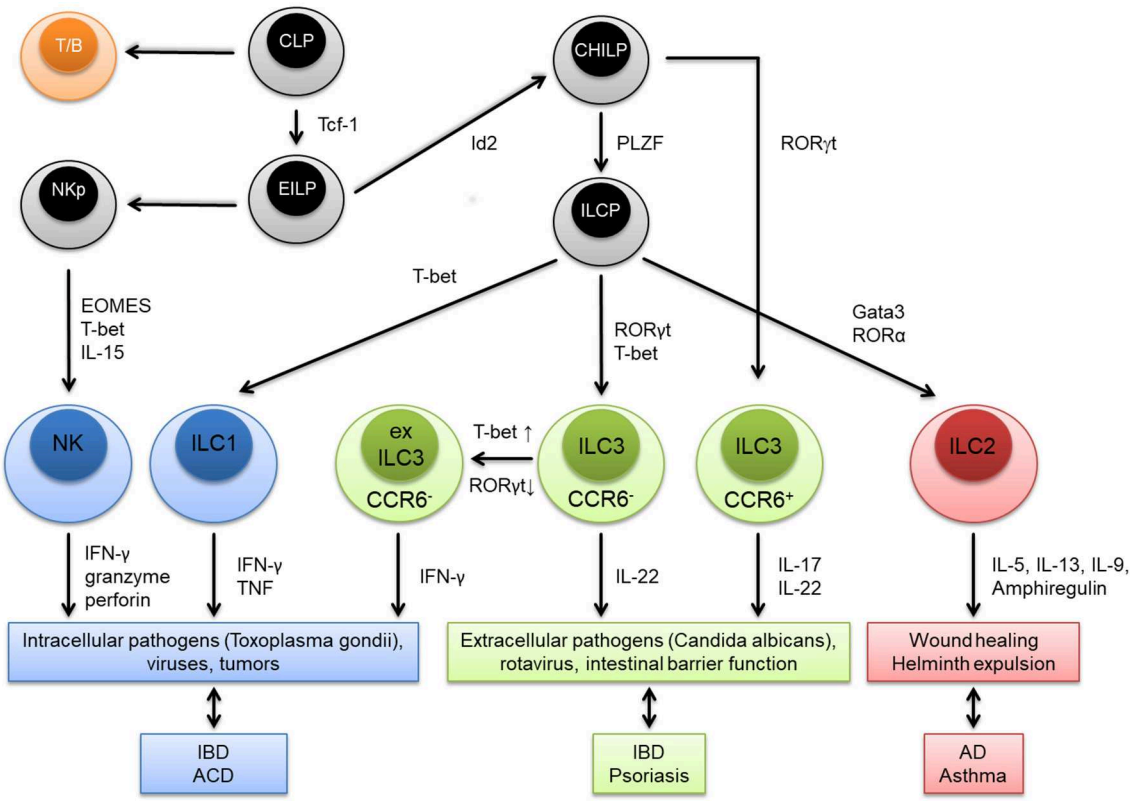

FIGURE 1 | Graphical summary of innate lymphoid cell (ILC) subtypes. ILC as well as T and B lymphocytes (T/B) derive from a common lymphoid precursor (CLP). The CLP gives rise to an early innate lymphoid precursor (EILP) that expresses the transcription factor (TF) T-cell factor 1 (Tcf-1). From this point natural killer (NK) cells develop via a NK precursor (NKp) and upregulate the TFs eomesodermin (EOMES) and T-box transcription factor TBX21 (T-bet). The helper-like ILC lineage derives from an Id2 expressing common helper-like ILC progenitor (CHILP) from which C-C chemokine receptor type 6 positive ($CCR6^+$) ILC3 can directly evolve depending on the expression of RAR-related orphan receptor (ROR)γt. All the remaining helper-like ILC subtypes, namely ILC1, ILC2, and ILC3, evolve from an innate lymphoid cell precursor (ILCP) which expresses the TF promyelocytic leukemia zinc finger (PLZF). $CCR6^-$ ILC3 can adapt a more ILC1-like phenotype by downregulating RORγt and upregulating T-bet. These cells are called ex ILC3. Production of their marker cytokines attributes certain physiological and pathological roles to the particular ILC subtype. IBD, inflammatory bowel disease; ACD, allergic contact dermatitis; AD, atopic dermatitis.

highlights the pathogenic role of ILC in the allergic skin diseases with a main focus on ILC2.

ILC CLASSIFICATION AND PLASTICITY

NK Cells and ILC1

NK cells are considered the innate counterpart of memory $CD8^+$ T cells. They share similar functions such as cytotoxicity and interferon-γ (IFN-γ) production and both express the transcription factors Eomes and T-bet. ILC1 on the other hand closely resemble T_H1 cells. Both express and depend on T-bet but lack EOMES and produce IFN-γ (16–19). NK cells and ILC1 are involved in protecting the organism against pathogens, viruses and tumors (16, 20, 21). Intraepithelial ILC1 can be found in Crohn's disease patients and contribute as a proinflammatory IFN-γ-producing population in an anti-CD40-induced colitis model in mice (22). NK cells are suggested to be important in enhancing inflammatory responses in a hapten based contact hypersensitivity mouse model and human allergic contact dermatitis (23, 24). Taken together these cell types are mainly involved in mounting a type 1 immune response.

ILC2

ILC2, like T_H2 cells, highly express the transcription factor GATA3 and produce type 2 cytokines including interleukin-5 (IL-5), IL-13 and the epidermal-growth-factor-like molecule amphiregulin (7). ILC2 mediate pathology in a mouse model of atopic dermatitis and promote wound healing in an IL-33-dependent manner (25, 26). ILC2 promote type 2 driven immune responses by promoting T_H2 differentiation of naïve $CD4^+$ T cells through production of IL-13, and by expression of MHC class II on their cell surface induce T cell priming (27–29). In addition, the inducible T-cell costimulatory (ICOS) molecule is highly expressed on ILC2 regulating their activation status and proliferation (30, 31). Moreover, activated ILC2 can express the TNF receptor superfamily ligand OX40L, which promotes local T_H2 cell proliferation and adaptive type 2 inflammation (32). Increased ILC2 numbers are linked to human allergic airway and skin diseases like allergic asthma atopic dermatitis (25, 33–36). Thus, type 2 immune responses are profoundly shaped by ILC2.

ILC3

ILC3 share RORγt expression with T_H17 cells and can produce IL-17 and IL-22 thereby helping the organism to fight against bacteria and fungi and viruses, such as *Citrobacter rodentium*, *Salmonella enterica*, *Candida albicans*, and rotavirus (2, 7, 37–41). There are ILC3 expressing the chemokine receptor CCR6 which comprise lymphoid-tissue-inducer (LTi) cells and can be $CD4^+$ or $CD4^-$. These cells are crucially important in the embryonic development of many lymphoid organs, whereas in adult mice they reside mainly in cryptopatches of the intestine with low proliferation (42–45). In mice, $CCR6^-$ ILC3 can express natural killer cell receptor such as NKp46 (NCR^+ ILC3), loose RORγt expression and upregulate T-bet, finally leading to IFN-γ production (46–50). These "ex-$ROR\gamma t^+$ ILC3" closely resemble ILC1. A large population of ILC3 can be found in the intestine where they are essential for maintaining barrier integrity and immunologic tolerance to commensal bacteria of the gut (51–53). IL-17 producing ILC3 are proposed to be involved in plaque formation in a psoriasis mouse model based on the topical application of the Toll-like receptor 7 (TLR7) agonist imiquimod (54). Finally, elevated numbers of ILC3 are found in blood and affected skin of psoriasis patients (55–57). Given this data ILC3 are part of type 3 immune responses and intestinal immunopathology.

ROLE OF ILC IN ATOPIC DERMATITIS

Impaired barrier function of the skin is a hallmark in the pathogenesis of atopic dermatitis (AD). Loss-of-function-mutations in the gene coding for the epidermal structure protein filaggrin is strongly associated with an elevated risk to develop atopic dermatitis by allowing elevated trans epidermal water loss, higher prevalence of *Staphylococcus aureus* on the skin and facilitated penetration of allergens (58–61). The type 2 inflammatory response in AD is known to involve innate and adaptive immune cells like mast cells, eosinophils, and $CD4^+$ T_H2 cells, the latter producing type 2 cytokines like IL-4, IL-5, and IL-13 (62). Since ILC2 are described in the skin (63) this led to the hypothesis that innate lymphoid cells, especially ILC2, may contribute to the pathogenesis of this frequently occurring atopic disease (**Figure 2**).

ILC in Human Atopic Dermatitis

Significantly more ILC2 can be found in lesional skin biopsies from patients suffering from atopic dermatitis in relation to skin from healthy individuals (25, 36). These ILC2 produce high amounts of the type 2 cytokines IL-5 and IL-13 and express the membrane bound IL-33 receptor ST2 as well-receptors for IL-25 and thymic stromal lymphopoietin (TSLP) (25, 36). These changes are even more profound when ILC2 are isolated from skin of house dust mite (HDM) allergic individuals that have been challenged epicutaneously with HDM extract. IL-33 is able to strongly enhance the expression of IL-13 and IL-5 and to increase the migratory capacity of isolated skin-derived ILC2 *in vitro* (36). Interestingly, ILC2 from atopic patients also express higher amounts of the killer cell lectin-like receptor G1 (KLRG1), which is even further elevated after stimulation with IL-33 or TSLP (36).

Human ILC2 express the prostaglandin D_2 (PGD_2) receptor chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2) (64, 65). PGD_2 which is mainly produced by mast cells induces ILC2 migration, production of type 2 cytokines and upregulation of the expression of IL-33 and IL-25 receptor subunits (ST2 and IL-17RA) *in vitro* (66). The effects of PGD_2 on ILC2 can be mimicked by the supernatant from activated human mast cells (through IgE-mediated degranulation) and inhibited by a CRTH2 antagonist highlighting a cross-talk between mast cells and ILC2 (66).

ILC2 respond to further mast cell mediators like cysteinyl leukotrienes, particularly LTE_4 (67). Human ILC express the functional leukotriene receptors $CysLT_1$ and its expression is increased in patients with atopic dermatitis (67). LTE_4 not only induces migration, promotes cytokine productions

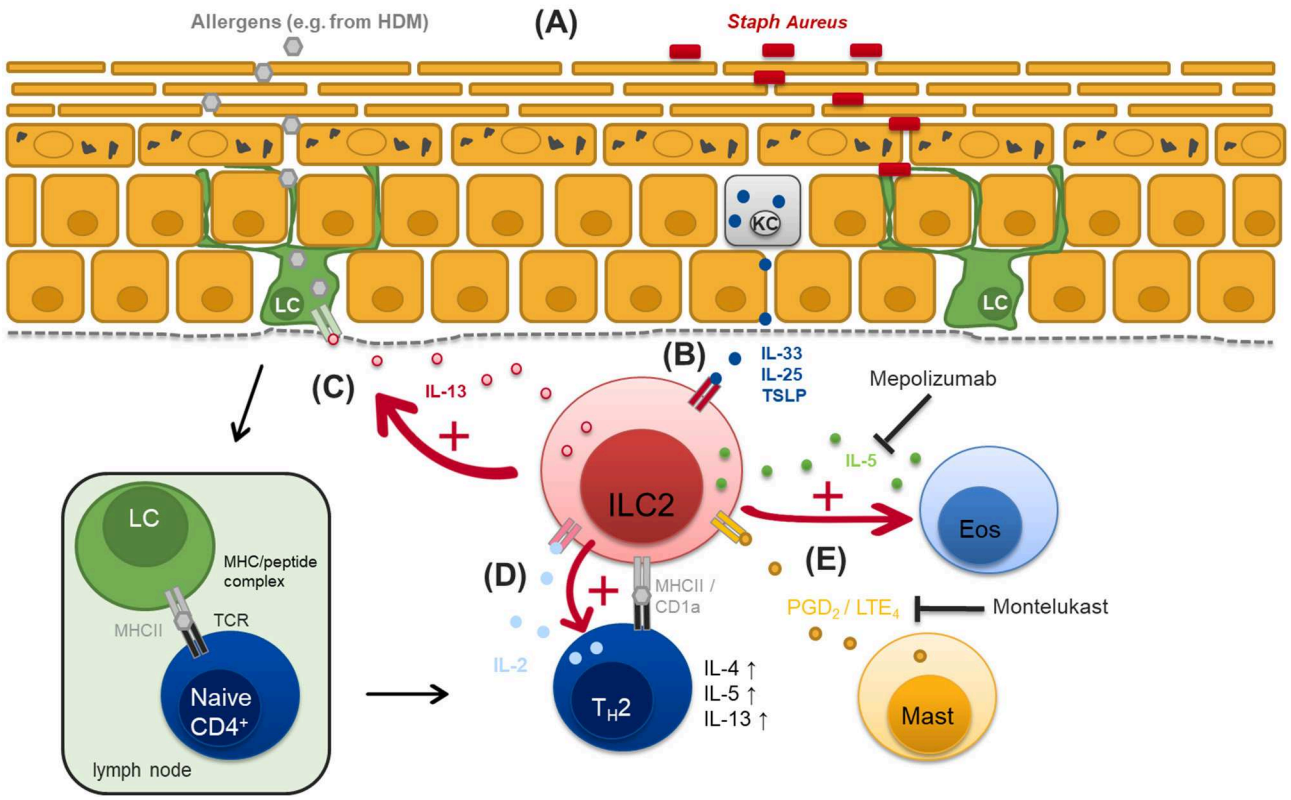

FIGURE 2 | Suggested pathogenic role of ILC2 in atopic dermatitis. **(A)** Loss-of-function-mutations in the gene coding for the epidermal structure protein filaggrin allow elevated transepidermal water loss (TEWL), higher prevalence of *Staphylococcus aureus* (Staph Aureus) on the skin and facilitated penetration of allergens, e.g., from house dust mite (HDM). **(B)** Damaged keratinocytes (KC) release cytokines like interleukin-33 (IL-33), IL-25, and thymic stromal lymphopoietin (TSLP) which activate dermal ILC2. **(C)** Activated ILC2 produce high amounts of IL-13 which stimulates epidermal Langerhans cells (LC). LC migrate to regional lymph nodes to prime naïve T cells by antigen presentation via MHCII to promote development of T_H2 cells that produce type II cytokines like IL-4, IL-5, and IL-13. **(D)** ILC2 can act as antigen presenting cells for T_H2 effector cells through antigen presentation via MHCII and/or CD1a prompting them to produce IL-2 which in turn sustains ILC2 activation and survival. **(E)** ILC2 can be activated by mast cell (Mast) derived prostaglandin D_2 (PGD_2) and cysteinyl leukotrienes LTE_4. ILC2 in turn produce IL-5 which promotes eosinophil (Eos) activation. Administration of montelukast can block LTE_4-mediated activation of ILC2. IL-5 function can be therapeutically blocked by specific monoclonal antibodies like mepolizumab. MHCII, major histocompatibility complex II; TCR, T cell receptor.

and upregulation of IL-33/IL-25 receptors in human ILC2 *in vitro*, but also enhances the pro-inflammatory effect of the epithelial cytokines IL-25, IL-33, TSLP, and of PGD2 as seen by increased production of IL-5 and IL-13. This effect of LTE_4 can be partially inhibited by adding the leukotriene antagonist montelukast. Finally, addition of IL-2 to LTE_4 and epithelial cytokines significantly further amplifies the activation of ILC2 (67). These findings clearly suggest a pathogenic role of ILC2 in the pathogenesis if atopic dermatitis in humans (**Figure 2**).

ILC in Atopic Dermatitis Mouse Models

Topical application of a synthetic form of active vitamin D3 (MC903) to the skin of mice can mimic atopic dermatitis-like inflammation with a type 2 signature (68). Using the MC903 AD mouse model Salimi et al. and Kim et al. investigated inflammatory responses in the presence and absence of ILC2. When ILC2 are depleted in $Rag1^{-/-}$ mice by administering an anti-CD90.2 and/or anti-CD25 monoclonal antibody this leads to an dramatically decreased ear swelling response (25, 36). Furthermore, using $Ror\alpha^{sg/sg}$ (Rorα-knockout) bone marrow chimeric mice which lack ILC2, a markedly reduced inflammatory response in the skin can be seen, highlighting ILC2 as a main proinflammatory cell in this type 2 inflammatory model (36). An increase in IL-33 and IL-25 expression has been reported in lesional skin of patients with AD compared with healthy individuals underlining an important role for these cytokines as proinflammatory ILC2 activating cytokines in AD (36, 69, 70). Strikingly, when flow cytometry assisted cell sorting (FACS)-purified ILC2 from MC903-treated C57BL/6 wild-type mice are adoptively transferred by intradermal injection into naïve C57BL/6 wild-type recipient mice, the recipient mice develop AD-like skin reactions with a type 2 T cell response indicating that these innate cells alone are capable of eliciting an AD-like skin response (25).

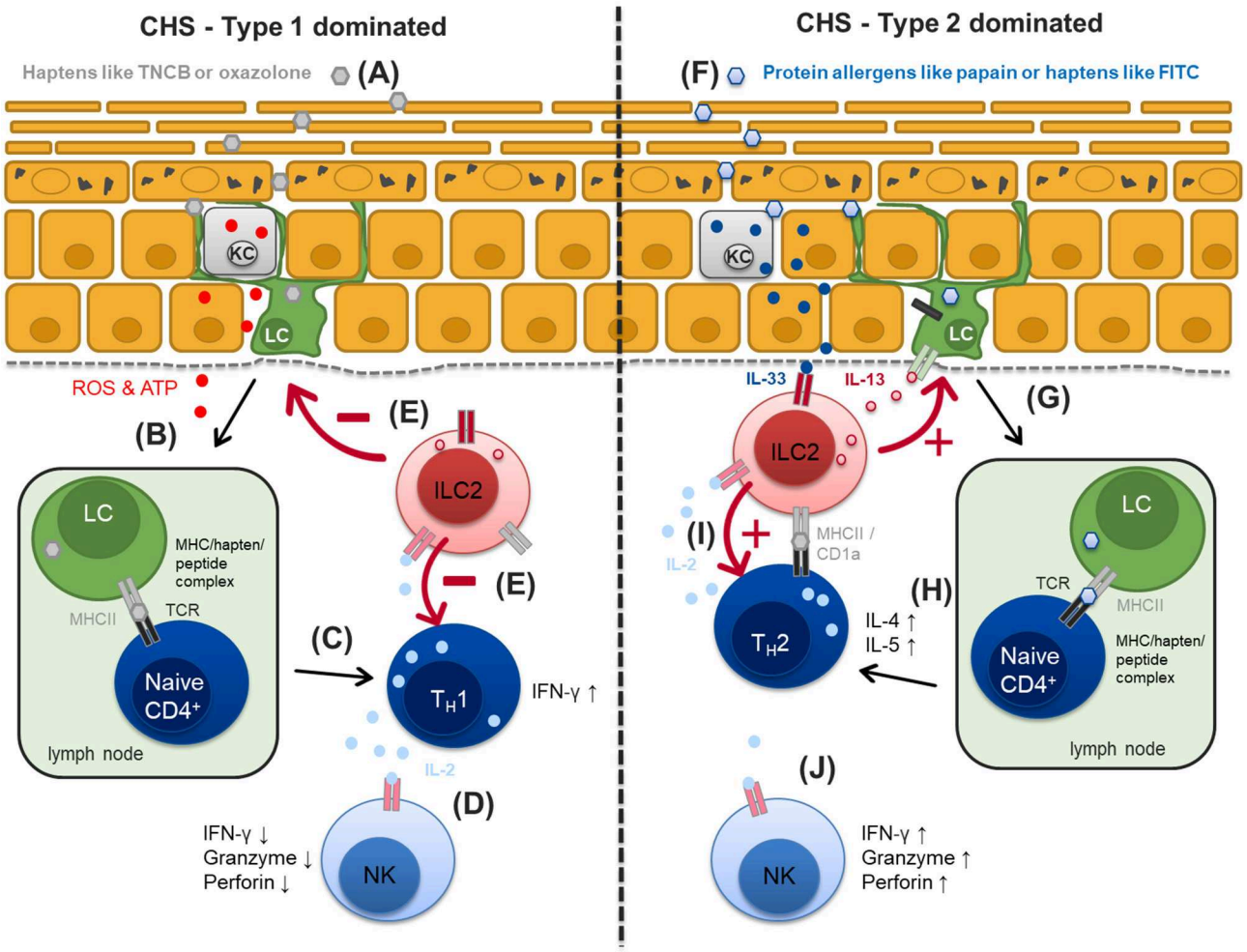

FIGURE 3 | Context dependent role of ILC2 in type 1 and type 2 dominated contact hypersensitivity. Contact allergens irritate and penetrate the upper skin layers. **(A)** Typical contact allergens like TNCB or oxazololone cause ROS and ATP release from damaged keratinocytes (KC) and uptake by epidermal Langerhans cells (LC). **(B,C)** LC migrate to skin draining lymph nodes and promote a type 1 driven immune response mediated by T_H1 and NK cells resulting in increased IFN-γ and IL-2 production. **(D)** Higher availability of IL-2 for NK cells results in their enhanced activation and effector cytokine production (IFN-γ, granzyme, perforin). **(E)** ILC2 are likely to suppress LC migration, T_H1 polarization and NK cells activation in this type 1 CHS response via mechanisms that are currently not well-understood. **(F)** Protein allergens like papain or haptens like FITC cause IL-33 release from keratinocytes (KC) which in turn activates dermal ILC2 to produce large amounts of IL-13. **(G,H)** ILC2 derived IL-13 promotes trafficking of Langerhans cells (LC) to regional lymph nodes where they prime naïve T cells by interaction of MHCII/peptide/hapten complex and T cell receptor promoting the development of T_H2 cells and a type 2 biased immune response. **(I)** ILC2 can act as antigen presenting cells for T_H2 effector cells prompting them to produce IL-2 which in turn sustains ILC2 activation and survival. **(J)** ILC2 compete with other innate effector cells like NK cells for the survival factor IL-2 leading to a reduced/moderate NK cell activation. FITC, Fluorescein isothiocyanate; MHCII, major histocompatibility complex II; ROS, reactive oxygen species; TCR, T cell receptor; NK, natural killer cell; ATP, Adenosine triphosphate.

Another possible mouse model to study eczema like skin reactions are the "flaky tail" mice. These mice bear a frameshift mutation in the murine filaggrin gene (flg) resulting in expression of a truncated profilaggrin (~215 kDa) instead of the normal high-molecular-weight profilaggrin (>500 kDa) (71). Topical application of allergen to mice homozygous for this mutation results in cutaneous inflammatory infiltrates and enhanced cutaneous allergen priming with increased development of allergen-specific antibody responses (71). Saunders et al. characterized changes of ILC2 numbers and their cytokine production in flg-mutant mice (72). These mice show spontaneous atopic dermatitis-like inflammation and develop compromised pulmonary function. In the skin and skin draining lymph nodes of these mice, there is a significant increase in the frequency of IL-5-producing ILC compared to wild type animals. However, no differences in cell numbers are seen for ILC1 and 3. Furthermore, flg-mutant mice show higher skin infiltrates of eosinophils, mast

cells and basophils (72). Even more astonishing, when flg-mutant mice are crossed with Rag1$^{-/-}$ mice (Flg$^{ft/ft}$Rag1$^{-/-}$) skin lesions but not lung inflammation occur as shown by cutaneous expansion of IL-5-producing ILC2, indicating that skin inflammation can develop independently of the adaptive immune system in these mice (72). Regulation of ILC responses by adaptive immune cells is also reported in other tissues (73). Finally, increased frequency of ILC2 can be found in skin blisters taken from non-lesional skin of patients with filagrin mutations compared with the skin of filagrin wildtype subjects (72). Taken together, loss of filagrin function in humans and mice is clearly linked to increased ILC2 activation and disease progression in atopic dermatitis.

This latter model, however, has been challenged recently by the work of Schwartz et al. which provides evidence that atopic dermatitis like lesions can evolve independent of ILC2 and ILC2-derived cytokines in Filaggrin-mutant (Flg$^{ft/ft}$) mice bred on an ILC2-deficient background (74). Interestingly, inflammation in these mice following MC903 treatment requires IL-1β and IL-1R1-signaling but is independent of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation and results in elevated numbers of IL-1β-responsive connective tissue mast cells (74). Finally, Flg$^{ft/ft}$ mice do not develop skin inflammation under germ-free compared to SPF conditions indicting a crucial role for the microbiome in promoting proinflammatory immune responses in this mouse model (74). This issue will be discussed in more detail in a later section.

ILC2 as Possible Therapeutic Targets in AD

Development of ILC2 depends on the transcription factor receptor-related orphan receptors alpha (RORα) and lack of RORα results in impaired lung inflammation in response to protease allergen in mice despite normal T_H2 cell responses (75). Dai et al. provide evidence that a synthetic RORα/γ inverse agonist (SR1001) is able to suppress inflammation in the MC903-induced atopic dermatitis mouse models. Topical treatment with SR1001 reduces epidermal and dermal inflammation, suppresses the production of type 2 cytokines and TSLP, and reverses impaired keratinocyte differentiation (76). Since SR1001 also inhibits RORγ signaling it is quite possible that RORγt$^+$ ILC3 functions may also be impaired (42). If topical inverse agonists for RORα may have anti-inflammatory functions in humans remains to be elucidated.

A crucial role for the IL-33/ILC2 axis in the pathogenesis of AD has been proposed by Imai et al. The authors generated a transgenic mouse line which overexpresses IL-33 in keratinocytes. These mice spontaneously develop an itchy dermatitis closely resembling AD at age 6–8 weeks with thickened epidermis, skin infiltration of eosinophils and mast cells, and high histamine and IgE levels in the blood (77). Moreover, IL-5 and IL-13 expressing ILC2 numbers are significantly increased in lesional skin, peripheral blood, and regional lymph nodes. Administering a neutralizing monoclonal anti-IL-5 antibody results in a marked reduction of the inflammatory response as shown by a decreased peripheral blood eosinophil count, milder thickened epidermis and lower inflammatory infiltrates including eosinophils (77). Unfortunately, a randomized, placebo-controlled parallel group design study in patients with AD could not detect a clinical improvement by administering a monoclonal antibody to human interleukin-5 (mepolizumab) in two single doses of 750 mg, given 1 week apart, despite a significant decrease in peripheral blood eosinophils (78).

ROLE OF ILC IN ALLERGIC CONTACT DERMATITIS

Allergic contact dermatitis (ACD) is a prevalent inflammatory skin disease triggered by low molecular weight organic chemicals or metal ions which penetrate the skin and bind covalently or by complex formation to proteins thereby activating the innate and adaptive immune response. ACD can be separated into two phases. The sensitization phase, were antigen upon first encounter with the skin is taken up by dendritic cells and transferred to the regional draining lymph nodes to be presented to antigen specific T-cells for priming. And the elicitation phase that is induced by subsequent antigen contact and leads to an infiltration of antigen-specific T-cells into the skin peaking 24–48 h after second antigen contact. In the mouse model of ACD, the contact hypersensitivity (CHS) model, hapten-specific CD8$^+$ cytotoxic T-cells are thought to be the key effector cells in the elicitation phase rendering CHS a classical type 1 driven adaptive immune response (**Figure 3**). Typical haptens used in these models comprise oxazolone, 2,4,6-Trinitrochlorobenzene (TNCB) or 2,4-dinitrofluorobenzene (DNFB) (79–81). In addition, we and others have previously demonstrated that sensing of danger signals by cells of the innate immune system including dendritic cells, neutrophils, and mast cells represent a crucial element in the initiation and elicitation of CHS responses (82–86).

NK Cells in Type 1 Dominated CHS Responses

Group 1 ILCs consisting of NK cells and ILC1 are involved in inflammatory bowel and allergic skin diseases in mice (12, 24, 87, 88). Regarding ACD Carbone et al. were able to characterize CD56^{high}CD16$^-$CD62L$^-$ NK cells in an *ex vivo* human model which accumulate in affected skin of hapten allergic human individuals and these NK cells release type 1 cytokines and induce keratinocyte apoptosis *in vitro* (23). In mice NK cells can be further subdivided into two distinct subsets: CD49a$^+$DX5$^-$ liver-resident (Trail$^+$) and CD49a$^-$DX5$^+$ conventional NK cells (cNK) (12). Furthermore, cNK cells seem to express much higher amounts of the transcription factor EOMES (87). Liver-resident NK cells can mediate long-lived, antigen-specific adaptive recall responses to haptens like DNFB and oxazolone independent of B cells and T cells (24). Preceding was the finding that a CHS response to several haptens can be elicited in Rag2$^{-/-}$ mice lacking T- and B-cells but not in mice that either contain dysfunctional NK cells (SCID × beige mice) or completely lack NK cells (Rag2$^{-/-}$ Il2rg$^{-/-}$ mice). A proper CHS response can be transferred by FACS-purified antigen-specific Thy-1$^+$ Ly49C-I$^+$ liver-resident NK cells from sensitized Rag2$^{-/-}$ mice

when transferred into naive Rag2$^{-/-}$ Il2rg$^{-/-}$ recipients (24). The same NK cell type seems to mount antigen specific immunity against certain viral pathogens as well (88). Our own investigations using the hapten TNCB support the role of EOMES^{+} cNK cells as the dominant proinflammatory innate cell type in the early phase of contact hypersensitivity. NK cell numbers increase significantly 24 h in the ear skin of mice after allergen challenge and produce type 1 marker cytokines like IFN-γ and TNF (89). Taken together, NK cells seem to represent a major driving force of the innate immune system in CHS pathogenesis pathogenesis (**Figures 3A–D**).

Helper-Like ILC in Type 1 Dominated CHS Responses

Very little is known about the involvement of helper-like ILC in the pathogenesis of CHS, however there has been some indirect evidence for it in the past. ILC2 are known to be a major source of IL-13 production thus playing a crucial role in innate type 2 immune responses to worms and inhaled allergens (90, 91). IL-13-deficient mice (Il13$^{-/-}$) show impaired T_H2 responses induced by epicutaneous ovalbumin (OVA) exposure whereas i.p. sensitization is normal and results in responses equivalent to wild type mice (92). Interestingly, Il13$^{-/-}$ mice display an even enhanced ear swelling responses to the hapten DNFB, which is also known to elicit a type 1 T-cell driven immune response (93), compared to wild type mice. At the time, this finding was interpreted as a lack of T_H2-mediated suppression but it's tempting to speculate that impaired ILC2 function in this mouse model may also have contributed to a disinhibited and thus exaggerated type 1 immune response. We recently characterized cell numbers and cytokine production of all ILC subgroups (ILC1-3 and NK cells) during the elicitation phase of a CHS mouse model based on the hapten TNCB using an ILC reporter system (89). Numbers of ILC are elevated in skin draining lymph nodes, show an activated phenotype and produce elevated amounts of their marker cytokines IL-13 and IL-5 at late time points (48 and 72 h), i.e., during the resolution phase of the inflammatory response in the skin. On the other hand, NK cell numbers and their production of IFN-γ and TNF are highest 24 h after allergen challenge paralleling the strongest skin inflammation period (89). The latter is expected since TNCB is known to elicit a type 1 driven immune response (93, 94). However, lack of ILC achieved by either antibody mediated depletion using an anti CD90.2 mAb in Rag1$^{-/-}$ mice or by using mice that selectively lack ILC2 [Ror$\alpha^{sg/flox}$Il7r$^{Cre/+}$mice (29)] results in a significantly enhanced and long lasting inflammatory response (89). The ear infiltrate of ILC depleted mice show a tendency toward a more type 1 biased immune response indicated by increased numbers of T-bet^{+} CD4^{+} T-cells (89). This data supports the concept of a counter regulatory role for ILC2 in CHS (**Figures 3A–D**).

Helper-Like ILC in Type 2 Dominated Allergic Skin Responses

Some allergens like Fluorescein isothiocyanate (FITC) and papain rather induce allergic type 2 immune responses with increased IL-4 producing T_H2 cell infiltrates in murine skin when reapplied topically or intradermally (28, 95, 96), suggesting that ILC2 might rather play a proinflammatory role in these models. Along this line we demonstrated in a papain skin challenge model that lack of IL-13-producing ILC2 leads to a marked reduction of inflammation with less skin infiltrating T_H2 cells [(28); (**Figures 3F–J**)]. A first therapeutic approach in type 2 dominated allergic skin responses has been proposed by Bao et al. They demonstrate that ILC2 numbers are increased in the skin of FITC-challenged mice. In addition, intraperitoneal injection of the cycloartane triterpene saponin Astragaloside IV during the sensitization phase leads to a reduction of the inflammatory response as seen by a decreased ear swelling response, less production of pro-allergic cytokines like IL-33 and TSLP, and significantly reduced numbers of ILC2 in the skin of these mice (97). Thus, ILC2 seem to have contrary roles in type 1 and type 2 dominated allergic skin reactions, respectively (**Figure 3**).

ROLE OF DERMAL ILC2 IN INNATE AND ADAPTIVE IMMUNE CROSS TALK

Antigen Presentation by MHCII

ILC2 and ILC3 express MHCII molecules on their surface and can act as antigen presenting cells for helper T cells (29, 51, 52). Our own analysis of MHCII expression on ILC2 revealed that in skin draining lymph nodes of mice ∼50% of the ILC2 express MHCII, while in the skin only ∼3% express MHCII. Antibody mediated depletion of ILC leads to a significant reduction of MHCII positive ILC2 both in skin and LN (89). Currently, we can only speculate that ILC2 might regulate effector T cells in a direct fashion via MHCII. In line with this, Oliphant et al. recently demonstrated that MHCII expression on ILC2 and subsequent antigen presentation to CD4^{+} T cells is crucial for successful helminth expulsion in mice (29). The crosstalk between ILC2 and CD4^{+} T cells seems to involve IL-2 since activated CD4^{+} T cell-derived IL-2 has been shown to synergize with IL-33 to stimulate ILC2 (29). Thus, lack of ILC2 may lead to a higher availability of IL-2 for proliferation of other effector cells like NK cells leading to an augmented response in CHS.

Antigen Presentation by CD1a

Another way how ILC2 might crosslink innate and adaptive immunity is by expressing the lipid-presenting molecule CD1a. Other than classical MHC proteins that present peptides, CD1 molecules present endogenous and exogenous lipid antigens to T lymphocytes (98). In a CHS model using the poison ivy-derived lipid contact allergen urushiol, CD1a expressing Langerhans cells are important to promote CD1a-restricted CD4^{+} T cells to produce IL-17 and IL-22. Furthermore, treatment with blocking antibodies against CD1a alleviates skin inflammation dramatically (99). More recently Hardman et al. demonstrated in a human skin challenge model that skin-derived ILC2 not only express CD1a but are also capable of helping CD1a-reactive T cells to sense *S. aureus* components in an cytosolic phospholipase A2 (PLA2G4A) and TLR-dependent–dependent manner, suggesting a new role for ILC2 in lipid surveillance of the skin (100). Currently, it is unclear whether this also applies for the adaptive immune response against

urushiol. Taken together CD1a expression on ILC2 seems to be clearly involved in shaping the phenotype of adaptive T cell responses.

Crosstalk With Basophils and Macrophages

Mashiko et al. reported significantly elevated frequencies of basophils, ILC and T_H2 cells in the lesional skin of AD patients compared to patients suffering from psoriasis. Interestingly, basophils and ILC2 are positively correlated in skin, whereas skin basophils are inversely correlated with blood ILC2 suggesting that skin basophils may attract circulating ILC2 to skin of AD patients by IL-4 production (101). Kim et al. detected elevated numbers of basophils and ILC that form clusters in inflamed human AD skin compared to control skin. Using the MC903-based AD mouse model in IL-4/GFP reporter mice, they demonstrated that murine basophil responses preceded ILC2 responses and those basophils are the dominant IL-4-producing cell type in inflamed skin. In addition, ILC2 express the IL-4 receptor IL-4Rα and proliferate in an IL-4-dependent manner. Finally using $Il4^{-/-}$ mice Kim et al. provide evidence that especially basophil-derived IL-4 is necessary for proinflammatory ILC2 responses in the skin (102).

Most notably, Egawa et al. have shown that basophil-derived IL-4 converts $Ly6C^+CCR2^+$ inflammatory monocytes into anti-inflammatory M2 macrophages in an IgE-mediated chronic allergic inflammation (IgE-CAI) mouse model, a model where basophils rather than mast cells and T cells play a critical role for the elicitation of allergic response (103, 104). In this model, skin infiltrating monocytes acquire an M2-like phenotype in an IL-4R- and basophil-dependent manner and adoptive transfer of $Ly6C^+CCR2^+$ inflammatory monocytes dampens the exacerbated IgE-CAI in $CCR2^{-/-}$ mice which also requires IL-4R signaling (103). Thus, it is tempting to speculate, that basophil-derived IL-4 may promote pro-inflammatory responses via ILC2 and anti-inflammatory signals via M2 macrophages at the same time, leading to a counterbalanced immune response. However, the role of ILC2 in the IgE-CAI model is not known so far.

On the other side, ILC2 have been shown to promote polarization of the anti-inflammatory M2 macrophages by producing type-2 cytokines (IL-4, IL-5, and IL-13) in an renal ischemia-reperfusion injury model and experimental cerebral malaria (105, 106). Furthermore, in obese mice PD-1^{high} ILC2 are inhibited by PD-L1 expressing M1 macrophages which is promoted by TNF. PD-1 blockade improves ILC2 function, reinforces type 2 innate responses and promotes adipose tissue homeostasis (107, 108). Interestingly, in an serum-induced arthritis mouse model ILC2 were indispensable for dampening proinflammatory IL-1β secretion by bone marrow-derived macrophages (109). Finally, basophil-derived IL-4 seems to be essential for M2 macrophage mediated trapping of Nippostrongylus Brasiliensis larvae in the skin during second infection of mice thereby leading to reduced worm burden in the lung (110). However, basophils had no apparent contribution to worm expulsion from the intestine highlighting their crucial role in the skin (110).

Taken together, there seems to be an intense crosstalk between basophils, ILC2 and macrophages involving cytokines like IL-4, IL-13, and IL1β and resulting in differential polarization of macrophages dependent on the disease model. How these three cell type interact in AD and CHS remains to be elucidated.

Crosstalk With Dendritic Cells

Using the protease-allergen papain which induces type 2 allergic airway and skin inflammation we showed that ILC2 are necessary for mounting an appropriate antigen specific T_H2 memory response and that ILC2 activation clearly precedes T_H2 involvement in papain induced airway and skin inflammation (28). Furthermore, ILC2-derived IL-13 is needed for the activation and expansion of an allergen-induced subset of dendritic cells ($CD11b^+CD103^-IRF4^+$) which produce the T_H2 cell chemoattractant CCL17. Using ILC2-deficient mice, we demonstrated that dermal ILC2 are crucial to mediate expansion of $CCL17^+$ dendritic cells after skin challenge with papain finally leading to an effective T_H2 memory response. Thus, ILC2 licensing of dendritic cells is a critical component of the memory T_H2 cell response to certain allergens at barrier sites (28).

INFLUENCE OF SKIN MICROBIOTA ON ILC2 IMMUNITY

As mentioned earlier, filaggrin mutant mice significantly differ in their microbiome composition compared to wild type mice and do not develop skin inflammation under germ-free conditions prompting a crucial role for the microbiome in shaping this setting (74). Several studies have investigated the role of skin commensal bacteria in shaping the host immune cell functions of this organ (111–113). This mostly involves skin derived dendritic cells as sensors of bacterial antigens which promote development of commensal-specific T cells. These T cells help to improve tissue repair and protection to pathogens rendering them as important players in the skin homeostasis (111, 112).

When analyzing different skin-derived bacterial strains in a pediatric AD cohort over time, Byrd et al. were able to detect certain clonal *S. aureus* strains which are associated with more severe disease (113). Interestingly, heterogeneous *Staphylococcus epidermidis* strains were found in patients with less severe disease indicating that clonal expansion of certain bacterial strains can trigger proinflammatory responses in human AD. Furthermore, *S. aureus* isolates from AD patients with more severe flares can induce epidermal thickening and expansion of cutaneous T_H2 and T_H17 cells in a murine AD model (113).

These findings are suggesting a role of the microbiome to shape ILC2 functions as well. Interestingly, ILC2 distribution and homeostatic function in bone marrow, fat, lung, gut, and skin seems to be independent of commensal microbiota when comparing SPF to germ free mice (114). However, in mouse model of chronic obstructive pulmonary disease (COPD), challenge with *S. aureus* or *Haemophilis influenzae* lead to loss of GATA-3 expression in ILC2 and a subsequent increase in the

expression of IL-12Rβ2, IL-18Rα, and T-bet giving them an ILC1-like phenotype (115). This ILC2 plasticity can also be influenced by viral stimuli especially influenza A virus (115).

Taken together, there is substantial evidence that the microbiome is involved in shaping ILC2 function and plasticity, especially in inflammatory lung diseases. Whether this concept also applies to the pathogenesis of inflammatory skin disease like AD and CHS remains to be determined.

TYPE 1 AND TYPE 2 COUNTER REGULATION IN CHS

Type 1 and type 2 immune responses are known to tightly counter-regulate each other (116). T_H1 cytokines such as IFN-γ have been shown to antagonize the function of ILC2 and type 2 innate immune responses in mouse models of allergic lung inflammation and viral respiratory tract infections (13, 117). ILC2-mediated lung inflammation is enhanced in the absence of the IFN-γ receptor on ILC2 cells *in vivo* and IFN-γ effectively suppresses the function of tissue-resident ILC2 cells, two observations that clearly suggest a suppressive function of type 1 cytokines on ILC2 (13). Our own investigations reveal that TNCB based CHS in a mouse model is counter regulated by activated ILC, since lack of all ILC or ILC2 alone leads to a dramatic increase in the inflammatory response with a type 1 immune response bias (89). More recently, it has been reported that in the early stage of papain-induced lung inflammation in mice, depletion of NK cells results in increased numbers and cytokine production of ILC2, suggesting that NK cells negatively regulate ILC2 (118). Hapten based CHS experiments in $Il15^{-/-}$ mice, which lack NK cells, demonstrate dramatically reduced ear swelling responses and at the same time increased numbers of ILC2 in skin and skin draining lymph nodes (89). Thus, a mutual balance between type 1 and type 2 immunity may also exist in CHS, in which NK cells negatively regulate ILC2 and ILC2 counter regulate type 1 immune responses mainly driven by NK cells, T_H1, and T_C1 cells.

Recently, Kim et al. identified IL-10-producing lineage negative lymphoid cells that show elevated numbers in the axillary as well as inguinal lymph nodes and ear tissues of Oxazolone challenged mice suggesting a possible regulatory role of ILC (119). These cells were designated "ILC10" and identified by expression of markers like CD45, CD127, and Sca-1, while detailed characterization of the exact ILC subpopulation was not provided. Along the same line, an IL-10 producing ILC2 effector cell population has recently been described in murine lung and suggested to regulate immune responses in a papain induced allergic lung inflammation model (4). These studies prompted us to address the presence of IL-10 producing ILC. Using highly sensitive IL-10 transcriptional reporter mice (120) we, however, could not identify relevant numbers of IL-10 transcribing lineage negative cells in different tissues (skin, lymph nodes, blood, and spleen) in the TNCB induced CHS model (89). Thus, at least in our hands ILC derived IL-10 does not appear to be responsible for the regulatory effects of ILC in type 1 dominated CHS of the skin.

Nevertheless, ILC2 are reported to promote regulatory T (Treg) cell expansion, thus framing the hypothesis that ILC2 can regulate inflammation indirectly. Molofski et al. demonstrated that ICOSL expression by ILC2 can stimulate $ICOS^+$ Treg cells, providing a potential indirect link between IL-33 and Treg cells (121). In line with this, Rauber et al. could demonstrate that IL-9 producing ILC2 are crucial in promoting Treg driven anti-inflammatory effects in an antigen-induced arthritis mouse model. This ILC2/Treg interaction was dependent on direct cell contact involving ICOS–ICOSL interaction (122).

We recently showed that IL-33-induced OX40L expression by ILC2 is critical for tissue-specific expansion of Treg cells (32). Moreover, our data indicates that OX40L/OX40-driven interactions between ILC2 and Treg cells preferentially expands $GATA3^+$ Treg cells, which are thought to be tissue-resident and functionally primed (123). IL-33-induced OX40L expression by ILC2 and the associated Treg cell expansion seems to be restricted to specific anatomical locations such as the airway and adipose tissue but not LN or gut (32). Thus, it remains unknown if a similar mechanism or alternative ILC2-independent suppressive pathways are involved in the skin.

Malhotra et al. recently found skin resident RORα-expressing Tregs to dampen ILC2-driven inflammation in a mouse model for atopic dermatitis (124). This effect is thought to be based on the enhanced expression of TNF ligand–related molecule 1 (TL1A) and death receptor 3 (DR3) on ILC2 as well as suppressed IL-4 expression. RORα-expressing Tregs are found in higher numbers in human skin compared to peripheral blood suggesting a possible counter regulatory role for these cells in ILC2-driven allergic skin diseases (124).

Taken together, these data show that ILC2 can act as modulators of the adaptive immune response and that the functional outcome very much depends on the context of the inflammatory reaction that is analyzed. In type 2 dominated skin inflammation ILC2 seem to be primarily proinflammatory while in the context of a type 1 dominated immune response ILC2 can act as regulators that help to counterbalance the inflammatory reaction (**Figure 3**).

CONCLUDING REMARKS AND OUTLOOK

Innate lymphoid cells are increasingly emerging as important effectors of the innate immune system finally shaping a distinctive adaptive immune response. This includes on the one side important physiological functions in promoting wound healing, adipose tissue homeostasis, protection from pathogens and dampening of certain inflammatory disorders via Treg induction. On the other side, ILC2 have been shown to be important proinflammatory players in diseases like allergic asthma and atopic dermatitis. In the case of atopic dermatitis ILC2 have been described to be the major proinflammatory ILC subtype accountable for the production of marker cytokines like IL-13 and IL-5, cross-talk with other innate cells like basophils and dendritic cells, and finally promoting the development of

T_H2 cells. ILC2 will continue to be of high interest as possible targets in AD therapy, especially concerning their potential to produce high amounts of cytokines.

Immunologic reaction in allergic contact dermatitis can differ depending on the type of hapten used. Haptens like TNCB or oxazolone inducing type 1 responses clearly favor NK cells and T_H1 cells as the driving proinflammatory force. In these models ILC2 may have counter regulatory functions as our own investigations suggest. On the other side, in allergic type 2 responses of the skin, induced by distinct haptens like FITC or protein allergens like papain, ILC2 seem to have a proinflammatory role. These observations clearly emphasize a context dependent function of ILC2 which is determined by the type of model analyzed (type 1 or type 2 dominated).

Additionally, ILC2 have recently been shown to be part of a neuro-immune interface. ILC2 function can be influenced by the neuropeptide neuromedin U (NMU) secreted by cholinergic neurons in the mucosal tissue of the gut and lungs. This goes in line with other studies showing that further neuroendocrine factors like norepinephrine, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and acetylcholine can modify ILC2 function as well (125–131). Furthermore, challenge of mouse skin with the poison ivy compound urushiol leads to an increase in IL-33 expression which can act on small to medium-sized dorsal root ganglion neurons that innervate the skin and express the IL-33 receptor ST2 (132). Strikingly, targeting IL-33 by either neutralizing antibodies or intrathecal application of ST2 siRNA results in significantly reduced itching and subsequently less scratching behavior in these mice, suggesting a new therapeutic approach in poison ivy ACD (132). Since pruritus is a hallmark symptom of ACD in humans and mice which is mediated by certain sensory neurons (133) it is tempting to speculate that this new identified "neuron-ILC2 unit" may also be important in the pathogenesis of AD and ACD. This hypothesis is further supported by studies showing that type 2 cytokines like TSLP and IL-4 can enhance itching (134, 135).

Taken together, the picture of ILC function in allergic skin diseases is far from complete. Further investigations especially on the mode of action of how ILC modify immune responses in a context dependent fashion are needed to fill this gap of knowledge.

AUTHOR CONTRIBUTIONS

DR-S did the main research and wrote the first draft of the manuscript. CK, TH, and YT provided substantial contributions to acquisition, analysis, and interpretation of the scientific content of this work. TJ provided the main contribution to the conception and design of the work. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

Research in the lab of YT was funded by the DFG (TA 436/4-1 and P06/SFB1160) and the Else Kröner-Fresenius Stiftung (2017_EKES.34). DR-S was supported by the clinician scientist programme Excellent Clinician Scientists in Freiburg—Education for Leadership (EXCEL) of the Faculty of Medicine, University of Freiburg, funded by the Else Kröner-Fresenius-Stiftung. This work was supported by grants from the German Research Foundation (DFG; KL 2963/2-1 and SPP1937 - KL 2963/3-1 to CK) and the European Research Council (ERCEA; ERC Starting Grant #803087 to CK).

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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.

frontiers in Immunology

REVIEW
published: 11 December 2019
doi: 10.3389/fimmu.2019.02897

Interleukin-7 Receptor Alpha in Innate Lymphoid Cells: More Than a Marker

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Innate lymphoid cells (ILCs) are a group of immune cells that are important for defense against pathogens, tissue repair, and lymphoid organogenesis. They share similar characteristics with various subsets of helper T cells but lack specific antigen receptors. Interleukin-7 (IL-7) and thymic stromal lymphopoietin (TSLP) are cytokines that engage the IL-7Rα and have major roles in dictating the fate of ILCs. Recent advances in the field have revealed transcriptional programs associated with ILC development and function. In this article, we will review recent studies of the role of IL-7 and TSLP in ILC development and function during infection and inflammation.

Keywords: IL-7, TSLP, innate lymphoid cells, mucosal immunity, lymphopoiesis, inflammation

OPEN ACCESS

Edited by:
Arthur Mortha,
University of Toronto, Canada

Reviewed by:
Cyril Seillet,
Walter and Eliza Hall Institute of Medical Research, Australia
Ai Ing Lim,
National Institute of Allergy and Infectious Diseases (NIAID), United States

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *25 September 2019*
Accepted: *26 November 2019*
Published: *11 December 2019*

Citation:
Sheikh A and Abraham N (2019) Interleukin-7 Receptor Alpha in Innate Lymphoid Cells: More Than a Marker. Front. Immunol. 10:2897. doi: 10.3389/fimmu.2019.02897

INTRODUCTION

Innate lymphoid cells (ILCs) are a recently discovered subset of immune cells critical for the development of innate immunity against external pathogens, facilitating tissue repair, and mediating inflammation at multiple mucosal sites (1). It has become clear that these cells are major contributors despite being rare in proportion among immune cells. Although they lack antigen receptors, ILCs share multiple developmental circuitries with and are ancestral to the more abundant adaptive lymphoid cells. Both populations of lymphoid cells are known to develop from the same stem cell precursors in the bone marrow called common lymphoid precursors (CLPs) (2–5). In addition to sharing common progenitors with adaptive cells in the bone marrow, ILCs also require similar growth factors and cytokines to develop and function. The interleukin-7 receptor α (IL-7Rα or CD127) dependent cytokines, IL-7 and thymic stromal lymphopoietin (TSLP) are examples of such cytokines and they play an important role in determining the fate and function of ILCs (1, 6, 7). IL-7 is canonically important for the early development of B and T cells from bone marrow precursors and the thymic development of T cells (8, 9). Mature T cells also require IL-7 for survival, proliferation and multiple effector functions during infections and tumor infiltration (10–13). TSLP is mainly and constitutively produced by epithelial cells of the skin, gut and lungs, and shapes the response of dendritic cells and T cells against invading pathogens in a typical type 2 "weep and sweep" response that when misdirected, may also contribute to asthma and allergic inflammation (14–17).

Due to the heterogeneity in ILC populations and their multiple precursors, and our incomplete understanding of the biological factors (transcription factors, cytokines, disease states, etc.) that dictate ILC lineage commitment and function, we lack the knowledge to use ILC biology to develop new treatments or understand the precise role of ILCs in response to current therapeutics. For example, cytokines that govern adaptive lymphoid cells such as rhIL-7 are in multiple clinical trials

for treatment of HIV, solid tumors, T cell reconstitution, and enhancing CAR-T cell therapy for B-cell lymphomas, yet the effects this may have on ILCs and other disease outcomes is understudied (18). In this review we will discuss relevant findings on the roles of IL-7 and TSLP in ILC development and function at various tissue sites as well as the mechanisms involved downstream of their signals. Where appropriate, we will also identify the significant gaps in the field and possible future directions.

IL-7 AND TSLP

IL-7Rα is found on multiple subsets of lymphoid cells during their developmental and mature states. Both IL-7 and TSLP use IL-7Rα to initiate the formation of a heterodimeric receptor. IL-7 is a common gamma chain (γc) cytokine and requires the heterodimerization of IL-7Rα with the γc receptor (CD132) for signaling (19), whereas TSLP signaling requires heterodimerization with the TSLP receptor (TSLPR) (20, 21) (**Figure 1**).

IL-7 is produced mainly by stromal cells in the bone marrow and thymus under steady state where it plays an indispensable role in the development of both pre- and pro-B cells in the bone marrow (22). The dynamic expression of IL-7Ra was shown to be critical for IL-7 responsiveness for specific stages of maturation in the thymus in shaping T cell development and survival (23–25). In addition to being an essential factor for B and T cell development, IL-7 can also influence effector T cells. For instance, exogenous IL-7 treatment enhances cytotoxic CD8 T cell anti-tumor activity and reverses T cell exhaustion caused by chronic LCMV infection, and thus, preventing liver pathology (10, 11).

While IL-7 is known to be produced mainly in the stromal and epithelial cells of the bone marrow and thymus respectively, the cells in the various other tissues that are capable of producing this cytokine are elusive (26). It is clear that the main producers are radio resistant cells of non-hematopoietic origin (27). IL-7 can be detected at low levels in the small intestine, lungs, liver, and skin where it can modulate T cell responses (28–32). In addition, through use of IL-7-eGFP mice, it became apparent that while stromal and epithelial cells contribute to IL-7 production, lymphatic endothelial cells are the key producers of IL-7 throughout the body including mucosal tissues such as the lung (33, 34). However, whether these cells are the primary source of IL-7 during inflammation, and if they play a role in ILC responses, remains a question.

What regulates the expression or availability of IL-7 in mucosal barrier tissues is not entirely clear. In the liver, LPS induced TLR stimulation leads to TRIF dependent expression of IL-7 in hepatic cells (28). Whether this mechanism is conserved in other tissues is not known. Interestingly however, while both ILCs and T cells express IL-7Rα, the expression of this receptor is significantly higher on ILCs due to an increased resistance to IL-7 mediated internalization (27). As such, ILCs are key regulators of the availability of IL-7 in lymphoid tissues acting as a cytokine sink and limiting IL-7 availability to other cells such as T cells which depend on IL-7 for homeostatic proliferation (27). Considering T cells heavily outnumber ILCs, further investigation is necessary to determine the extent to which ILCs limit the availability of IL-7 in peripheral tissues and how that affects other immune cells during homeostasis and inflammation.

TSLP was originally identified in a conditioned murine thymic stromal cell line supernatant and reported to play a role in the *in vitro* development of B cells (35–37). However, loss of function *in vivo* studies have demonstrated minimal or secondary role for TSLP in lymphoid development (22, 38, 39). IL-7 and TSLP are often compared with each other owing to their

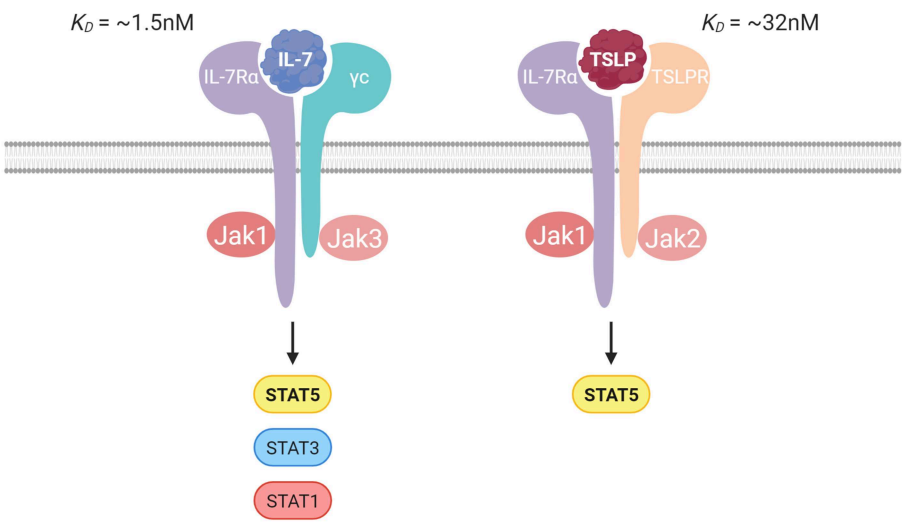

FIGURE 1 | Illustration of the IL-7 and TSLP receptor complexes with the binding affinities. IL-7 signals through IL-7Rα paired with the γc receptor, and TSLP signals through IL-7Rα paired with the TSLP receptor. Jak/STAT signaling is crucial in transduction of signal for both cytokines.

shared dependency on IL-7Rα and ability to activate STAT5, albeit through different JAK proteins (40). However, despite its discovery from thymic cells and its nomenclature, TSLP is mainly produced in a constitutive manner by epithelial cells, notably by keratinocytes and that of mucosal organs such as the intestine and lungs (14, 15, 41, 42). There is strong evidence suggesting its importance in maintaining barrier integrity in these locations upon infection and inflammation through tissue remodeling and by conditioning dendritic cells (DCs) toward a tolerogenic phenotype. This supports the development of regulatory T cells and polarization of activated helper T cells to exhibit type 2 (Th2) characteristics (14–17). Altogether, these findings suggest roles for TSLP that are not only segregated spatially and possibly temporally from that of IL-7 but also serve a unique function in developing immune responses.

ILC SUBSETS AND THEIR ORIGIN

Terminally differentiated ILCs closely resemble helper T cells, such that a version of a helper ILC mirrors each of Th1, 2, 17 helper T cells in terms of key transcription factor dependency, cytokine output, and resulting pathologies (43, 44). In fact, most of what we have learned about the function and development of ILCs since their discovery has been aided by our knowledge of T cells.

ILCs have been documented and categorized into three general helper ILC groups. Group 1 ILCs (ILC1s) are found in various tissues including the small intestine and liver, are dependent on T-bet and produce the Th1 cytokines IFN-γ and TNF- α (4, 45). Natural killer (NK) cells are similar to ILC1s but are not considered part of the helper ILC subset and generally do not express the IL-7Rα (45). However, some tissue NK cells express a range of IL-7Rα levels, like in the thymus, colon, and small intestine lamina propria (siLP) (46, 47). Group 2 ILCs (ILC2s) express GATA3 prominently, similar to Th2 cells, and likewise, produce IL-5, IL-13, and in some conditions IL-9 when activated by the alarmins TSLP, IL-33, and IL-25 (6, 48–50). They are also the major ILC subset found in the lungs and play an important role in airway immunity (51, 52). Group 3 ILCs (ILC3s) are RORγt-dependent, like Th17 cells, and consist of a major subset that produces IL-17 and IL-22 in response to IL-23 and are critical for intestinal immunity against pathogens (1, 53). Another subset of ILCs are the Lymphoid Tissue inducer cells (LTi), which are also RORγt-dependent group 3 ILCs, and are considered important for the development of secondary lymphoid organs (54).

In mice, the fetal liver serves as the earliest known source of ILCs where IL-7 is known to be produced and support the development of other lymphoid cells (5, 55). The various precursors at different stages of ILC development are primarily studied using mouse models and are classified based on their surface markers and the transcription factors that lead to their lineage restriction. Common lymphoid progenitors (CLPs) are descendants of lymphoid-primed multipotent progenitors (LMPPs) which largely do not express IL-7Rα and are the source of all lymphoid cells (56). CLPs can develop into all lymphoid cell progenitor subsets including α-lymphoid progenitors (αLP), early innate lymphoid progenitors (EILPs), and the common helper innate lymphoid precursors (CHILPs) (57, 58). αLP and EILPs can develop into all of the known ILC subsets including ILCs 1, 2 and 3 and conventional NK cells (57, 58). These cells are also known as global innate lymphoid progenitors (GILPs). The more restricted precursors, $Id2^+$ common helper innate lymphoid precursor (CHILPs), can generate all helper ILC subsets (ILC1, 2, 3 and LTi) but not NK cells (4). LTi cells arise from $PLZF^-$ precursors while the rest of the helper ILCs 1, 2, and 3 arise from $PLZF^+$ innate lymphoid precursors (5). However, it is important to note that a hierarchical model of development is prone to revision based on new studies. The developmental stages and potentials of the various ILC precursors are more nuanced and complex, and can change depending on the organism, age, sex, inflammation, and the tissues examined (55).

When helper ILCs were discovered as a new subset of immune cells and reported to play important roles in immunity, they were described as $IL\text{-}7R\alpha^+$ cells. Aside from being an important defining marker for a major subset of ILCs, IL-7Rα is important in mediating IL-7 and TSLP signaling in these cells to promote their development and function.

IL-7Rα IN THE DEVELOPMENT OF ILCs

IL-7 and ILC Development

IL-7 is indispensable for the development of all helper ILCs. The role of IL-7 in ILC development was initially discovered in LTi cells. It is now well-established that IL-7 is important for LTi cell development and therefore the architecture of secondary lymphoid organs (59–61) [reviewed in (62).] The more recent discovery of the helper-ILC groups extended the importance of IL-7 to the development of other ILCs. It was first reported that "natural helper" cells (now called ILC2s) associated with the adipose tissue depend on IL-7 to survive and maintain their numbers in the tissue (6). Despite these findings, we have been unaware of how IL-7 instructs the development of ILCs. The recent unveiling of the heterogeneity in ILC precursors and their transcription factor dependency has now led to a better understanding of the factors that mediate the development of ILCs. Nuclear factor IL-3 (NFIL3) is a transcription factor required for NK cell development, and it was more recently found to be critical for the development of all other ILCs (4, 58, 63–66). The expression of NFIL3 in CLPs requires IL-7, which directs STAT5 activation and binding of pSTAT5 to the NFIL3 promoter (63). NFIL3 expression is specifically required for Id2 expression and generation of CHILPs, and hence the development of all helper-ILCs (63). While it is better established that IL-7 controls pan helper-ILC development through control of a common precursor from CLPs to CHILPs, it is less clear how IL-7 controls development of more committed $PLZF^+$ ILC precursors (ILCPs) from CHILPs. It is known that expression of GATA3 at the CHILP stage is required for the development of ILCPs that give rise to the majority of the ILC lineage (67). Since GATA3 is downstream of STAT5, it is possible that IL-7 signaling is important if not indispensable in the development of ILCPs and their ILC2 progenies through related pathways

(68, 69). In addition to inducing GATA3 in ILC2s, a recent studies have found STAT5 to be a major regulator of ILC homeostasis by regulating multiple networks ranging from survival factors such as Bcl-2 and transcription factors such as T-bet, RORγt, and Sall3 which each play a role in the function and differentiation of various ILC subsets (70, 71) (**Figure 2**).

In addition to the BM, IL-7 is produced in the fetal liver where earliest progenitors of ILCs can be found (72). Moreover, IL-7 is known to orchestrate the development of lymphoid cells in this tissue (73). IL-7 is also abundant in the thymus where T cells develop from early thymic progenitors (ETPs). Increasing evidence suggests thymic development of ILCs, however, it is unclear to what degree the thymus serves as a source of ILCs and whether ILCs and T cells share the same thymic progenitors. Early studies describe a mouse thymic NK cell subset that expressed the IL-7Rα depends on IL-7 for their development and has the capability to seed peripheral tissues (47). These murine thymic NK cells also shared similar characteristics to human $CD56^{+}$ NK cells (47). More recent studies have shown E-proteins E2A and HEB to be specifiers of T cell commitment in thymic precursors and their deletion leads to a skew toward ILC2 development, suggesting that thymocytes explore multiple fates before commitment (74, 75). Furthermore, IL-7Rα expression is inversely correlated with the expression of E-proteins in early ILC progenitors in the BM but correlates directly with that of Id2. This suggests an increased dependence on IL-7 for ILC

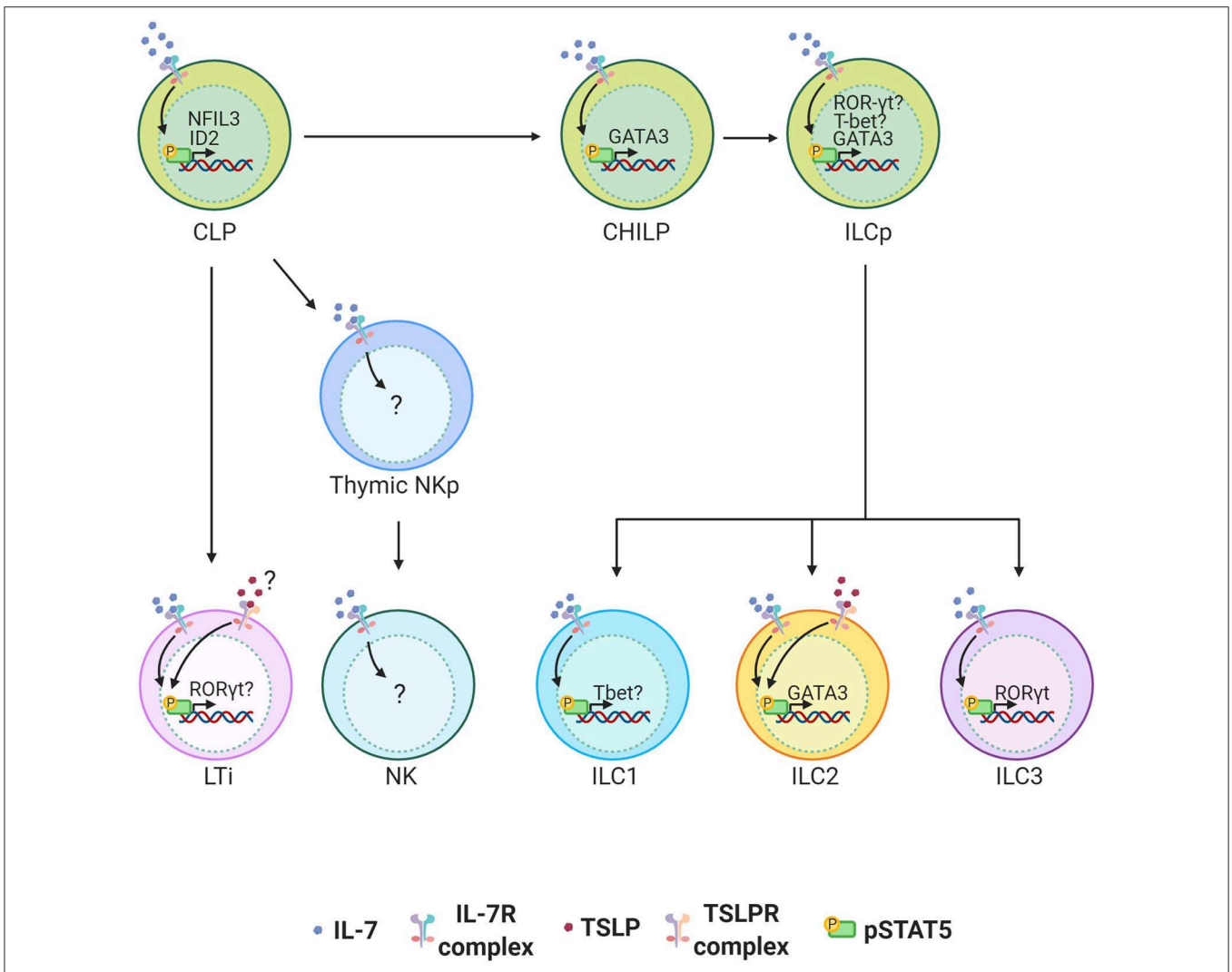

FIGURE 2 | ILC development in the bone marrow (BM) is highly dependent on IL-7 signaling. IL-7 engagement with the IL-7 Receptor complex activates STAT5. TSLPR expression on BM ILC precursors is not clear and its functional role in the development of ILCs is minimal. STAT5 activation by IL-7 induces NFIL3 in common lymphoid progenitors (CLPs) in the BM of adult mice and this is required for the expression of ID2 and generation of all helper ILCs. STAT5 is also important for the induction of GATA3 which can play an important role in the development of all ILCs at any stage between common helper innate lymphoid precursors (CHILP) and innate lymphoid precursors (ILCPs) to group 2 innate lymphoid cells (ILC2s). In ILCs STAT5 also induces RORγt and Tbet. The extent to which this plays role in terminal differentiation of ILCP to ILC 1, 2 and 3 is not clear. While IL-7 is critical for the development of LTi cells and thus lymph nodes, TSLP can partially compensate for the loss of IL-7.

development. To better understand the role of IL-7 in thymic ILC development, it is important that we elucidate its downstream signaling in various ILC precursors in the BM and thymus.

IL-7 has recently been found at the center of ILC differentiation. For instance, the quantity of IL-7 that is available combined with the strength and duration of Notch signaling can dictate the fate of mouse fetal liver derived CLPs (76). Whereas, high IL-7 and medium Notch signaling favors ILC2 and ILC1/NK cell differentiation, low IL-7 and high Notch signaling favors T cell differentiation in mice (76). The results are however different when human hematopoietic progenitor cells are treated in a similar manner, where IL-7, and Notch signaling induce ILC3 differentiation while suppressing IL-15 induced NK cell differentiation (77). These findings reveal contrasting roles for IL-7 in ILC differentiation which can be explained by differences in mouse and human hematopoiesis and progenitor sources. Further studies are needed to identify other contributing factors that create divergent roles for IL-7.

While IL-7 is clearly crucial to the development of ILCs, other cytokines can partially compensate for IL-7 or drive IL-7-independent maintenance of ILCs. For instance, IL-15 (a γc cytokine) can partially complement IL-7's defects in ILCs in IL-7Rα$^{-/-}$ mice (46). This is based on a study that found residual ILCs in IL-7Rα$^{-/-}$ mice express the IL-15Rβ and respond variably to IL-15 *in vitro* through increased survival depending on the type of ILCs (46). Further assessment of IL-15$^{-/-}$, IL-7Rα$^{-/-}$, and IL-7Rα$^{-/-}$ IL-15$^{-/-}$ mice revealed IL-7-independent sustenance of ILCs by IL-15 that varied in degrees depending on the type of ILCs and the tissue examined. Most notably, NK cells were normal in numbers in IL-7Rα$^{-/-}$ mice but greatly reduced in IL-15$^{-/-}$ mice as expected (46). However, IL-7Rα$^{-/-}$ IL-15$^{-/-}$ mice had greater loss of NK cells compared to IL-15$^{-/-}$ mice, more so in the colon than the siLP. Consistent with this is the higher expression of IL-7Rα observed by colon NK cells compared to siLP NK cells. This suggests a supportive role for IL-7 in NK cell development in a tissue specific manner. ILC1s were only marginally reduced in IL-7Rα$^{-/-}$ and IL-15$^{-/-}$ mice compared to WT mice, while IL-7Rα$^{-/-}$ IL-15$^{-/-}$ mice experienced a multi-fold reduction in ILC1s in the colon and the siLP, suggesting a synergistic contribution by both cytokines in development/maintenance of ILC1s (46). While IL-15$^{-/-}$ mice have normal number of NKp46^{+} and CCR6^{+} ILC3s in the siLP and colon, loss of both IL-7 and IL-15 signaling results in even greater loss of these cells compared to IL-7Rα$^{-/-}$ mice. Similarly, IL-7Rα$^{-/-}$ IL-15$^{-/-}$ mice had greater reduction in number of ILC2s in the siLP and colon than IL-7Rα$^{-/-}$ mice. However, IL-7Rα$^{-/-}$ and IL-7Rα$^{-/-}$ IL-15$^{-/-}$ mice have equal numbers of ILC2Ps suggesting a supportive role for IL-15 in survival of ILC2s in the periphery (46). The subset of ILC2s most affected in IL-7Rα$^{-/-}$ mice were ST2^{+} KLRG1^{+} ILC2s while ST2^{-} ILC2s were unaffected. The functional importance of these residual ILC2s has yet to be determined. Since they lack ST2 (IL-33 receptor) and are IL-7Rα deficient, they are non-responsive to IL-33, IL-7, and TSLP—the most potent activators of ILC2s—what are the cytokines that activate these cells? Common gamma chain cytokines including IL-7 and IL-15 use the γc receptor which relies on Jak3 to transmit signals. Loss or mutation of this receptor leads to loss of multiple ILC subsets in mice and inhibition of Jak3 using tofacitinib abrogates human ILC1 and 3 proliferation and development *in vitro* (78). Investigating downstream signaling factors can help identify overlapping pathways that are necessary for the development of ILCs.

FLT3 ligand (FLT3L) can also compensate for IL-7 in ILC development (79). IL-7$^{-/-}$ mice present with normal numbers of NK cells in the small intestine but have reduced ILC2s and ILC3s. Loss of FLT3L depletes all ILCs including NK cells suggesting a role for FLT3 that is earlier than that of IL-7 in ILC development (79). Treatment with rFLT3L for 10 days can restore all ILC populations in of IL-7$^{-/-}$ mice except for ILC2s which suggests either a greater dependence on IL-7 by ILC2s or that FLT3L mediated rescue of ILCs occurs at later stages of ILC development, perhaps after commitment to specific groups.

TSLP and ILC Development

The influence of TSLP in the development of ILCs is minimal, as previous reports using TSLPR$^{-/-}$ mice have shown normal numbers of ILC2s in the lungs (80). This is not surprising since its effect in lymphopoiesis is insubstantial as well. Although loss of TSLP signaling has little effect on lymphopoiesis, addition of TSLP to *in vitro* cultures can enhance mouse and human B cell as well as mouse T cell expansion from hematopoietic progenitors sourced from fetal liver (37, 81–83). It is however, unclear if ILC progenitors express the TSLPR and if significant levels of TSLP are produced in the fetal liver and bone marrow.

Both IL-7 and TSLP use the IL-7Rα but the increased importance that IL-7 has compared to TSLP in lymphoid development may stem from the increased binding affinity of IL-7 to its receptors, or more likely due to the established importance of γc/Jak3 in ILC development. Understanding how well TSLPR and/or Jak2 facilitate ILC development will be important to make a definitive statement.

Most studies of TSLP are in the context of ILC2s since no other ILCs have been reported to express the receptor for TSLP. Nonetheless, ILC research is in its preliminary stages and identification of the transcriptional dependencies of the various ILC subsets is in progress. It is possible that TSLP can mediate aspects of ILC development through unidentified pathways that are possibly masked by our current method of grouping ILCs. Supporting this hypothesis, over expression of TSLP has been shown to support the development of LTis in a compensatory manner in IL-7-deficient mice (61) (**Figure 2**). It is unknown whether these effects by TSLP are direct or indirect. Since the manipulation of TSLP and/or IL-7 signaling is integral for drugs that treat several conditions including allergies, cancers and infectious diseases, it is important that we have a better understanding of the interplay between the two cytokines in ILC development to design more efficient drug treatments (18, 84).

IL-7Rα IN ILC HOMEOSTASIS AND FUNCTION

IL-7 and ILC Function

While studies on IL-7's developmental roles in ILCs are substantial, research in its effector functions are relatively modest. Indeed, IL-7 was considered an important factor for the

development of T cells long before its role in effector functions was examined. IL-7 is mainly produced in primary lymphoid tissues such as the bone marrow and thymic stromal cells where immune cell development occurs (26, 85). However, this cytokine is also produced in secondary lymphoid organs and can be induced in skin, lung, intestinal epithelial cells, and liver as shown by fluorescence microscopy of IL-7 reporter mice and ELISA (28, 29, 32, 85, 86). The extent of our knowledge on IL-7 and ILC function is based on a series of *in vitro* experiments.

IL-7 alone or with IL-33 can stimulate the production of Th2 cytokines, IL-5 and IL-13, from murine ILC2s (6, 48, 50, 87) (**Figure 3**). These Th2 cytokines are important factors produced by ILC2s that promote helminth expulsion, antiviral effects, and tissue repair. A recent study showed that mice lacking T-bet had increased number of ILC2s and production of IL-5 and IL-13 leading to enhanced worm clearance during a *Trichinella spiralis* infection (88). This activity in ILC2s correlated with higher expression of IL-7Rα leading to increased activation of STAT5 (88). This suggests that T-bet is a regulator of IL-7Rα expression, and that IL-7 may enhance ILC2 function (88). This necessitates further *in vivo* examination of the role of IL-7 in ILC2 function.

RORγt$^+$ ILC3 derived IL-22 plays an important role in defense and regulation of pathogenic and non-pathogenic bacteria in the intestine by maintaining barrier integrity and inducing anti-microbial peptide expression by epithelial cells such as RegIIIγ and RegIIIβ (89–91). RORγt$^+$ ILC3s can however lose these abilities and differentiate into RORγt$^-$ cells through stimulation with IL-12 and IL-15 (92). This leads to their conversion from IL-22 producing ILC3s to IFN-γ producing ILC1-like cells. Together with the microbiota, IL-7 is able to counteract this transition by stabilizing RORγt expression (92) (**Figure 4**). IL-23 is the key cytokine responsible for RORγt-mediated IL-22 production by ILC3s (93). This finding suggests that IL-7 may in part be important for maintaining the IL-22 production status in ILC3s, which is pivotal for host defense and barrier integrity during bacterial infection. Indeed, IL-7 stimulation can induce RORγt expression and play a supportive role in IL-23 mediated IL-22 production in ILC3s (94). Furthermore, *in vitro* co-culture of IL-7 producing mesenchymal stromal cells (MSCs) with ILC3s led to IL-22 production and enhanced IL-2 induced proliferation of ILC3s (95). This was due to IL-7 derived from MSCs as measured by ELISA (95).

IL-7, together with retinoic acid (RA), is also important for homing of ILCs to gut-associated tissues by upregulating ILC3-intrinsic expression of α4β7 and selectin ligands, an effect mediated by IL-7 in T cell homing as well (96, 97) (**Figure 4**). Interestingly, IL-7 is also required for survival of ILC3s in the LN post-development, which in turn is important for homing naïve T cells to lymphoid tissues (98). It should be noted however, that while the RA/IL-7 axis has a positive effect on ILC3s, treatment with RA reduces IL-7Rα expression in ILC2s and ILC2Ps and this leads to reduced numbers of ILC2s possibly through reduced survival, homing or development (99) (**Figure 4**). Taken together, IL-7 plays diverse roles in dictating ILC function but new approaches are necessary to clarify them and clearly distinguish them from IL-7's developmental roles.

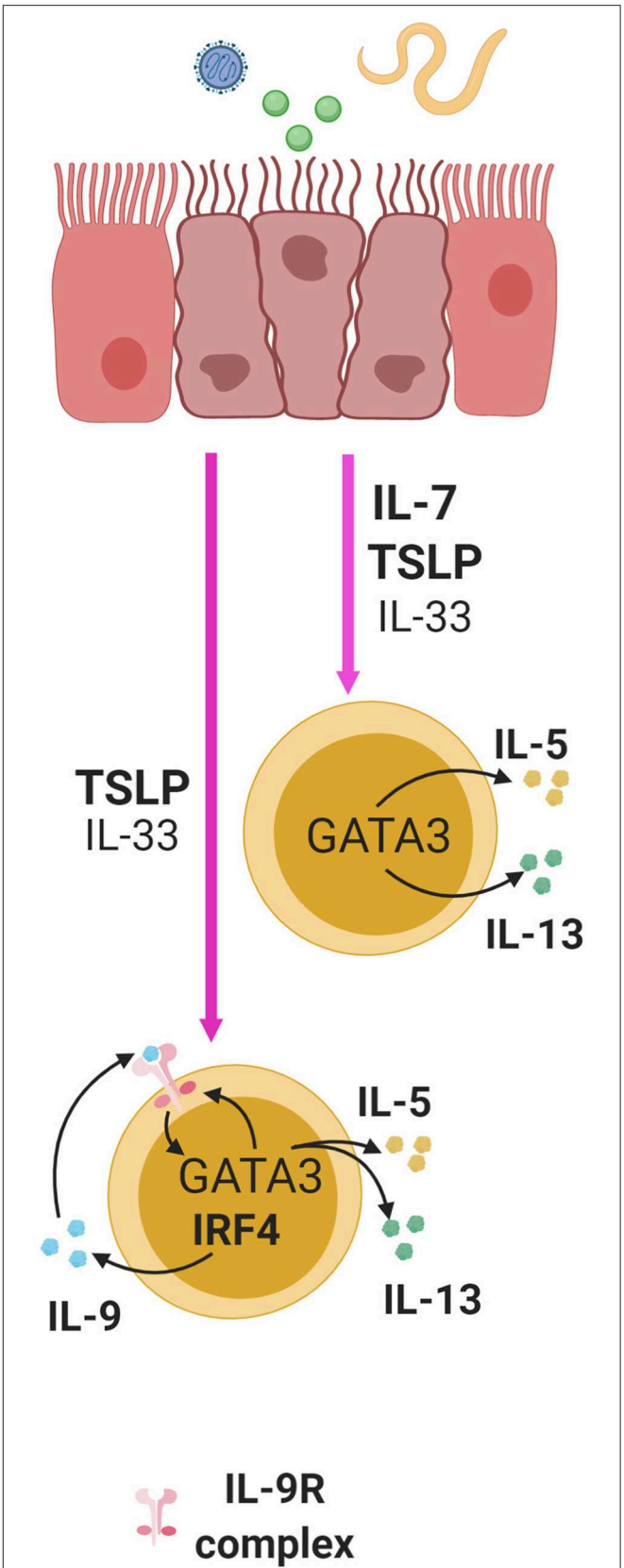

FIGURE 3 | Continued

FIGURE 3 | ILC2s in the lung, small intestine, and skin are activated by alarmins following exposure to virus, helminthes, or allergens and this induces cytokine production in ILC2s. IL-7 and TSLP significantly enhance the effect of IL-33 in multiple mucosal sites. TSLP and IL-33 synergistically induce the expression of IL-9 in an IRF4 dependent manner in ILC2s. Through autocrine signaling, this can enhance the production of IL-5 and IL-13 and IL-9R in a GATA3 dependent manner, thus creating a positive feedback loop to further enhance cytokine production.

TSLP in ILC2 Function

TSLP, along with IL-33 and IL-25, are produced by mucosal epithelial cells, and as alarmins, these cytokines are important activators of immune responses. Stimulation of lung epithelial cells with virus, allergens or helminths can lead to enhanced production of TSLP, IL-33 and IL-25, and stimulation of lung ILC2s with TSLP alone or in combination with IL-33 can induce IL-4, IL-5 and IL-13 secretion (48–50, 80). Interestingly, while IL-33 alone is able to induce such secretion *in vitro*, adding small doses of TSLP to the stimulation cocktail is sufficient to significantly enhance the secretory program, indicating its potency (50) (**Figure 3**). Furthermore, TSLP is able to mediate skin inflammation through ILC2s in mice independent of IL-33 and IL-25 (100). In summary, TSLP can greatly enhance ILC2 responses with or without help from IL-33 and IL-25.

The TSLP-GATA3 Axis in ILC2 Function and Homeostasis

TSLP can activate multiple signaling pathways in T cells and ILC2s alike. In T cells, TSLP as well as IL-7, are able to activate STAT5 signaling which activates pro-survival signals mediated by Bcl-2 (40). Similarly, ILC2s respond to TSLP by activating STAT5, and this triggers IL-13 production (68). The transcription factor GATA3 is indispensable for the development of all ILCs, an important identifying marker for ILC2s, and a direct target of STAT5 (67, 68). In addition, TSLP can induce GATA3 in ILC2s which in turn mediates IL-4, IL-5 and IL-13 production *in vitro* in these cells, and silencing GATA3 alone greatly reduces this TSLP response (68). This signifies the importance of GATA3 for TSLP-mediated ILC2 function. Interestingly, GATA3 induction can lead to enhanced expression of TSLPR and IL-33 receptor (ST2) which suggests that TSLP signaling through GATA3 can enhance responsiveness to IL-33 and TSLP (68). This model is consistent with studies that have suggested TSLP and IL-33 having synergistic effects on ILC2s (50). Moreover, GATA3 can directly bind to exon 2 of the gene encoding IL-7Rα and induce its expression thus enhancing IL-7 and TSLP signaling (101). Altogether, assuming linearity, this suggests a positive feedback loop whereby TSLP and GATA3 signaling rely on each other to amplify early innate responses by relatively rare cells in an environment with limited cytokine availability.

TSLP in Support of IL-9 Programming of ILC2 Function

ILC2s can produce IL-9 in response to TSLP in a manner dependent on the transcription factor interferon regulatory factor 4 (IRF4) (50). IL-9 derived from ILC2s can also serve to enhance early ILC2 responses by signaling in an autocrine fashion to increase IL-5 and IL-13 production during helminth infection in mice with *Nippostrongylus brasiliensis* (**Figure 3**). This subsequently promotes the expression of genes important for mucus production and tissue repair in lung epithelial cells (50, 102). Additionally, IL-9 receptor expression has been found to be positively regulated by GATA3 through RNA-sequencing transcriptomic analysis of ILC2s (67). Since TSLP can induce GATA3 expression in ILC2s, this suggests that TSLP may shape ILC2 responses through control of IL-9 receptor and ligand expression (68) (**Figure 3**). This further demonstrates that TSLP can act through multiple pathways in regulating ILC2 function.

The Dark Side: TSLP Duality

Notwithstanding its importance at barrier sites, TSLP is well-established as an inducer of Th2 cytokine driven allergic inflammation such as atopic dermatitis, eosinophilic esophagitis, asthma and allergic rhinitis, all mediated by a variety of cells including eosinophils, basophils, and ILC2s (16, 48, 100, 103, 104). TSLP supports the production of IL-5 and IL-13 by lung ILC2s, which contributes to eosinophilia and elevated mucus production in papain and chitin models of allergy induction (48, 50). Corticosteroids are a common treatment for asthma, but TSLP can enhance the survival and proliferation of IL-13$^+$ ILC2s through STAT5 activation, thus limiting therapeutic effectiveness (105–107). Similarly, respiratory syncytial virus (RSV) infection in mice induces airway hyper responsiveness (AHR) and airway obstruction, characterized by enhanced ILC2 proliferation and production of IL-13, and this effect is significantly reduced in TSLPR-deficient mice (49). Interestingly, TSLPR protein expression can be upregulated in ILC2s early during RSV infection. Proliferating ILC2s that produce IL-13 (but not IL-5) had higher expression of TSLPR mRNA and protein. (49). This differential expression by subpopulations of ILC2s may explain how TSLP can have both beneficial and detrimental effects on ILC2s. Nonetheless, comparative analysis of AHR, allergy, and infection models is necessary for a definitive statement.

There are other circumstances that can lead to duality in TSLP's effect on ILC2s or other TSLPR expressing cells. Relevant clinical studies have implicated the over-production of TSLP, either due to mutation(s) or constant exposure to allergen(s), as the main culprit in TSLP-mediated allergic inflammation (108, 109). Another model suggests that TSLP may have diverse roles due to the presence of two transcript variants for TSLP producing a long and short isoforms (110, 111). The short isoform of TSLP is expressed constitutively during homeostasis and is important for anti-inflammatory, barrier integrity and anti-microbial responses, while the long isoform is expressed during inflammation and supports inflammatory cytokine production (110, 111). Lastly, it is possible that the combined effects of other cytokines in the environment with TSLP can influence the outcomes, and these varying compositions may define a certain threshold. One or more of these scenarios may occur simultaneously making

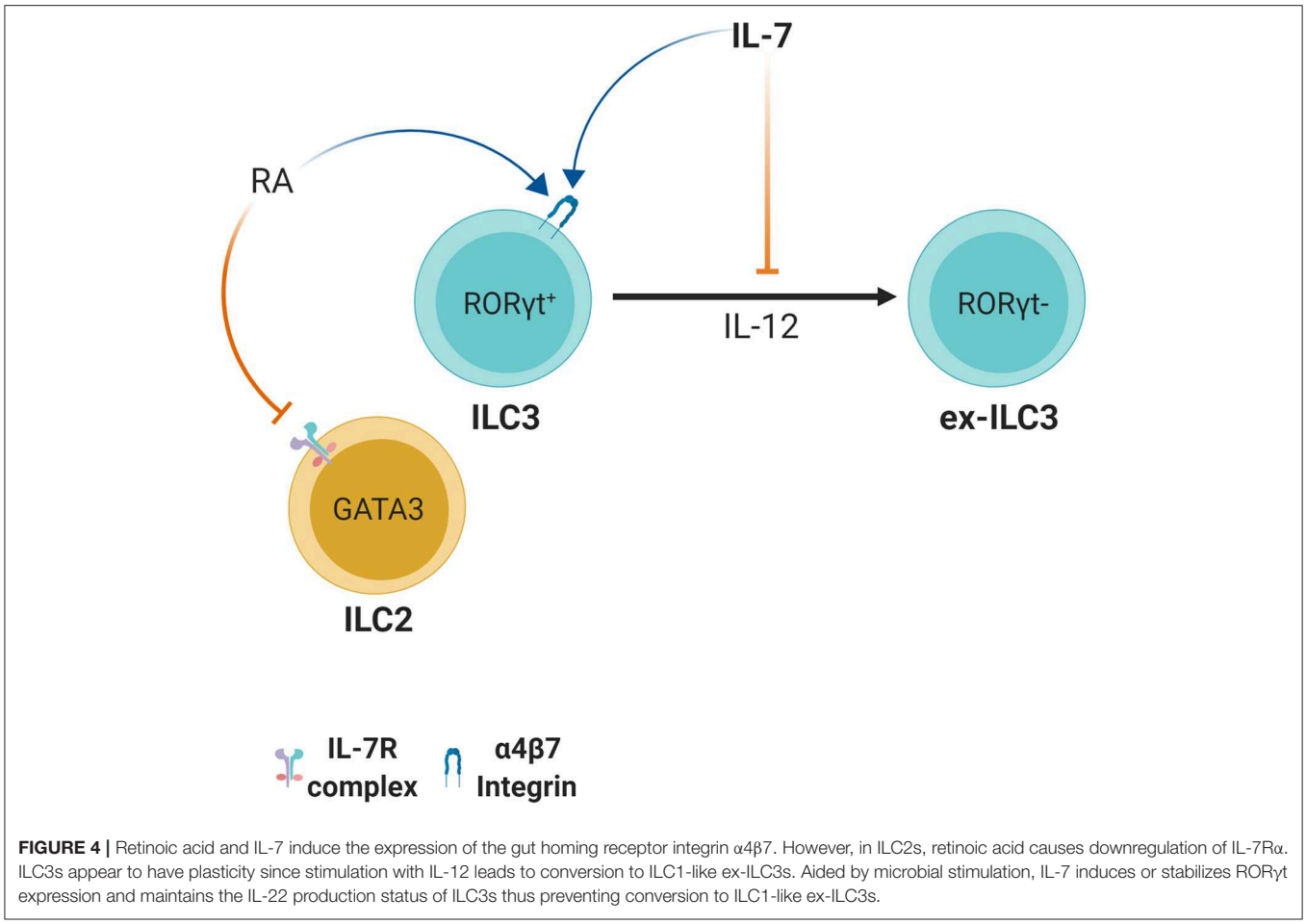

FIGURE 4 | Retinoic acid and IL-7 induce the expression of the gut homing receptor integrin α4β7. However, in ILC2s, retinoic acid causes downregulation of IL-7Rα. ILC3s appear to have plasticity since stimulation with IL-12 leads to conversion to ILC1-like ex-ILC3s. Aided by microbial stimulation, IL-7 induces or stabilizes RORγt expression and maintains the IL-22 production status of ILC3s thus preventing conversion to ILC1-like ex-ILC3s.

multi-faceted approaches a preferred route of treatment for allergic inflammations.

The negative effects of TSLP have been noted in ILC3s as well. In a report, loss of IKKα in murine intestinal epithelial cells (IECs) led to an overproduction of TSLP during *Citrobacter rodentium* infection (112). This resulted in reduced IL-22 production by ILC3s, impaired bacteria clearance and increased mortality. *In vivo* blockade of TSLP was sufficient to restore anti-bacterial immunity. The inhibitory effect of TSLP on ILC3 function was confirmed in *in vitro* experiments. Addition of TSLP impaired the ability of IL-23 to stimulate IL-22 production from ILC3s in bulk splenocyte cultures, however this phenomenon was not seen with sort purified ILCs, suggesting that TSLP acted indirectly. This finding is unprecedented and warrants further investigation to clearly map the connection between TSLP and ILC3s, and provide more insight into their implications in mucosal and barrier health.

CONCLUDING REMARKS

IL-7Rα is a cytokine receptor whose expression is tightly regulated throughout the development and life of lymphoid cells. IL-7 and TSLP signal through IL-7Rα and play multiple roles in determining the fate of ILCs and T cells. Since their discovery, research on ILCs have led to great insights in mucosal immunology and lymphoid development. Their resemblance to adaptive lymphoid cells has enabled us to study their biology more efficiently. Despite the current progress, we have yet to fill significant gaps of knowledge in ILC development and function. It is still unclear when commitment to the ILC fate occurs during hematopoiesis and how IL-7 controls this program. The development of ILCs *in vivo* was found to rely on key transcription factors such as NFIL3 whose activation is dependent on IL-7 signaling. However, without a complete picture of the source of ILCs, it is hard to pinpoint dependencies on any given single cytokine. In addition, recent studies have allowed us to better appreciate the complexity of hematopoiesis, and in doing so, the differences between murine and human lymphopoiesis. Although studies with mouse models have provided great insight in lymphopoiesis, we should be cautious in our interpretations. Recent advances in understanding ILC development can be credited to transcriptomic studies and single cell resolution analysis that have provided a complex view of ILC heterogeneity. Further studies utilizing similar methods can be conducted to identify ILC precursors in lymphoid and non-lymphoid tissues and examine the factors that regulate their development. Multiple studies that have shown a role for IL-7 and TSLP in ILC function have

used *in vitro* treatment with cytokines. While these studies have provided great insights on how these cytokines can influence ILCs, it is important to validate and extend these findings through various transgenic animal models to reveal any physiologically relevant and indispensable roles of IL-7 and TSLP in ILC biology.

AUTHOR CONTRIBUTIONS

AS reviewed the literature and wrote the manuscript, edited it and generated the figures. NA reviewed the drafts, provided critical input and edited the text and figures.

FUNDING

Work in the laboratory was supported by a grant to NA from the Canadian Institutes for Health Research (MOP-126060 and PJT-156119). AS was supported by the John Richard Turner Fellowship.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. Lisa Osborne, Julia Lu, and Etienne Melese at the University of British Columbia for their critical revision of this manuscript. All figures were made in ©BioRender - biorender.com.

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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.

frontiers in Immunology

REVIEW
published: 24 January 2020
doi: 10.3389/fimmu.2020.00042

Regulation of γδ T Cell Effector Diversification in the Thymus

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γδ T cells are the first T cell lineage to develop in the thymus and take up residence in a wide variety of tissues where they can provide fast, innate-like sources of effector cytokines for barrier defense. In contrast to conventional αβ T cells that egress the thymus as naïve cells, γδ T cells can be programmed for effector function during development in the thymus. Understanding the molecular mechanisms that determine γδ T cell effector fate is of great interest due to the wide-spread tissue distribution of γδ T cells and their roles in pathogen clearance, immunosurveillance, cancer, and autoimmune diseases. In this review, we will integrate the current understanding of the role of the T cell receptor, environmental signals, and transcription factor networks in controlling mouse innate-like γδ T cell effector commitment.

Keywords: γδ T cells, thymus, TCR signal strength, transcriptional regulation, innate-like lymphocyte, IL-17A, IFNγ

OPEN ACCESS

Edited by:
Arthur Mortha,
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Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *22 November 2019*
Accepted: *08 January 2020*
Published: *24 January 2020*

Citation:
Parker ME and Ciofani M (2020) Regulation of γδ T Cell Effector Diversification in the Thymus. Front. Immunol. 11:42. doi: 10.3389/fimmu.2020.00042

INTRODUCTION

γδ T cells are part of the three evolutionary conserved lymphocyte lineages (with αβ T cells and B cells) that undergo somatic gene rearrangement for the generation of antigen receptors (1). While immune cells can broadly be divided by adaptive vs. innate, γδ T cells straddle this classification by having properties of both. Although γδ T cells are capable of generating unique T cell receptors (TCRs), many γδ T cells express TCRs with limited diversity (2). Innate-like γδ T cells, also referred to as "natural" γδ T cells, are endowed with their effector functions early during development in the thymus and consequently do not require clonal expansion or differentiation from a naïve cell for their effector responses (3, 4). Importantly, innate-like γδ T cells exhibit the four hallmark characteristics of tissue-resident lymphocytes; (1) self-renewal and long-term maintenance, (2) enrichment at barrier tissues, (3) tissue sensing capabilities, and (4) rapid effector responses (5). These tissue-resident properties combined with early seeding during fetal life enable innate-like γδ T cells to act as a first line of defense in the skin, gut, and reproductive tract while other lymphocytes are still being developed.

γδ T cells play innumerable roles in pathogen clearance, wound healing, autoimmunity, and cancer, largely through the production of soluble mediators (6). The two major effector subsets of γδ T cells can be distinguished based on cytokine production: IFNγ producers (Tγδ1) and IL-17A producers (Tγδ17), although γδ T cells are capable of producing many other cytokines (6). IFNγ production by γδ T cells is associated with clearance of intracellular pathogens and anti-tumor responses, while IL-17A production is linked to clearance of extracellular bacteria and fungi (7, 8). Although protective against infectious diseases, cytokine production by γδ T cells is involved in many immune pathologies and autoimmune diseases when dysregulated (9). Remarkably, the presence of γδ T cells within tumors was found to be the most significant favorable cancer-wide prognostic population in humans (10). While enriched at mucosal and barrier tissues, γδ T cells are also present in many other non-lymphoid tissues where they support steady-state tissue

homeostasis (6, 11). Recent studies have shown that IL-17A production by γδ T cells regulates adipose tissue immune cell homeostasis and thermogenesis (12), bone regeneration (13), and the promotion of short-term memory in the brain meninges (14). As innate-like lymphocytes, γδ T cells sense their local environment and are regulated through a combination of the TCR, cytokine receptors, co-stimulatory receptors, inhibitory receptors, and natural killer receptors (15). These receptors recognize various environmental ligands or stimuli that induce signaling cascades that lead to expression of key transcription factors (TFs) that can then dictate the identity and effector function of γδ T cells. This review will focus on the integration of TCR and environmental cues with downstream TF modules that govern the effector fate of mouse innate-like γδ T cells.

γδ LINEAGE COMMITMENT IN THE THYMUS

In the thymus, double-negative $CD4^-$ $CD8^-$ (DN) thymocytes give rise to two distinct T cell lineages defined by the expression of either an αβTCR or a γδTCR (16). DN thymocytes are a heterogeneous group of developmentally linked progenitor cells distinguished by the expression of CD44, CD117 (also known as c-kit), and CD25 that encompass the transition of early thymocyte progenitor cells (ETP/DN1) through the DN2, DN3, and DN4 cell stages (16). Rearrangement of the TCRβ, TCRγ, and TCRδ gene loci begin in DN2 cells and are completed in DN3 cells (17), a time frame that coincides with the divergence of the αβ and γδ lineages (18, 19). Indeed, the DN3 stage represents an obligatory checkpoint at which productive rearrangement and expression of either a pre-TCR (TCRβ + invariant pTα) or γδTCR complex signals the rescue of cells from apoptosis, proliferation, and αβ or γδ lineage differentiation (17). β-selected cells undergo further development to the $CD4^+CD8^+$ double positive (DP) stage, where TCRα rearrangement and additional selection events yield mature $CD4^+$ or $CD8^+$ single positive αβ T cells (16, 20). Unlike αβ T cells, γδ T cells develop following a single γδ-selection step mediated by the γδTCR, do not progress through to a DP stage, and rather most γδ T cells remain DN instead (16).

Developing DN thymocytes integrate signals from the TCR complex expressed on their cell surface along with myriad environmental cues. As such, two models were proposed to explain αβ vs. γδ lineage choice: the signal strength model and the stochastic-selective (pre-commitment) model (16). The major difference between these models is the importance placed on TCR signaling and the timing of its influence. The pre-commitment model is founded on the idea that lineage fate is determined prior to rearrangement of TCR loci. The expression of γδTCR on γδ T cell precursors or pre-TCR on αβ precursors simply confirms their fate and cells pre-committed to one fate with a mismatched TCR were hypothesized to die. Initial studies supporting this model showed that DN thymocytes lacking TCR expression but expressing high levels of IL-7Rα (21) or the high mobility group (HMG) box TF Sox13 (22) were predisposed to becoming γδ T cells. However, more recent evidence that Sox13 is not required for the generation of all γδ T cells, but rather only for a select subset of IL-17-producing γδ T cells marked by Vγ4 usage (23) [Tonegawa nomenclature (24)], is at odds with the pre-commitment model.

In contrast, the signal strength model of αβ vs. γδ lineage commitment has garnered widespread support. It posits that the strength of TCR signal that DN thymocytes receive dictates the lineage decision; weak signals promote αβ fate, while strong signals promote the γδ fate. The extensive evidence in favor of this model has been previously reviewed in detail (16, 25). Most notably, key support was provided by elegant experiments demonstrating that a single γδTCR transgene can mediate both γδ and αβ lineage fates, dependent on the signal strength of the TCR (26, 27). In particular, lineage fate toggled between αβ and γδ outcomes when TCR signal strength was tuned by genetic alterations in TCR ligand availability, TCR surface expression levels, or in expression of TCR signaling factors (26, 27). Enhanced or prolonged activation of the extracellular signal-regulated kinase (ERK) pathway and downstream Egr, and Id3 targets are important mediators of strong γδTCR signals that promote γδ lineage commitment (25, 26, 28). More recent work has begun to shed light on the mechanism by which DN cells translate differences in signal strength and ERK signaling into alternative lineage fates. γδ T cell development is dependent on a non-canonical mode of ERK action mediated by its DEF-binding pocket (29). This domain is favored by strong and more prolonged signals and enables ERK to bind a distinct set of proteins required for γδ lineage adoption. Thus, strong signals mediated primarily by γδTCR complexes are required for DN cell commitment to the γδ T cell lineage.

EFFECTOR PROGRAMMING OF γδ T CELLS

Waves of γδ T Cell Development

A distinctive and poorly understood feature of γδ T cell ontogeny is the development of γδ thymocytes in a series of "waves" that are defined by γ-chain variable regions (Vγ) usage (**Table 1**). Interestingly, the waves of Vγ subsets are highly correlated with homing abilities to specific tissues early in life, where they become long-lived tissue-resident cells. This process begins when the fetal thymus is seeded as early as embryonic day 13.5 (E13.5) by fetal liver progenitors to generate the first wave of γδ T cells, known as $Vγ5^+Vδ1^+$ dendritic epidermal T cells (DETCs) that exclusively home to the epidermis of the skin (30). The second wave of γδ T cells, expressing an invariant Vγ6Vδ1 TCR, develop around E16 and primarily seed epithelial layers of the female reproductive tract, lung, and tongue (31). Next, the late fetal stages give rise to $Vγ4^+$ and $Vγ1^+$ γδ T cells that express more varied TCRs due to pairing with several Vδ chains and can be found in many tissues such as peripheral lymphoid organs, blood, lung, liver, and dermis (2, 31). Unlike $Vγ5^+$ and $Vγ6^+$ γδ T cells, these subsets are not restricted to the fetal window and can also develop during neonatal and adult life (2, 31). Of note, the $Vγ7^+$ γδ T cells that reside in the intraepithelial layer of the small intestine are thought to mature extrathymically (2, 32). While the link between

TABLE 1 | Waves of γδ T cell development.

Subset	V(D)J diversity	Timing of development	Tissue residence	Major cytokines produced
Vγ1	High (NKT γδ T cells = $Vγ1^+Vδ6.3^+$)	Perinatal and adult	Liver, lymphoid tissues	IFNγ (IFNγ and IL-4)
Vγ4	Variable	E18 to adult	Dermis, lung, liver, lymphoid tissue	IL-17A or IFNγ
Vγ5	Invariant ($Vγ5^+Vδ1^+$)	E13-E16	Epidermis	IFNγ
Vγ6	Invariant ($Vγ6^+Vδ1^+$)	E16-birth	Uterus, lung, tongue, liver, placenta, kidney	IL-17A
Vγ7	Intermediate	Neonatal	Epithelial layer of small intestine	IFNγ

E, embryonic day.

Vγ usage and tissue homing can be explained in DETCs with upregulation of CCR10 in the thymus before trafficking to the epidermis (33, 34), this association is not yet understood for other Vγ subsets. Moreover, the molecular mechanisms governing the unique sequential development of Vγ subsets are unknown, however features of both the fetal progenitors and environment have been implicated (35–38).

Effector Diversification of γδ Thymocytes

In contrast to αβ T cells that leave the thymus as naïve cells and acquire their effector function in the periphery, γδ T cells can commit to an effector fate during development in the thymus. The pre-programming in the thymus allows γδ T cells to be early innate-like responders to infection and tissue-damage, without the delay that is required for αβ T cell responses. While this review focuses on "pre-programmed" innate-like or "natural" γδ T cells, some γδ T cells exit the thymus as naïve cells and acquire effector function following activation in the periphery; these are referred to as "inducible" γδ T cells (4, 39). Similar to αβ T cells, innate lymphoid cells (ILCs), and other lymphocyte lineages, γδ T cells can be divided into effector subsets based on the expression of either T-bet/IFNγ (Tγδ1) or RORγt/IL-17A (Tγδ17). During ontogeny, effector γδ T cell subsets differentiate in functional waves encompassing DETCs, IL-17A producers, and NKT γδ T cells, which are also partially associated with Vγ usage (40). Specifically, $Vγ5^+$ DETCs preferentially produce IFNγ, while $Vγ6^+$ γδ T cells mainly produce IL-17A (41). Later waves, such as Vγ4 and Vγ1, are more heterogenous in their capacity to produce various effector cytokines. While IL-17A production is not limited to a specific Vγ subset, innate-like Tγδ17 cell generation is restricted to a window of time during fetal life, approximately E16 to birth, that enriches for $Vγ6^+$ and $Vγ4^+$ γδ T cell subsets (42). Within the third functional wave, $Vγ1^+Vδ6.3^+$ NKT γδ T cells express PLZF and are capable of producing both IL-4 and IFNγ (43, 44). Therefore, the fate decisions of developing thymocytes during fetal life impacts the adult reservoir of innate-like γδ T cell effectors.

γδ T cell effectors can be defined by various cell surface markers: IFNγ producing γδ T cells typically express CD27, CD122, NK1.1, and high levels of CD45RB, while IL-17A producing γδ T cells lack expression of CD27, CD122, and NK1.1 but usually express CCR6 and low levels of CD45RB (41, 45, 46) (**Figure 1**). Nevertheless, the study of γδ effector diversification has been hampered by the lack of definitive markers that distinguish Tγδ1 and Tγδ17 precursors. Before effector commitment, CD25 is expressed by the earliest γδ T cells in the thymus (47), as γδ-selected thymocytes are derived from $CD25^+$ DN2 and DN3 T cell precursors (18, 48). Post-selection γδ thymocytes are also distinguished by CD27 upregulation (48), and these $CD25^+CD27^+$ are the earliest progenitors of IL-17A and IFNγ γδ effectors (46). Emerging γδ thymocytes with low levels of γδTCR also express intermediate levels of CD45RB, and have molecular signatures and developmental potential consistent with being precursors to both Tγδ17 and Tγδ1 cells (41, 49). Indicative of their immature status, these pioneer γδ T cells are marked by high levels of CD24 expression, which is later downregulated upon maturation (50).

Several recent studies have provided clarity regarding the developmental trajectories of innate-like γδ T cell effector subsets beyond the precursor stage (49, 51). Recent work by Sumaria and colleagues identified $CD45RB^-CD44^-$ γδ thymocytes as precursors of both type 1 and type 17 effectors, suggesting that all γδ T cells downregulate CD45RB prior to effector diversification (**Figure 1**) (52). Consistent with this view, the absolute block in Tγδ17 development in the absence of c-Maf revealed an effector specialization checkpoint at the immature $CD45RB^-CD24^+$ γδ thymocyte stage (49). This block also provides genetic support for a model in which effector programming is molecularly distinct from γδ-selection (3). Among mature $CD24^-$ γδ thymocytes, CD45RB and CD44 distinguish effector lineages: $CD44^{hi}CD45RB^{lo}$ γδ T express high levels of RORγt and IL-7Rα and are committed to IL-17A production, whereas $CD44^+CD45RB^+$ γδ T cells express T-bet, but lack RORγt or IL-7Rα expression and are committed to IFNγ production (**Figure 1**) (51). Additionally, CD73 expression, which is linked to strong ligand-dependent γδTCR signaling (53), is significantly more expressed on IFNγ-committed than IL-17A-committed γδ thymocytes (51), and $CD73^-$ γδ thymocytes are enriched for those undergoing type 17 differentiation in the perinatal thymus (54). Interestingly, although $CD24^+$ γδ thymocytes are considered "immature," they nonetheless express key TFs necessary for their effector acquisition, such as RORγt for Tγδ17 cells (49, 54, 55), and are surprisingly also functionally competent to produce IL-17A (51). The application of global single cell transcriptomic analysis to fetal γδ thymocytes is likely to add significant granularity to the developmental trajectories of effector programming [preprint (56)].

ROLE OF γδTCR

Similar to the role of TCR in αβ vs. γδ lineage choice, the γδTCR is important for determining the effector fate of γδ T cells. The current understanding supports a model with two sequential

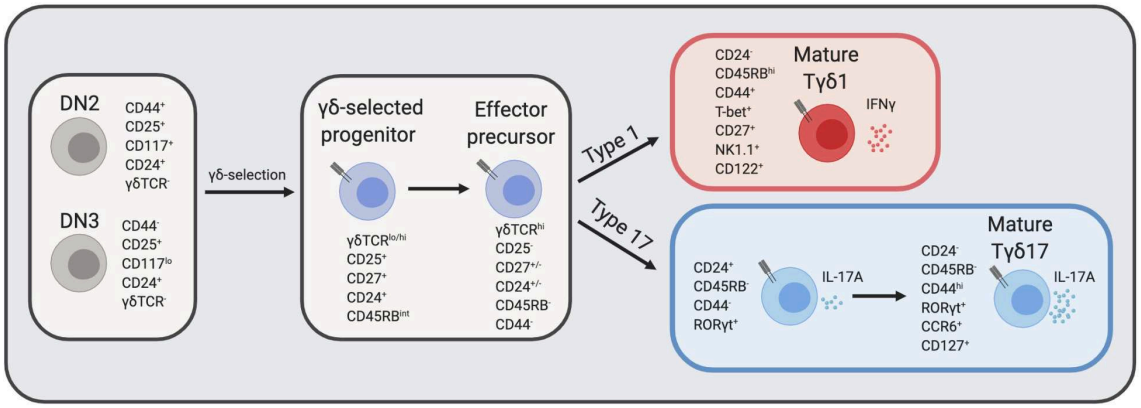

FIGURE 1 | γδ T cell development in the thymus. DN thymocytes undergo γδ-selection and become immature γδ thymocytes that eventually diverge into either IFNγ producers or IL-17A producers. The expression of cell surface markers and transcription factors that define transitional precursors and mature effector γδ T cells are listed next to each cell type. CD24 and CD27 expression at the "effector precursor" stage is heterogenous and is marked by +/–, however, cells transition from $CD24^+$ to $CD24^-$. DN, double negative; TCR, T cell receptor. Figure made with biorender.com.

steps in commitment; first, the decision of αβ vs. γδ, and second, the decision to become an IFNγ- or IL-17A-secreting γδ T cell (3). Both steps in development are dependent on TCR signal strength integrated with numerous environmental signals. The idea that thymic selection determines the effector fate of γδ T cells was first supported by the finding that γδ T cells exposed to a TCR ligand leading to a strong TCR signal become IFNγ producers, whereas the absence of ligand or weak γδTCR signal result in the IL-17A effector fate (57). Further supporting the notion that ligand-dependent strong γδTCR signals promote the type 1 fate, DETCs, known to produce IFNγ, adopt an IL-17A producing γδ T cell fate in the absence of their selecting ligand, Skint-1 (discussed further below) (41). Conversely, enhancing γδTCR signal strength through the addition of crosslinking γδTCR antibody GL3 to fetal thymic organ cultures (FTOC) significantly reduced the number of $CD44^{hi}CD45RB^-$ IL-17A-committed cells while increasing type 1-associated $CD44^+CD45RB^{hi}$ cells (51). A similar outcome was achieved when strong TCR signals were mimicked by transduction of T cell progenitors with a constitutively active form of the kinase Lck (Lck^{F505}) (49). Together, these studies suggest that the type 17 program is the default effector pathway that is otherwise repressed by strong or ligand-dependent TCR signals. Whether Tγδ17 development supported by weak TCR signaling is truly or universally ligand-independent remains to be determined.

γδ T cell effector fate choice is also influenced by specific TCR signal transduction pathways. For example, ERK signals support the type 1 program as ERK-deficient $TCR\beta^{-/-}$ mice have an increased frequency of $CD27^-$ γδ T cells, and ERK-deficient KN6 γδ TCR transgenic thymocytes are skewed toward IL-17A production compared to the controls that predominately produce IFNγ (29). More recently, it was revealed that the tyrosine kinase Syk is selectively required for Tγδ17 development, through activation of the PI3K/Akt pathway downstream of γδTCR signaling (58). Studies show that impairment of TCR signal strength with SKG [Zap70 mutant (59)] and CD3DH (CD3γ and CD3δ double heterozygous) mice both have reduced frequencies of IL-17A-producing $V\gamma6^+$ γδT cells (60, 61). Notably, the defect in Zap70 signaling impacts $V\gamma4^+$ Tγδ17s as well, just to a lesser extent, while the $V\gamma4^+$ γδT cells in the CD3DH mice are not impaired (60, 61). These findings imply that while we group Tγδ17s into one effector class, the Vγ subsets may require specific signal strengths and downstream signaling molecules for their effector programs. Taken together, these findings also support the model that IFNγ producing γδ T cells require strong TCR signals, while IL-17A producing γδ T cells generally require weaker TCR signal strength (41, 46, 51).

ENVIRONMENTAL CUES

Environmental cues in the thymus are derived from both thymic epithelial cells (TECs), developing thymocytes, and other hematopoietic cells. Timing is also a critical factor, as the developmental windows in which progenitors seed the thymus influence their exposure to signals integrated from both the stromal microenvironment and resident developing thymocytes. Therefore, γδ T cell effector specialization can be influenced by various environmental cues during ontogeny.

Lymphotoxin Signaling

One of the best-studied examples of such signals is a process called "trans-conditioning." This phenomenon was initially discovered in $TCR\beta^{-/-}$ mice that have an altered γδ T cell gene profile and significantly reduced secretion of IFNγ by splenic γδ T cells (62). The authors concluded that αβ T cells are required for the normal development of γδ T cells (62). Subsequent work identified lymphotoxin production by DP thymocytes as the mechanism, in part, responsible for the regulation of γδ T cell maturation and differentiation toward an IFNγ-producing fate (63). Mechanistically, this was extended with the finding that CD27, a tumor necrosis factor (TNF) receptor superfamily member, engages CD70 and positively upregulates

lymphotoxin beta receptor (LTBR) expression on γδ T cells (46). Accordingly, the function of CD27 in supporting IFNγ production coincides with its selective expression by mature Tγδ1 as compared to Tγδ17 cells (**Figures 1**, **2**) (46). The role of lymphotoxin signaling in γδ T cell effector commitment is complex as the thymic differentiation of IL-17A-producing γδ T cells is also dependent on this pathway (64). Indeed, by way of the lymphotoxin signaling pathway, the NF- κb family members, RelA and RelB, play distinct roles in the thymic preprogramming of Tγδ17 cells. RelA regulates lymphotoxin ligand expression in accessory thymocytes, thereby indirectly controlling IL-17A production by γδ T cells. On the other hand, γδ T cell precursors require RelB downstream of LTBR to maintain *Rorc* expression for differentiation into mature Tγδ17 cells (**Figure 2**) (64). Taken together, lymphotoxin signaling regulates the effector fate acquisition of γδ T cells through integration of γδ T cell-intrinsic and extrinsic pathways.

Cytokines and Notch Signaling

IL-7 is known for being a non-redundant, key regulator of lymphocyte homeostasis through promotion of survival and proliferation (65–68). The IL-7/IL-7R pathway plays essential roles at distinct stages in the development of multiple lymphocyte lineages (69). In particular, γδ T cells require IL-7Rα for their development, as IL-7R-deficient mice lack all γδ T cells (70). Follow-up work by several groups demonstrated that IL-7Rα-deficient mice have a block in V-J recombination of the TCRγ genes (71), and that IL-7R controls the accessibility of the TCRγ locus (72–74). While IL-7 signaling is required for all γδ T cell development, high levels of IL-7Rα expression and IL-7 signaling preferentially favor the differentiation of IL-17A-producing γδ T cells (75, 76). In line with this notion, *Aire*-deficient mice have increased production of IL-7 by medullary thymic epithelial cells (mTECs) that results in expanded populations of IL-17A-producing $V\gamma6^+V\delta1^+$ T cells in the thymus and the periphery (77). The IL-7 signaling pathway also integrates with additional environmental signals and transcriptional regulators, most notably, the Notch signaling pathway. The Notch target and transcriptional repressor, Hes1, is specifically expressed in IL-17A-producing γδ T cells and Hes1 ablation significantly decreases IL-17A production with no effect on IFNγ secretion in peripheral γδ T cells (**Figure 2**) (78). Notch also regulates Tγδ17 differentiation in a Hes1-independent, but RBPJκ-dependent manner (79). Mechanistically, Notch signaling and RBPJκ are required for IL-7Rα expression, and IL-7Rα-mediated signaling is indispensable for the homeostasis of $IL\text{-}17^+$ γδ T cells (**Figure 2**) (79). Future studies further exploring the transcriptional activators and repressors of *Il7r* will help elucidate how IL-7 signaling integrates with other environmental cues to control γδ T cell fate.

IL-17 is another interesting example of a soluble mediator produced in the thymus that regulates the development of γδ T cells. The development of innate-like Tγδ17 cells is restricted to a functional embryonic wave during fetal life from E16 to birth, resulting in long-lived, self-renewing cells that are found in adult mice (42). Surprisingly, it was found that IL-17 production in the thymus influences the development of Tγδ17 cells through a negative feedback loop such that $CCR6^+CD27^-$ Tγδ17 cell numbers are increased in *Il17af*$^{-/-}$ mice (mice with deletion of the entire *Il17a* and *Il17f* locus) compared to wild-type controls (42). Interestingly, IL-17-producing $Thy1^+$ cells resembling group 3 innate lymphoid cells (ILC3s) were found in the thymus of Rag1$^{-/-}$ mice (42). Therefore, the restriction of Tγδ17 cell development may be attributed to IL-17 production from both innate lymphoid cells and $IL\text{-}17^+$ αβ and γδ T cells (42).

TGF-β signaling has pleiotropic effects on immune cells. Among type 17 lineages, a specific role for TGF-β was first defined for the differentiation of naïve $CD4^+$ T cells into Th17 cells. Specifically, TGF-β1$^{-/-}$ mice have severely diminished Th17 cells in peripheral lymphoid organs (80). Despite major distinctions between Th17 cells and Tγδ17 cells, IL-17A-producing γδ T cells are also significantly reduced in mice deficient for either TGF-β1 or Smad3, the TGF-β signaling adaptor molecule, suggesting a similar dependence of TGF-β signaling for IL-17 production in the γδ lineage (81). However, this study was performed in neonates at a time point when innate-like Tγδ17 cells have left the thymus, therefore, the precise role of TGF-β signaling in Tγδ17 cell development is still unclear. In this regard, TGF-β may support Tγδ17 cells as a driver of Ras signaling (82), a signaling cascade that strongly promotes the type 17 program in γδ T cells (49).

Butyrophilins

Whether γδ T cells undergo thymic selection analogous to αβ T cells has been a major question in the field. In order to explain the domination of tissue-specific γδ T cell compartments by particular Vγ subsets, it was hypothesized that the same γδTCR-specific ligands expressed in both the fetal thymus and target tissues could mediate positive selection during ontogeny and thereafter, tissue localization and maintenance cues for long-term residence (83). FVB-Tac mice harboring a spontaneous mutation that selectively disrupts the DETC compartment was reported to map back to a single gene expressed by TECs and keratinocytes, representing the first support for the hypothesis that DETCs undergo positive selection in the thymus (84). A few years later, the phenotype of FVB-Tac mice was attributed to a mutation in the *Skint1* gene (85). *Skint1* is a member of the butyrophilin-like (Btnl) family that structurally resembles the B7 superfamily molecules CD80 and PD-L1 (86–88). *Skint* gene expression is restricted to the thymus and skin, therefore, the broader applicability of this mechanism of selection for other intraepithelial γδ T cells was questioned (85). Recently, expression of Btnl1 by villus epithelial cells in the small intestine was shown to mediate the extrathymic selection of $V\gamma7^+$ intraepithelial lymphocytes (IELs), driving their expansion and maturation (89). In particular, joint expression of Btnl1 and Btnl6 by intestinal epithelial cells regulates the TCR-dependent responses of $V\gamma7^+$ IELs (89). Importantly, human intestinal epithelium co-expressing BTNL3 and BTNL8 selectively regulated $V\gamma4^+$ γδ T cells, indicating an evolutionary conserved mechanism of γδ T cell regulation across mouse and human (89). While extensive progress has been made,

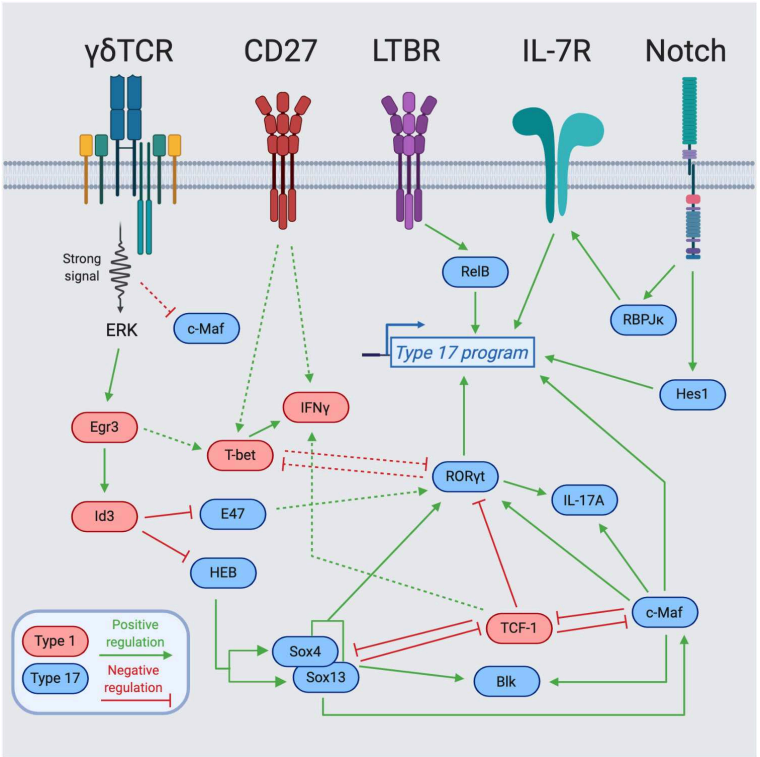

FIGURE 2 | Transcription factor network regulating γδ T cell effector programming. Integration of cell surface receptors [TCR, Lymphotoxin Beta Receptor (LTBR), CD27, and Notch] with downstream transcription factors for the programming of γδ T cell effector function. Blue-colored TFs support the type 17 program, while red-colored TFs support the type 1 program. The dotted lines represent indirect regulation or that the supporting data was described in another cell type. The solid lines represent more direct regulation. Figure made with biorender.com.

much remains unknown regarding the identity of γδTCR ligands that drive specific γδ T cell subset selection for tissue homeostasis (90).

γδ T Cell Crosstalk With mTECs

Aire-expressing mTECs are necessary for central tolerance through expression of tissue-restricted antigens (91). Previous work identified the importance of RANKL-RANK signaling for induction of mTEC Aire expression by lymphoid tissue inducer (LTi) cells (92, 93). Notably, the timing of Aire expression on mTECs coincides with the first wave of $V\gamma5^+$ DETC precursors seeding the thymus (94). Interestingly, RANKL-RANK interactions between $RANKL^+$ $V\gamma5^+$ DETC thymocytes and $RANK^+$ mTECs also induce Aire expression and mTEC maturation. Such RANKL-RANK signaling is additionally required for Skint-1 expression by mTECs, and thus is reciprocally necessary for $V\gamma5^+$ DETC development. Taken together, this study elegantly demonstrates the crosstalk between developing DETC progenitors and immature mTECs that each rely on shared RANKL-RANK signals for maturation. While DETCs are the first γδ thymocytes to emerge in ontogeny, similar crosstalk between resident immune cells and TECs may account for the discrete developmental windows of other innate-like γδ T cell subsets.

TRANSCRIPTIONAL NETWORKS REGULATING γδT CELL IDENTITY

γδ T cell effector acquisition is regulated by a highly-integrated network of transcriptional regulators. The lineage-defining transcription factors (LDTFs), RORγt and T-bet, promote the effector fates of IL-17A vs. IFNγ producers in various lymphocyte lineages, respectively (95–97). Although these LDTFs are integral to programming γδ T cell effector function, many other signal-dependent and collaborating TFs play essential roles in establishing and maintaining γδ T cell identity downstream of TCR signaling and various environmental signaling cascades (**Figure 2**).

In order to better understand the effector diversification of γδ T cells from a global perspective, the Immgen consortium performed gene-expression profiling of isolated *ex vivo* γδ T cells subsets (55). Among these, distinct clusters of immature γδ T cells could be distinguished based on their transcriptomes, reflecting three unique effector programs: IL-17A producers ($V\gamma6^+$ and $V\gamma4^+$), IFNγ producers ($V\gamma1^+$, $V\gamma1^+V\delta6.3^+$, $V\gamma7^+$), and DETCs ($V\gamma5^+$) (55). Importantly, key TFs are enriched in specific γδ effector subsets, such as *Rorc*, *Maf*, *Sox13*, and *Sox4* for the IL-17A producers and *Tcf7* (TCF-1), *Lef1*, *Tbx21* (T-bet), *and Eomes* for the IFNγ producers (55). The dual action of many of these TFs in both promoting one effector fate, while

repressing the alternative fate leads to a complex TF network in γδ T cells (**Figure 2**). Interestingly, TFs associated with type 17 programming in adaptive Th17 cells—namely, IRF4, BATF, and STAT3—are dispensable for Tγδ17 cells (64, 98–100).

TCR-Independent Transcriptional Regulators

Independent of conventional TCR signaling, innate-like γδ T cell effector programming is regulated by a quartet of HMG box TFs including Sox4, Sox13, TCF-1, and Lef1 (101). Among these, Sox13 and Sox4 are essential for the differentiation of Vγ4$^+$ IL-17A-producing cells (101). This Vγ-specific requirement is intriguing as it implies that discrete regulators drive the specification of distinct subsets of Tγδ17 cells, although it remains possible that redundancy between Sox13 and Sox4 masks a global role for Sox TFs in γδ T cell type 17 programming. Within the Vγ4$^+$ subset, Sox13 and Sox4 regulate key Tγδ17 program genes such as *Rorc* and *Blk* (23, 101), a tyrosine protein kinase that is selectively required for the development of Tγδ17 cells (102). While Sox proteins positively regulate type 17 fate, TCF-1 and Lef1 function to restrain Tγδ17 cell generation and gene expression (101). TCF-1 is targeted by multiple environmental signals; it is a Notch-induced TF that plays critical stage-specific roles in T cell differentiation (103, 104), and is also influenced by the Wnt signaling pathway through its β-catenin interaction domain, which is required to ensure DP thymocyte survival (104). In γδ T cells, TCF-1 promotes the expression of Lef1 and the IFNγ producing fate (101). Sox13 may also counteract the type 1 program through direct antagonism of TCF-1 via its β-catenin interaction domain (22), and indirectly via TCF-1 targets, as evidenced by Sox13 Tg mice expressing greatly diminished levels of Lef1 (101). The mutually opposing functions of Sox proteins and TCF-1/Lef1 in Tγδ1 and Tγδ17 differentiation likely reinforces and stabilizes effector fate. Together, TCR-independent HMG box TFs represent key interconnected nodes in the transcriptional network of γδ T cells.

TCR-Dependent Transcriptional Regulators

A crucial question in γδ T cell biology is how distinct functional potentials arise from differential TCR signal strengths? (41). Broadly, effector commitment to an IFNγ-producing fate through strong TCR signaling requires both promotion of drivers of the type 1 program, and simultaneous neutralization of drivers of the type 17 program. TCR signaling can be linked to γδ T cell lineage and effector commitment through the Egr-Id3 pathway. Downstream of strong TCR signaling, Erk induced Egr1 promotes the development of γδ T cells through activation of the E protein inhibitor Id3 (26, 28). Induction of Id3 is also required for functional IFNγ production, providing a mechanism by which signal strength is translated into downstream effectors (28). This signal is key in suppression of E proteins that otherwise support Tγδ17 features (**Figure 2**). Indeed, it has been demonstrated in DP thymocytes that E proteins enhance RORγt expression, while Egr3 negatively regulates RORγt expression by inducing Id3 (105). Similarly, Id3 can antagonize the type 17 program by forming an inactive heterodimer with HEB, an E protein TF that is required for direct promotion of Sox13 and Sox4 expression and CD73$^-$ Tγδ17 cell development (54). Along these lines, Egr3 is highly expressed in Vγ5$^+$Vδ1$^+$ thymocytes and upregulation of Egr3 after Skint-1-mediated selection or strong TCR signal represses *Rorc* and *Sox13* but supports *Tbx21* expression and commitment toward an IFNγ producing fate (41). Therefore, Egr3 downstream of Skint-1-mediated selection directs the TF balance necessary for proper DETC development through restraint of the "default" type 17 program. These findings highlight that TCR-dependent and TCR-independent TFs both antagonize and promote each other to regulate the effector fate of γδ T cells.

Regulation of Type 17 Commitment

In contrast to Tγδ17 specification factors important for type 17 differentiation of distinct Vγ subsets [e.g., Sox13, Sox4, and HEB (54, 101)], the AP-1 factor c-Maf was recently identified as universally required for the generation and maintenance of all IL-17A-producing γδ T cells (49). As a canonical commitment factor, c-Maf directly activates *Rorc* and key Tγδ17 effector genes (*Il17a* and *Blk*), while also antagonizing the expression or function of negative regulators of the type 17 program (TCF-1 and Lef1) that promote the alternative Tγδ1 fate (**Figure 2**) (49). c-Maf globally supports a Tγδ17 chromatin accessibility landscape, with a particularly important role in the establishment of an active regulatory status at *Rorc* involving the recruitment of the histone acetyltransferase p300, and H3K27 acetylation (49). The signals that directly activate c-Maf in γδ thymocytes remain to be defined, but may involve known Tγδ17-promoting factors such as Notch, TGF-β, and IL-7 that have been described as c-Maf activators in CD4$^+$ T cells or ILCs (75, 78, 79, 81, 106–108). There is some evidence that Sox TFs function upstream of c-Maf and can regulate its protein expression (49). Interestingly, unlike Sox13 expression that is independent of TCR signaling (101, 109), c-Maf expression is tuned by TCR signal strength in fetal γδ thymocytes; strong TCR signals lead to low c-Maf and weak signals result in high c-Maf protein levels, providing a mechanism by which weak γδTCR signals can be translated into Tγδ17 regulatory programming (49).

Integration of Type 17 Regulators

A highly-integrated network of regulators control type 17 programming (**Figure 2**). Sox13 and Sox4 collaborate with c-Maf in the direct activation of *Rorc* and other key Tγδ17 genes such as *Blk* and *Il17a* (49, 101). The close proximity of Maf recognition element (MARE) and HMG box consensus sites in the c-Maf-dependent *Rorc* enhancer (CNS+10) suggests that c-Maf and Sox TFs may bind and function cooperatively in γδ T cells (49), as has been described in multiple other cell types (110–112). Of particular relevance, Sox5 and c-Maf can cooperatively bind the *Rorc* promoter and drive its expression in Th17 cells (112). Additionally, c-Maf and RORγt collaborate in the activation of *Il17a* and potentially other type 17 signature genes, however, c-Maf also functions independently of its direct target RORγt in regulating key Tγδ17 lineage-modulating factors (e.g., *Blk, Lef1,* and *Syk*) (49). Aside from activation of the type 17 program, both Sox13 and c-Maf repress the alternative type 1 fate by targeting

TCF-1/Lef1 (49, 101). TCF-1 negatively regulates the *Rorc* locus (101), and its occupancy at *Rorc* CNS+10 is antagonized by c-Maf in γδ thymocytes (49). As TCF-1 harbors intrinsic HDAC activity (113), this antagonism may represent another mechanism by which c-Maf promotes H3K27 acetylation at the *Rorc* locus (49). Intriguingly, c-Maf also restrains the expression and function of TCF-1 in ILC3s (106), while TCF-1 represses the c-Maf/RORγt axis to limit the formation of Tc17 cells in $CD8^+$ T cells (114). This suggests that c-Maf/TCF-1 antagonism is conserved across multiple lymphocyte lineages to regulate the balance of the type 1 vs. type 17 specialization.

The integration of various signals in the effector programming of γδ thymocytes suggests several tiers of regulators in specialization. In building a model, this includes: (1) specification factors (e.g., RelB, Notch, HEB, Sox13, and TCF-1) that perceive environmental signals to support type 1 or type 17 programming either universally or in the establishment of discrete Tγδ17 subsets; (2) commitment factors (e.g., c-Maf, Egr-Id3) that impart or reinforce effector identity programs, and (3) LDTFs (e.g., RORγt, T-bet) that control genes for key canonical effector functions (**Figure 2**). As γδ T cell selection and effector diversification occur across various DN and γδ thymocyte developmental intermediates, with numerous thymus and TCR-derived signals likely occurring over a protracted period, the temporal contributions of such inputs with respect to effector commitment remains unclear. In this regard, a recent intriguing study employing a Sox13 reporter mouse, identified DN1-like ($CD117^-CD24^+CD25^+$) precursors in the perinatal to day 10 thymus that are prewired for the expression of the Tγδ17 gene network (e.g., *Rorc*, *Sox4*, *Tcf7*, *Tcf12*, *Maf*, *Il7r*, *Scart2*, and *Blk*) and are generated in a TCR-independent manner (109). Remarkably, such $Sox13^+$ DN1d cells are predisposed to become $CCR6^+$ IL-17A-producing cells, suggesting they are pre-committed to the Tγδ17 fate (109). Future work focused on how such effector-committed precursors intersect with the rearrangement of particular Vγ TCRs and signal strengths will broaden our understanding of the integration of environmental and TCR inputs in the effector programming of γδ thymocytes during ontogeny.

CONCLUDING REMARKS

The last decade of research has led to enormous leaps in the understanding of tissue-resident lymphocytes, with newfound appreciation for the diversity of innate lymphocytes. Although dependent on the same LDTFs, innate-like γδ T cells and ILCs have unique transcriptional networks that control their effector fates. Such underlying distinctions in regulatory programming may translate into functional differences or non-redundant roles for innate-like γδ T cells vs. ILCs. Indeed, γδ T cells possess a TCR complex that endow them with additional environmental sensing capacities. Thus, uniquely, innate-like γδ T cell effector commitment can be controlled, in part, by the fine-tuning of key transcriptional regulators downstream of TCR signaling to both promote one fate while repressing the other. However, there is still much to be learned with respect to the establishment of transcriptional programs independent of TCR signaling and the elements that predispose γδ thymocytes to an effector fate prior to TCR expression. In the future, taking advantage of advances in single-cell sequencing and genomics techniques will lead to a higher resolution picture of γδ T cell trajectories and lineage decisions.

AUTHOR CONTRIBUTIONS

MP prepared and wrote the manuscript. MC edited the manuscript.

FUNDING

MP and MC were supported by National Institutes of Health grant RO1 GM115474. MC was additionally supported by NIH grant UM1 HG009428 and a Whitehead Charitable Foundation Scholar Award.

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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.

frontiers in Immunology

MINI REVIEW
published: 29 January 2020
doi: 10.3389/fimmu.2020.00072

Innate Lymphoid Cells in Renal Inflammation

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Since their identification as a separate family of leukocytes, Innate lymphoid cells (ILCs) have been shown to play crucial roles in immune-mediated diseases and repair mechanisms that restore tissue integrity after injury. ILCs mainly populate non-lymphoid tissues where they form intricate circuits with parenchymal cells to regulate tissue immunity and organ homeostasis. However, the specific phenotype and function of ILC populations that reside in specific anatomical locations, such as the kidney, still remains poorly understood. In this review, we discuss tissue-specific properties of kidney-residing ILCs and summarize recent advances in the understanding of ILC biology in kidney diseases that might pave the way for development of novel treatment strategies in humans.

Keywords: innate lymphoid cells, chronic kidney disease, acute kidney injury, glomerulonephritis, ILC modulation

OPEN ACCESS

Edited by:
Claudia U. Duerr, McGill University, Canada

Reviewed by:
Malcolm Ronald Starkey, University of Newcastle, Australia
Steven J. Van Dyken, Washington University School of Medicine in St. Louis, United States

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *21 November 2019*
Accepted: *13 January 2020*
Published: *29 January 2020*

Citation:
Becker M, Gnirck A-C and Turner J-E (2020) Innate Lymphoid Cells in Renal Inflammation. Front. Immunol. 11:72. doi: 10.3389/fimmu.2020.00072

INTRODUCTION

Chronic kidney disease (CKD) affects ∼10% of the population in industrialized countries and is a major risk factor for cardiovascular mortality (1). CKD often shows a progressive course leading to end stage renal disease with the need for renal replacement therapy (dialysis or kidney transplantation), resulting in substantial morbidity and mortality of affected patients. Diabetes mellitus and arterial hypertension are the most common diseases that lead to chronic renal injury with subsequent dysfunction, but immune-mediated kidney diseases, such as glomerulonephritis and interstitial nephritis, are also frequent causes of CKD cases (∼20%) (2).

In addition to CKD, acute impairment of kidney function (acute kidney injury = AKI) is a common clinical problem that affects up to 25% of hospitalized patients worldwide and represents an important risk factor for in-hospital mortality (3). AKI can result from various clinical conditions, including ischemia, sepsis, and nephrotoxic agents, and usually resolves after successful treatment of the underlying condition or withdrawal of the toxin. However, it has become evident that previous episodes of AKI increase the risk for development of CKD, underlining the importance of AKI for long-term patient outcome (4, 5).

Regardless of the underlying etiology, the local immune response in renal tissue critically contributes to initiation and progression of acute and chronic kidney damage. However, if activated appropriately, regulatory components of the immune system can also promote kidney tissue regeneration and limit renal inflammation (6, 7). Thus, immunomodulatory strategies that are aimed at shifting the balance from a pro-inflammatory, tissue destructive immune response in the kidney to an anti-inflammatory, pro-regenerative response are promising candidates for the development of novel therapies for kidney diseases. In this context, several recent studies identified kidney-residing Innate lymphoid cells (ILCs) as potential therapeutic targets in the attempt to promote tissue regeneration in AKI and/or slow progression of CKD (8).

INNATE LYMPHOID CELLS

Innate lymphoid cells (ILCs), as a separate family of leukocytes, are considered to represent the innate counterpart of conventional T cells. Similar to T cells, ILCs exhibit lymphoid morphology and produce large amount of cytokines, but in contrast to adaptive lymphocytes, they do not rely on rearranged antigen receptors for activation. Instead, ILCs are equipped with a wide array of receptors to sense, integrate and respond to local cues provided by haematopoietic and non-haematopoietic cells of the tissue niche they reside in.

ILCs are now subdivided into cytotoxic NK cells (or "killer" ILCs) and four groups of "helper" ILCs: ILC1s, ILC2s, ILC3s, and Lymphoid tissue inducer (LTi) cells, based on their expression of specific transcription factor and cytokine profiles, mirroring the classification of $CD4^+$ T helper cell subsets into T_H1, T_H2, and T_H17 cells (9–11).

NK cells are the innate cytotoxic counterpart of $CD8^+$ T cells, depend on the transcription factors Tbx21 (Tbet) and eomesodermin (Eomes) and produce IFN-γ, granzymes, and perforin after activation. ILC1s resemble T_H1 cells and, similar to NK cells, express T-bet and IFN-γ, but not Eomes and are less cytotoxic. ILC2s are defined by GATA-3 expression and produce the T_H2 cytokines IL-13, IL-5, and IL-4, as well as IL-9 and the epidermal growth factor amphiregulin. ILC3s represent the innate T_H17 counterpart and are characterized by expression of RORγt and AHR, as well as the production of IL-17 and/or IL-22, GM-CSF, and lymphotoxin. Within the ILC3 subset, the expression of Natural cytotoxicity receptors (NCRs, e.g., NKp46, NKp44) further differentiates ILC3s into NCR^+ and NCR^- ILC3s, exhibiting different effector functions (12). Similar to ILC3s, LTi cells, that are essential for the formation of secondary lymphoid organs during embryonic development, depend on RORγt and produce IL-17, IL-22, and lymphotoxin, but recent studies indicate that they develop from a different precursor (11).

In the past decade, helper ILCs were extensively studied and are now recognized as important regulators of immune responses in a variety of organs and inflammatory conditions (13, 14). As largely tissue-resident cells (15), ILCs are adapted to the microenvironment they reside in Ricardo-Gonzalez et al. (16), thus showing organ-specific subset distribution, phenotype, and functional regulation. While the critical function of helper ILCs in barrier organs, such as the intestine, lung, and skin has been elucidated in great detail, knowledge about their tissue-specific properties in the kidney is still emerging. The role of NK cells in kidney health and disease has been recently reviewed elsewhere (17) and will therefore not be discussed here.

DISTRIBUTION, PHENOTYPE, AND REGULATION OF HELPER ILC SUBSETS IN THE KIDNEY

First evidence that ILC2s represent a major ILC subset in the murine kidney came from a study using IL-5 reporter mice to investigate the distribution of IL-5-expressing ILCs in various tissues. In these analyses up to 7% of all $CD45^+CD90.2^+$ cells were $IL\text{-}5^+$ non-T cells, representing the kidney-residing ILC2 population (18). A more detailed characterization of the total IL-7Rα $(CD127)^+Lineage^-$ lymphocyte population in the kidney of naïve mice revealed that, depending on the mouse strain (19, 20), ∼1–6% of total $CD45^+$ lymphocytes are helper ILCs. Among these, IL-5/IL-13-producing GATA-3^+ ILC2s are indeed the most abundant ILC subset in the kidney (∼80%), while $ROR\gamma t^+$ ILC3s and $Tbet^+Eomes^-$ ILC1s represent only minor fractions (19). Kidney-residing ILC2s share important characteristics with ILC2s in other anatomical locations, such as tissue residency (21) and expression of specific surface receptors that determine their responsiveness to activating and inhibitory stimuli (see below) (19, 20, 22, 23). However, there are first indications of kidney-specific features of the local ILC2 population (24), warranting further investigation.

The healthy human kidney also harbors a $CD127^+CD161^+Lineage^-$ helper ILC population that accounts for ∼0.5% of total lymphocytes. In line with the mouse data, the kidney-residing ILC population in humans contains a considerable percentage of ILC2s (∼35%) defined by expression of CRTH2 and the receptors for IL-33 (T1/ST2) and IL-2 (CD25) (19, 21). However, unlike in the mouse, $cKit^+NCR^+$ ILC3s (∼15%) and $cKit^+NCR^-$ ILC3s (∼40%, possibly containing some ILC precursors (25), are also abundant in the human kidney in non-inflammatory conditions (19).

Strategic positioning of ILCs within barrier tissues is especially important for their function. ILC2s can be detected by immunohistochemical staining in the glomerular and tubulointerstitial compartments of the mouse kidney (19), but it was shown recently that under homeostatic conditions a majority of renal $IL\text{-}5^+$ ILC2s reside in the perivascular adventitial cuff surrounding the main arterial vessels where they co-localize with kidney dendritic cells (24, 26). Although the functional relevance of this finding for kidney homeostasis is still unclear, it can be speculated that, similar to the lung, stromal adventitial cells provide cytokines, such as IL-33 and TSLP, that might promote ILC2 maintenance in the healthy kidney tissue (27).

ILCS IN ACUTE KIDNEY INJURY

Acute kidney injury is characterized by a rapid decrease of kidney excretory function, resulting in elevation of serum creatinine levels and/or decreased urine output (28). Renal ischemia is one major cause of AKI in humans and is induced by various clinical conditions that lead to hypoperfusion of the kidney, such as severe volume depletion, circulatory shock, or renal vascular occlusion. The widely used ischemia/reperfusion injury (IRI) model applies surgical clamping of the renal artery for a defined time period with subsequent reperfusion of the kidney to mimic the pathomechanism of ischemic AKI (29). Similar to ILC2s in other organs, kidney-residing ILC2s express the receptors for IL-25 (IL-17RB) and IL-33 (T1/ST2) and can be activated and expanded *in vivo* by administration of these cytokines in mice (19, 22). Application of ILC2-expanding cytokines has been used to investigate the *in vivo* role of ILC2s in the IRI mouse model of AKI (21, 22). In this model, systemic intraperitoneal

application of IL-25 or IL-33 previous to IRI induction resulted in significant renal tissue protection, as indicated by lower serum creatinine levels and reduced tubular damage, accompanied with increased renal expression of the type 2 cytokines IL-4, IL-5, and IL-13 produced by local $Lin^{-}CD127^{+}CD90^{+}CD25^{+}ST2^{+}IL\text{-}17RB^{+}$ ILC2s and, in case of IL-25, by an additional smaller population of $Lin^{-}CD127^{-}CD90^{-}ST2^{-}CD25^{-}IL\text{-}17RB^{+}c\text{-}Kit^{+}$ Multipotent Progenitor Type 2 Cells (**Figure 1**). Whether the latter are a separate cell type (30) or represent IL-25-responsive inflammatory ILC2s with low expression of the IL-7 receptor (CD127) (31) remains to be elucidated. The beneficial *in vivo* effects of IL-25 and IL-33 application were indeed mediated by ILC2s, since transfer of IL-25- or IL-33-elicited ILC2s was sufficient to ameliorate renal impairment in mice with IRI (21, 22). Moreover, partial depletion of ILC2s with anti-CD90 antibodies in IL-33-treated $Rag1^{-/-}$ mice abolished the protective IL-33 effect, while depletion of Tregs in immunocompetent mice, which have also been described to be IL-33-responsive (32), did not (21). In line with the enhanced intrarenal type 2 response after IL-25 or IL-33 treatment, kidney-residing macrophages were shifted toward a M2 phenotype. Furthermore, neutrophil accumulation in the kidney was reduced by a yet unknown mechanism. The authors could further demonstrate, that *in vitro* differentiated M2 macrophages protected tubular epithelial cells (the primary target cells of ischemic AKI) from apoptosis, providing a potential downstream mechanism for ILC2-mediated tissue protection via alternative activation of macrophages (22). In addition, it was shown that IL-33-activated ILC2s require production of the epidermal growth factor amphiregulin to mediate their protective effects in renal IRI (21), indicating that ILC2s might employ multiple pathways to shift the intrarenal microenvironment from a pro-inflammatory to an anti-inflammatory, pro-regenerative state (**Figure 1**). Importantly, the therapeutic effect of IL-33 application was maintained when cytokine therapy was started after induction of IRI in mice and was also observed in mice with a humanized immune system that were treated with human recombinant IL-33 (21).

Although these results highlight the therapeutic potential of ILC2-directed therapies in AKI, so far there is no evidence for a role of endogenous ILC2 activation and expansion during AKI. A recent study addressed this issue by comparing tissue injury and renal function impairment between control IRI mice and IRI mice that are reduced or deficient in ILC2s, either constitutively ($Il7r^{cre/+}Rora^{fl/fl}$) or after DTx-mediated depletion ($Cd4^{cre/+}Icos^{dtr/+}$). In these experiments, the authors did not observe a substantial difference in histopathologic tubular injury and inflammatory marker expression in the kidney, leading to the conclusion that endogenous ILC2s that are not previously expanded by cytokine therapy are redundant in IRI (24). Moreover, a previous study provided conflicting evidence for a pro-inflammatory role of IL-33 in AKI by showing that its application in a mouse model of nephrotoxic AKI induced by the cytostatic drug cisplatin aggravates renal injury (33), suggesting that action of IL-33 and IL-33-induced ILC2s, although not specifically addressed in this study, might be highly context-dependent.

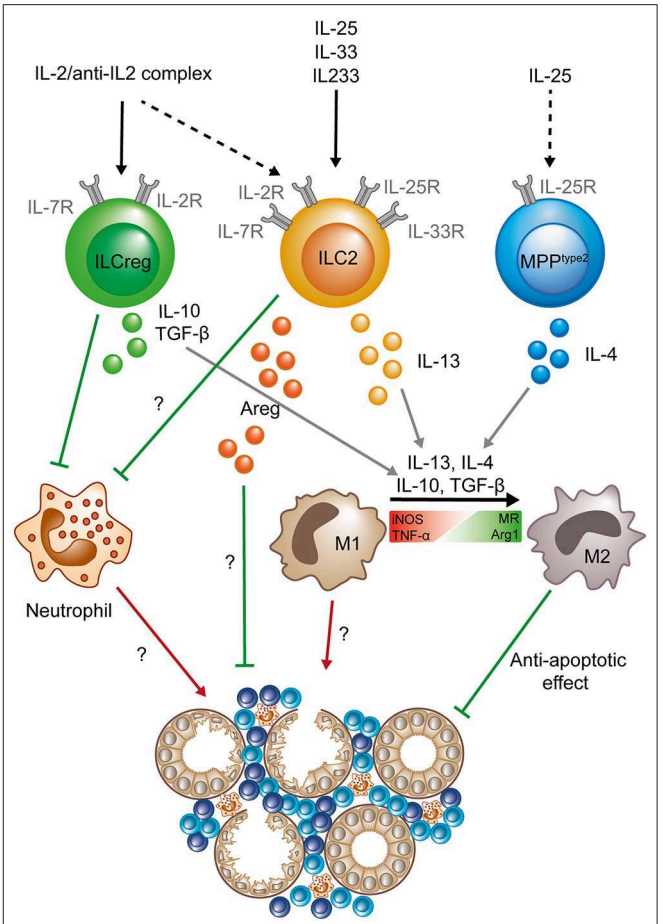

FIGURE 1 | Protective role of ILC2s, MPPtype 2 cells, and "ILCregs" in acute kidney injury. After activation by an IL-2/anti-IL-2 complex (IL2C) ILC2s and "ILCregs" (whether the latter are a separate lineage or IL-10 producing ILC2s is still a matter of debate) prevent neutrophil accumulation in the kidney. "ILCregs" produce IL-10 and TGF-β upon activation. ILC2s can be activated by IL-33, IL-25, the hybrid cytokine IL233, or IL2C and secrete IL-13 and Areg to promote tissue protection. IL-25 can stimulate MPP^{type2} cells to produce IL-4, which in addition to IL-13, IL-10, and TGF-β, has been shown to promote the shift from a pro-inflammatory M1 phenotype (expression of iNOS and TNF-α) to an anti-inflammatory M2 phenotype (expression of MR and Arg1) in macrophages. The exact mechanisms of how ILC2s (and "ILCregs") prevent neutrophil accumulation and Areg-dependent tissue protection are still unknown. Question marks indicate mechanisms that are so far not completely understood and need to be further elucidated. Green lines symbolize protective and beneficial effects, whereas red arrows indicate proinflammatory effects. (Areg, amphiregulin; Arg1, Arginase 1; iNOS, Inducible nitric oxide synthase; MR, mannose receptor; M1, classical macrophage; M2, alternatively activated macrophage; TNF-α, tumor necrosis factor α; TGF-β, Transforming growth factor β).

A recent study by Cao et al. demonstrated that a small population of IL-10-producing ILCs (2–3% of total ILCs, representing ∼0.06% of total lymphocytes) can be detected in the murine and human kidney (34). Definition of these cells was based on a previous report of a similar ILC population in the intestine that was termed "ILCregs" (35), but, since ILC2s can produce large amounts of IL-10 under certain stimulatory conditions (36), it is still a matter of debate if these $IL\text{-}10^{+}$

ILCs indeed represent a separate ILC subtype (37). However, the authors went on to show that IL-10-producing ILCs in the kidney can be expanded by IL-2/anti-IL-2 complex (IL2C) treatment and mediate protective effects in the IRI-AKI model by downstream mechanisms similar to IL-25- or IL-33-elicited ILCs (**Figure 1**) (34), underlining the therapeutic potential of kidney ILCs in AKI.

In the attempt to translate this concept into a therapeutic approach for potential use in human renal disease, a novel hybrid cytokine linking IL-33 with IL-2 has been designed to activate cell types that express a combination of the respective receptors, such as $ST2^+CD25^+$ Tregs and $ST2^+CD25^+$ ILC2s. This hybrid cytokine, termed IL233, was recently shown to be effective in protection from nephrotoxic and IRI-induced AKI by expansion of Tregs and ILC2s (38) and might provide a valuable basis for further development of ILC-directed therapies toward first in-human studies.

ILCS IN CHRONIC KIDNEY DISEASE

Progressive scarring of the glomeruli (glomerulosclerosis) and fibrosis of the tubulointerstitial compartment are the histopathological hallmarks of CKD. In BALB/c mice, application of the cytostatic drug Adriamycin induces podocyte damage and breakdown of the glomerular filtration barrier, leading to proteinuria, progressive glomerulosclerosis, and chronic tubulointerstitial injury. This "Adriamycin-induced nephropathy" (AN) shares main histopathological features with human CKD and has been widely used as a model to study the effect of therapeutic interventions in proteinuric CKD (39). It was shown previously that, similar to the AKI model, repeated application of IL-25 after AN induction ameliorates its clinical course by induction of M2 macrophages, but the IL-25-responsive cell type responsible for this effect was not addressed in the initial study (40). More recently, our own group showed that a short course of IL-33 treatment in mice (400 ng i.p. on four consecutive days) leads to a massive and sustained increase in kidney ILC2s for up to several month and effectively improved histopathological and clinical parameters of renal injury in the AN model (19). Mechanistically, IL-33-mediated kidney protection in AN was accompanied by an accumulation of eosinophils and a reduction of neutrophil and inflammatory mononuclear phagocyte infiltration. Analysis of ILC-deficient $Rag^{-/-}Il2rg^{-/-}$ mice and eosinophil-deficient ΔdblGATA mice confirmed that the IL-33 effect depended on the presence of ILCs and eosinophils (19) (**Figure 2**). In line, pre-emptive treatment with the above-mentioned, novel hybrid cytokine IL233 protected mice from progressive glomerulosclerosis in AN (38).

In contrast to the beneficial effects of the IL-33/ILC2 axis in glomerulosclerosis, a potential deleterious role of endogenous IL-33 in kidney fibrosis was reported by Chen et al., demonstrating partial protection from tubulointerstitial fibrosis induced by unilateral urinary obstruction (UUO) in $Il33^{-/-}$ and $Il1rl1^{-/-}$ mice (41). Accordingly, administration of high-dose IL-33 (500 ng i.p. daily for 14 days) promoted tubulointerstitial fibrosis at week two after IRI-AKI, while inhibition of IL-33 reduced AKI-induced fibrosis. Although the exact cellular mediators and downstream mechanisms of this deleterious IL-33 effect in renal fibrosis were not explored in these studies (41, 42), pro-fibrotic effects of chronically activated ILC2s via production of IL-13 were described in the liver and lung (43, 44), indicating that systemic ILC2-directed therapies might comprise a substantial risk for side effects which are likely to be determined by dose, duration, and context of cytokine application. While higher amounts (1 μg per injection) and/or prolonged application of IL-33 (14 days) might have disadvantageous effects (33, 42), lower doses (0.3–0.5 μg IL-33 or IL-25 per injection) and short-term treatment (3–5 days) were shown to be beneficial (19–22, 34, 40) in various models. Whether systemic ILC2 expansion after i.p. treatment with these cytokines also contributes to the tissue protective effects in the kidney is still unclear and warrants further studies.

Since two independent studies suggested increased numbers of ILC2s and type 2 cytokines (IL-4, IL-5, IL-13) in the peripheral blood of patients suffering from CKD due to type 2 diabetes (45, 46), it can be speculated that ILC2s might be a marker for renal fibrosis in human CKD. However, technical limitations in the flow cytometry gating strategy used to identify ILC2s in these studies preclude valid conclusions from these data and further research is clearly needed to assess a potential role of ILC subsets in human CKD.

ILCS IN GLOMERULONEPHRITIS

Glomerulonephritides (GNs) are a major cause of CKD and are characterized by a pathogenic immune response against renal autoantigens or by renal manifestations of systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) or anti-neutrophil cytoplasmic antibody (ANCA)-associated small vessel vasculitis. A potential role of ILCs in the pathogenesis of GN is just beginning to be unraveled. In a recent study, our group provided first evidence that kidney-residing ILC2s are decreased in frequency and number with progression of autoimmune renal inflammation in the MRL/MpJ-Fas^{lpr} (MRL-lpr) mouse model of SLE (20). Progression of lupus nephritis in MRL-lpr mice was characterized by marked increase in IFN-γ and IL-27 expression in the inflamed kidneys that were produced by T cells and inflammatory myeloid cells, respectively (20). We and others could further show that, similar to ILC2s in the lung (47, 48), kidney ILC2s express the IFN-γR and IL-27R and are extremely sensitive to IFN-γ/IL-27-mediated inhibition of IL-33-induced proliferation and cytokine production *in vitro* (20, 23), providing a mechanism for inflammation-induced reduction of ILC2s in the kidney (**Figure 2**). Most importantly, treatment with IL-33 restored kidney ILC2s, increased type 2 cytokine expression and eosinophil accumulation, reduced severity of lupus nephritis, and improved survival of MRL-lpr mice (20), indicating that ILC2s might be protective in immune-mediated glomerular diseases.

While in the MRL-lpr model the other helper ILC subsets were unaltered (20), a recent study suggested that a previously unknown ILC1 subtype expressing CD8 might infiltrate glomeruli in rat and potentially also in human anti-GBM nephritis (49). However, if this $CD8^+$ cell subset indeed

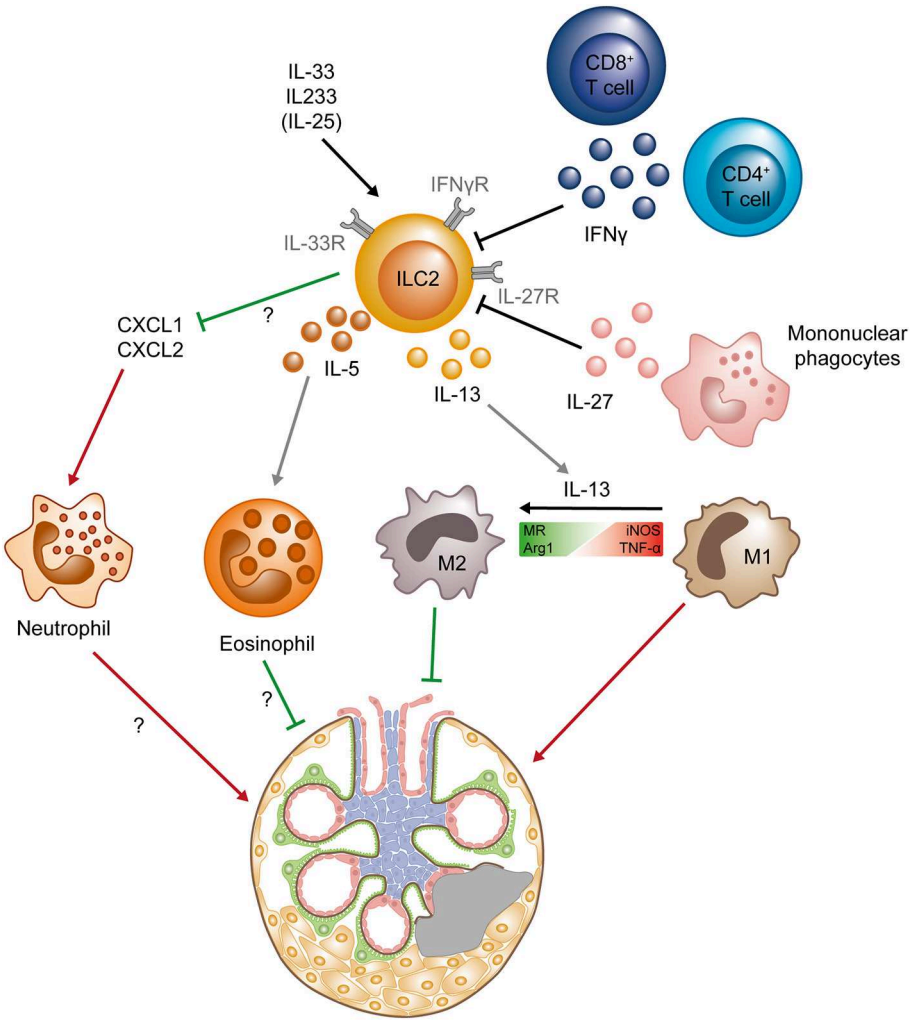

FIGURE 2 | Protective role of ILC2s in chronic kidney diseases. ILC2s can be activated by the cytokines IL-33 and IL-25, as well as the hybrid cytokine IL233, whereas IFNγ (secreted by $CD4^+$ and $CD8^+$ T cells) and IL-27 (produced by mononuclear phagocytes) suppress ILC2s. Activated ILC2s produce IL-5 and IL-13, leading to the accumulation of eosinophils and the shift from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype in macrophages. M2 macrophages have been shown to directly protect the tissue, whereas the exact mechanisms of tissue protection mediated by eosinophils are still unclear. The activation and expansion of ILC2s also results in decrease of the chemokines CXCL1 and CXCL2 in the kidney, preventing neutrophil accumulation that mediate renal injury. Question marks indicate mechanisms that are so far not completely understood and need to be further elucidated. Green lines symbolize protective and beneficial effects, whereas red arrows indicate proinflammatory effects. (Arg1, Arginase 1; iNOS, Inducible nitric oxide synthase; MR, mannose receptor; M1, classical macrophage; M2, alternatively activated macrophage; TNF-α, tumor necrosis factor α).

represents a novel ILC subset needs to be confirmed in future studies.

Initial studies in patients suffering from ANCA-associated vasculitis showed that total ILC numbers in the peripheral blood were reduced in the acute phase of the disease, as compared to healthy controls, which was due to a reduction of both ILC2s and ILC3s (50). Moreover, the authors could demonstrate a significant correlation between a reduction in ILC numbers and high disease activity, supporting the conclusion from the murine SLE model that ILCs might have a protective effect in chronic autoimmunity (20, 50). However, another study analyzing peripheral blood ILC numbers in ANCA vasculitis patients and in appropriate disease controls with a similar impairment of renal function was unable to detect a vasculitis-specific reduction, indicating that a decrease in peripheral ILCs might be a non-specific manifestation of CKD (51).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In the last decade ILCs have emerged as important effector cells of the innate immune system in a variety of chronic inflammatory and autoimmune conditions. A number of recent studies in preclinical models demonstrate a role of ILC2-directed therapies in promoting kidney regeneration

after acute injury and in shifting the intrarenal immune milieu toward a tissue protective type 2 response. However, chronic and systemic over activation of ILC2s might comprise the risk of pro-fibrotic and pro-allergic side effects in the kidney and other organs which have to be considered in the attempt to translate these findings into specific ILC-directed treatment strategies for inflammatory kidney diseases in humans.

So far, there are no comprehensive studies addressing kidney-specific ILC properties, but first data indicate a specific phenotype of the local ILC2 population in the kidney (24). In the future, it will be critical to elucidate the specific molecular pathways that drive kidney ILC activation and to obtain a detailed understanding of their localization and interaction with other immune cells and parenchymal cells within the kidney tissue. These analyses will help to identify pathways that allow for specific targeting of kidney-residing ILCs in the attempt to exploit their tissue protective properties, without causing potential deleterious ILC activation in other anatomical locations.

AUTHOR CONTRIBUTIONS

All authors have participated sufficiently in the work to take public responsibility for the content. MB, A-CG, and J-ET drafted, revised, and approved the final version of the manuscript.

ACKNOWLEDGMENTS

J-ET was supported by an Emmy Noether Grant (TU 316/1-2), the Priority Programme 1937 Innate Lymphoid Cells (TU 316/4-1), and the Collaborative Research Center 1192 (TP A6), all funded by the German Research Foundation.

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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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REVIEW
published: 06 February 2020
doi: 10.3389/fimmu.2020.00116

Immunoregulatory Sensory Circuits in Group 3 Innate Lymphoid Cell (ILC3) Function and Tissue Homeostasis

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Recent years have seen a revolution in our understanding of how cells of the immune system are modulated and regulated not only via complex interactions with other immune cells, but also through a range of potent *inputs* derived from diverse and varied biological systems. Within complex tissue environments, such as the gastrointestinal tract and lung, these systems act to orchestrate and temporally align immune responses, regulate cellular function, and ensure tissue homeostasis and protective immunity. Group 3 Innate Lymphoid Cells (ILC3s) are key sentinels of barrier tissue homeostasis and critical regulators of host-commensal mutualism—and respond rapidly to damage, inflammation and infection to restore tissue health. Recent findings place ILC3s as strategic integrators of environmental signals. As a consequence, ILC3s are ideally positioned to detect perturbations in cues derived from the environment—such as the diet and microbiota—as well as signals produced by the host nervous, endocrine and circadian systems. Together these cues act in concert to induce ILC3 effector function, and form critical sensory circuits that continually function to reinforce tissue homeostasis. In this review we will take a holistic, organismal view of ILC3 biology and explore the tissue sensory circuits that regulate ILC3 function and align ILC3 responses with changes within the intestinal environment.

Keywords: innate lymphoid cells, ILC, mucosal immunology, neuroimmune, circadian, immune circuits

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *12 November 2019*
Accepted: *16 January 2020*
Published: *06 February 2020*

Citation:
Domingues RG and Hepworth MR (2020) Immunoregulatory Sensory Circuits in Group 3 Innate Lymphoid Cell (ILC3) Function and Tissue Homeostasis. Front. Immunol. 11:116. doi: 10.3389/fimmu.2020.00116

GROUP 3 INNATE LYMPHOID CELLS—SENTINELS OF THE GASTROINTESTINAL TRACT

Innate lymphoid Cells (ILCs) are a family of innate immune effectors that localize mainly to mucosal surfaces and which play critical roles in regulating tissue immunity and homeostasis. The ILC family can be divided into three main subsets—group 1 ILC (ILC1), ILC2, and ILC3 based on their expression of master transcription factors and associated effector cytokine profiles [Reviewed extensively elsewhere (1–8)]. In this review we will focus on group 3 ILC (ILC3), a group of ILC that act constitutively to maintain intestinal health through regulation of the intestinal barrier and commensal microbiota, and through protective immune responses against extracellular microbial pathogens.

ILC3s are characterized by the expression of the retinoid-related orphan receptor γt (RORγt) (1, 5, 6) and they can be further sub-divided into at least two sub-groups in adults (9).

These subsets are developmentally, transcriptionally and functionally heterogeneous and include lymphoid tissue inducer cells (LTi)-like ILC3s; characterized by surface expression of CCR6, c-kit (CD117), Neuropilin-1, and variable expression of CD4, in addition to natural cytotoxicity receptor expressing (NCR)+ ILC3s—which lack LTi-associated markers but express a range of NCR (e.g., NKp46 in mice) while further co-expressing the transcription factor T-bet (10, 11). The characteristics and differences between ILC3 subsets have been discussed in detail elsewhere (9) and as such, for the sake of clarity, we will largely refer to ILC3 cumulatively in this review without distinguishing the specific subset.

As discussed in detail below, ILC3s are at the center of multiple tissue regulatory circuits in which a variety of *inputs* (in the form of environmental and host-derived cues) are sensed and interpreted by ILC3 and give rise to functional *outputs* that culminate in the downstream modulation of tissue physiology to maintain health and homeostasis. While the *inputs* of these sensory circuits vary, and will be discussed in detail below, a major common ILC3-associated *output* is the secretion of effector cytokines including IL-22, IL-17A, IL-17F, and GM-CSF and lymphotoxin (LT) (1, 4, 7, 8) (**Figure 1**). These soluble mediators in turn act upon both neighboring tissue-resident immune cells and non-hematopoietic cells—such as epithelia and stroma. In this review, we will comprehensively discuss the major tissue circuits through which ILC3 function is regulated, and through which ILC3 propagate these signals to regulate and orchestrate the wider immune response and to promote optimal tissue function, mediate protective immune responses and maintain health.

ILC3 CIRCUITS IN THE REGULATION OF INTESTINAL HOMEOSTASIS

Host-Microbiota Sensory Circuits

Mammals have evolved multiple complimentary immunological mechanisms to promote the anatomical containment of commensal bacteria. These mechanisms enforce tolerance, suppress inflammation and maximize mutualism with the microbiota, and ILC3s have key roles in this process (12–15). ILC3s are enriched within gastrointestinal (GI) tract where they are ideally positioned to promote barrier repair and to prevent bacterial translocation (15). ILC3 produce a range of soluble mediators that enable them to continually reinforce the barrier and maintain the containment and physical segregation of commensal microorganisms. Chief amongst these mediators is the cytokine interleukin (IL)-22, which binds to the heterodimeric receptor IL22RA1-IL10RB (IL-22R) expressed by cells of the non-haematopoietic lineage, most notably intestinal epithelial cells (**Figure 1**: *outputs*). IL-22 signaling induces the production of antibacterial peptides such as RegIIIβ and RegIIIγ and S100 family members, which in turn regulate the commensal microbiota and limit access to the epithelial and mucosal niche (16, 17). IL-22 also promotes the physical exclusion of commensal bacteria through induction of mucins and goblet cell hyperplasia, and by regulating the expression of tight-junction components (15, 17, 18). Moreover, ILC3s induce fucosylation of intestinal epithelial cells through an IL-22 and LTα driven process, which in turn favors colonization by mutualistic bacterial species at the expense of potential pathogens (**Figure 1**: *outputs*) (19–21). In addition, IL-22 produced by ILC3s acts to regulate epithelial turnover and intestinal crypt stem cell maintenance, and has been ascribed both pro- and anti-tumorogenic functions, most recently being shown to promote DNA damage response (DDR) mechanisms in order to prevent tumor formation (22–25). IL-22 also modulates nutrient uptake via the intestinal epithelia, in particular lipid uptake (26). In line with this central role for ILC3 and IL-22 in maintaining intestinal barrier function and tissue homeostasis, loss of IL-22 production by ILC3s in mice results in dysbiosis, barrier disruption and an increased susceptibility to experimental induced colitis (27, 28). Moreover, depletion of intestinal ILC3 leads to peripheral dissemination of intestinal bacteria and systemic inflammation that can be rescued by providing exogenous IL-22 (15). Thus, a central function, and key *output,* of ILC3-mediated effector responses is the orchestration of host-microbiota interactions (**Figure 1**: *outputs*).

Intestinal homeostasis and host-commensal interactions are also modulated by the type 3 cytokines IL-17A and IL-17F, both of which are also produced by ILC3 (1, 4, 7, 8). Similar to IL-22, IL-17A/F promote tissue integrity by enhancing the synthesis of tight junctions and antimicrobial peptides, including β-defensins, REG proteins, S100 proteins, lipocalins and lactoferrins (29). Additionally IL-17A/F act in part to attract myeloid cells to the tissue site, through the induction of chemokines and growth factor expression by epithelial cells (30, 31). While ILC3 have been reported to be a potent source of IL-17A/F in early life, expression of these cytokines appears to be somewhat limited at steady state in adult tissues (28, 32). In contrast, during infection and inflammation ILC3 produce IL-17 in response to myeloid-derived cues including IL-23 and IL-1β (33, 34), and ILC3-derived IL-17 has been attributed critical roles in immunity to fungal and bacterial pathogens (34–37). In particular, IL-17 production by ILC3s has been implicated in immunity against fungal pathogens, specifically in response to *Candida albicans* (34). Interestingly, HIV patients commonly manifest oropharyngeal candidiasis, and loss of IL-17 production by ILC3s was observed in tonsils and buccal mucosa during SIV infection in macaques (38, 39).

While homeostatic IL-17 production has been attributed protective functions in intestinal health and host-commensal microbe interactions, elevated IL-17A/F production has also been associated with the pathogenesis of inflammatory bowel disease (IBD). Indeed, ILC3-derived IL-17A and IL-17F are increased during intestinal inflammation in both mice and humans (40, 41). Together, IL-17A/F production by intestinal ILC3—in addition to Th17 and γδ T cell populations—has highly contextual roles in intestinal health, immunity and inflammation.

Conversely, the microbiota itself is also increasingly appreciated to act reciprocally to modulate ILC3 function (**Figure 1**: *inputs*). Indeed, early studies suggested microbial colonization of the neonatal intestine regulates the composition and size of the ILC3 pool within the intestinal

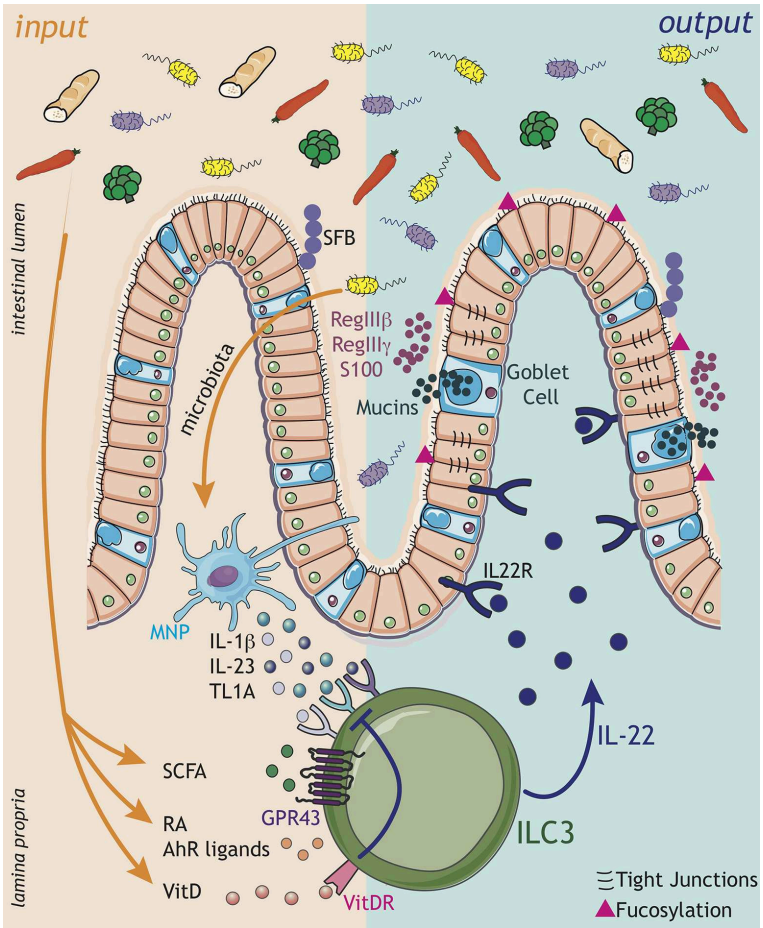

FIGURE 1 | ILC3 engage in complex sensory circuits in order to integrate microbial and dietary cues and enforce mucosal homeostasis. **Inputs** (orange arrows): ILC3s act as innate immune sentinels of the gastrointestinal tract, and respond rapidly to changes in the tissue environment. Environmental signals, comprising microbial and dietary cues, are sensed either via myeloid cell intermediaries [e.g., dendritic cells (DC), macrophages, also known as mononuclear phagocytes (MNP)], which release cytokine cues (IL-1β, IL-23, TL1A) to modulate ILC3 function, or through direct sensing of metabolites and dietary ligands. Microbial metabolites, such as short chain fatty acids (SCFA), signal directly to modulate ILC3 function though the receptor GPR43. Additionally, ILC3 integrate dietary cues in the form of the vitamin A metabolite retinoic acid (RA) and AhR ligands, which together promote ILC3 development and effector cytokine responses. In contrast, vitamin D acts as a negative regulator of ILC3 activation by suppressing the ability of ILC3 to sense myeloid cues—such as IL-23. Within the complex tissue microenvironment ILC3 are likely exposed to multiple signals in parallel, which must be appropriately integrated to maintain intestinal homeostasis. **Outputs** (dark blue arrows): Signals translated by ILC3 are propagated in the form of ILC3-derived *outputs*—most notably cytokine signals, which are received by other immune and non-immune cells within the local environment. In particular, ILC3-derived IL-22 acts on epithelial cells to enforce intestinal barrier integrity and induce the production of antimicrobial peptides (AMPs) such as RegIIIβ, RegIIIγ, and S100 family proteins, secretion of mucins by goblet cells, modulation of tight junctions and epithelial cell fucosylation. IL-22-dependent pathways further regulate the growth of specific commensal bacteria species that are intimately associated with the host, such as segmented filamentous bacteria (SFB). Together, the balance of signals perceived by ILC3 determine the strength of the effector response, regulate the balance of the commensal microbiota and ensure their spatial segregation from the underlying intestinal tissue. In the context of disease, dysregulation of these signals may dramatically alter ILC3 responses and result in a loss of barrier function and translocation of the bacteria from the lumen, thus precipating or exacerbating inflammatory disease.

tract. Pups born to germ free mothers were reported to have reduced frequencies of ILC3s—indicating a role of microbial signals in promoting tissue seeding by ILC3 subsets (28). However, in contrast to these findings IL-22 producing ILC3 numbers were found to be suppressed in a microbiota dependent manner through epithelial expression of IL-25 (32). Despite these discrepancies, the dialogue between the microbiota and ILC3s within the intestine has emerged as a critical circuit of intestinal immunity and tissue homeostasis.

Recent studies have begun to shed light on the microbial-derived metabolites that mediate this immune regulatory on ILC3. For example, ILC3s have the capacity to sense and respond to short chain fatty acids (SCFA)—including butyrate, acetate and propionate—critical regulators of immune responses which are metabolized from dietary fiber by commensal microbes (**Figure 1**: *inputs*) (42, 43). Levels of butyrate differ along the intestinal tract, in line with differing densities of commensal microbes, and were previously correlated with reduced ILC3 cell number and cytokine production in distal regions of the small

intestine (44). SCFA can signal via multiple G-coupled protein receptors, as well as via histone deacetylase enzymes (HDAC) (43), and despite these advances the mechanisms through which SCFA regulate ILC3 are still being delineated. The SCFA receptor GPR109a was implicated in the microbiota-associated regulation of ILC3 cytokine production via the modulation of dendritic cell (DC)-derived IL-23 in the colon, although these studies largely utilized a GPR109a agonist—leaving the precise contribution of endogenous SCFA unclear (45). Interestingly, a recent study highlighted ILC3-intrinsic expression of the SCFA receptor *Gpr43* (*Ffar2*) in the modulation of intestinal ILC3 responses (**Figure 1**: *inputs*) (46). Triggering of GPR43 with the SCFAs propionate and acetate (but not butyrate) selectively promoted colonic ILC3 proliferation and expansion and production of IL-22, subsequently protecting mice from chemically induced colitis and from enteric bacterial infection (46).

Dietary Circuits

Cues derived from mutualistic microbiota establish a critical dialogue between the host and it's environment and regulate the intestinal immune system—including ILC3. In addition to the microbiota, the intestine is also continually exposed to metabolites and phytochemicals derived from the diet (**Figure 1**: *inputs*). As highlighted above, the availability and liberation of many dietary metabolites is also determined in part by mutualistic, commensal microbes within the intestine—while conversely the diet itself can modulate microbial composition and thus, determine the nature of host-commensal interactions. For example, the feeding of high fat diet (HFD) to pregnant mice was found to modify the expansion of ILC3 in the intestines of progeny through the modification of the mothers microbiota (47).

Similarly, Aryl hydrocarbon receptor (AhR) ligands are normally liberated from cruciferous vegetables in the diet—such as broccoli and cabbage, but they can also be microbially derived (48). AhR is a dietary-sensing nuclear receptor that is expressed by ILC3s and has critical roles in the development, transcription and function of these cells (**Figure 1**: *inputs*). Indeed, ILC3 are highly AhR dependent, and present severe functional impairments in the absence of cell-intrinsic AhR expression (14, 49–51). As a result of ILC3 defects, AhR-deficient mice fail to form tissue-associated lymphoid structures, such as cryptopatches (CP) and are unable to control infections with the extracellular pathogen *Citrobacter rodentium* (49, 52). Intriguingly, the development and seeding of intestinal ILC3 in neonates was demonstrated to be dependent upon the mothers microbiota and the transfer of antibody-bound AhR ligands through the mothers milk (48), suggesting maternal transfer of dietary ligands to neonates may play critical roles in the development of the immune system, microbial colonization and protection from infections in early life.

Indeed, maternal transfer of dietary ligands is increasingly appreciated to be a determinant of neonatal immunity and ILC3 development. *In utero* exposure to the Vitamin A metabolite retinoic acid (RA) impacts directly on secondary lymphoid organ development with long-term immunological consequences (53). Mice genetically modified to have hematopoietic cell-intrinsic deficiency in RA lacked PP or exhibited impairment in LN formation and maturation as a result of defective ILC3 differentiation (**Figure 1**: *inputs*). Moreover, it was shown that RA directly regulates the master transcription factor of ILC3, RORγt, and in the absence of maternal retinoids ILC3 failed to develop correctly (53). In addition to maternally derived RA signals, deprivation of vitamin A in adulthood also results in the collapse of the intestinal ILC3 populations and, as a consequence, results in susceptibility to *Citrobacter rodentium* infection (54, 55). In addition to direct effects of RA on ILC3 development, RA produced by DCs was also found to regulate the homing properties of ILC3s by imprinting expression of the intestinal homing markers CCR9 and α4β7 (56).

The importance of dietary vitamins in ILC3 effector circuits is further supported by evidence that vitamin D also plays a role in intestinal ILC3 homeostasis (**Figure 1**: *inputs*). ILC3 numbers in the small intestine of mice deficient for the vitamin D receptor (VDR—KO mice) were shown to be increased, as was IL-22 expression, resulting in enhanced resistance to infection with *Citrobacter rodentium* (57). Consistently, human ILC3s stimulated with IL-23 and IL-1β upregulate the VDR, and VDR signaling subsequently acts to downregulate the IL-23 signaling pathway—suggesting vitamin D acts as a negative regulator and suppressive feedback loop to control ILC3 activation (**Figure 1**: *inputs*) (58). Vitamin D availability has also been implicated in the pathogenesis of IBD, as patients are reported to have lower plasma levels of vitamin D than healthy subjects, and exhibit an upregulation of the IL-23 signaling pathway which could potentially explain exacerbated ILC3 responses that are associated with intestinal inflammation in IBD (58). In contrast to these studies, mice lacking *Cyp27B1*—an enzyme required for the conversion of vitamin D to it's chemically active form—exhibit reduced colonic ILC3 numbers and IL-22 production suggesting a more nuanced role for vitamin D in the regulation of ILC3 function (59). Together these findings highlight the importance of dietary cues in regulating ILC3 function and intestinal homeostasis. An increased understanding of the complex dialogue between diet, microbiota and host is likely to reveal novel immune regulatory circuits and clarify how environmental cues act as risk factors, and contribute to the onset of metabolic and inflammatory disorders.

ILC3 IMMUNE CROSSTALK IN THE ORCHESTRATION OF INTESTINAL HEALTH

Translating Microbial Cues: Myeloid—ILC3 Circuits

While ILC3 are potently regulated by the microbiota and diet within the intestinal environment, it remains unclear the extent to which they are able to directly sense these cues, beyond the pathways detailed above. Indeed, the majority of evidence suggests third party sensory cells of the myeloid lineage are required to directly sense, translate and communicate

environmental information to ILC3. Classically, tissue-resident mononuclear phagocytes (MNPs) act as key intermediaries and signal to ILC3 via the release of cytokine mediators during both homeostatic and protective immune responses (60, 61). Indeed, intestinal myeloid populations are well-equipped to directly sense microbial metabolites, pathogen associated molecular patterns (PAMPs) and danger signals and to transfer this information to ILC3 (**Figure 1**: *inputs*). In particular, CX3CR1$^+$ intestinal MNPs cluster with ILC3 in distinct, organized lymphoid structures, such as CPs (62, 63). Microbiota sensing by CX3CR1$^+$ MNPs was shown to result in local production of IL-1β and IL-23, which are key activating cytokines of ILC3 and which potently induce IL-22 secretion (**Figure 1**: *inputs*) (63). Depletion of CX3CR1$^+$ MNPs resulted in impaired IL-22 production by ILC3 and failure to control *Citrobacter rodentium* infection (62, 64, 65). In addition to the provision of the activating signals IL-23 and IL-1β, CX3CR1$^+$ MNP-derived TL1A further acts to augment IL-22 production from ILC3 (**Figure 1**: *inputs*) (62).

NEUROIMMUNE CIRCUITS

While microbial sensing by intestinal MNP and conserved crosstalk with ILC3 appear to be a major sensory circuit of intestinal immunity, emerging evidence suggests diverse sensory mechanisms across multiple biological systems provide additional *inputs* to regulate ILC3 function. In particular, the central and enteric nervous systems are rapidly being appreciated as critical sensory and immunoregulatory systems.

It has been suggested that the immune and nervous systems are evolutionary linked, since they share functional similarities (66, 67). Both nervous and immune systems rely on similar processes to for cellular communication; such as cell-cell contact and synapse formation, release of soluble mediators and sensing of circulating metabolites. Recent evidence suggests immune and neuronal cells are positioned in close proximity, and form conserved interactions that have been termed "neuro-immune cell units" (NICUs) (67). NICUS can form through interactions with both the central and peripheral nervous system and are increasingly being described in peripheral tissues such as the gastrointestinal tract and lung.

Neuroimmune interactions are evident very early in life—and during the embryonic period the development of the enteric nervous system (ENS) and SLO organogenesis share many parallels. Notably, the neurotrophic factor receptor RET is essential for the development of Peyer's patches (PP) and also the ENS (68, 69). Moreover, RET expression by CD11c$^+$ cells present in the anlagen initiates a cascade of immune cell recruitment, in particular of fetal ILC3s, through sensing of neurotrophic factors that drive the formation of primordial lymphoid clusters (68, 69). Moreover, increasing evidence suggests ILC3 can directly sense these neuronal derived *inputs* and respond during both development as well as in the adult intestine (**Figure 2**: *inputs*). As mentioned previously, fetal and adult ILC3 development and function relies on RA signaling (53). Intriguingly, neurons have been suggested to be a physiological source of RA (70), surprisingly suggesting RA may be derived not only from the diet but also from the host nervous system.

The ENS is increasingly appreciated to regulate tissue-resident immune functions (71), include those of ILC3. One pioneer study demonstrated that a glial-ILC3-epithelial axis is required to regulate enteric defense against bacterial infection (72). Like myeloid cells, intestinal glial cells also have the capacity to sense microbial cues and alarmins in a *Myd88*-dependent manner; thus, implicating the enteric nervous system as a key player in environmental sensing circuits. In response to these cues glial cells secrete neurotrophic factors, which directly act on adult ILC3 cytokine production via cell-intrinsic RET expression (**Figure 2**: *inputs*). Ablation of *Ret* in ILC3s led to a reduction in IL-22, consequently impairing epithelial function and host defense to enteric bacterial infection (72). In addition to ENS cues, CNS–derived signals propagated by the vagus nerve—via release of acetylcholine—have also been implicated in the regulation of ILC3 responses to bacterial infections in the peritoneal cavity (73). Vagal disruption was shown to lead to dysregulated ILC3 cell numbers in the peritoneal lavage (73). Mechanistically, acetylcholine acted to promote the release of pro-resolving lipid mediators—generated via ILC3-intrinsic expression of the PCTR biosynthetic pathway—which subsequently promoted protective immunity during *E. coli*-driven sepsis (73). Together, these studies illustrate the importance of neuronal *inputs* in regulating ILC3 *outputs* during infection (**Figure 2**).

Recent studies suggest that the number of neuropeptides with immunoregulatory activity may be broader than previously appreciated. Vasoactive intestinal peptide (VIP) release by enteric neurons was also shown to regulate ILC3-derived IL-22 production through signaling via VIPR2, triggered in part by feeding and dietary cues (**Figure 2**: *inputs*) (74, 75) (discussed in detail below). However, despite the strategic location of ILC3s within the CPs, which are enveloped by glial cell nervous fiber bundles and neuronal projections, the full extent of neuroimmune interactions that regulate ILC3 function are still to be determined. Indeed, recent years have seen an explosion in our understanding of neuroimmune signals that regulate other immune cells, including other members of the ILC family—most notably ILC2s (76–82). These studies have opened up new avenues of research and expanded our understanding of crosstalk between diverse biological systems, thus provoking the need for further studies to fully elucidate neuroimmune sensory circuits in the regulation of ILC3 responses, intestinal immunity and host-microbiota interactions.

ANTICIPATORY ILC3 RESPONSES AND CIRCADIAN CIRCUITS

In addition to local environmental cues, mammals are also constantly exposed to a range of external stimuli and pressures such as fluctuations in temperature, oxygen levels and the daily light cycle. As a result many organisms have evolved circadian rhythms to align core biological processes with time of day, which are imprinted by an internal biological clock. Specifically,

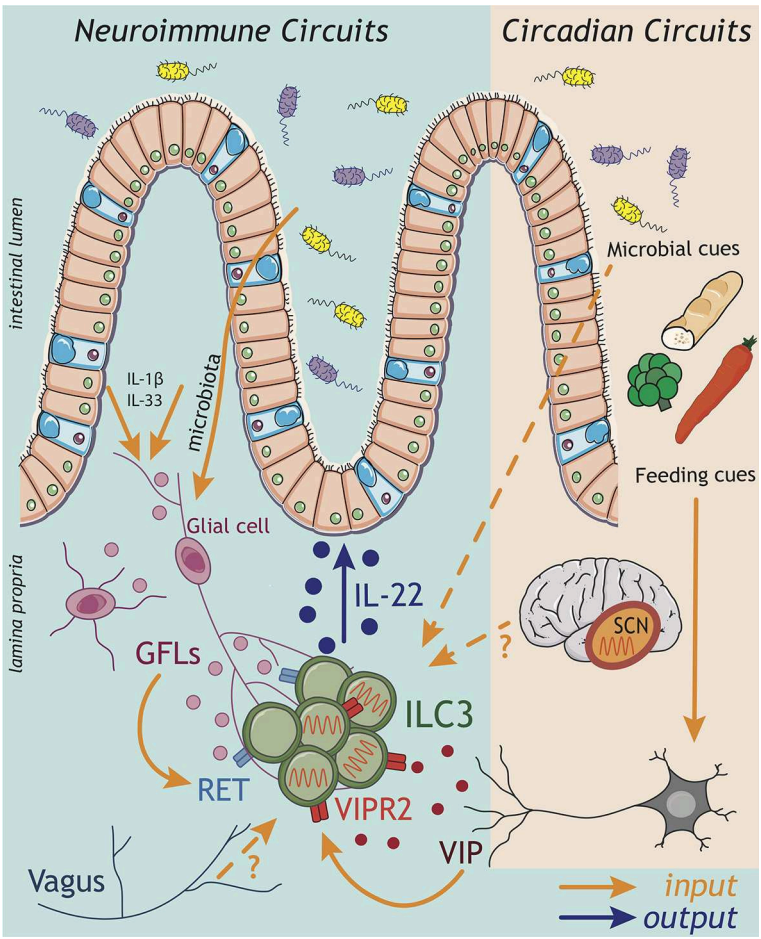

FIGURE 2 | ILC3 neuroimmune and circadian circuits. Emerging findings implicate *inputs* from the nervous system in the regulation of ILC3 circuits within the gastrointestinal tract. Both the central (CNS) and enteric (ENS) nervous systems have the capacity to sense perturbations within the intestinal environment and relay this information via the release of neuropeptides to influence the ILC3 response. Strikingly, enteric glial cells are able to directly sense microbial patterns and alarmins released within the tissue, and respond by producing glial-derived neurotrophic factors (GDNF family of ligands; GFL) that directly activate the production of IL-22 by ILC3 through the tyrosine kinase RET. Indeed, recent evidence suggests a broader spectrum of neuropeptides may act to regulate ILC3 function including vasointestinal peptide (VIP) produced by enteric neurons in response to feeding cues. Signals transmitted by the nervous system also play critical roles in aligning ILC3 effector function with periods of activity and high risks of environmental exposure and pathogen encounter over the course of a 24 h day. In this regard, circadian rhythms entrained by light—and sensed via the suprachiasmatic nucleus (SCN) of the brain—trigger a cascade of molecular transcriptional-translational feedback loops of clock genes, which orchestrate rhythms in the ILC3 response. While the "central clock" within the CNS appears to be a central entrainer of ILC3 oscillatory function in the gut, the mechanisms through which the CNS transmits this information to regulate ILC3 function peripherally remain unknown. Nonetheless, *inputs* from the CNS have previously been shown to be relayed to ILC3 via the vagus nerve. Together cues from both the CNS and ENS have the potential to entrain intestinal ILC3 function, while circadian rhythms in ILC3 may be imprinted through a combination of central clock-mediated light entrainment, feeding-associated neuronal feedback and environmental cues from the microbiota.

circadian rhythms are driven by cell-autonomous transcriptional feedback loops ("clocks"), which enable organisms to anticipate and adapt to temporal changes in their environment (e.g., changing seasons, jet lag, shift work) and regulate metabolically demanding biological processes including body temperature, locomotor activity, endocrine responses, and feeding behavior—while on the cellular level circadian clocks regulate cellular metabolism and cell cycle (83, 84). In line with this, it is increasingly appreciated that circadian rhythms also regulate immune cell responses (85), and immune cells exhibit circadian oscillations in leukocyte trafficking, priming, effector function and host-pathogen interactions (85).

In mammals, circadian rhythms are controlled by the central circadian pacemaker or master clock—located in the suprachiasmatic nucleus (SCN) of the brain (86). The SCN acts to interpret and propagate light cues received via the optical nerve and subsequently, cell autonomous circadian rhythms are imprinted by systemic signals that act to align oscillations in a tripartite system of transcriptional-translational feedback-loops (85, 87, 88). The induction of the loop starts with the transcriptional activators CLOCK and BMAL1 promoting the expression of the repressors Period (*Per*) and Cryptochrome (*Cry*), which in time translocate back into the nucleus and inhibit their own expression (85, 87, 88). The second loop is

composed by nuclear receptors RAR-related orphan receptors (RORs) (α, β, γ) and REV-ERBs (α, β), which exert opposing effects on the clock through transcription factor binding to the promoter of *Arntl* (encoding BMAL1) (85, 87, 88). Finally, the third loop consists of transcriptional activator albumin D-box binding protein (DBP) and the repressor nuclear factor for interleukin 3 (NFIL3), which act synergistically to regulate the expression of D-box genes including that of *Per* (85, 87, 88). Upon establishment of the transcriptional loops, the SCN keeps peripheral clocks in synchrony via neuronal sympathetic/parasympathetic transmission and through the hypothalamus pituitary adrenal (HPA) axis, including the release of catecholamines (epinephrine and norepinephrine) and glucocorticoids (84). Remarkably, similar circadian molecular mechanisms are found in the periphery. However, while the SCN network allows for the generation of sustained oscillations and time-of-day alignments, perturbations from environmental *inputs* such as temperature changes, the microbiota and feeding cues can also impact on peripheral, cell-intrinsic clocks (84).

Many constitutive innate immune processes, including the maintenance of intestinal barrier function via steady state IL-22 release from ILC3, come with significant metabolic costs for the host. Thus, circadian rhythms are thought to have evolved to align these processes with anticipated challenges and times of highest risk—most notably during waking activity and feeding where exposure to microbes, dietary antigens and potential pathogens is highest. Intriguingly, several components of the transcriptional circadian clock machinery including NFIL3 and RORγ/α are also key transcriptional regulators of ILC3 development and function, suggesting the possibility that these cells may also be regulated in a circadian manner (89–92). Moreover, ILC3 and IL-22 are critical regulators of the intestinal microbiota, with oscillations also reported amongst levels of commensal microbes in the intestinal tract (93, 94).

In line with this, several recent studies have demonstrated circadian control and oscillatory ILC3 responses, which are regulated by the master clock gene *Arntl* (Bmal1) in a cell-autonomous fashion (95, 96). Deletion of *Arntl* in ILC3s resulted in an altered epigenetic landscape, dysregulated cell numbers and IL-22 expression, and subsequently contributed to alterations in steady-state oscillations in the microbiome itself (95–97). Moreover, disrupted ILC3 responses resulted in altered epithelial responses and disrupted lipid uptake within the intestine (74, 95, 96). Of note, while deletion of *Arntl* led to a broad impairment of total ILC3 numbers (95), deletion of the related clock gene *Nr1d1* (also known as *Rev-erbα*) resulted in altered ILC3 subset development—with mice exhibiting a marked reduction NCR^+ ILC3s, while LTi-like ILC3 were unperturbed (97). Moreover, lack of *Nr1d1* increased expression of *Il17* in ILC3s, a mechanism previously reported in in Th17 cells (97, 98). Interestingly, ILC3s isolated from the inflamed intestine of patients with IBD presented with alterations in expression of several circadian-related genes, including *Nr1d1*, suggesting circadian clock disruptions—such as those seen in shift workers—may act to disrupt normal immune function and have relevance in the onset and/or pathogenesis of chronic inflammatory diseases (96). Of note however, the role of *Nr1d1* as a transcriptional regulator of both *Nfil3* and *Rorc* suggests clock-associated genes may have additional roles that are independent of circadian regulation (97).

Circadian rhythms may be imprinted by a range of systemic and environmental cues. Within the intestinal tract feeding cues were shown to contribute to the entrainment of oscillatory function in ILC3 (**Figure 2**: *inputs*). Time-specific feeding altered daily circadian rhythms in clock related genes (95) and IL-22 expression oscillated across the day between active and resting phases (74, 75). Interestingly, signals from the ENS appear to be critical in sensing feeding cues to entrain circadian rhythms in ILC3 (**Figure 2**) (74, 75). Feeding was found to induce VIP release from enteric neurons, consequently triggering VIPR2 signaling in ILC3s and *enhancing* IL-22 production and the barrier function of the epithelium. In contrast during fasting this neuropeptide cue waned, resulting in decreased IL-22 production by ILC3s and thus, imprinting diurnal rhythms onto intestinal ILC3s (74). In contrast, another study (pre-print currently under review) reported that VIP release from enteric neurons upon feeding rather *decreases* production of IL-22 by ILC3s, allowing for the outgrowth of the epithelial-associated commensal microbe SFB (75). Despite these discrepancies, both studies clearly implicate the sensing of feeding cues by the enteric nervous system as a key entrainer of circadian rhythmicity in ILC3. One possible explanation for the apparent differences in these findings is that complex interplay with the host microbiota may further augment ENS cues or act directly on ILC3 to provide complimentary or competing *inputs*, which then combine with cues from the central clock to tune anticipatory rhythms. In line with this, the microbiota was also shown to have an impact on circadian gene expression in ILC3s—adding another layer of complexity in the crosstalk between ILC3s and the commensal microbiota (95, 96).

While these studies all implicate peripheral cues in the entrainment of anticipatory ILC3 responses, light signals derived from the central clock (in the brain) are also known to be central in aligning many biological processes and in imprinting circadian rhythms. Indeed, signals from the central clock were shown to be a key regulator of ILC3 rhythmicity (**Figure 2**: *inputs*) (95). Utilizing mice in which the central clock was surgically ablated, or mice genetically deficient for *Arntl* only in the SCN, ILC3s developed disrupted cytokine oscillations and an altered phenotype—including the downregulation of intestinal homing markers which could partially explain time of day differences in ILC3 numbers within the gastrointestinal tract (95). The mechanisms through which the central clock in the SCN mechanistically aligns biological processes with light cues vary, but can include the release of hormonal cues—most notably glucocorticoids (84). While it remains to be determined whether this mechanism acts on ILC3 in the context of the central circadian clock, glucocorticoids have been shown to suppress ILC3s IL-22 production *in vitro* (99). Together these findings suggest that long-range and local circadian cues may directly regulate ILC3 numbers and function during homeostasis or following infection, mediating ILC3 interactions with the microbiota and regulation of intestinal barrier function.

LYMPHOID ORGANOGENESIS: ILC3-STROMAL CIRCUITS

Unlike cells of the adaptive immune system, ILC3 are one of the first immune cells to colonize the intestine during the embryonic period and are critical for the formation of SLOs (100). In this regard one of the most fundamental circuits through which ILC3 contribute to barrier immunity is through the orchestration of organized interactions between the innate and adaptive immune system. In contrast to the sensory circuits described above, where *inputs* derived from third party cells stimulate *outputs* in ILC3, during both embryogenesis and adult life ILC3 provide the *input* and stimulatory cues to stromal cells to initiate a cascade of events that lead to the formation of secondary and tertiary lymphoid tissues.

The formation of LN and PP is initiated via specialized stromal cells, known as lymphoid tissue organizer cells (LTo) that start to express chemokines such as CXCL13, CCL19, CCL21, as well as the adhesion molecules VCAM-1, ICAM-1, and MadCAM-1 (101, 102). The expression of these factors creates a gradient to recruit *bona fide* fetal lymphoid tissue inducer cells (LTis; fetal members of the ILC family, referred to here as fetal ILC3), which cluster with the LTo forming the primitive anlagen of the SLO (103). Fetal ILC3s at this stage express CXCR5, CCR7, and α4β7; homing markers that are important for fetal ILC3 recruitment and which were shown to mediate migration toward LTo-derived chemokines and adhesion molecules, respectively. In fact, full maturation of LTo and development of lymphoid tissue is dependent on recruitment of fetal ILC3s and provision of lymphotoxin (LT) (103, 104). Conversely, LTo also provide critical survival signals for fetal LTi/ILC3 with IL-7 expression shown to be necessary for ILC3 maintenance, while IL-7R blockade in adults also resulted in a rapid loss of normal migration of B and T cells to the LN (105). This stromal IL-7 circuit is likely also active at other sites such as in the fetal liver and bone marrow, where stroma derived IL-7 signaling could trigger the expression of NFIL3 (91). In addition, the same stromal-ILC3 circuit acts to restore normal lymph node architecture following infection-induced disruption of lymphoid microanatomy (106). Therefore, the crosstalk between ILC3s and lymph node-associated stroma is reactivated in in adulthood and crucial to enable adaptive immune responses during secondary infections (106). Thus, a key sensory circuit and stimulatory loop formed between ILC3 and stromal cells is critical for the formation of lymphoid tissues, and to facilitate the action of the broader innate and adaptive immune system.

Postnatally, a large number of organized lymphoid structures designated as tertiary lymphoid structures start to form under the influence of environmental stimuli. These immune cell clusters include cryptopatches (CP), which are confined to bottom of the crypts within the intestinal lamina propria. CP formation is driven through similar molecular mechanisms to SLO, including via interactions between ILC3-associated LTα1β2 with the LTβR expressed by stromal cells and IL-7 signaling (107, 108). CPs can further give rise to isolated lymphoid follicles (ILFs) in a CCR6 and LTα1β2-dependent manner (109, 110), resulting in up-regulation of secretory antibody (Immunoglobulin A; IgA) synthesis in response to changes in the composition of microbiota (111, 112).

A unique feature of ILF development in comparison to LN, PP, and CPs is the requirement for microbial exposure. Intestinal bacteria are sensed by myeloid cells which increase the interactions between ILC3s and LTos, also via a LTα1β2 dependent axis, leading to increased expression of adhesion molecules by the stroma and recruitment of B cells to these structures (113, 114). ILFs are largely absent in a microbiota free environment, and are restored upon recolonization with commensal microbes (115). Similarly inflammation and intestinal barrier disruption results in increased numbers of ILFs in the colon, and intriguingly mice deficient in the transcription factor RORγt develop more ILFs than their wild type counterparts in the context of intestinal inflammation, suggesting a potential regulatory role for type 3 immune responses, such as ILC3, in this setting (116).

Interactions between ILC3 and stroma also provide important cues to localize ILC3 to defined tissue microenvironments, and to facilitate interactions with adaptive immunity (discussed in detail below). Within the intestine-draining mesenteric lymph node multiple distinct stromal populations have been identified with differential capacities to attract immune populations and orchestrate immune cell crosstalk (117). One such population expresses the enzyme *Ch25h,* which acts to generate the cholesterol metabolite 7,α25-OH—a key migratory ligand for multiple immune cells including ILC3 (117–120). This stromal cue is sensed by ILC3 via the receptor EBI2 (*Gpr183*), and facilitates localization of not only ILC3 but also T follicular helper cells, DCs and B cells to the follicular border of lymph nodes (118, 120–125). Similarly, within the intestinal tissue stromal generation and breakdown of cholesterol ligand cues create a migratory gradient required to recruit ILC3 to CP in a *Gpr183*-dependent manner (119). Together these studies indicate that a stromal ILC3 circuit is a key regulator not only of lymphoid organogenesis but also of ILC3 localization and function, which together facilitate the interactions between ILC3 and adaptive immune cells and foster modulatory crosstalk.

CIRCUITS OF IMMUNE ORCHESTRATION: CROSSTALK BETWEEN ILC3 AND ADAPTIVE IMMUNITY

ILC3s are also emerging as key orchestrators and regulators of adaptive immune responses [Reviewed in detail in (126)]. This regulation is mediated by ILC3 either through indirect modulation of bystander cells that subsequently modulate the adaptive immune response or directly via both soluble mediators and cell contact-dependent interactions with adaptive lymphocytes.

As discussed above, ILC3s contribute to the formation of lymphoid structures and were found to be strategically positioned in clusters within lymph nodes where they have potential to interact with both T and B cells both directly and indirectly

(127). Many of the same mechanisms employed by ILC3 to induce lymphoid organogenesis during early life are similarly employed in adult tissues to regulate the adaptive immune system. For example, ILC3s can support the production of IgA by B cells in the PP, in part through both soluble $LT\alpha_3$ and surface bound LTα1β2 interactions with DCs (128, 129). Similarly, in the spleen, production of LTα1β2, GM-CSF, and BAFF/APRIL production by ILC3s also acts to support B cell responses (**Figure 3**: *outputs*) (130).

In line with these findings, ILC3s have the capacity to crosstalk both directly and indirectly with the adaptive immune system through the production of multiple soluble factors. Following exposure to the commensal microbiota IL-22 produced by ILC3s acts to support homeostatic tissue Th17 responses through the induction of serum amyloid protein A (SAA) from epithelial cells (**Figure 3**: *outputs*) (131). Interestingly, ILC3 derived IL-22 can also prevent the activation of T cells in an AhR-dependent manner to limit immune activation or tissue damage (132). Conversely, T cells may also regulate the magnitude of ILC3-derived IL-22 production (26, 133), suggesting complex crosstalk between T cells and ILC3 in determining the level of IL-22 produced in the tissue.

As highlighted previously, sensing of the microbiota by myeloid cells is a critical regulator of ILC3 responses, and has consequences for adaptive immunity. IL-1β induction of GM-CSF production by ILC3s feeds back on tissue-resident MNP to trigger IL-10 and RA production by intestinal macrophages and DCs—resulting in the induction and maintenance of tissue regulatory T cells (Treg) and reinforcing immune tolerance (**Figure 3**: *inputs*/*outputs*) (134). Similarly, IL-1β produced by intestinal MNP further induces ILC3 to produce IL-2, a critical growth signal that helps to support peripherally induced Tregs in the small intestine and to maintain intestinal tolerance (**Figure 3**: *inputs*/*outputs*) (135). Conversely Treg interactions with MNP may limit IL-23 production to prevent ILC3-driven inflammation via a LAG3-dependent mechanism (**Figure 3**: *outputs*) (136), implicating a bidirectional axis involving ILC3, MNP, and Treg in determining the immune tone of the intestinal tract.

ILC3s are increasingly appreciated to also act as a direct orchestrator of tissue immune responses through their ability to act as antigen-presenting cells. ILC3 are also endowed with a broad array of accessory co-activating and co–inhibitory molecules that enable further modulation and tuning of adaptive immune cell function. Thus, when coupled with their strategic localization within lymphoid structures, ILC3 have the potential to potently regulate adaptive immune responses. At steady state, ILC3s in the mLNs and large intestine constitutively express MHC class II (MHCII) molecules at levels comparable with other professional antigen-presenting cells and can acquire, process and present antigens (**Figure 3**: *inputs*/*outputs*) (137). However, under homeostatic circumstances these interactions do not induce T cell proliferation, due in part to the absence of classical co-stimulatory molecules such as CD40, CD80, and CD86 on the cell surface (137). In contrast $MHCII^+$ ILC3s were found to suppress effector $CD4^+$ T cell responses toward the microbiota in the intestine (137–139). In line with a suppressive function for ILC3-associated antigen presentation, deletion of ILC3-intrinsic MHCII also disrupts crosstalk between ILC3 and adaptive immune cells at the interfollicular border of the mLN resulting in a spontaneous T follicular helper response that subsequently drives increased IgA responses against mucosal-dwelling commensals, and results in an altered intestinal metabolome (**Figure 3**: *outputs*) (120). While these findings suggest a suppressive and regulatory role for antigen-presenting ILC3 in the context of health, additional reports suggest that in contrast during immunization or infection tissue-specific inflammatory cues act to alter the nature—and consequences—of ILC3 antigen presentation. Indeed, activation of ILC3 by IL-1β resulted in antigen-presentation dependent *promotion* of T cell responses as a result of upregulated expression of classical co-stimulatory molecules (CD80/CD86) on ILC3 (140). In addition to antigen-presentation to $CD4^+$ T cell subsets, ILC3 also express CD1d—conferring the ability to present lipid antigens to invariant (i) NKT cell populations, and promote their functionality (141).

Indeed, ILC3 have the capacity to modulate a broad variety of specialized adaptive immune responses through cell-cell interactions via additional non-classical co-stimulatory and co-inhibitory molecules. Seminal early studies in the field demonstrated a critical role for ILC3-associated CD30L and OX40L in the modulation of T cell memory through cognate interactions with CD30/OX40 (**Figure 3**: *inputs*/*outputs*) (126, 142, 143). Recent studies have expanded upon these observations to demonstrate a role for tissue-resident MNP-derived TL1A in regulating the expression of OX40L on ILC3, which was subsequently demonstrated to enable ILC3 to promote inflammatory effector T cell responses in the context of colitis (144). In addition ILC3 have been reported to express co-inhibitory and immune checkpoint molecules (e.g., PD1, PDL1) suggesting further immunoregulatory functions for these cells—although further investigation is required to determine the functional relevance of this receptor repertoire (145, 146). As investigation into this aspect of ILC3 function increases, the nature and breadth of interactions with both innate and adaptive immunity are likely to expand and present new intervention possibilities for the modulation of tissue immune responses.

CONCLUSIONS AND FUTURE PERSPECTIVES

The maintenance of mucosal homeostasis is mediated through a complex interplay between the host and its environment, between immune and non-immune cells and by the balance of pathogenic and commensal microbes. Here we have highlighted the contributions of sensory circuits within the intestinal tract, which culminate in the activation and regulation of ILC3s. ILC3 display connectivity with an increasing number of physiological systems, many of which are likely to act simultaneously within the tissue in the context of health and disease—and ultimately to regulate the same range of ILC3-derived *outputs*. Thus, despite recent advances, one future challenge will be to

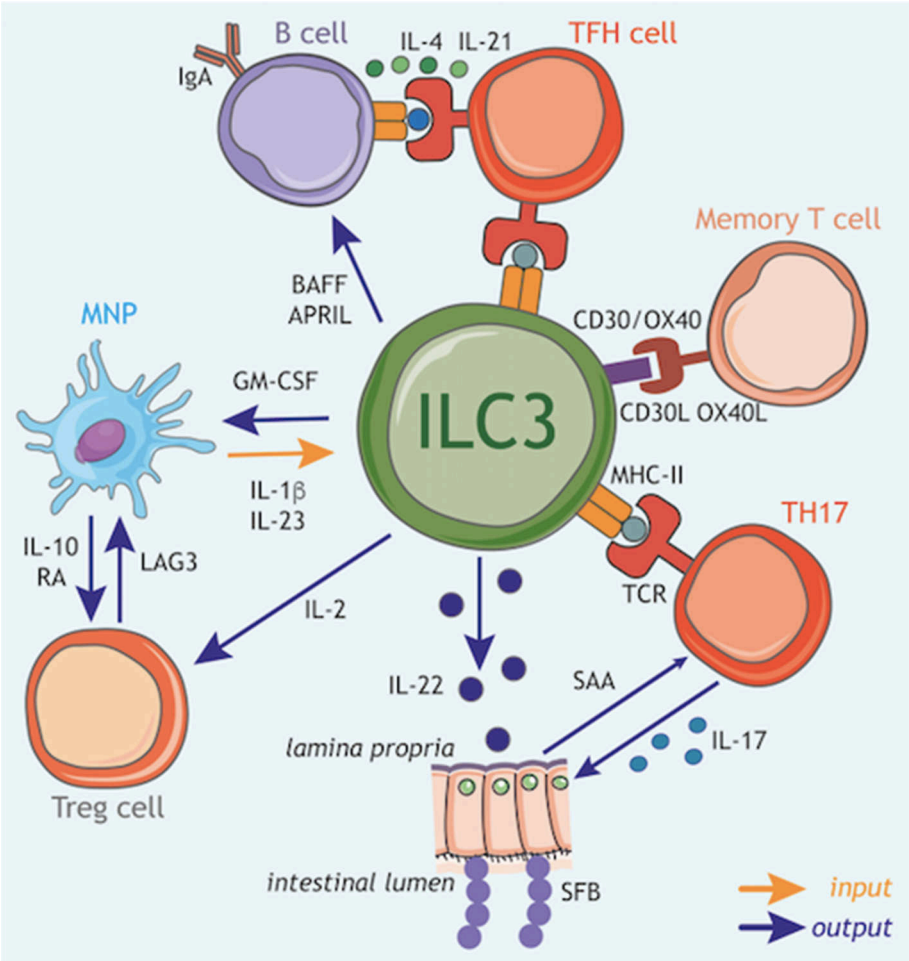

FIGURE 3 | ILC3 circuits orchestrate adaptive immune responses. In addition to their function as tissue-resident cytokine producing cells, ILC3s have the capacity to participate in multiple cellular circuits through direct cell–cell modulation of T cell responses, as well as the release of soluble mediators that augment adaptive immune function and development. ILC3s can control the magnitude and quality of the $CD4^+$ T cell response via antigen presentation in the context of MHC class II (MHCII). At steady state ILC3s lack co-stimulatory molecule expression and appear to limit $CD4^+$ T cell responses, however this interaction may be altered in inflammatory scenarios via upregulation of co-stimulatory molecules such as CD40, CD80, and CD86, which favor the promotion of T cell response. Furthermore, ILC3s act to modulate the survival of recirculating memory $CD4^+$ T cells via interactions via OX40L and CD30L, although it is unknown whether this process also requires MHCII-dependent antigen presentation. In addition, ILC3 regulation of T follicular helper (TFH) cell responses has consequences for the priming of germinal center B cells and the induction of T-dependent IgA responses toward colon-dwelling commensal microbes. ILC3s can also modulate adaptive immune cells through the production of regulatory cytokines and growth factors. In line with this, ILC3 directly support B cell responses in the spleen through provision of critical growth factors such as BAFF/APRIL. Similarly, ILC3 also modulate the magnitude of the T cell response within the intestinal tract through the production of soluble mediators. For example, ILC3-derived IL-22 induces epithelial serum amyloid A (SAA) protein, which subsequently promotes local Th17 responses and acts to limit colonization with segmented filamentous bacteria (SFB) via the induction of antimicrobial peptides. In addition, ILC3 facilitate the establishment of a regulatory and tolerogenic environment in the gut by promoting regulatory T cell (Treg) responses. ILC3 crosstalk with tissue-resident myeloid cell populations establishes a feedback circuit whereby ILC3-derived GM-CSF promotes IL-10 and RA production by myeloid cells to promote Treg conversion. Conversely, Treg, myeloid cells and ILC3 may feedback on each other through a variety of soluble and cell-cell interactions suggesting a dynamic and malleable communication loop to ensure tolerance and tissue homeostasis. Finally, ILC3 subsets are a potent source of IL-2 in the small intestine that provides survival signals for Treg. Together these tissue-resident immune circuits place ILC3 at the center of a number of pathways through which they regulate adaptive immune responses to promote tissue health and homeostatic interactions with the microbiota.

understand how ILC3 integrate multiple concurrent signals from varying biological systems within a given tissue niche, and to determine how these cues are translated into cell fate decisions to determine the magnitude or quality of an ILC3 response. Many signaling pathways downstream of both cytokine and neuropeptide receptors converge upon core regulators of cell function—such as the mammalian target of rapamycin (mTOR) (147). Moreover, the appropriate licensing and modulation of anabolic cell metabolism pathways in order to generate new cellular biomass, effector proteins and facilitate proliferation is a central checkpoint of cellular function, critical to regulate immune cell function and controlled in part through mTOR activation (148). In line with this, a recent report demonstrated the induction of an mTOR complex 1-dependent programme

of glycolytic metabolism as a central rate-limiting step in the production of ILC3-derived cytokines and proliferation (149). Engagement of glycolysis was also associated with the expression of the oxygen-sensing transcription factor HIF1α, suggesting other tissue-specific environmental factors may augment ILC3 responses via licensing of glycolysis and anabolic metabolism.

Ultimately, an increased knowledge of the network of *inputs* and *outputs*—and importantly the mechanisms through which these multiple sensory circuits are integrated and interpreted—will allow for new approaches to target this mucosal immune sentinel in the context of health and disease. Indeed, while ILC3 mediate many protective processes at homeostasis, dysregulated ILC3 responses have been implicated in a wide range of chronic inflammatory and metabolic diseases and have increasingly been suggested to play roles in cancer development and progression. Most notably, disruption of ILC3 responses is associated with the pathogenesis of inflammatory bowel disease (IBD) (1, 27, 40, 41, 62, 138, 150). Interestingly, lifestyles associated with disruption of sleep cycles and circadian rhythms (e.g., shift work, jet lag) have been suggested as potential triggers for IBD flares (151). Thus, while there have been recent major achievements in the understanding of how ILC3 sense signals from the CNS and ENS and perceive circadian cues (72, 74, 75, 95–97), the physiological impact of these systems on ILC3 function in the context of IBD could prove important in beginning to decode the multitude of factors that lead to disease onset and progression.

In conclusion, ILC3 are strategically positioned within mucosal sites where they act as a hub of multiple distinct, yet complementary, sensory circuits. Together, these circuits act to continually survey the intestinal tract for perturbations in microbial, dietary and external environmental cues and enable the rapid communication and translation of this information, resulting in protective effector responses that continually reinforce normal tissue function and health. Strategies aimed at exploiting these cues and sensory circuits to promote or restore homeostatic ILC3 function, while simultaneously suppressing the dysregulated signaling associated with maladapted immune function, may lead to novel therapeutic intervention strategies in a number of human diseases.

AUTHOR CONTRIBUTIONS

MH and RD conceived of and contributed to the writing of the manuscript. RD constructed the figures.

FUNDING

The Hepworth Laboratory was supported by a Royal Society and Wellcome Trust Sir Henry Dale Fellowship (Award 105644/Z/14/Z) and a Lister Institute of Preventative Medicine Prize.

ACKNOWLEDGMENTS

We thank members of the Hepworth laboratory for the discussions and critical reading of the manuscript.

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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.

frontiers in Immunology

REVIEW
published: 21 February 2020
doi: 10.3389/fimmu.2020.00242

Innate Lymphocytes in Psoriasis

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Skin is a fundamental component of our host defense system that provides a dynamic physical and chemical barrier against pathogen invasion and environmental insults. Cutaneous barrier function is mediated by complex interactions between structural cells such as keratinocytes and diverse lineages of immune cells. In contrast to the protective role of these intercellular interactions, uncontrolled immune activation can lead to keratinocyte dysfunction and psoriasis, a chronic inflammatory disease affecting 2% of the global population. Despite some differences between human and murine skin, animal models of psoriasiform inflammation have greatly informed clinical approaches to disease. These studies have helped to identify the interleukin (IL)-23-IL-17 axis as a central cytokine network that drives disease. In addition, they have led to the recent description of long-lived, skin-resident innate lymphocyte and lymphoid cells that accumulate in psoriatic lesions. Although not completely defined, these populations have both overlapping and unique functions compared to antigen-restricted αβ T lymphocytes, the latter of which are well-known to contribute to disease pathogenesis. In this review, we describe the diversity of innate lymphocytes and lymphoid cells found in mammalian skin with a special focus on αβ T cells, Natural Killer T cells and Innate Lymphoid cells. In addition, we discuss the effector functions of these unique leukocyte subsets and how each may contribute to different stages of psoriasis. A more complete understanding of these cell types that bridge the innate and adaptive immune system will hopefully lead to more targeted therapies that mitigate or prevent disease progression.

Keywords: innate, psoriasis, lymphocyte, skin, disease

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *30 October 2019*
Accepted: *29 January 2020*
Published: *21 February 2020*

Citation:
Polese B, Zhang H, Thurairajah B and King IL (2020) Innate Lymphocytes in Psoriasis. Front. Immunol. 11:242. doi: 10.3389/fimmu.2020.00242

INTRODUCTION

The skin is the largest barrier organ. The most superficial layer of mammalian skin consists of an avascular, stratified epithelial layer that provides a physical and chemical barrier to environmental insults, is responsible for hair formation and supports a diverse commensal microbial community that promotes colonization resistance to invasive pathogens. Underlying the epidermis is the dermis composed of a fibroblast network providing structure for a complex neurovascular system that regulates heat transfer, pain sensation, and host defense (1). The epidermis and dermis harbor unique leukocyte subsets that are not only central to cutaneous immunity, but also contribute to basic skin physiology including wound healing, hair follicle cycling, and lipid production by sebaceous glands. Given the intimate relationship between immune-structural cell interactions, it stands to reason that aberrant communication within this compartment can lead to altered host defense mechanisms and/or dysregulated skin inflammation and disease. One of the most common cutaneous inflammatory diseases is psoriasis. Affecting between 2 and 5% of the adult

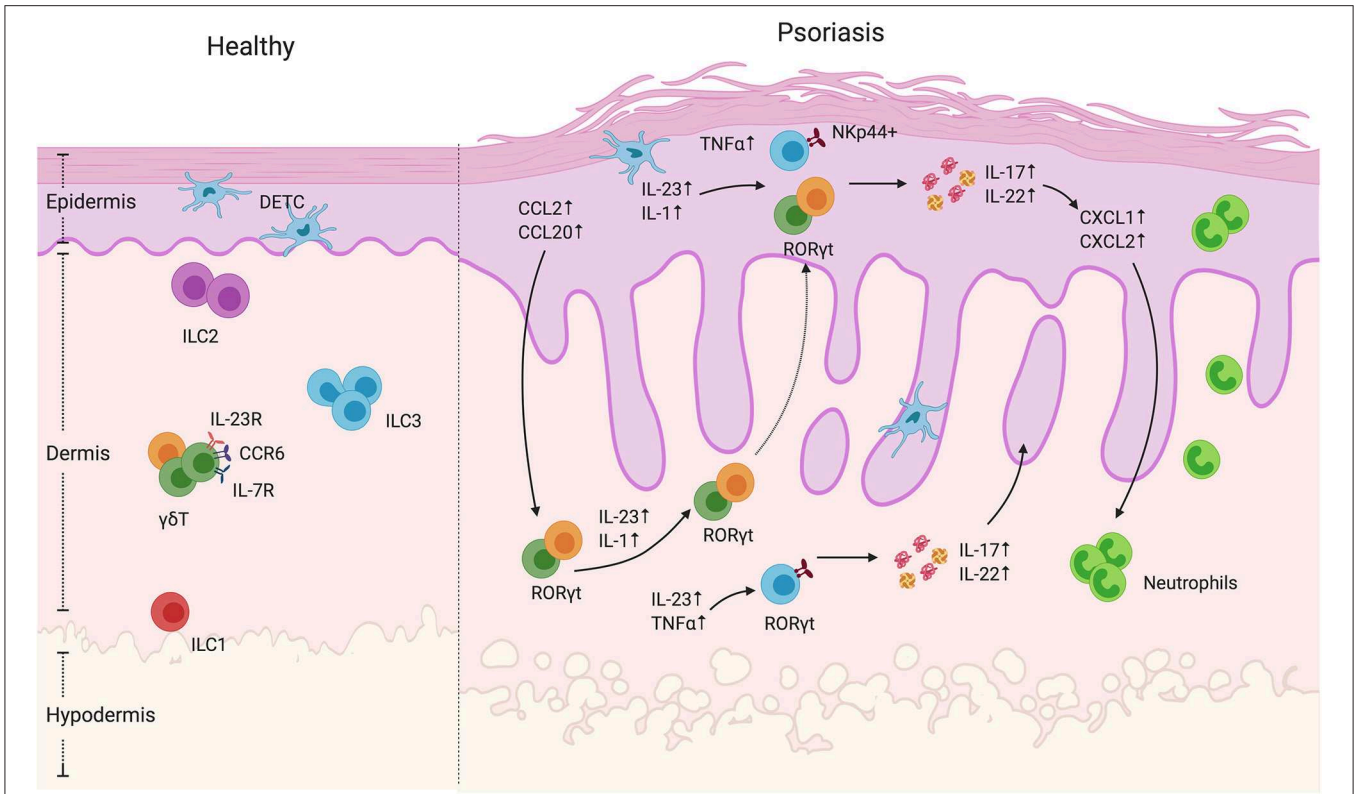

FIGURE 1 | γδ T cells and ILCs in psoriatic skin. Diverse subsets of γδ T cells colonize the skin. Under homeostatic conditions, the mouse epidermis contains dendritic epidermal T cells, which are a monoclonal population of $V\gamma5^+$ cells. The dermis contains $V\gamma4^+$ and $V\gamma6^+$ γδ T cells enriched for expression of IL-23R, CCR6, and IL-7R. In mouse models of psoriasiform inflammation, activated keratinocytes produce chemokines such as CCL2 and CCL20, which subsequently recruit dermal γδ T cells to the epidermis. Among these γδ T cells, there is a subgroup that express the transcriptional factor RORγt, that are capable of producing IL-17 and IL22 upon IL-1 and IL-23 stimulation. Both mouse and human studies have shown that, upon cytokine stimulation, dermal-derived γδ T cells secrete IL-17 and IL-22 that drives keratinocyte hyperplasia, neutrophil recruitment and disease progression. ILCs are also present in the healthy skin. They are divided into three groups based on transcription factor expression and effector functions. Under steady-state conditions, ILC2 are the largest population. ILC3 are currently thought to be the dominant population that contribute to disease progression. In human skin lesions, $NKp44^+$ ILC3s are able to produce IL-22 and IL-17 that exacerbate disease progression.

population in developed countries, psoriasiform inflammation varies in severity but is most commonly characterized by red, scaly plaques across the surface of the body in a form referred to as psoriasis vulgaris. Although the etiology of psoriasis has not been identified, both environmental and genetic factors have been shown to contribute to incidence and severity of disease (1–3). Importantly, psoriasis is associated with comorbidities such as atherosclerosis and metabolic syndrome suggesting systemic dysregulation of the immune response in these patients providing further motivation for understanding disease pathogenesis (1, 4). Despite some differences between human and rodent skin, animal models of "psoriasiform" inflammation have been instrumental in identifying the immunological mechanisms underlying psoriasis development. For example, the models described in more detail below have helped to determine the interleukin (IL)-23/IL-17 axis as central to disease progression (1, 5). The essential role of these cytokines has been validated by the clinical efficacy of humanized monoclonal antibodies targeting TNFα, IL-23, IL-17A, and the IL-17 receptor (6, 7). However, these treatment approaches have limitations. First, they are not curative; symptoms reappear upon cessation of treatment. Thus, biologics must be given throughout the patient's lifetime. Second, the IL-23/IL-17 immune axis plays an important role in protection against cutaneous pathogens such as *Candida* and pathobiotic *spp.* of *Staphylococcus areus* (8, 9), thus raising questions about the long-term use of these treatments regarding susceptibility to infection. Furthermore, these biologics do not specifically target the skin and may compromise host defense at other barrier sites such as the intestine. Therefore, further investigation into the initiating factors that drive psoriatic disease will not only enrich our knowledge of skin biology in general, but lead to more targeted, tissue-specific treatments for this chronic inflammatory disease.

The recent discovery of immune cell subsets that are resident to the skin such as γδ T cells and innate lymphoid cells (ILCs) has prompted a growing interest in how these and other better known cell types that blur the separation between the innate and adaptive immune system such as Natural Killer (NK) cells and NKT cells contribute to psoriasiform inflammation. Indeed, these cells serve as acute sensors of infection and tissue

injury without the need for specific recognition of antigen. While these properties have likely evolved to respond rapidly to tissue changes, their non-specific activation requirements leave them susceptible to hyperreactive responses against innocuous stimuli. In this review, we describe the diversity of innate lymphocyte lineages present in the skin and our current understanding of how each subset contributes to the pathogenesis of psoriatic disease.

THE CUTANEOUS γδ T CELL COMPARTMENT

Of the innate T lymphocytes in the skin, γδ T cells, defined by expression of gamma (γ) and delta (δ) TCR subunits, are the most studied. Their innate classification comes from two main characteristics: first, the repertoire of γ and δ chains possess less diversity than their more classical αβ TCR counterparts. Second, γδ T cells do not require TCR engagement in order to expand and exert their effector functions. Rather, cytokines alone are sufficient to endow γδ T cells with cytotoxic and cytokine-producing ability (10).

In mice, γδ T are usually distinguished based on the γ chain expression. It is worth mentioning that two nomenclatures are often used but rarely specified in the literature, namely the Heilig and Tonegawa vs. the Garman classification. In this review, we will use the Heilig and Tonegawa nomenclature only, which includes the Vγ1–Vγ7 subtypes (11). Each subtype has a propensity to localize to specific organs as well as exert unique effector functions. Their development and migration to the epithelial tissues starts during fetal life (12–14) with consecutive waves associated with different γδ T subsets migrating from the thymus to their specific tissue (10, 15). From day E13, the Vγ5 subtype is produced in the thymus and migrates to the epidermis (**Figure 1**). Vγ5 γδ T cell development is exclusively fetal and occurs only in mice. These cells are called dendritic epithelial T cells (DETC) due to their morphology, are non-migratory and are maintained by self-renewal (16, 17). As DETC seem to be most relevant for maintaining skin homeostasis and wound repair and have been reviewed extensively elsewhere, we will not be discussing this subset further. On the other hand, Vγ4 and Vγ6 subtypes constitute the dermal γδ T cell compartment (**Figure 1**). Unlike DETCS, dermal γδ T cells are motile with $V\gamma6^+$ cells seeding the dermis during fetal life and $V\gamma4^+$ cell recruitment limited to the first days of life (18). Accordingly, the dermal γδ T cell compartment can be replenished after irradiation, but only if neonatal thymocytes are transferred (19).

Vγ usage is also associated with a specific effector function profile. In fact, γδ T cells can be largely defined based on their expression of lineage-restricted transcription factors and effector functions. The most prominent subsets include IFNγ (γδ1) and IL-17 producing γδ T cells (γδ17) that rely on the transcription factors T-bet and RORγt, respectively, for their differentiation (20). Interestingly, however, γδ T cell effector functions are uniquely imprinted in the thymus where SOX13 drives γδ T cell lineage commitment and subsequent TCR dependent and independent mechanisms that dictate effector functions (21). For example, CD27 is a thymic determinant of γδ T cell fate by promoting γδ1 over γδ17 cells and inducing IFNγ-associated genes (22). Additionally, strong TCR engagement favors IFNγ-producing γδ T development (23) while limiting γδ17 development (24). As a result, IFNγ and IL-17-producing γδ T subsets can be identified on the basis of CD27 and CCR6 expression, amongst other markers (22, 25). Dermal $V\gamma4^+$ and $V\gamma6^+$ γδ T cells express several hallmarks similar to Th17 cells including RORγt, IL-7R, CCR6, and IL-23R expression as well as ability to produce IL-17 (19, 26). They can be stimulated by IL-23, which leads to their expansion and IL-17 production (26) (**Figure 1**). Dermal γδ T cells have been associated with immunosurveillance functions. In the context of mycobacterial infections, they have been shown to be the dominant source of IL-17 and their absence was correlated with diminished immune response to BCG immunization (27). Furthermore, IL-17 production by dermal γδ T can be stimulated by various microbe-derived products (26), further emphasizing their immune sentinel role. As $V\gamma6^+$ cells are rarely found in secondary lymphoid organs, MacKenzie et al. suggested that this subset might have specifically evolved for immunosurveillance of non-lymphoid tissues while the more migratory, lymphoid organ-skewed Vγ4 subset might serve as a pool that is rapidly mobilized to barrier sites following challenge (28).

In humans, γδ T cells are usually distinguished based on δ chain expression including Vδ1, Vδ2, and Vδ3 (i.e., Vδ1-Vδ2-) subtypes. Vδ1 cells seed barrier tissues while Vδ2 and Vδ3 are observed in the blood of healthy patients (29). Similar to murine γδ T cells, human γδ T cells are potent cytokine-producing cells, but the regulatory mechanisms are less understood. Unlike murine γδ T cells, human γδ T cells are more dependent on TCR engagement for activation and appear to produce a greater diversity of effector cytokines. For example, human γδ17 cell differentiation, which likely occurs in the periphery since they are absent from the human mature thymus (30), requires IL-23 and TCR activation. Furthermore, Vγ9Vδ2 cells that represent the majority of the Vδ2 subset, exhibit remarkable heterogeneity in term of surface markers and cytokine production. These plastic cells are able to produce IFNγ, IL-4, or IL-17, which contrasts with murine γδ T cell commitment (31).

As opposed to mice, human γδ T cells are rare in the skin with Vδ1-expressing cells being the dominant subtype observed in healthy skin, mainly in the dermis. With the help of αβ T cells (16), Vδ1 seem to recapitulate the role of DETC given that they present a restricted repertoire (32), can be observed in the epidermis, produce keratinocyte growth factors and exert anti-tumor activity (31, 33). $V\delta1^+$ cells are also usually associated with IFNγ production and a cytotoxic profile (34). Notably, human dermis-derived γδ T cells have been shown to produce IL-17A. In fact, Cai et al. found IL-17-producing γδ T cells to be enriched in psoriatic skin lesions. However, the full repertoire of cutaneous γδ T cells has yet to be investigated (26).

γδ T Cells Are Major Contributors to Murine Psoriasiform Inflammation and Implicated in Human Disease

Two mouse models of cutaneous inflammation are most commonly used to study the mechanisms underlying psoriasiform inflammation. The imiquimod (IMQ) model that consists of topically applying a TLR7/8 agonist emulsified in a cream or intradermal injection of recombinant IL-23 (5, 35). Both approaches lead to epidermal hyperplasia, parakeratosis, and expansion of rete ridges, all features of psoriasiform inflammation (36). These preclinical models have been shown to depend on the presence of IL-17 for fulminant inflammation and motivated clinical trials the development of neutralizing antibodies targeting IL-23, IL-17A, or IL-17RA (the receptor for both IL-17A and IL-17F) (5–7, 26). The incredible clinical success of these biologics has validated these models and led to further investigation into the cell types driving IMQ and IL-23-induced inflammation (6, 7). Importantly, both models revealed decreased inflammation and psoriasiform symptoms inflammation in mice genetically lacking γδ T cells (TCRδ$^{-/-}$) compared to mice deficient in αβ T cells (TCRα$^{-/-}$) mice (26, 37). Importantly, TCRδ$^{-/-}$ mice reconstituted with Vγ4 and Vγ6 subpopulations restored disease susceptibility (18). Similarly, selective depletion of Vγ6^{+} or Vγ4^{+} γδ T cells using antibody-mediated or genetic depletion approaches indicate that both subsets are necessary and sufficient for IMQ-induced inflammation (38, 39). Interestingly, Vγ4^{+} γδ T cells have been shown to have memory-like capacity. Indeed, two papers have demonstrated that this γδ T cell subset persists in the skin after termination of IMQ treatment and exhibits classical features of memory cells upon secondary IMQ challenge (i.e., a more rapid response with greater magnitude) in the same area or even distant sites (40, 41). Ramirez-Valle et al. further demonstrated that the migration and recruitment to distant sites was mediated via CCR2 signaling (41). They showed that IMQ-activated Vγ4^{+} T cells expressed less CCR6 than unchallenged γδ T cells and that the former subset demonstrated increased responsiveness to IL-1. Downregulation of CCR6 was unexpected as it was previously shown that both models of psoriasis induce CCL20 (42), the chemokine recognized by CCR6, and that a CCL20/CCR6 axis was essential for disease progression (38, 43) (**Figure 1**). Induction of CCL20 leads to dermal IL-17^{+}γδ T cell recruitment into the epidermis, exacerbating inflammation. Accordingly, an anti-CCL20 antibody treatment reduced IL-23-induced inflammation by decreasing the γδ T trafficking into the epidermis (42). In the latter study, the source of chemokine secretion was not identified but it has been demonstrated that IL-1β can stimulate keratinocyte production of CCL2 and CCL20, which might impact γδ T cell recruitment (18). In addition, activated dermal γδ T cells increase expression of X-linked IL-1 receptor accessory protein-like 1 (IL1RAPL1) which promotes a feedforward system inducing more IL-17 production by these cells. IL-38, a cytokine of the IL-1 family secreted by keratinocytes at steady state, is able to restrict γδ T cell activity by inhibiting IL1RAPL1 on the surface of γδ T cells (44). Accordingly, the levels of IL-38 secreted by the keratinocytes is decreased in psoriatic lesions as well as in mouse skin following IMQ treatment (44, 45). These results underline the loop that exacerbates psoriasis, where inflammation induces keratinocytes secretion of chemokines, which in turn triggers γδ T cell recruitment. The pro-inflammatory environment leads to cytokine production by γδ T cells, which promotes keratinocyte hyperproliferation and epidermal thickening.

TABLE 1 | Cytokines produced by innate immune cells during psoriasis.

Cytokine	Cell types	References
IL-17A	γδT cell, ILC3, NK cell	(18, 26, 37, 38, 46–49)
IL-22	γδT cell, ILC3, NK cell	(37, 47, 50)
IL-25 (IL-17E)	γδT cell	(51)
IFNγ	NK and NKT cell	(52–55)
TNFα	NK and NKT cell	(53)

Although γδ T cells are capable of cytotoxic activity, their potent cytokine production seems to play a dominant role in psoriasiform inflammation (**Table 1**). In the IMQ model, both IL-17 and IL-22 production by RORγt $^{+}$ γδ T cells, Vγ4^{+} cells in particular, is greatly increased (37) (**Figure 1**). Consistent with these results, IL-17R deficient mice showed reduced and delayed signs of psoriasiform inflammation such as ear thickness and erythema after IMQ treatment (56). However, disease was not completely abolished in IL-17R deficient mice and increased levels of TNFα, IL-6, and IL-22 as well as IL-17-producing cells were observed in the skin. This demonstrates the importance of IL-17 signaling for psoriasiform inflammation, but also suggests an alternative pathway for IMQ-induced inflammation. Similarly, IMQ-induced inflammation was strongly reduced in mice with a keratinocyte-specific deletion of the IL-17 receptor (57). In another study using the IMQ model, the main producer of IL-22 was also γδ T cells. However, in Rag-deficient mice that lack mature T and B cells, levels of IL-22 in response to IMQ remained elevated suggesting an alternative source of cutaneous IL-22 (50). Although it was shown that, in addition to IL-17, IL-22 is required for IL-23 induced inflammation, the failure of clinical trials using anti-IL-22 antibodies have kept the focus on the effector functions of IL-17 and its associated family members. In fact, a recent report showed that IL-17E (better known as IL-25) signaling via IL-17RB also plays an important role in IMQ-induced psoriasiform inflammation (51). This work was recently followed up by studies demonstrating that IL-17A can signal via an alternative receptor, IL-17RD, to drive psoriasiform inflammation (58). To conclude, γδ T cells are major contributors to murine psoriasiform inflammation via the production of IL-17 and IL-22 (**Figure 1**, **Table 1**). The Vγ4 subtype is particularly implicated in the disease due to its quick cytokine response, migration capacities and long-lasting memory capacity.

Such as in mice, γδ T cells are expanded in human psoriatic skin and produce IL-17A (26) (**Table 1**). A population of Vγ9Vδ2^{+} cells that express IL-17A, IFNγ, TNFα and CCR6 has been specifically observed in human psoriatic lesions (59). These cells were able to activate keratinocytes and stimulate

chemokine, cytokine and defensin production. Laggner et al. also showed that Vγ9Vδ2$^+$ cells were increased in psoriatic skin compared to healthy skin and, even more, increased in lesional skin compared to non-lesional skin of the same patients (59). In addition, Vγ9Vδ2$^+$ cells were reduced in psoriatic patient blood. Finally, they showed a negative correlation between blood levels of Vγ9Vδ2$^+$ cells and psoriasis severity. These results suggest that the Vγ9Vδ2$^+$ population is recruited from the peripheral blood to the skin where they activate keratinocytes and contribute to psoriasis development. On the other hand, it has been recently shown that the majority of IL-17A producing T cells observed in human psoriatic lesions are oligoclonal αβ T cells and not γδ T cells (60). Furthermore, mast cells have been shown to produce IL-17A and IL-22 in human psoriatic plaques (61). The diverse subsets previously found to be expressing and/or producing IL-17 cytokines in human psoriasis and disparate results between groups continues to fuel a controversy over the most relevant cytokine-producing cells for psoriatic disease development and progression. Longitudinal studies using large, diverse patient cohorts may help reconcile these differences.

THE INNATE LYMPHOID CELL SKIN POPULATION

ILCs are bone marrow-derived tissue-resident lymphocytes that, although arising from common lymphoid progenitors, do not express rearranged antigen-specific receptors. ILC nomenclature is largely analogous to CD4$^+$ T helper effector cell subsets: ILC1s express the transcription factor T-bet and secrete IFNγ, ILC2s express GATA3 and produce the Th2 cytokines IL-5 and IL-13 and ILC3s express RORγt and secrete IL-17 and IL-22. Although ILCs are thought to be largely tissue-resident cells (62), ILCs have been detected in the circulation that express high levels of cutaneous leukocyte-associated antigen (CLA), a skin homing marker (63). In both mice and humans, all three groups of ILCs have been observed in the skin with ILC2s being the largest population (63–65). Furthermore, a study examining the cutaneous ILC population in mice showed that different layers of the skin are populated differentially by ILCs: the epidermis is mainly populated by ILC3s, the subcutaneous layer is populated by ILC2s and the dermis contains both ILC2s and ILC3s (66) (**Figure 1**). However, the signals that result in the differential homing of ILCs in the skin and whether this is representative of human ILC populations is not completely understood. ILC1s, although present in the skin, are a rare population with unknown functions. Although sharing several features with natural killer (NK) cells, ILC1s do not exert cytotoxic activity—lack perforin and granzyme expression—and do not express traditional NK cell antigens such as CD56, CD16, or CD94. However, the cytokine profile of ILC1s, most notably IFNγ, resembles NK cells and has been shown to play a role in the protection against intracellular pathogens (62, 67, 68). As ILC1s are thought to contribute to Crohn's disease and inflammation in a mouse model of colitis (69, 70), they could potentially play similar roles in the skin both in terms of protection as well as autoimmune-like pathology, however this has not been thoroughly investigated. ILC2s on the other hand are much more common in the skin and are thought to play a role in maintaining skin homeostasis. For example, ILC2s have been shown to promote wound healing in the skin through the production of IL-13 (71, 72). Skin-resident ILC2s can also produce high levels of amphiregulin, a molecule regulating wound healing (73). In dermatitis, amphiregulin has been shown to play a role in wound healing by acting as an epidermal growth factor receptor (EGFR) ligand (74). However, other evidence indicates the involvement of ILC2s in allergic-type or type 2 inflammation of the skin, namely atopic dermatitis likely through dysregulated production of type 2 cytokines such as IL-5 and IL-13 (73, 75, 76). Lastly, ILC3s are one of the subtypes of immune cells in the skin capable of producing IL-17A and IL-22 and are therefore of specific interest when discussing psoriasis (**Figure 1**).

ILC3s Are Observed in Human Psoriatic Skin and Correlate With Disease Severity

While ILC3s seem to play a role in the development and maintenance of psoriasis, the role of ILC1 and 2 subsets is a matter of debate (**Figure 1**). Some groups found a reduction in ILC2 numbers in psoriatic patients (64) while others saw no difference in frequencies. Notably, different methods of tissue processing from skin biopsies in these studies may explain the differences (63–65). Given that ILC2s are known to play a role in maintaining skin homeostasis and wound healing (71, 72), they may also be playing a protective role during the development of psoriasis. Second, these studies did not indicate involvement of ILC1s (63–65). However, one group reported a significant increase in the number of ILC1s in psoriatic skin (77); this latter group detected the number of ILCs using imaging of whole skin whereas the other groups performed flow cytometry which may explain the difference. Since ILC1s in the gut seem to play a role in inflammatory pathologies, it is possible that ILC1s may also be paying a role in inflammatory pathologies in the skin such as psoriasis. When looking at the cells in circulation, both healthy individuals and psoriatic patients have a similar mean frequency of ILCs in total peripheral blood mononuclear cells (PBMCs) (65). However, there seems to be an overall increase of ILCs in psoriatic skin (**Figure 1**). This increase in ILCs is mainly due to an increase of ILC3s (63, 64, 77). NKp44 has been associated with pro-inflammatory functions in ILC3s, its activation leading to TNFα production (78). ILC3s in the skin of healthy patients were shown to be mainly NKp44–, whereas NKp44$^+$ ILC3s were barely detectable in the skin and blood (63). NKp44 expression is induced in NKp44-ILC3s upon IL-1β and IL-23 stimulation, cytokines commonly present in psoriatic inflammation (63) (**Figure 1**). In psoriasis patients, the levels of NKp44$^+$ ILC3 but not NKp44– ILC3s were increased in the blood, lesional, and non-lesional skin. Furthermore, psoriasis severity as measured by the PASI scoring system positively correlated with the number of cutaneous NKp44$^+$ ILC3s (63–65). These data suggest that the amount of NKp44$^+$ ILC3s in the blood or the skin can potentially be used as a biomarker for disease severity. Furthermore, ILCs in psoriatic skin were seen to be in close proximity to T cells, suggesting a crosstalk between ILCs and T cells during the development of psoriasis (77). Given the innate features of ILC3s

and their largely tissue-resident nature, these cells may contribute to the initiation of psoriatic inflammation. Indeed, ILC3s alone were able to induce psoriasis in a human skin xenotransplant mouse model to a degree similar to αβ T cells (79). Furthermore, patients with psoriatic arthritis, a disorder with similar features of psoriasis but with joint involvement, also had an increased ILC3: ILC2 ratio (80).

As mentioned above, IL-17 producing γδ T cells have been shown to be important drivers of IMQ-induced inflammation (81). However, it has been shown that Rag-deficient mice are still susceptible to psoriasiform inflammation via IMQ (37, 43), indicating that cells other than T cells play a role in the pathogenesis. Using Rag1/IL-2R deficient mice lacking T cells and ILC, Pantelyushin et al. showed that RORγT$^+$ γδ T cells and RORγt$^+$ ILC contribute to IMQ-induced psoriasiform inflammation (37). Furthermore, anti-TNFα or TNFα inhibitor treatment has been demonstrated to be a very effective treatment for psoriasis (82). TNFα plays a role in psoriasis development by synergizing with IL-23 to induce IL-17 producing cells, including ILC3s (46). Individuals undergoing successful anti-TNFα (adalimumab) treatment for psoriasis had a reduction in the number of pathogenic NKp44$^+$ ILC3s and an increase in NKp44– ILC3s in the circulation (65), suggesting that a major role of TNFα in the pathogenesis of psoriasis includes potentiating pathogenic ILC3s. However, it was elegantly demonstrated that γδ17 were non-redundant effector cells in murine skin pathology (81). Indeed, when γδ17 cells were deleted from birth, they were replaced by IL-17 producing ILC3s that promoted IMQ-induced inflammation. However, acute depletion of γδ17 cells did not lead to ILC3 accumulation and mice remained resistant to psoriasiform inflammation. In summary, ILC2s appear dominant in healthy skin whereas NKp44$^+$ ILC3s are the major ILC subset associated with psoriatic disease. Although ILC3s and γδ17 cells may play overlapping roles in murine models of psoriasis, more studies are needed to discern their relative contributions to human disease.

CUTANEOUS NK AND NKT CELLS

Natural Killer (NK) cells are a group of innate immune cells with both cytotoxic and cytokine producing effector functions and have been recently classified as one of two ILC1 subsets (83, 84). Through germ-line encoded activating and inhibitory receptors, NK cells can respond quickly following activation, releasing pro-inflammatory cytokines particularly IFNγ, chemokines, or specialized cytotoxic granules to infected or tumor cells (85). In human and mice, there are two distinct populations of NK cells, circulating NK cells (cNK, CD49a$^+$CD103$^-$ or CD56^{dim}CD16$^+$ in human and CD49a$^-$DX5$^+$ in mice) and tissue-resident NK cells (trNK, CD49a$^-$CD103$^+$, or CD56^{bright}CD16$^-$ in human and CD49a$^+$DX5$^-$ in mice) (**Figure 2**); both can induce cytotoxicity and produce cytokines (86–89). Murine skin is composed of both trNK cells and cNK cells (87) (**Figure 2**). However, the cNK and trNK cells do not share the same development pathways. cNK cells are derived from the bone marrow, continue their maturation in the thymus and then the spleen, tonsils and lymph nodes (90, 91). In mice, the transcription factors T-bet and Eomes are required for the maturation of cNK cells (92). In humans, both T-bet and Eomes are co-expressed in mature cNK cells (93). T-bet is expressed at lower levels in cytokine-producing CD56^{bright}(CD56^{hi}CD16$^-$) NK cells than the highly cytotoxic CD56^{dim} (CD56^{lo}CD16$^+$) NK cells, while CD56^{bright} NK cells have higher frequency of Eomes$^+$ cells than CD56^{dim} NK cells (93), indicating that there is a gradual loss of Eomes expression during the development of CD56^{bright} cells to T-bet^{hi}Eomes$^+$ CD56^{dim} cells. trNK cells were first discovered in the murine liver, strictly require T-bet, Hobit and PLZF for their development, however do not express Eomes (87, 89). Murine liver trNK cells are capable of degranulation and produce similar IFNγ levels to cNK cells. However, both the liver IFNγ$^+$ and degranulating trNK cells produce TNFα, which is rarely seen among responding cNK cells (87). Unlike mouse trNK cells, human liver trNK cells have high Eomes expression rather than T-bet (94). Of note, the features and developmental pathways of trNK cells differ from one organ to another. In the murine gut and dermis, the development of NKp46$^+$CD3$^-$ trNK cells is reported to be dependent on the transcriptional factor RORγt and RORγt$^+$ trNK cells are capable of producing IL-22 (95). The origin of skin trNK cells is unclear, but murine studies show that skin trNK cells share some features with liver trNK cells, in terms of phenotype, function and developmental requirements. They are CD49a$^+$DX5$^-$ with no Eomes expression, and their development is dependent on IL-15 and IL-15R. Human CD56^{bright}CD16$^-$NK cells are present in the dermis at steady state and disease conditions such as psoriasis, while CD56$^+$CD16$^+$ cNK cells are rare (52, 96, 97). These CD56^{bright}CD16$^-$ dermal NK cells lack perforin and NKG2D expression but are capable of lysing melanoma cells after activation *in vitro* (97). Recently, studies have found IL-17 and IL-22 producing NK cells in both humans and mice, which indicates the potential for NK cell participation in the development of psoriasis (47–49).

Natural Killer T (NKT) cells are present in both human and mouse skin (**Figure 2**). However, the composition of NKT cells is not well-defined. In human allergic contact dermatitis, for example, NKT cells range from 1.72 to 33% of the T lymphocyte infiltrate and in human atopic dermatitis patients, the proportion of NKT cells in CD3$^+$ T cells is ∼5% (98, 99). In murine skin, they compose ∼0.03% of total healthy skin cells and ∼0.6% of total hyperplastic skin cells (100). NKT cells are a unique hybrid between αβ T cells and NK cells as they co-express an αβ TCR and NK cell lineage markers. NKT cells are divided into four categories with type 1 (referred to as invariant NKT cells) being the vast majority (101). Compared to conventional T cells, they express a semi-invariant TCR α chain (Vα14-Jα18 in mice and Vα24-Jα18 in human), which allows specific recognition of glycolipids presented on an atypical MHC Class I molecule, CD1 (102–104). α-galactosylceramide (α-GalCer), a compound derived from marine sponges, has a strong CD1d binding affinity and is a potent stimulant for iNKT cells. Potential endogenous ligands of NKT cells were previously believed to be glycosphingolipids (GSLs) and phospholipids that are derived from bacterial, plant, protozoan, and mammalian

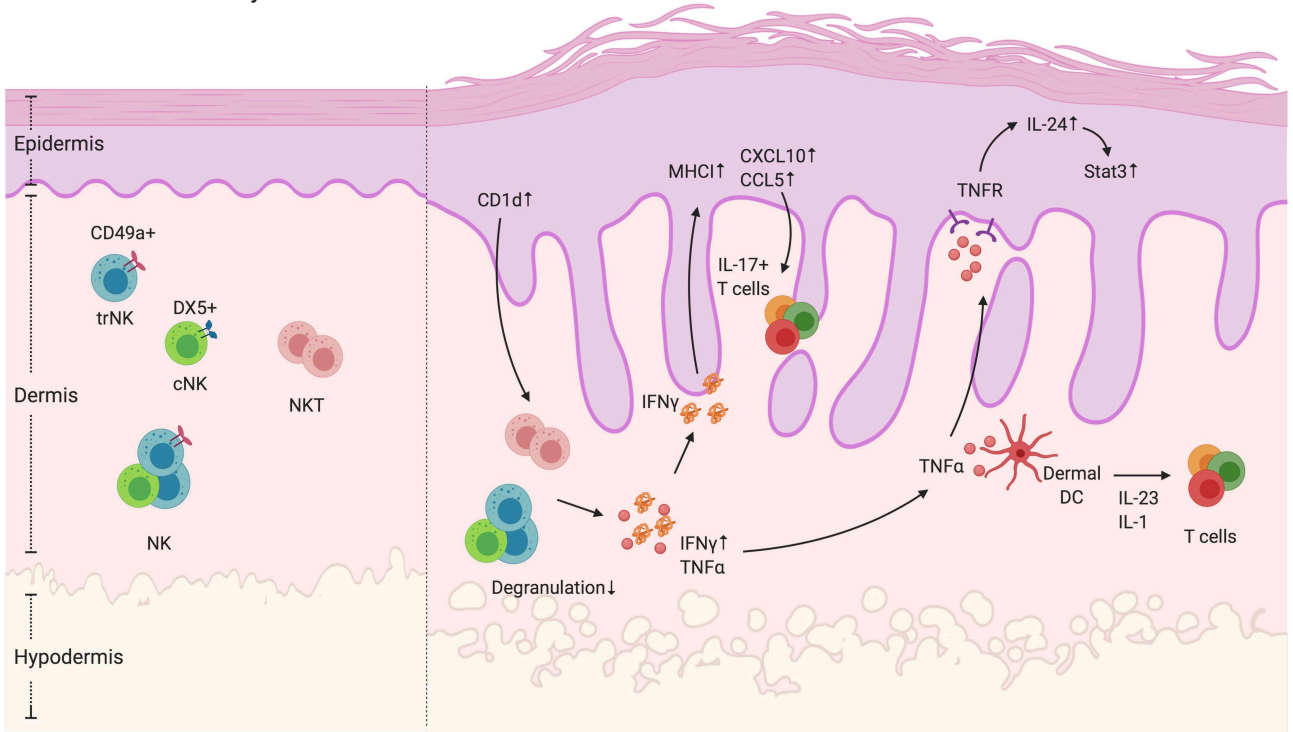

FIGURE 2 | NK and NKT cells in psoriatic skin. NK and NKT cells are innate immune cells that have cytokine-producing and cytotoxic functions. They both reside in the dermis. The NK cells can be divided into two groups, namely cNK and trNK cells, based on the receptors CD49a and DX5. Unlike NK cells, NKT cells also express an antigen-specific TCR that recognizes glycolipid through CD1 presentation by antigen-presenting cells. In psoriatic skin lesions, NK and NKT cells are rare. However, CD1d expression is reported to be elevated in keratinocytes in inflamed skin. In addition, NK and NKT cells have decreased degranulation ability, but display increased IFNγ production. High IFNγ production can contribute to an increase in keratinocyte-derived chemokines such as CXCL10 and CCL5, and the elevated expression of MHCI, both of which increase cell recruitment and presentation of autoantigens. In addition, NK and NKT cells produce TNFα that activate keratinocytes in an IL-24/Stat3-dependent manner as well as indirectly enhance dermal IL-17$^+$T cell activation by facilitating dendritic cells to produce IL-1 and IL-23.

species. However, more recent studies suggest that NKT cell ligands are more diverse and not limited to GSLs (105, 106). Thus, the endogenous ligands of NKT cells are still being clarified. When stimulated with α-GalCer or its analogs, NKT cells rapidly produce pro- and anti-inflammatory cytokines including IFNγ, TNFα, IL-10, IL-4, IL-13, IL-17 and GM-CSF, and participate in the regulation of infection, autoimmunity, and tumor immunity (107). Unlike NK cells, NKT cells undergo positive and negative selection within the thymus, but emerge later in development than most other T cell subsets (108, 109). During the selection process, NKT cells are only selected when CD1 is expressed on double positive ($CD4^+CD8^+$) thymocytes, which segregates the NKT cell (CD161 low in human and NK1.1- in murine at this stage) from the conventional T cell developmental pathway (110–114). The transcriptional factors Ras, Mek, Fyn, and Ets1 are reported to participate in the development of murine NKT cells, and the cytokine IL-15 and its receptor IL-15R are important during NKT cell development (115–118). After selection, the immature human CD161low or murine NK1.1- NKT cells either stay in the thymus or migrate to peripheral tissues, where they undergo a maturation process with the upregulation of CD161 (human) or NK1.1 (murine) expression (108, 109). The transcription factor T-bet was shown to participate in the terminal maturation of NKT cells (119). Both mouse and human NKT cells can exert cytotoxicity and produce seemingly antagonistic IL-4 and IFNγ cytokines upon TCR stimulation (120, 121). However, cytokine production may be developmentally regulated as mature NKT cells produce high levels of IFNγ while IL-4 is dominantly produced by immature NKT cells (108, 109). Recent data showed that NKT cells can also secrete Th17-related cytokines such as IL-17A, IL-17F, and IL-22 (107, 122, 123). A murine CD4-NK1.1- NKT cell group, which is the precursor of CD4-NK1.1$^+$ NKT cell, has been found to constitutively express RORγt and IL-23R and is a major source of IL-17$^+$ NKT cells (107). In addition, α-GalCer-activated murine NKT cells, that can express RORγt and IL-17, but not IFNγ or IL-4, develop in a c-Maf dependent way. These IL-17$^+$NKT cells are essential for inducing neutrophil-rich airway inflammation (122). In humans, even though RORγt$^+$ T-betloPLZF- NKT cells are found in the circulating PBMCs, the IL-23R expression is almost completely absent on circulating NKT cells. These NKT cells show poor IL-17 release after IL-23 stimulation. However, TCR

stimulation (e.g., α-GalCer or αCD3/CD28Ab) in the presence of IL-2, IL-23, IL-1β and TGFβ1, NKT cells successfully produce IL-17 but not IFNβ or TNFα (123). Interestingly, there are more IL-23R$^+$ NKT cells in the PBMCs and joint compartment of Spondyloarthritis patients than healthy controls, showing an IL-17 signature (123), which suggests that NKT cells could participate in the development of psoriasis. Although cutaneous NKT cells are important for the anti-microbial response due to their ability to recognize the bacterial glycolipids via CD1d presentation (124), they may function differently in cutaneous diseases, a result that may depend on the microbial and/or self-antigen repertoire of the skin. It has been shown that large numbers of NKT cells can be recruited into human skin during contact dermatitis, producing mainly IFNγ (98, 99) however, results in animal studies are controversial. Murine NKT cells were previously reported to suppress this response by producing IL-4 and IL-13 in response to CD1d-presented haptens (125), while it was also reported that murine NKT cells enhance the contact sensitivity reaction (126–128). Different results may be explained by the animal model studied, which shape the NKT cell cytokine repertoire. Studies have found decreased number of circulating Vα24$^+$ NK T cells in atopic dermatitis patients, and they produce both IL-4 and IFNγ (99, 129). NKT cells were also shown to suppress skin transplant rejection, through the production of IL-4 (130–132). To conclude, even though the proportion of NK and NKT cells is rare, they do participate in cutaneous immunity through diverse effector programs.

NK and NKT Cells Are Rare in Psoriatic Skin

The role of NK and NKT cells in psoriasis development is not clear. Even though studies showing involvement of NK cells in psoriasis are rare, NK cells have been shown to be present in psoriatic skin. Human studies show that NK cells are recruited in psoriatic plaques, particularly in the dermis (52, 133) (**Figure 2**). The psoriatic lesion-isolated NK cells exhibited low degranulation ability. However, their cytokine-producing ability is dependent on the source of NK cells (52, 53). Ottaviani et al. observed higher IFNγ production by NK cells isolated from psoriatic lesions and showed that IFNγ was able to induce keratinocyte chemokine production (such as CXCL10 and CCL5) and MHC-I expression (52) (**Figure 2**, **Table 1**). Consistent with the human data, mice treated with IMQ had increased NK1.1$^+$ cells in the skin, which suggests that either NK or NKT cells were recruited into the skin during psoriasiform inflammation (134). Another study showed that NK cells from PBMCs of patients with psoriasis vulgaris have reduced cytotoxicity and lower levels of pro-inflammatory cytokines IFNγ and TNFα (53). However, questions remain about NK cells in the context of psoriasis. Psoriasis was initially thought to be a IFNγ related disease but more recent studies—and the success of biologics targeting the IL-17 pathway—indicate a more dominant role for TNFα and IL-17 driven disease (1, 135, 136). As suggested above, TNFα and its associated receptors have been reported to be elevated in psoriatic lesions compared to non-lesional skin and TNF-R is abundantly expressed by keratinocytes (137, 138). It has been reported that TNFα signaling is involved in IL-24-induced psoriasis like inflammation in mice (139). In addition, both TNFα inhibitors and blocking antibodies show efficacy in alleviating psoriatic arthritis symptoms (140). Since both IFNγ$^+$ and degranulating skin trNK cells produce TNFα (87), it is possible that skin NK cells participate in the progression of psoriasis by the production of TNFα rather than IFNγ. To address this question, TNFα production by NK cells in the skin of healthy control and psoriasis patients needs to be addressed. To date, there is no direct link between IL-17 signaling and NK cell function in psoriasis. However, NK cells have been implicated in protection from oral and dermal Candidiasis infections that requires IL-23 and IL-17 signaling (8, 141, 142). Whether NK cells participate in psoriasis via IL-17 signaling needs to be further explored. A concern about human NK cell studies is that CD56 is routinely used as a marker for NK cells, however, CD56 is also found on human IL-17 and IL-22-producing ILCs (47, 143, 144). Therefore, these studies do not exclude other CD56$^+$ ILCs in the involvement in psoriasis.

The NKT frequency within the psoriatic lesions is very low—<0.1%—indicating that they are an unlikely determinant of psoriasis development (52). However, Nickoloff et al. showed that *in vitro* co-culture of NKT cells with CD1d-overexpressing keratinocytes is able to directly induce NKT production of IFNγ and IL-13. In addition, the *in vivo* injection of psoriasis lesion-derived NKT cells into the pre-psoriatic engrafted skin in SCID mice could successfully induce psoriatic plaques (54), indicating a potential role of NKT cells in the psoriasis progression. Of note, the previous attempts to use IFNγ$^+$ CD3$^+$/CD4$^+$ T cell lines to induce psoriasis using this experimental approach were unsuccessful (145). This effect may be due to increased skin-infiltrating CD8 T cells (54), which predominantly generate IL-17 responses in human psoriasis lesions (146). This result is consistent with a human study showing that in psoriatic lesions, CD1d expression was highly enhanced in keratinocytes, which may activate the NKT cells to produce more IFNγ, thus contributing to the progression of psoriasis (55) (**Figure 2**). However, as previously mentioned, IL-17, TNFα, and GM-CSF production by NKT cells should also be also examined. Finally, the frequency of NKT cells expressing inhibitory receptors rather than activating receptors (CD158b$^+$ and/or CD94/NKG2A$^+$) was elevated in the circulation of psoriasis patients and correlated with disease severity (147). To conclude, even though they are rare in psoriatic lesions, NKT might contribute to plaque development by IFNγ production, thus recruiting more immune cells such as IL-17 producing T cells to exacerbate the disease progression.

CONCLUSION

γδ T, ILC, NK, and NKT cells have all been shown to be increased in psoriasiform inflammation in humans and mice. Consistently, evidence suggests a correlation between disease severity and peripheral blood levels of γδ T, ILCs, and NKT. In addition, murine models lacking γδ T and/or ILCs demonstrated their essential role in psoriasiform inflammation

development suggesting that NK and NKT cells likely play a more subtle role, a finding largely supported by studies of plaque psoriasis in humans. One fundamental characteristic of innate cells is their ability to respond rapidly and produce comparatively large amounts of inflammatory mediators in the absence of cognate antigen. Consistent with these traits, γδ T, ILCs, and NKT are all able to produce cytokines that have established pathogenicity in psoriasis. These results suggest that despite the relative rarity of these populations in psoriatic lesions, they may be more amenable to non-specific dysregulation with important consequences for disease. Interestingly, the emerging concept of "innate memory" (148), as implicated in γδ T cell-driven psoriasiform inflammation, increases the complexity of these unique leukocytes and raises new questions about their roles in complex diseases such as psoriasis.

AUTHOR CONTRIBUTIONS

HZ and BT wrote the manuscript. BP and IK determined the topic and wrote the manuscript.

FUNDING

Support for this publication is provided by the Canadian Institutes of Health Research, fund PJT-148552.

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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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REVIEW
published: 20 March 2020
doi: 10.3389/fimmu.2020.00308

Neuro-Immune Circuits Regulate Immune Responses in Tissues and Organ Homeostasis

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The dense innervation of the gastro-intestinal tract with neuronal networks, which are in close proximity to immune cells, implies a pivotal role of neurons in modulating immune functions. Neurons have the ability to directly sense danger signals, adapt immune effector functions and integrate these signals to maintain tissue integrity and host defense strategies. The expression pattern of a large set of immune cells in the intestine characterized by receptors for neurotransmitters and neuropeptides suggest a tight neuronal hierarchical control of immune functions in order to systemically control immune reactions. Compelling evidence implies that targeting neuro-immune interactions is a promising strategy to dampen immune responses in autoimmune diseases such as inflammatory bowel diseases or rheumatoid arthritis. In fact, electric stimulation of vagal fibers has been shown to be an extremely effective treatment strategy against overwhelming immune reactions, even after exhausted conventional treatment strategies. Such findings argue that the nervous system is underestimated coordinator of immune reactions and underline the importance of neuro-immune crosstalk for body homeostasis. Herein, we review neuro-immune interactions with a special focus on disease pathogenesis throughout the gastro-intestinal tract.

Keywords: neuro-immune interactions, chronic inflammatory diseases, autonomous nervous system, enteric nervous system (ENS), tissue homeostasis

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *13 November 2019*
Accepted: *07 February 2020*
Published: *20 March 2020*

Citation:
Jakob MO, Murugan S and Klose CSN (2020) Neuro-Immune Circuits Regulate Immune Responses in Tissues and Organ Homeostasis. Front. Immunol. 11:308. doi: 10.3389/fimmu.2020.00308

INTRODUCTION

Immune responses at mucosal barriers are of particular interest because the mucosa is the primary entry port for many pathogens as well as the major site for chronic and sometimes detrimental immune responses. The interaction between the immune and the nervous system at mucosal barriers is attracting more attention from researchers worldwide. Recent advances in understanding the role of the interplay between both systems have uncovered a pivotal role of the nervous system in modulating immune responses and vice versa. The notion that the immune system and the nervous system share many commonalities emerged the idea of strong cross-interactions (1). Evolutionary similarities, such as signaling via transmitters, information delivery to distant body regions and migratory behavior, link the nervous with the immune system and together they coordinate the integration of danger signals to external environmental stimuli (1). The nervous system *per se* is a large interface that is strongly involved in maintaining body homeostasis (2). On the one hand, autonomic neurons sense a broad variety of parameters such as mechanical distortion, physicochemical attributes, secretions, nutrients and toxins (3). On the other hand,

the autonomic nervous system controls effector functions such as intestinal motility, blood flow, and secretory functions (4). Specifically, the enteric nervous system (ENS) controls and dictates the motor function of smooth muscle cells throughout the gastrointestinal tract. Such coordinated muscular activity results in squirting of ingested food, allows for mixing with digestive enzymes and eventually commands the aboral transport of non-digestible products (5). Of note, the intestine is densely innervated by the autonomous nervous system and populated by hematopoietic cells, therefore providing opportunities for neuro-immune interactions (4). Advances in the understanding of neuro-immune interactions has uncovered the immune-modulatory properties of neurons and emerged an interesting treatment approach for inflammatory conditions (6).

Current treatment modalities for autoimmune diseases, such as inflammatory bowel diseases (IBD), are often insufficient. These therapies target specific molecules on the surface of immune cells or, more general, dampen immune responses. However, this strategy fails to control disease-activity in many patients (7). There is strong evidence that modulation of the autonomic nervous system can exert strong anti-inflammatory effects, even after exhausted therapeutical modalities (8, 9). Current biologics targeting immune cells for example in IBD are often insufficiently effective and associated with severe side effects (10). There is a strong need for novel therapeutics with low side effects that have immune-modulatory functions rather than solely dampening effector functions. Thus, treatment strategies that harness neuro-immune interactions may be a promising approach because it is known that the nervous system is able to exert strong anti-inflammatory effects in mice and humans (9, 11). Herein, we review current knowledge in neuro-immune-interactions that maintain body homeostasis with a special focus on disease entities and the translational relevance as a potential therapeutic target in inflammatory diseases within the intestine.

ANATOMICAL ORGANIZATION OF THE AUTONOMOUS NERVOUS SYSTEM

The term "gut-brain-axis" illustrates the bidirectional communication between the central nervous system (CNS) and the intestine that includes the autonomous nervous and the neuroendocrine system via the hypothalamic-pituitary-adrenal-axis. The autonomous nervous system provides an anatomical cue connecting the CNS with the peripheral tissues (**Figure 1**). Generally, the innervation of tissues can be classified as intrinsic, if the neuron's cell body lies within the respective tissue and extrinsic, if the cell body of the neuron is located outside the tissue (**Figure 2**) (12). The ENS belongs together with the sympathetic and parasympathetic nervous system to the autonomous nervous system. Even though the ENS receives input from the CNS, it largely functions independently suggesting a hierarchical structural organization. As a matter of fact, the majority of neurons in the vagal nerve are afferent and thus transmit signals from the intestine to the CNS suggesting that the brain is rather a signal receiver that perceives and integrates signals arising from the gut in order to quickly react to potential danger, damage, or threat (13). The pivotal role of the ENS is highlighted in Hirschsprung's disease, a disorder characterized by congenital lack of enteric neurons. The consequential lack of coordinated propulsive motility pattern in the colon mediated by the ENS results in high morbidity and mortality (14). The crucial role of the ENS for body homeostasis is also illustrated in enteric infections that affect enteric neurons, such as in Chagas disease, which may cause acquired loss of enteric neurons resulting in megaviscera with potential life threatening complications (15).

The ENS is organized in afferent/sensory neurons that transfer information to the CNS, and efferent/motor neurons that transmit signals from the CNS to the periphery. Upon stimulation, somatosensory information is further processed/integrated by dorsal root ganglia located in proximity to the spinal cord. The effector function of the nervous system can be categorized into a somatic and an autonomous arm. The somatic efferent system originates from the brainstem and spinal cord and forms motor neurons that innervate skeletal muscles. The effector function of the somatic neuronal system can be consciously controlled. The autonomous nervous system on the other hand is largely independent of CNS control and can be further subdivided into the sympathetic nervous system, parasympathetic nervous system and ENS (**Figure 1**, right vs. left panel). The sympathetic and parasympathetic nervous system are anatomically distinct and in many aspects designed to be biochemical and functional counter players (16). The conserved function of sympathetic neurons is to elicit a fight-or-flight reaction, whereas parasympathetic neurons activate a rest-and-digest reaction. The cell bodies of the preganglionic neurons from the sympathetic nervous system are localized in the thoraco-lumbar region, which receive input from the brain stem, hypothalamus and the formation reticularis. Preganglionic sympathetic neurons synapse with postganglionic neuron located in the latero-dorsal thoracolumbar region, which is also referred to as sympathetic trunk. After signal transmission, the long postganglionic sympathetic neurons innervate the gastro-intestinal tract and maintain tissue homeostasis. The cell bodies of parasympathetic preganglionic neurons on the other hand are located in the brainstem and the pelvic sacral nerves. Similar to the sympathetic, the parasympathetic nervous system transmits signals from the brainstem to the respective organs via two neurons. However, the postganglionic parasympathetic neurons are localized in immediate proximity to the target organ. The parasympathetic nervous system innervates the gastro-intestinal tract with nerve fibers from the vagal nerve that end just before the splenic flexure of the transverse colon (also known as Cannon's point) and afterwards with fibers originating from pelvic sacral nerves. Both, the sympathetic and parasympathetic preganglionic neurons are cholinergic and predominantly express and secrete the neurotransmitter acetylcholine (**Figure 1**; neurotransmitters highlighted in red/green/yellow). The parasympathetic postganglionic neurons are also cholinergic whereas the sympathetic postganglionic sympathetic neurons are catecholaminergic and predominantly express and secrete norepinephrine as a neurotransmitter. The identification of the

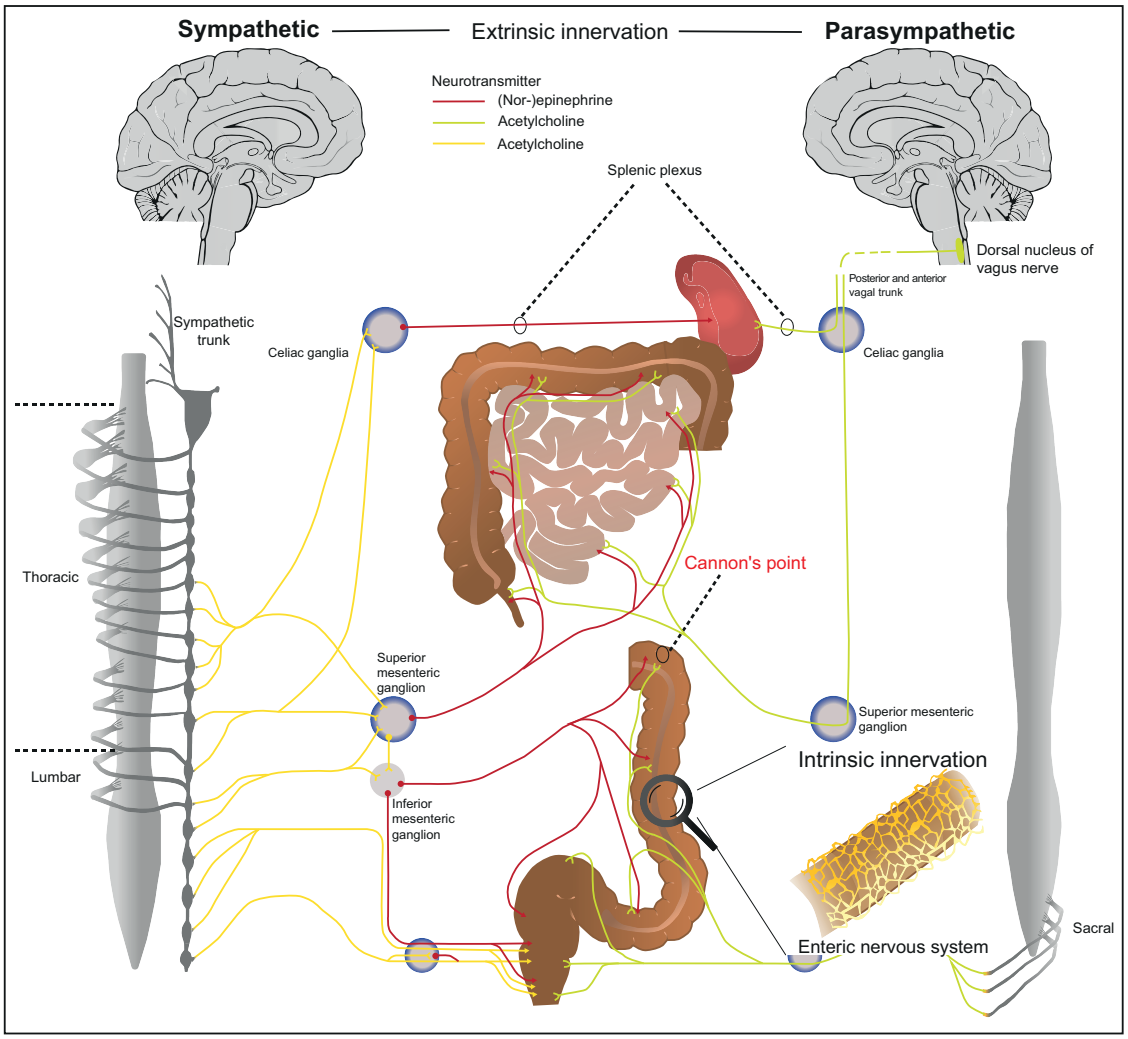

FIGURE 1 | Anatomical organization of the autonomous nervous system. The autonomous nervous system is organized in three anatomical and biochemical distinct systems. (1) The sympathetic nervous system has its preganglionic cell bodies in the thoraco-lumbar region (sympathetic trunk). The pregangliotic sympathetic neurons synapses with the postgangionic neuron in the sympathetic trunk, whereas the long postganglionic neuron (red) innervates the respective part of the gastro-intestinal tract. (2) The cell bodies of the parasympathetic nervous system are located in the brainstem and the pelvic sacral nerves. The vagal nerve includes preganglionic fibers from parasympathetic nervous system (green) that innervates the gastro-intestinal tract and ends just before the splenic flexure of the transverse colon (also known as Cannon's point). After Cannon's point, the colon is innervated by the pelvic sacral plexus. The postganglionic neuron is localized in immediate proximity to the target organ. (3) The enteric nervous system is located within intestinal tissues (Auerbach plexus, Meissner plexus) and has a characteristic architecture (details see **Figure 2**).

respective neuronal subsets is therefore based on the expression of enzymes involved in neurotransmitter synthesis such as tyrosine hydroxylase for sympathetic catecholaminergic neurons and choline acetyltransferase (Chat) for parasympathetic cholinergic neurons (17). Because many different immune cells express the receptor for norepinephrine, such as α- and β-adrenoreceptors, the sympathetic nervous system is tightly linked to immune regulation (18). Potentially as a part of the fight-and-flight reaction that need to ensure survival of the organism, the sympathetic nervous system initially has a pro-inflammatory function (19). In long term, the sympathetic nervous system rather suppresses inflammation via β-adrenergic receptors expressed on Neutrophils, Macrophages, innate lymphoid cells (ILCs) and other immune cells (20–25). The exact response of catecholamines, however, is also context-dependent for example on environment and local challenges, co-stimulatory factors and activation levels of cells (19). The parasympathetic nervous system acts via secretion of acetylcholine, which binds to muscarinic and nicotinic acetylcholine receptors. In general, acetylcholine has a rather anti-inflammatory effect following activation. This can be observed upon stimulation of the vagal nerve, which has been termed the "cholinergic anti-inflammatory reflex" (26). Apart from controlling vegetative functions, acetylcholine and norepinephrine regulate cytokine

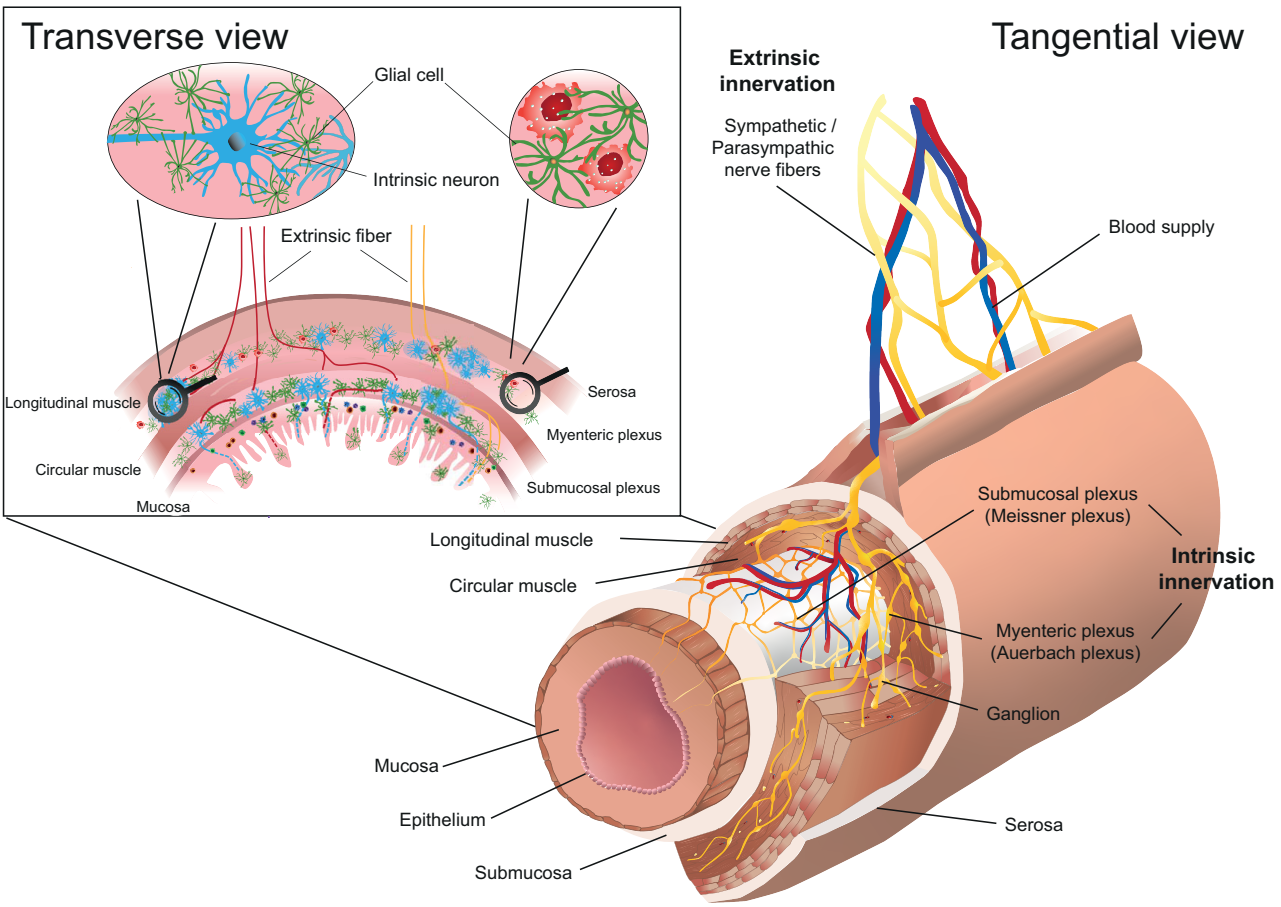

FIGURE 2 | Schematic representation of the enteric nervous system (ENS) in the intestine. The myenteric plexus (Auerbach plexus) lies within the longitudinal and the circular muscle layer, whereas the submucosal plexus (Meissner plexus) is located below the circular muscle layer. The transverse view of the intestinal wall shows the cellular composition of the ENS including neurons and glial cells. The innervation of the ENS is classified as intrinsic, if the neuron's cell body lies within the intestinal wall and extrinsic, if the cell body of the neuron is located outside the intestinal wall.

secretion of hematopoietic cells. Vice versa, neurons express cytokine receptors to adequately react on inflammatory stimuli (27). The effect of autonomic innervation of lymphoid organs has been highlighted in the spleen, which is innervated by the superior mesenteric ganglion and eventually the splenic nerve (**Figure 1**). Especially in the white pulp, T- and B-cell as well as macrophages are in close contact with neuronal innervation (28). Functionally, the interaction of the autonomous nervous system and immune cells control local and systemic inflammation via the cholinergic anti-inflammatory pathway (26). This anti-inflammatory pathway originates anatomically from the vagal nerve that innervates abdominal organs and controls the release of its predominant neurotransmitter acetylcholine. Activation of the vagal nerve lowers the systemic inflammatory response via inhibition of TNF production by myeloid cells (29).

The ENS is organized in plexuses throughout the intestine composed of neurons whose cell bodies lie within the intestinal wall (**Figure 2**). It forms a continuous modality and ranges from the upper esophagus to the internal anal sphincter. The ENS is the largest accumulation of neurons outside the CNS and contains 100 to 500 million neurons and is thus referred to as the "abdominal brain" (30, 31). The myenteric plexus (Auerbach plexus) lies between the longitudinal and circular muscle layer in the intestinal wall whereas the submucosal plexus (Meissner plexus) forms a network within the submucosal layer (**Figure 2**). The ENS forms a dense network that mainly includes neurons and glial cells and controls peristalsis, blood flow and maintains water and electrolyte homeostasis. Neurons in the ENS can be categorized based on their anatomy, function and neurotransmitter signature. Up to 20 functional classes of neurons can be identified in the guinea pig. Functionally and phenotypically, several types of enteric neurons are distinguished and can be further sub-classified: Excitatory neurons innervating intestinal muscles, inhibitory neurons innervating intestinal muscles (to circular and longitudinal muscles, respectively), secretomotor and vasodilator neurons, secretomotor neurons without vasodilator activity and neurons to enteroendocrine cells, sensory intrinsic primary afferent neurons, ascending and

descending interneurons and intestinofugal neurons (32–34). Single-cell sequencing experiments revealed nine clusters of enteric neurons in mice, which can be classified based on the two neurotransmitters nitric oxide (NO) (Nos1 expression, cluster 1–3) and acetylcholine (Chat expression, cluster 4–9) (35). Structurally, $Nos1^+$ neurons are preferentially type I neurons whereas among $Chat^+$ neurons type II neurons are overrepresented (34). Additional neurotransmitters include, gamma-Aminobutyric acid (GABA), epinephrine and dopamine, but also vasoactive intestinal peptide (VIP), neuromedin U (NMU), calcitonin gene-related peptide (CGRP), Substance P, Galanin, Tachykinin, and others (32, 35). With regard to neuronal regulation of immune responses, the biochemical signature of the neuron (neurotransmitters, neuropeptides) appear to be functionally most relevant, since many of the neuropeptides such as VIP, NMU, CGRP regulate immune responses via different subsets of immune cells (36–44). The in-depth characterization of enteric neurons may allow to identify neuronal subsets based on the expression of neurotransmitters/neuropeptides and to assign specific inflammatory functions analog to immune cells. However, how the immune modulatory function of neuronal factors is linked to their physiological function is still poorly understood. Such insights would provide an integrated view on the regulation of intestinal and immune homeostasis. For example, it is well-established that inhibitory motor neurons in the ENS are characterized by co-expression of NO and VIP as main neurotransmitters (34, 35). However, how the inhibition of motor activity is linked to regulation of immune cells and what are the respective stimuli for their release remains poorly understood.

NEURONAL AFFERENT SIGNALS MODULATE TISSUE IMMUNITY

Sensory neurons play an important role in detecting harmful environmental challenges, transmit these signals to the CNS and allow for an adequate reaction against potential pathogenic threats or tissue damage. Recent evidence suggests that the CNS receives direct neuronal afferent signals upon the input from gut enteroendocrine sensory cells. Enteroendocrine cells are gut epithelial cells that form a tight connection with vagal neurons. This interaction builds the basis of a neuro-epithelial circuit to the CNS that senses gut stimuli via glutamate as the main neurotransmitter (45). However, this finding is controversial because another study did not observe direct neuronal contact with epithelial cells (46). Thus, further experiments need to clarify the exact interaction between sensory neurons and epithelial cells. Sensory neurons respond to a broad variety of chemical and physical stimuli that can activate different ion channels, such as transient receptor potential vanilloid (TRPV1), transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential cation channel subfamily M member 8 (TRPM8) (18, 47). An important class of sensory neurons are nociceptors that are able to detect noxious stimuli such as heat, chemical and mechanical perturbations (48). The role of nociceptors in sensing a broad variety of stimuli, and in turn, regulating immunological functions has been proposed by several studies (49–54). For example, sensing of type 2 cytokines, such as Interleukin (IL)-4, IL-5, IL-13 directly activate sensory neurons and promote chronic itch that is dependent on neuronal IL-4Rα and JAK1 signaling (53). Interestingly, JAK inhibitors improved chronic itch in patients, even after failure of state-of-the-art immunosuppressive therapy and therefore represent a novel treatment option for atopic dermatitis (53). In addition to type 2 effector cytokines, the epithelial cell-derived alarmin, thymic stromal lymphopoetin (TSLP), which is an important initiator of type 2 immune responses, can activate $TRPA1^+$ sensory neurons in the skin and induce itch behavior in mice (50). Besides itch, also pain-sensitizations have been proposed to be induced by bacterial products following direct activation of nociceptor sensory neurons (49, 55). In fact, $Nav1.8^+$ neurons sense bacteria-derived N-formylated peptides and α-hemolysin suggesting that pain can be a direct consequence of neuronal sensing of bacteria during certain infections in addition to the reaction to immune activation or inflammation (49). Meseguer and colleagues found that lipopolysaccharide (LPS) was able to directly stimulate excitatory actions on $TRPA1^+$ neurons and thus eliciting nociceptor activity and eventually pain (55). The finding that bacteria directly induce pain-sensitizations is intriguing because subclinical, not overt low grade infections may be causative for different chronic pain syndromes in humans. Thus, blocking of specific, bacteria-derived neuronal sensitizations may be a valuable treatment option for such chronic pain syndromes (56). These studies further unraveled the potential of pathogen-sensing via the autonomous nervous system that has classically been attributed to pathogen-receptors expressed on immune cells. Therefore, the autonomous nervous system may be an important player in the establishment of host-microbial mutualism. Another fact that has not yet been deeply addressed is the expression of classical pattern-recognition receptors, such as toll-like receptors (TLRs) 2, 4, and 7 on enteric neurons, which have been well-studied on myeloid and epithelial cells (57, 58). If we consider the broad variety of existing TLR-ligands, the ENS may therefore be an unprecedented player in pathogen recognition. In fact, the ENS has recently been shown to directly recognize parasite-derived excretory-secretory products in a Myd88-dependent fashion during *Nippostrongylus brasiliensis* (*N. brasiliensis*) infection in mice underlining the concept of pathogen-sensing by the ENS (37). Furthermore, viruses can stimulate different TLRs, thus broadening the functional role of the ENS in mounting immune reactions against infections (59, 60). However, there is a fundamental lack in knowledge of how the ENS can sense these signals and consequently adapt immune effector functions. While functional studies investigating the role of TLRs in neurons are scarce, several reports highlight the importance of TLRs in glial cells. Deletion of the signaling adapter molecule MyD88 on glial cells, which transduces signals of many TLRs but also IL-1 cytokine receptors such as IL-1R, IL-33R, IL-18R, resulted in decreased ILC activation during DSS colitis and *N. brasiliensis* infection and suggests that there is TLR-mediated sensing of pathogens by the ENS and vice versa leading to immune activation (37, 61). Especially TLR2 seems to play an important role in controlling

ENS architecture and consequently intestinal inflammation via glial cell-derived neurotrophic factor (GDNF) (62). In fact, enteric neurons from TLR2$^{-/-}$ mice had smaller ganglia, fewer HuC/D^{+} and nNOS^{+} neurons as wells as shorter betaIII-tubulin axonal networks, whereas supplementation with GDNF corrected the observed phenotype (62). Nociceptors on the other hand release neuropeptides, such as CGRP, substance P, and VIP, which can adapt the local immune function depending on the milieu and the local challenge. That neurons have the ability to directly sense and integrate signals may also be represented by the fact that the microbial colonization has an important impact on neurophysiology and behavior (63, 64). In fact, germ-free mice, which are devoid of any microbial exposure, develop relevant alterations in behavior and the microbiota seem to be relevant to different neurodegenerative diseases (65, 66). A detailed discussion of the gut-brain axis is, however, beyond the scope of this review and the reader is kindly referred to excellent articles (64, 67).

THE ENTERIC NERVOUS SYSTEM INTEGRATES SIGNALS FROM COMMENSAL MICROBIOTA

The microbiota inhabit all mammalian body surfaces and play a pivotal role in the education of the host immune system (68). Recent evidence now suggests that the presence of commensal microbiota also shape the neuronal gene programs and eventually the extrinsic sympathetic activity (46, 69). Muller and colleagues proposed that the commensal microbiota shape intrinsic enteric-associated neuronal programs (EAN) region-dependent along the intestine, whereas intrinsic EAN are functionally adapted to the specific intestinal region and its associated microbial challenge. Interestingly, germ-free mice exhibited hyperactivation of sympathetic neurons whereas the microbial product, butyrate, suppressed sympathetic hyperactivation (46). These results reveal that a metabolite-mediated gut-brain circuit adapt autonomic nervous functions dependent on the local milieu. Because the sympathetic nervous system controls different autonomic nervous functions (blood pressure, heart rate and other) one may speculate that certain human diseases may be caused by alterations in the intestinal microbial composition. Furthermore, the aryl hydrocarbon receptor (*AhR*) has been shown to be expressed by virtually all myenteric neurons in the colon and the distal small intestine in specific-pathogen-free (SPF)-colonized mice whereas its expression was absent in the duodenum and jejunum of SPF-colonized mice or in the colon of germ-free mice suggesting that the microbial colonization dictates *AhR* expression. Enteric neuron-specific deletion of *AhR* resulted in an increase in gut-transit time whereas supplementation of the Ahr-ligand, I3C, restored intestinal transit time suggesting that neuron-specific and ligand-dependent activation of *AhR* controls intestinal motility (69). Taken together, these studies reveal a link between the intestinal microbiota and enteric neurons suggesting that enteric neurons constantly sense the commensal microbiota in order to maintaining body homeostasis.

CELLULAR MECHANISTICS OF NEURO-IMMUNE INTERACTIONS

Innate Lymphoid Cells (ILCs)

ILCs enclose diverse populations of innate immune cells, which are derived from the common lymphoid precursors, but which lack rearranged antigen-specific receptors and thus develop independently of Rag recombination (70, 71). Based on developmental and functional aspects, two different main groups of ILCs are distinguished, cytotoxic ILCs [conventional natural killer (NK) cells] and helper-like ILCs (ILC 1, 2, and 3) (72, 73). Conventional natural killer cells (NK cells) are known since the 1970s because they are well-represented in the blood and secondary lymphoid organs (74, 75). NK cells are developmentally dependent on the transcription factor Eomesodermin and mediate immunity to intracellular pathogens and tumors. The immunology of helper-like ILCs was mainly studied in the last 10 years (76) with the exception of lymphoid organ development mediated by lymphoid tissue inducer cells (LTi cells) (77). The reason for the late discovery has been discussed (78) but one reason might be the enrichment of helper-like ILCs at barrier surfaces, which were less in research focus at that time. Helper-like ILCs are characterized based on the expression of and developmentally dependency on lineage-specifying transcription factors and the effector cytokine profile: (i) ILC1s require T-bet and secrete IFN-γ and TNF and are involved in control of mainly intracellular pathogens, (ii) ILC2s require GATA-3 and BCL11b and secrete IL-5, IL-9, and IL-13 to combat helminth infections or to drive allergic reactions, and (iii) ILC3s are RORγt dependent IL-22 secreting cells, which maintain barrier integrity and protect from intestinal infections. ILC3s can be subdivided in CCR6^{+} LTi-like producing IL-17A and CCR6^{-} ILC3, which co-express T-bet and IFN-γ and have the potential to differentiate into ILC1-like cells. For a more detailed overview on ILCs biology the reader is kindly referred to more comprehensive reviews on this is topic (76, 79, 80).

ILCs are mainly located at barrier surfaces and act as a first line of defense against potentially invading microbes and are establishing host-microbial interactions. In addition to host-defense mechanisms, ILCs have also been implicated to contribute to tissue repair and maintenance of barrier integrity and organ homeostasis (81–84). In order to fulfill this function, ILCs need to be rapidly activated. However, in contrast to myeloid cells, the expression of pattern recognition receptors is very limited in ILCs suggesting that they do not sense danger signals directly by expression of pattern recognition receptors. Instead, they are activated indirectly by cytokines secreted by other cells in tissues, e.g. by alarmins and other cytokines such as IL-12 and IL-15 for ILC1s, IL-25, IL-33, and TSLP for ILC2s, IL-1β, IL-23, and TL1A for ILC3s (79, 85, 86).

Since ILCs are present in large numbers in the intestine, which is also densely innervated by the ENS and the other components of the autonomic nervous system, neuronal factors emerged as potential regulators of immune responses and sensors for danger signals. Indeed, recent research has provided evidence that ILCs integrate neuronal signals and express receptors for neuropeptides and neurotransmitters (**Figure 3**) (23, 36–42, 44,

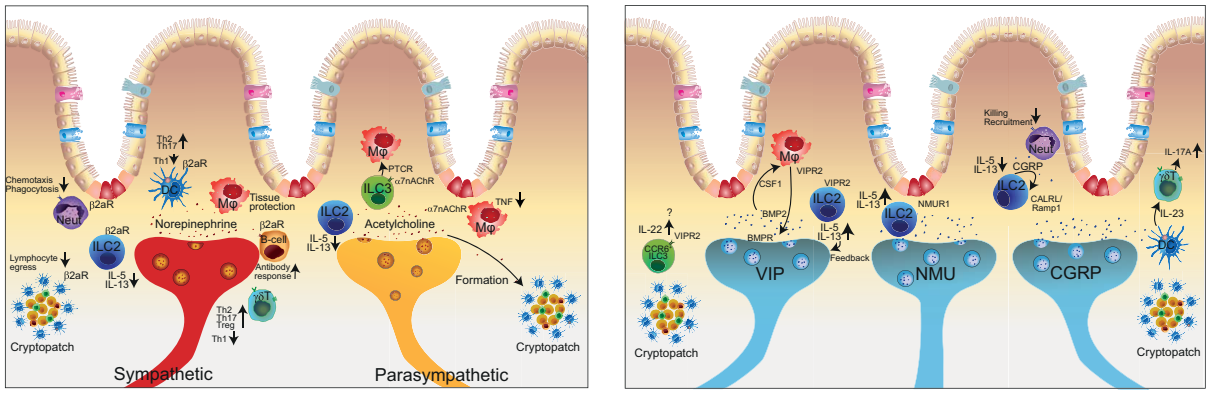

FIGURE 3 | The complex interactions of the enteric nervous system with innate and adaptive immune cells. **(A)** Representation of the close co-localization of extrinsic neuronal fibers and immune cells in the intestine. Sympathetic signal transmission (red axon) using (nor-)epinephrine can exert pro- and anti-inflammatory effects depending on the immune cell and respective receptor activation. The sympathetic nervous system in addition controls lymphocyte egress from lymph nodes. The parasympathetic nervous system (yellow axon) in general has anti-inflammatory effects but is also involved in the formation of tertiary lymphoid organs. **(B)** Schematic representation of intrinsic neuronal subsets of the enteric nervous system (VIP, NMU, CGRP) and the interaction with immune cells. Neut, neutrophil granulocyte; DC, dendritic cell; Mϕ, macrophage; ILC, innate lymphoid cell; γδT, gamma-delta T cell; α7nAChR, alpha7 nicotinic acetylcholine receptor; β2aR, beta2 adrenergic receptor; VIP, vasoactive intestinal peptide; NMU, neuromedin U; CGRP, calcitonin-gene related peptide; VIPR2, vasoactive intestinal peptide receptor 2; NMUR1, Neuronmedin U receptor 1.

87, 88). ILC responses are regulated by the ENS as well as the sympathetic and parasympathetic nervous system, glial cells and by endocrine loops (89).

The sympathetic arm and the respective neurotransmitter norepinephrine has been shown to inhibit ILC2s and consequently decrease type 2 responses via β2-adrenergic receptors (**Figure 3A**). Absence of the β2-adrenergic receptor on ILC2s in a mouse model of *N. brasiliensis* magnified the type 2 immune reaction and resulted in improved worm clearance (23). Similar to the cholinergic anti-inflammatory pathway, regulation of acetylcholine on ILC2s has been shown to bind on alpha7-nicotinic acetylcholine receptors (α7nAChR) (**Figure 3A**). Administration of a specific agonist for α7nAChR on ILC2s reduced ILC2 effector function and eventually dampened allergic lung inflammation (88). In contrast, vagotomy and lack of acetylcholine results in a delayed resolution of Escherichia coli infection via peritoneal ILC3 (90). Mechanistically, abrogation of vagal neuropeptides functionally decreased secretion of the immunoresolvent PCTR1 by peritoneal ILC3 whereas supplementation of either PCTR1 or ILC3 restored host responses against *E.coli*. These results suggest that the cholinergic modulation has a tissue protective role and shapes the ILC3 compartment to regulate tissue homeostasis (91). Furthermore, signals from the vagal nerve regulate the formation of tertiary lymphoid tissue during chronic inflammation (92). However, how much LTi cells are involved as receivers of neuronal signals in this process requires further investigation.

ILC3 are critical in host-defense at mucosal sites and regulators in inflammation. Recent data show that adult CCR6$^+$ ILC3 express the neurotrophic receptor RET and ILC3-autonomous RET ablation decreased IL-22 production and increased the susceptibility to bowel inflammation and infection suggesting a modulatory interaction of the nervous system with ILC3 (61). Microbial sensing of the microenvironment is mediated by glial cells adjacent to cryptopatches, in which the CCR6$^+$ ILC3s are located. Upon sensing of microbial-associated molecular patterns, glial cells released the RET-ligand glial cell-derived neurotrophic factor (GDNF) to stimulate IL-22 production of CCR6$^+$ ILC3s in the cryptopatch. (61). Within intestinal tissues, ILCs and nerves show a close co-localization, which presumably supports neuronal regulation of ILC responses. Enteric neurons express the neuropeptides NMU, VIP, and CGRP whereas the receptors for these neuropeptides are expressed on ILCs (**Figure 3B**) (37–44).

NMU is a highly conserved neuropeptide, which is generated by proteolytic cleavage of a pro-protein by unknown proteases into bioactive small peptide fragments (93). NMU is mainly expressed in the thalamus in the CNS and enteric neurons within the gastrointestinal tract. NMU binds to two large G-protein coupled receptors, coined NMUR1 and NMUR2. While NMUR2 is mainly expressed by neurons, NMUR1 was found to be selectively expressed by ILC2s (**Figure 3B**) (37–39, 90, 93). Furthermore, NMU was a very strong stimulator of ILC2s and triggered type 2 immune responses promoting anti-helminth immunity in the intestine or in the context of lung inflammation via NMUR1 (37, 38). Interestingly, NMU was shown to be upregulated during helminth infection and enteric neurons were shown to directly sense worm-derived excretory/secretory products in a Myd88-dependent manner and react to that stimuli with the production of NMU as an immune effector molecule. Altogether these data support a model where cholinergic neurons regulate type 2 inflammation via production of NMU and engagement of NMUR1 on ILC2s.

While NMU from cholinergic neurons stimulate ILC2s, the neuropeptide CGRP is also secreted by neurons with a cholinergic signature but in contrast inhibits ILC2 activation (**Figure 3B**). Interestingly, ILC2 also produce CGRP themselves in addition to being equipped with the receptors for CGRP CALCRL/Ramp1. Therefore, CGRP might act as a negative feedback loop to control ILC2 activation. CALCRL/Ramp1 engagement by CGRP binding triggers a signaling cascade in ILC2s, which signals via Gαs proteins and regulate intracellular cAMP levels. ILC2 activation is suppressed by CGRP and genetic deletion of components of the CGRP—CALCRL pathway resulted in elevated ILC2s responsiveness and type 2 inflammation in the context of helminth infection, lung inflammation and food allergy (40–43). Other findings uncovered the regulation of ILC2s by neuroendocrine cells (94) and tuft cells that share many commonalities with neurons (Chat-expression, sensing, signal transmission). Pulmonary neuroendocrine cells on one hand secrete CGRP and GABA and reside in close proximity to ILC2 in the lung. In models of allergic asthma, neuroendocrine cells are pivotal players in regulating ILC2s and consequently mucosal type 2 responses (94). Tuft cells on the other hand are chemosensory cells in the intestine and the major source of IL-25 and can activate ILC2 to mount type 2 responses (95–97). The commonalities of these cell types with the nervous system however requires further investigation.

While Nmur1 was reported to be selectively expressed by ILC2, Vipr2, one receptor for VIP, is expressed on both ILC2 and ILC3 (**Figure 3B**). VIP, known as the circadian synchronizer, has been shown to stimulate ILC2 via Vipr2 resulting in the release of IL-5. (87). Nav1.8^{+} nociceptors in the lung secrete VIP upon stimulation and the resulting induction of IL-5 has been linked to eosinophil accumulation and consequently worsened ovalbumin-induced lung inflammation (87). The ILC2-mediated production of IL-5 further increased the nociceptor stimulation in the sense of a backward loop (36). VIP can also adapt ILC3 function in the intestine dependent on the day and oscillating between active and resting phases (41). ILC3 expressed high levels of *Vipr2* whereas VIP induced the IL-22 production that has been shown to be an important player in maintaining bowel integrity (98). In fact, genetic deletion of the VIP-Vipr2 pathway by using *Vipr2*$^{-/-}$ mice resulted in an increased susceptibility to DSS-colitis (41). A study by Talbot and colleagues found that CCR6^{+} ILC3s but not CCR6^{-} ILC3s, in cryptopatches expressed Vipr2 and additional molecules related to neuro-immune interaction. CCR6^{+} ILC3s are an important source of IL-22, which regulates epithelial function including production of antimicrobial peptides and lipid absorption and thereby adapting the immune control to nutrient uptake (44). While the VIP–VIPR2 pathway links antimicrobial immunity, circadian rhythm and food adsorption, whether ILC3 are stimulated or inhibited by VIP is controversial. Seillet and colleagues measured that VIP induces ILC3s and IL-22 production whereas Talbot et al. found inhibition of ILC3s and IL-22 by VIP (41, 44). Therefore, further experiments need to be conducted to investigate context-dependent effects of VIP.

The hypothalamic-pituitary-adrenal axis (HPA) regulates the immune system via control of glucocorticoid secretion, which is key negative regulator of hematopoetic cells. Although secreted by the adrenal gland, the production of glucocorticoids is under control of the CNS and, therefore, linked to neuronal regulation of immune responses. Quatrini and colleagus recently showed that the regulation of natural killer (NK) cell function is dependent on the glucocorticoid receptor (GR) for resistance to sepsis and for immunopathology in the context of murine cytomegaly virus infection. Mechanistically, endogenous glucocorticoids induced the expression of PD-1 on NK cells and limited the production of IFN-ɣ eventually preventing mortality in infected mice (99, 100). These results highlight the importance of further studies that investigate the functional role of the HPA axis in tuning or downregulating immune functions.

In summary, neuronal regulation of ILCs via multiple neuropeptides and neurotransmitters and the corresponding receptors expressed by ILCs, emerged as an important signaling hub in tissues for integration of body homeostasis and immunity at barrier surfaces.

Dendritic Cells

Dendritic cells (DC) are classical antigen-presenting cells that express pattern recognition receptor (PRRs) to sense the environment for the presence of danger signals and if necessary initiate an immune response against the pathogenic encounter. In addition to PRRs, DCs express adrenergic receptors and receptors for neuropeptides suggesting a modulative effect of the autonomous nervous system in mounting immune responses (22). In fact, β2-adrenergic stimulation of DCs results in skewing the T cell response toward Th2 and Th17 responses at the costs of Th1 promotion (**Figure 3A**) (22). However, conclusions drawn from these findings are limited because experiments, which demonstrate the importance of DC-neuron interaction via β2-adrenergic receptors *in vivo* are still missing (25).

The interaction of neurons and DCs has recently been highlighted in the skin in the context of Candida albicans (*C. albicans*) infections and Psoriasis-like inflammation (51, 52). Nociceptive signals by *C. albicans* in the skin can directly induce the secretion of CGRP. Such stimuli lead to the production of IL-23 by DCs further resulting in activation of ɣδT cells and secretion of IL-17A (**Figure 3B**). Notably, the absence of sensory neurons increased the susceptibility to *C. albicans* infections suggesting that neurons sense pathogens in order to control infections in close interaction with DCs (52). Another study showed that DCs are in close contact to nociceptive neurons and express the ion channels TRPV1 and Nav1.8 in the skin. Ablation of nociceptors led to failure of IL-23 production by DCs and consequently did not induce inflammatory cytokine production by ɣδT cells. Interruption of this neuro-immune cue failed to recruit inflammatory cells upon infection suggesting that TRPV1^{+} Nav1.8^{+} nociceptors regulate the IL-23/IL-17 pathway and control cutaneous immune responses (51). These experiments suggest a clear link between the neuro-DC interaction and skin disease pathogenesis. However, the expression of a broad variety of receptors for neuropeptides/neurotransmitters on DCs remains a blackbox and studies need to delineate the

role of the DC-neuron interaction in steady-state and other disease models.

Neutrophils

Neutrophils express and release a large variety of cytokines to regulate inflammatory reactions, and to recruit and activate other cells of the immune system. In addition, they have the ability for engulfment and intracellular killing and thus are players at the front-line of defense against invading pathogens (101). Because these cells act at the fore-front of tissue damage, the neuronal signaling may be obvious because of the urgency of infections and the need for cell recruitment.

In the context of Streptococcus pyogenes infection in the skin, bacteria can directly activate nociceptive neurons via secretion of streptolysin S. Activation of nociceptors resulted in the release of the neuropeptide CGRP that inhibited the recruitment of neutrophils and phagocytic killing that can be seen as a hide-me signal of bacteria (**Figure 3B**) (56). Interestingly, Botulinum neurotoxin A and CGRP antagonists reversed the suppressed immune-reaction suggesting that this may be a valuable strategy to overcome the pathogenicity of highly invasive bacterial infections. In line with this data and in a model of Staphylococcus aureus pneumonia, $TRPV1^+$ nociceptors suppressed the recruitment of neutrophils and altered γδT cells whereas this inflammatory suppression worsened survival, cytokine production and bacterial clearance (54). Another study that highlights the role of neuronal-neutrophil crosstalk has shown that noradrenalin suppressed chemotaxis and phagocytosis in a stroke model (**Figure 3A**) (24).

Taken together, there is good evidence that neuronal sensing of microbes shapes immune responses at barrier surfaces such as the skin and the lung. However, there is a fundamental lack of knowledge on the complex interaction of neurotransmitters and neuropeptides at other barrier surfaces such as the intestine. For example, because the receptor for CGRP CALCRL/Ramp1 is relatively broadly expressed on immune cells, future studies need to address if the adaptation of neutrophil function is a rather direct effect mounted by CGRP itself or an indirect effect through functional changes of other cells. A direct effect of neurons on neutrophil function would be intriguing because it could explain rapid cell recruitment during inflammation. However, further studies need to address the expression and function of specific neuropeptide or neurotransmitter receptors on neutrophils.

Macrophages

Macrophages are specialized phagocytes that are located in most body tissues. As a part of the innate immune system, macrophages help keeping the organism clean and restore tissue damage. Thus, they process dead cells, debris, foreign bodies, and initiate inflammatory processes via antigen-presentation. The expression pattern of receptors for neuropeptides and neurotransmitters on macrophages suggests that neurons and macrophages are closely linked in order to regulate tissue homeostasis and to fight infections.

The pivotal role of macrophages in integrating cholinergic signals resulting in a profound anti-inflammatory effect has been shown by the group of Tracey, which has been termed the "cholinergic anti-inflammatory pathway" (**Figure 3A**) (102). The initial observation that vagus nerve stimulation prevented the development of septic shock in mice implies neuronal control of macrophage function in acute disease (11). Later on, the group of Tracey discovered that vagal signals are transmitted via acetylcholine that binds α7 nicotinic acetylcholine receptors (α7nAChR) expressed on macrophages and results in dampening of TNF production (102). If we consider the speed of neuronal conductance, central stimuli are capable of instantaneous cell recruitment and modulatory signals to the site of inflammation (8). Another example of cholinergic vagal control of inflammation via macrophages has been shown in a model of postoperative ileus. The α7nAChR was expressed on muscularis macrophages and controlled postoperative ileus formation whereas stimulation of the vagal nerve attenuated surgery-induced intestinal inflammation (103). The interplay and the tight connection of macrophages located within the longitudinal and circular muscle layer in close contact with the myenteric plexus (**Figure 2**) not only controls postoperative ileus formation, but is also involved in the pathogenesis of diabetic-induced gastroparesis (104). There are clear parallels between the autonomous nervous-macrophage interaction in the periphery and the interaction of tissue-resident macrophages within the CNS (105). This close proximity of biologic functions in different tissues has been suggested because neuronal signals in the CNS keep tissue-resident macrophages at a quiescent state and macrophages in the CNS express high levels of CX3CR1, a pattern that has been postulated to be unique for tissue resident-macrophages in the CNS, the microglia (105).

In the intestine, the growth factor for macrophage development, colony stimulatory factor 1 (CSF1), is secreted by the nervous system and controls gastrointestinal motility. Reciprocally, macrophages sense the microbiota and change the pattern of smooth muscle contractions via bone morphogenic protein 2 (BMP2) binding on the BMP receptor expressed on enteric neurons (**Figure 3B**) (106). These results suggest a reciprocal tight regulation of gastrointestinal motility via the interaction of muscularis macrophages and enteric neurons that in turn depend on signal input from the intestinal microbiota.

The anatomic location of intestinal macrophages is highly specialized dependent on the proximity to the gut-lumen. In fact, lamina propria macrophages represent a rather pro-inflammatory phenotype in comparison to macrophages located in the muscularis that represent a rather tissue-protective phenotype. Extrinsic sympathetic neurons mediate tissue-protective effects via activation of β2-adrenergic receptors expressed on macrophages in the muscular sheet (**Figure 3A**) (21). Furthermore, intestinal muscularis macrophages protect neurons from cell-death via β2-adrenergic mediated upregulation of neuroprotective programs (107). Taken together, macrophage function is highly dependent on the signal input from the autonomous nervous system and vice versa to rapidly react to infectious stimuli and tissue damage.

Mast Cells

Urticaria, a common psycho-dermatological disorder, is the result of vascular dilation, edema, and the immediate release of

histamine by mast cells in the skin (108). It has been suggested that psychological stress is strongly involved in the pathogenesis of urticaria underlining the role of neuronal triggers to effector cells such as mast cells (109). Throughout the gastrointestinal tract, mast cells are located in close proximity to sensory nerve fibers (110). Mast cells contain granules rich in histamine and heparin, which can be immediately released and trigger rapid responses such as allergic reactions or anaphylaxis.

Mast cells have also been implicated in atopic dermatitis, where dermal lesions are hyper-innervated with a high abundance of substance P fibers and an increased respective receptor expression on mast cells (111). Nerve-derived substance P induced the rapid release of histamine, TNF, leukotriene B4, and vascular endothelial growth factor by mast cells suggesting a close interaction of neurons and mast cells in allergic diseases (18, 112, 113). This neuronal-mast cell connection has been underlined in the context of allergic skin disease models in mice. House dust mites directly activated $TRPV1^+$ nociceptive sensory neurons driving the development of allergic skin inflammation via the secretion of substance P that eventually resulted in degranulation of mast cells (113). This data provides an important signaling pathway that may be the mechanistical basis for a broad variety of allergic diseases. Psychological stress additionally triggers the release of neuropeptides (Substance P, Corticotropin Releasing Hormone) that act on mast cells and promote the release of mast cell mediators (114). Interestingly, mast cells but not eosinophils or T-cells were associated with asthmatic diseases in patients underlining the importance of these cells for allergy development in humans (115).

T- and B-Cells

Autonomous nervous fibers innervate lymphoid organs such as mesenteric lymph nodes and Peyer's patches (116, 117). There is evidence that lymph nodes may receive neural afferent innervation in addition to the sympathetic efferent innervation that may suggest neuronal sensing of imminent immunologic threats whereas such coordinated actions direct the immune system to sites of injury and infection (118, 119). The close proximity to adaptive immune cells suggests that nerve fibers participate in neuro-immune cross-talk and modulate signals from the adaptive immune system. Sympathetic neurotransmitters such as epinephrine and norepinephrine predominantly bind β2-adrenergic receptors that are highly expressed on B cells and to a lower level in $CD4^+$ T cells (**Figure 3A**) (20). Activation of β2-adrenergic receptors in general increase intracellular cAMP that activates protein kinase A. Such activation of the B-cell compartment via β2-adrenergic receptors seems to be needed for maintenance of an optimal antibody response suggesting that the autonomous nervous system controls and shapes the magnitude of immune responses (120). In line with the effects observed in B-cells, T cells and the release of their effector cytokines are controlled via sympathetic activity whereas sympathetic innervation suppresses Th1 and promotes Th2, Th17 and Treg responses (20, 25). Another finding that supports the notion that the autonomous nervous system controls adaptive immune functions and recruits cells to effector sites is that activation of β2-adrenergic receptors enhanced retention-promoting signals and inhibited lymphocyte egress from lymph nodes (121). Such migratory effects are dependent on circadian regulation in the T-cell compartment suggesting that the magnitude of adaptive immune responses can depend on neuronal-regulated signaling input from the CNS (122). It should be noted that the β2-adrenergic receptor was reported to control ILC2 and macrophage activation. Thus, further experiments need to clarify if the modulation of T-cell function is rather a consequence of the release of cytokines by other cells or delineate the exact downstream effects upon β2-adrenergic receptor activation in steady-state and disease.

NEURO-IMMUNE INTERACTIONS IN DISEASE

Inflammatory Bowel Disease

Psychologic disorders show a lifetime prevalence of up to 30% in the general population and major depression may become the most important disease in Western societies (123, 124). In line with the constant increase of psychologic disorders, the incidence of IBD increase as well emerging an unprecedented link between a potential nervous dysregulation and overwhelming immune activation (125). In fact, many patients with IBD have alexithymia that is characterized by the impossibility to verbalize emotions. Such endogenous stress may interfere with body homeostasis and lead to a distorted integrity of the neuro-immune axis that may be causative or at least worsen the clinical course of IBD (126). In mouse models, catecholamines acting on α2-adrenoreceptors led to pro-inflammatory cytokine production worsening dextran sodium sulfate (DSS) colitis. Paradoxically, sympathetic denervation induced clinical signs of colitis (**Figure 4A**) (127, 128). *In vitro* experiments have shown that norepinephrine blocks the secretion of a variety of proinflammatory cytokines and mice lacking the beta-2-adrenergic receptor were more susceptible to DSS-colitis (129). These studies reveal that sympathetic innervation can have pro- and anti-inflammatory effects and studies need to further clarify its role in IBD. In fact, a retrospective study in humans by using pharmacological inhibition of β-adrenergic receptors showed higher risk for IBD relapse suggesting that solely blocking the pro-inflammatory effect of sympathetic activation may have rather pro-inflammatory effects in long term (130). The parasympathetic tone via the vagal nerve also impedes with IBD. Studies in vagotomised mice showed increased susceptibility to develop colitis upon DSS treatment similar to the anti-inflammatory reflex observed in models of septic shock (131, 132). The absence of the vagal tone was associated with an increase in pro-inflammatory cytokines such as IL-1β, IL-6, and TNF (**Figure 4A**). These cholinergic signals seem to be transmitted via α7nAChR (131). Apart from the α7nACh receptor, also α5nAChR knockout mice had more severe colitis suggesting that vagal innervation acts in different acetylcholine receptor subunits and modulates immune functions (133). As mentioned above, the psychological distress profile of IBD patients focused the interest on the finding that mucosal levels of acetylcholine in a murine model of depression were associated

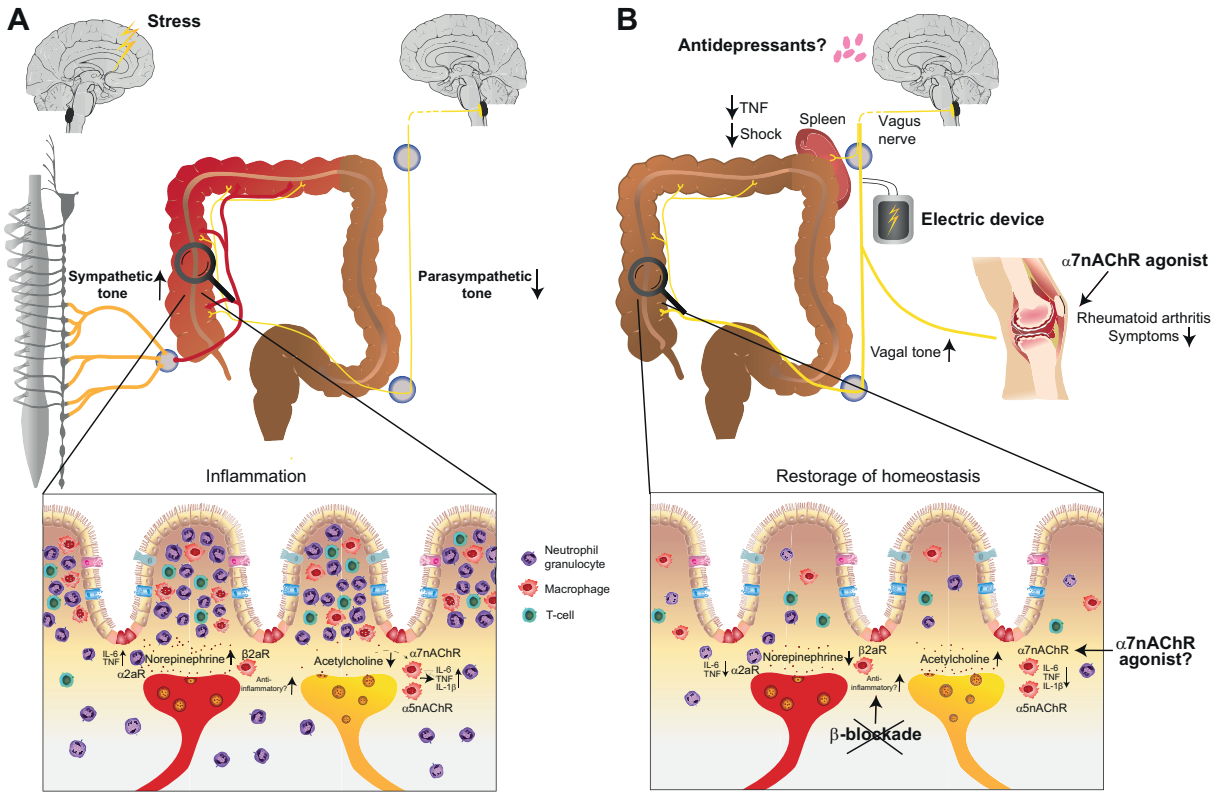

FIGURE 4 | Involvement of the autonomous nervous system in the pathogenesis of inflammatory bowel disease (IBD) and available treatment modalities that harness neuro-immune interactions. **(A)** Stress and the transmitted signals may enhance the sympathetic tone and lead to an additional pro-inflammatory reaction in the intestine that may be causal or at least worsening the course of IBD. Failure to maintain an adequate parasympathetic tone can further support the pro-inflammatory reaction. **(B)** Available treatment options in chronic inflammatory conditions include electric stimulation of the vagal nerve in rheumatoid arthritis, IBD and endotoxin-induced septic shock. Furthermore, the repertoire of treatment strategies for IBD and rheumatoid arthritis may be extended with α7nAChR-agonists. β-blockade should, however, be omitted in IBD. α7nAChR, alpha7 nicotinic acetylcholine receptor; α5nAChR, alpha5 nicotinic acetylcholine receptor; α2aR, alpha2 receptor; β2aR, beta-2 adrenergic receptor; TNF, tumor necrosis factor alpha.

with more severe colitis in response to DSS suggesting that chronic modulation of the vagal tone enhances the susceptibility to IBD (132). Interestingly, adoptive transfer of macrophages from depressive mice induced inflammatory markers and increased the severity of DSS colitis. These data identified the pivotal role of macrophage in linking stress and susceptibility to intestinal inflammation whereas this effect was reversible with antidepressants (**Figures 4A,B**) (134). Apart from the importance of macrophages as effector cells of the neuro-immune axis, transfer of $CD4^+$ T cells isolated from vagotomised animals resulted in an increased susceptibility to DSS colitis suggesting that more players are involved in cholinergic signal transmission underlining the need to study this interaction in more detail (135). Taken together, the sympathetic and parasympathetic nervous system play important roles in mounting pro- and anti-inflammatory immune reactions in the context of IBD. As a potential therapeutic target in a preclinical model of colitis, the α7nAChR agonist anabaseine, showed considerable effect and the mice developed less weight loss and less severe colitis in a DSS colitis model (**Figure 4B**) (136). Other reports showed opposite results. Although α7nAChR agonists reduced NF-κB transcriptional activity, IL-6 and TNF release, α7nAChR agonists worsened the effects of DSS-induced colitis or were ineffective in a model of TNBS-induced colitis (137). It is in addition of importance to emphasize that anti-inflammatory effects may lead to an increased susceptibility to infectious diseases (8). Following bacterial peritonitis, virtually all α7nAChR knock-out mice cleared the infection from their peritoneal cavities and had sterile blood cultures mediated via neutrophil recruitment, whereas wild type mice had high bacterial loads at the primary site of infection and were bacteremic (138). These data underline the potential importance of the α7nAChR in host defense. In line with this observations, acetyl-cholinergic agonists, such as nicotine, worsened bacterial clearance and survival upon abdominal sepsis (139, 140). Thus, translation into the clinical setting has to be obtained with caution because solely dampening

effector immune function and consequently immune suppression may lead to serious infectious complications. Another interesting approach for the treatment of IBD is the direct electric stimulation of the vagal nerve via an implantable device targeting the anti-inflammatory pathway (**Figure 4B**). First results have shown improvement in disease activity and endoscopic indices in patients following electric stimulation of the vagal-nerve (141). These results are promising because treatment failure of available biologics is not uncommon and effective treatment is associated with considerable side-effects and mortality in the long term (142). A deeper understanding is needed and may help to uncover novel therapeutic measures for treating IBD. Of note, depression and other psychological disorders may enhance the disease severity and either antidepressive medication or psychological co-therapy may adjust immune functions and lower the severity of the clinical course.

Furthermore, it is of importance to note that enteric glia cells, specialized macrophages in close proximity to neurons, outnumber neurons by 4- to 10-fold (**Figure 2**) (143). The pivotal role of glial cells in neuro-immune interactions was observed after ablation of enteric glia cells that led to fulminant jejuno-ileitis in mice (144). Enteric glial cells express a broad pattern of neurotransmitters and thereby protect neurons and regulate their activity (145). Enteric glial cells show abnormal behavior in IBD in humans but their role in its pathophysiology has to be further clarified.

Ileus

Postoperative Ileus is a serious concern in the surgical setting because patients fail to rapidly recover from an operative intervention and remain with symptoms such as nausea, vomiting, and constipation. Following a surgical procedure, postoperative ileus formation is characterized by an over-activation of inhibitory neuronal pathways that triggers inflammation beyond the distant untouched areas and leads to generalized impairment of gastrointestinal motility (146). In fact, low-grade inflammation due to macrophages residing in the intestinal muscularis is key in the induction of postoperative and endotoxin-induced ileus formation (147, 148). Activation of these macrophages mediated the influx of leucocytes at 3–4 days after surgery whereas the inflammatory response impaired normal propulsive neuromuscular function and consequently digestion (146). There is an urgent need to uncover novel pharmacologic targets in the early event of microscopic inflammation that may help to reduce ileus formation. Studies show that ileus onset can be reduced by modulating the cholinergic anti-inflammatory tone (149–151). Interestingly, vagal stimulation reduced surgery-induced inflammation and ameliorated postoperative ileus formation in a STAT3 dependent manner mediated by intestinal macrophages (149). Supportive literature showed that modulation of cholinergic neurons via α7nAChR agonists improved gastrointestinal transit time through inhibition of low-grade inflammation on the basis of macrophages (150, 151).

Sepsis

The cholinergic anti-inflammatory function via dampening of TNF synthesis has been shown in LPS-induced endotoxemia, whereas stimulation of the vagus nerve protected from the development of shock (11) (**Figure 4B**). Interestingly, splenectomy abolished the anti-inflammatory effect of the vagal nerve suggesting a pivotal role of the spleen in inflammatory reactions. This observation may explain why the organism is prone to the often fatal overwhelming post-splenectomy syndrome (OPSI) that may serve as an alternative hypothesis to the current thinking that OPSI is a result of impaired clearance of encapsulated bacteria (152). Advances in the mechanistic understanding of this observed phenotype exposed that acetylcholine signals via the α7 subunit of the acetylcholine receptor expressed on macrophages that controlled systemic TNF release (153). Since nerve fibers in the spleen lack the enzymatic machinery for acetylcholine production, systemic inflammation recruits vagus-primed T cells from the intestine to the spleen, which produce acetylcholine and mount the innate immune response (154). Other data suggest that the anti-inflammatory properties of cholinergic neurons also attenuate inflammation and injury during experimental pancreatitis and hepatitis (155, 156). In a mouse model of pancreatitis, pretreatment with the nicotinic receptor antagonist mecamylamine resulted in more severe pancreatitis increasing edema, plasma hydrolases, and IL-6 levels. Conversely pre-treatment with the selective α7nAChR agonist anabaseine strongly decreased the severity of pancreatitis suggesting that there may be a therapeutic role of the "cholinergic anti-inflammatory pathway" in the treatment of acute pancreatitis in order to attenuate inflammation and injury (155). As a matter of fact that cholinergic neurons have a systemic anti-inflammatory effect, vagotomy increased mortality in mice upon Fas-induced hepatitis whereas pretreatment with nicotine or α7nAChR agonist, inhibited this detrimental effect of vagotomy and rescued the mice (156).

Rheumatoid Arthritis

Rheumatoid arthritis is the most common inflammatory arthritis and affects up to 1.25% of the entire population (157). The pathogenesis is multidimensional and includes a genetic predisposition in addition to environmental challenges leading to synovial inflammation and eventually resulting in bone erosions, cartilage damage and eventually joint deformities and disabilities (158). Recent advances in the understanding of autoimmune diseases such as rheumatoid arthritis uncovered a pivotal role of the autonomous nervous system in disease pathogenesis (159). It has been shown that treatment with α7nAChR agonist improved arthritis scores in animal models of rheumatoid arthritis whereas α7nAChR knock-out mice showed worse disease outcome suggesting its therapeutic potential (**Figure 4B**) (160, 161). In fact, work provided by Koopmann and colleagues showed that electric stimulation via an implantable vagus nerve-stimulating device inhibits the production of TNF, IL-1β, and IL-6 and improved clinical scores of rheumatoid arthritis in patients (9). Together with similar data obtained in asthmatic patients, this study provides a proof-of-concept that treatment via activation of the cholinergic anti-inflammatory pathway is effective and may translate into the regular clinical setting.

SUMMARY AND FUTURE PERSPECTIVE

Preclinical studies targeting neuro-immune interactions upon stimulation of the vagus nerve, application of acetylcholine agonist, and β2 adrenoreceptor agonists have emerged the potential successful treatment in inflammatory diseases (155, 162, 163). Of note, the site specific control of immune functions by the nervous system via neurotransmitters/neuropeptides suggest that the nervous system can exert a rapid and local control of immune cells. Unlike the systemic effects of cytokines, neuronal regulation of immune responses allows for the selective and spatiotemporal control of immune functions without affecting the activity of distant cells. Based on this assumption, targeting neuro-immne interactions might allow for specific and targeted therapy at a cellular and compartmental level. The therapeutic potential of neuronal modulation of inflammation in humans was already demonstrated by stimulating the vagal nerve with electronic devices that has been successfully used for the treatment of rheumatoid arthritis and asthma (9, 164). Of note, some patients did no longer respond to a conventional anti-inflammatory treatment but developed disease improvement upon vagal nerve stimulation (9). Another pilot study showed the efficacy of vagal nerve stimulation in patients with Crohn's disease whereas its stimulation improved inflammatory parameters and clinical symptoms (141). This work provides a rationale for the potential of modulating neuro-immune interactions and shows promising results reflecting that vagal-nerve stimulation may be an alternative to pharmacological therapies. This observation is further supported by a clinical study that has shown asthma improvement during non-invasive vagal nerve stimulation (164). Current study enrolments of patients with a broad variety of diseases highlight the particular interest in neuro-immune interactions [Post-surgery Systemic Inflammation and Neuro-immune Interactions (POSINI) NCT03055325, Vagal Nerve Stimulation for Gastroparesis (VNS) NCT0312 NCT03908073, Transcutaneous VNS to Treat Pediatric IBD (STIMIBD) NCT03863704]. The increase of chronic inflammatory diseases in Western societies with a significant amount of non-responders to current treatment strategies underlines the need to uncover novel strategies/medications. Therefore, it is crucial to improve our understanding of how neurons interact with immune cells. Recent technical advances, such as the RiboTag system, imaging tools, genetic mouse models built the rationale to mechanistically understand neuronal-immune circuits in more detail and further uncover signaling pathways that could be therapeutically harnessed (165–168).

AUTHOR CONTRIBUTIONS

MJ and CK wrote the manuscript. SM designed figures, contributed significantly to the study and the writing of the article.

FUNDING

The work was supported by grants from the ARS Bridge to academic career grant (to MJ), the Swiss National Science foundation (Grant-ID: 184425; to MJ), the German Research Foundation (DFG; KL 2963/2-1 and KL 2963/3-1 to CK), and the European Research Council Starting Grant (ERCEA; 803087 to CK).

ACKNOWLEDGMENTS

We thank Mario Witkowski and Divija Deshpande for critically reading the manuscript.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 05 May 2020
doi: 10.3389/fimmu.2020.00802

Unique Phenotypes of Heart Resident Type 2 Innate Lymphoid Cells

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *22 January 2020*
Accepted: *07 April 2020*
Published: *05 May 2020*

Citation:
Deng Y, Wu S, Yang Y, Meng M, Chen X, Chen S, Li L, Gao Y, Cai Y, Imani S, Chen B, Li S, Deng Y and Li X (2020) Unique Phenotypes of Heart Resident Type 2 Innate Lymphoid Cells. Front. Immunol. 11:802. doi: 10.3389/fimmu.2020.00802

Innate lymphoid cells (ILCs), including ILC1s, ILC2s, and ILC3s, play critical roles in regulating immunity, inflammation, and tissue homeostasis. However, limited attention is focused on the unique phenotype of ILCs in the heart tissue. In this study, we analyzed the ILC subsets in the heart by flow cytometry and found that ILC2s were the dominant population of ILCs, while a lower proportion of type 1 ILCs (including ILC1 and NK cells) and merely no ILC3s in the heart tissue of mice. Our results show that ILC2 development kinetically peaked in heart ILC2s at the age of 4 weeks after birth and later than lung ILC2s. By conducting parabiosis experiment, we show that heart ILC2s are tissue resident cells and minimally replaced by circulating cells. Notably, heart ILC2s have unique phenotypes, such as lower expression of ICOS, CD25 (IL-2Rα), and Ki-67, higher expression of Sca-1 and GATA3, and stronger ability to produce IL-4 and IL-13. In doxorubicin-induced myocardial necroptosis model of mouse heart tissue, *IL-33* mRNA expression level and ILC2s were remarkably increased. In addition, IL-4 production by heart ILC2s, but not lung ILC2s, was also dramatically increased after doxorubicin treatment. Our results demonstrate that heart-resident ILC2s showed tissue-specific phenotypes and rapidly responded to heart injury. Thus, further studies are warranted to explore the potential for IL-33-elicited ILC2s response as therapeutics for attenuating heart damage.

Keywords: innate lymphoid cells, heart, ILC2s, IL-4, IL-33

INTRODUCTION

Innate lymphoid cells (ILCs), which are widely distributed in the body and lack the type of diversified antigen receptors, are the innate counterparts of T lymphocytes (1, 2). It is well accepted that ILCs are identified as lineage-negative (Lin^-) and interleukin-7 (IL-7) receptor α-positive ($CD127^+$) (3), emerging into three populations (ILC1s, ILC2s, and ILC3s) based on the signature transcription factors and effector cytokines. ILC1s require the transcription factor T-bet and

produce interferon-gamma (IFN-γ), ILC2s express the transcription factor GATA3 and produce the type 2 cytokines IL-4, IL-5, and IL-13, while ILC3s express the transcription factor RAR-related orphan receptor gamma t (RORγt) and have the ability to produce IL-22 and/or IL-17 (4, 5).

Growing evidence suggest that ILC subsets are involved in development of specific tissue tropisms, including the skin, intestine, liver and lung (1). For example, ILC1s are the dominant ILC population in intestinal intraepithelial layer (IEL) and liver, whereas ILC2s are the dominant population in the lung and skin. ILC3s are found in significant numbers in intestinal lamina propria layer (6–10). To date, the ILC subsets are poorly characterized in tissue homeostasis and tissue-specific response after injury in heart tissue. Most recently, a group of non-cytotoxic cardiac ILC progenitor was found in the heart tissue, suggesting that ILCs with specific-feature may also exist (11).

Here, we found that ILC2s are the dominant population of ILCs, while ILC1s are also present with a lower proportion and there are no ILC3s in the mice heart tissue. Compared with lung ILC2s, heart ILC2s have unique phenotypes in the identified markers and the ability of IL-13 and IL-4 cytokines secretion. Furthermore, ILC2s rapidly expanded and secreted IL-4 in response to myocardial necroptosis.

MATERIALS AND METHODS

Animals

Male or female C57BL/6 mice [vary from embryonic day (E) 18.5-8 weeks old] were maintained under specific pathogen free conditions, which were acclimatized at 22–25°C, 50 ± 10% relative humidity and had 12 h light/dark cycles, periodic air changes, and free access to water and food in the Experimental Animal Center of the Army Military Medical University (Chongqing, China). Congenic C57BL/6 CD45.1 mice strains were obtained from The Jackson Laboratory (Sacramento, CA, United States). All animal procedures and protocols were approved by the Animal Ethics Committee of the Army Medical University, and followed the guidelines of the Institutional Animal Care and Use Committees of the Army Military Medical University (Chongqing, China).

Parabiosis

Parabiosis were performed as previously described in the literature (12, 13). Briefly, mice were anesthetized by isoflurane vaporizer (4–5% v/v). Then skin incisions were made on the flanks of age-, sex- and weight-matched CD45.2$^+$ (C57BL/6), besides CD45.1$^+$ (C57BL/6) mice followed by gently detaching the skin from the subcutaneous fascia. The knee joints of two mice are clearly distinguishable, connected and then the incisions were joined with a continuous absorbable suture. 0.5 ml of 0.9% NaCl was administrated subcutaneously to each mouse to prevent dehydration and post-operatively. Mice received pain medication and antibiotics for the first week after parabiosis.

Doxorubicin (DOX)-Induced Myocardial Necroptosis

Eight weeks old C57BL/6 mice were injected with either DOX (20 mg/kg, i.p., Med Chem Express LLC, Shanghai, China) or saline, according to a previous study (14). Heart tissues were collected and single-cell suspensions were prepared by enzymatic digestion after 24 h or 96 h of DOX treatment.

Single-Cell Suspensions Preparation

Liver tissues were grinded and passed through a 70-μm stainless steel mesh. Then, cells were resuspended in 35% Percoll (GE Healthcare, Pittsburgh, PA, United States) and pellets were collected after centrifugation (450 × *g*, room temperature, 10 min). The liver mono-nuclear cells were separated from the pellets through lysing erythrocytes (15). For heart and lung lymphocyte isolation, the fresh mouse heart was perfused with cold PBS to remove peripheral blood cells. Briefly, mice were anesthetized by isoflurane vaporizer (4–5% v/v). The heart was slowly perfused with cold PBS from left ventricle by a 10 ml-syringe until the fluid was clear. Then heart and lung tissues were cut into pieces and then digested for 45 min at 37°C in Hank's solution containing 10% FBS and 1 mg/ml collagenase I (Sigma-Aldrich, St Louis, MO, United States), 1 mg/ml collagenase II (Gibco, Waltham, MA, United States) and 25 μg/ml DNase I (Sigma-Aldrich, St Louis, MO, United States). After digestion, the cells were then resuspended in 20% percoll in PBS (pH 7.4, Sigma-Aldrich, St Louis, MO, United States) and pellets were collected after centrifugation (450 × *g*, room temperature, 10 min) (16). For small intestines lamina propria layer lymphocyte isolation, luminal contents were flushed and peyer's patches were removed. Then the intestines were opened lengthwise and gently agitated for 20 min at 37°C in D-hank's solution (pH 7.4) containing 10 mM HEPES, 5 mM EDTA and 1 mM DTT. Tissues were then rinsed with Hank's solution prior to digestion with 1 mg/ml collagenase II for 40 min at 37°C under agitation. The collected digests were filtered through 100 micron mesh and subjected to centrifugation (450 × *g*, room temperature, 10 min) using 25% percoll solutions (17).

Antibodies and Flow Cytometry

Antibodies used for flow cytometry were commercially purchased and are listed in **Table 1**. We confirmed the species reactivity for all antibodies according to the official directions and performed preliminary experiments to determine the appropriate dilution for all antibodies. Standard protocols were followed for flow cytometry (18, 19). Briefly, single-cell suspensions were obtained from the heart, lung, liver and intestinal lamina propria tissue of mice. For surface markers, 2×10^6 cells were stained with anti-CD16/CD32 antibodies (eBioscience, San Diego, CA, United States) 15 min at room temperature, in the dark with staining buffer (phosphate-bufferd saline (PBS) containing 2% mouse serum, 2% horse serum, and anti-CD16/CD32 blocking antibodies). For intracellular IL-4, IL-5, and IL-13 staining, 2×10^6 cells were stimulated with IL-33 (eBioscience, San Diego, CA, United States) or PMA/ionomycin (BD Biosciences, San Diego, CA, United States) plus BD Golgi Plug protein transport

TABLE 1 | Antibodies used for flow cytometry.

Antibodies	Clone	Source	Dilution
Anti-mouse CD45	30-F11	BioLegend	1/200
Anti-mouse CD3e	145-2C11	BioLegend	1/200
Anti-mouse CD19	6D5	BioLegend	1/200
Anti-mouse B220	RA3-6B2	BioLegend	1/200
Anti-mouse Gr-1	RB6-8C5	BioLegend	1/200
Anti-mouse CD127	A7R34	BioLegend	1/100
Anti-mouse CD90.2	30-H12	BioLegend	1/100
Anti-mouse NK1.1	PK136	BioLegend	1/100
Anti-mouse NKp46	29A1.4	BioLegend	1/100
Anti-mouse CD49b	DX5	BioLegend	1/100
Anti-mouse KLRG1	2F1	BioLegend	1/100
Anti-mouse GATA3	16E10A23	BioLegend	1/20
Anti-mouse ICOS	15F9	BioLegend	1/100
Anti-mouse Sca-1	D7	BioLegend	1/100
Anti-mouse CD25	3C7	BioLegend	1/100
Anti-mouse F4/80	BM8	BioLegend	1/100
Anti-mouse CD11b	M1/70	BioLegend	1/100
Anti-mouse CD11c	N418	BioLegend	1/100
Anti-mouse MHC II	M5/114.15.2	BioLegend	1/100
Anti-mouse IL-4	11B11	BioLegend	1/50
Anti-mouse IgG2a	RTK2758	BioLegend	1/100
Anti-mouse IgG2b	RTK4530	BioLegend	1/100
Anti-mouse IgG	SHG-1	BioLegend	1/100
Anti-mouse IgG2a	RTK2758	BioLegend	1/100
Anti-mouse IgG2b	MPC-11	BioLegend	1/100
Anti-mouse CD49a	Ha31/8	BD Biosciences	1/100
Anti-mouse ST2	U29-93	BD Biosciences	1/100
Anti-mouse RORγt	Q31-378	BD Biosciences	1/100
Anti-mouse CD16/CD32	2.4G2	BD Biosciences	1/100
Anti-mouse CD4	RM4-5	BD Biosciences	1/100
Anti-mouse CD8a	53-6.7	BD Biosciences	1/100
Anti-mouse Ki-67	B56	BD Biosciences	1/66
Anti-mouse CD45.1	A20	BD Biosciences	1/100
Anti-mouse CD45.2	104	BD Biosciences	1/100
Anti-mouse IL-13	eBio13A	eBioscience	1/50
Anti-mouse IL-5	TRFK5	eBioscience	1/50

inhibitor (BD Biosciences, San Diego, CA, United States) for 4 h, then cells were fixed with Fixation/Permeabilization Solution Kit (BD Biosciences, San Diego, CA, United States) following the manufacturer's instructions. RORγt, GATA3 and Ki67 were stained as recommended by the manufacturer using Foxp3/Transcription Factor Staining Buffer Set Kit (eBioscience, San Diego, CA, United States). Lineage (Lin) markers included CD3e, CD19, B220 and Gr-1. Isotype-matched control antibodies were all purchased from Biolegend (Biolegend, San Diego, CA, United States) and BD (BD bioscience, CA, United States) and used at the same concentration as test antibodies. All flow cytometry experiments were carried out on a BD FACS Verse or BD FACS Canto (BD Biosciences, San Diego, CA, United States); 500,000 – 1,000,000 events were assessed per condition within 1 h. Data were analyzed with FlowJo software (version 10.0, FlowJo LLC, Ashland, OR, United States). The lines indicate median values for each group.

Histological Analysis

Histological structures of heart were determined by standard hematoxylin-eosin (HE) staining. Briefly, resected specimens were fixed in 10% neutral buffered formalin for at least 24 h, embedded in paraffin, and 4 μm-thick sections were cut. After processing the sections according to standard protocols, they were stained with hematoxylin and eosin. The coverslips were visualized under a Leica confocal laser-scanning microscope (Leica, Wetzlar, Germany). The investigators were blinded for acquiring the images.

RNA Isolation and qRT-PCR Analysis

To quantify the expression of mRNA, qRT-PCR was performed according to standard protocols as previously described (20). Total RNA was extracted from heart tissue using Trizol (Invitrogen, Waltham, MA, United States) and total RNA

(1 μg) was then reverse-transcribed into cDNA using a First Stand cDNA Synthesis Kit (DBI Bioscience, Ludwigshafen, Germany). Real-time PCR reactions were carried out with Bestar SYBR Green qPCR master mix (DBI Bioscience, San Diego, CA, United States) using an ABI Prism 7700 Sequence Detector. The cycle threshold (Ct) values were normalized by the internal control β-actin. Primer sequences for qRT-PCR, obtained from reported literatures or designed by Pubmed Primer-BLAST. The primer pairs used were as follows: *IL-33* forward, 5′- CCCTGGTCCCGCCTTGCAAAA-3′; *IL-33* reverse, 3′- AGTTCTCTTCATGCTTGGTACCCGA-5′; *IL-25* forward, 5′-ACAGGGACTTGAATCGGGTC-3′; *IL-25* reverse, 3'- TGGTAAAGTGGGACGGAGTTG-5′; β-*actin* forward, 5′-GCCAACCGTGAAAAGATGAC-3′; and β-*actin* reverse, 3′-CATCACAATGCCTGTGGTAC -5′ (21).

Statistical Analysis

All quantitative data were transferred to Excel and the statistical analyses were computed with SPSS software for Windows (Version 21, SPSS Inc., Chicago, IL, United States). Data are expressed as means ± S.E.M. For comparison between two independent experimental groups, an unpaired two-tailed Student's *t*-test when data were normally distributed. When three or more independent groups were compared, one-way ANOVA followed by Tukey's test was performed. A *p*-value less than 0.05 was considered to be statistically significant. In each analysis, there were $n = 3–11$ replicates per group and results were representative of at least two independent experiments. Sample size for each experiment is described in the corresponding figure legend. All graphs were produced by GraphPad Prism 5.0 for windows software (GraphPad Software Inc., La Jolla, CA, United States).

RESULTS

ILC2s Are the Predominant Subset Among ILCs in Mouse Heart Tissue

In order to investigate the subsets of ILCs in heart tissue, we collected heart lymphocyte mixture by lymphocyte separation from 8 weeks old mouse heart. Percoll-enriched pellets were resuspended and stained with surface and/or intracellular antibodies. Gate strategy of heart ILC subsets was shown in **Figure 1A**. We identified a population of lineage negative (Lin^-) and CD127 positive cells in the $CD45^+$ cells. Type I ILCs were identified by $CD45^+Lin^-CD127^+NK1.1^+NKp46^+$ (including ILC1 and NK cells), ILC2s were identified by $CD45^+Lin^-CD127^+CD90.2^+ST2^+$ and ILC3s were recognized by $CD45^+Lin^-CD127^+ROR\gamma t^+$ (8, 15, 22). We found that ILC2s were divided into $KLRG1^+$ ILC2s and $KLRG1^-$ ILC2s (**Figure 1B**). Among $CD45^+$cells, Type I ILCs accounted for about 0.2% (∼100 cells/per heart) and ILC2s accounted for about 1.7% (∼500 cells/per heart) (**Figures 1C,D**). Whereas, there were merely no ILC3s (∼18 cells/per heart) based on gate strategy used in the intestinal LPL ILC3s (**Figure 1E**). The ratios of ILC2s among $CD45^+$ cells were higher in the heart tissue in compared with lung ILC2s of 8 weeks old mice (∼1.7-fold) (**Figure 1F**). As some studies reported that some ILC1 subsets, such as liver ILC1s and salivary ILC1s (23, 24), did not express CD127, we also used $CD45^+Lin^-NK1.1^+NKp46^+CD49a^+CD49b^-$ to gate ILC1s. and $CD45^+Lin^-NK1.1^+NKp46^+CD49a^+CD49b^-$ ILC1s accounted for about 0.4% of $CD45^+$cells, which suggested that part of ILC1s also did not express CD127 in murine heart tissue. Besides, conventional NK cells accounted for about 3.0% of $CD45^+$cells in the mouse heart (**Figure 1G**). Together, these data demonstrated that ILC2s were the most predominant subset of ILCs in mouse heart tissue, even greater than in lung tissue.

Heart ILC2s Peak at the Age of 4 Weeks After Birth

All ILCs initially generate in E13.5 fetal liver and seed tissues during fetal development (25). To explore the kinetics of heart ILC2s during development, we determined the ratios of total and each subset of ILC2s at the age of 1, 2, 4, 6, and 8 weeks in both heart and lung tissue. The data revealed that the frequencies of heart total and each subset of ILC2s (including $KLRG1^+$ILC2 and $KLRG1^-$ILC2) peaked at the age of 4 weeks after birth (**Figures 2A,B**), while the frequencies of lung ILC2s and subsets peaked at the age of 2 weeks after birth (**Figures 2C,D**). We also determined the ratio of Type I ILCs, ILC2s and ILC3s in mouse heart at E18.5 and post-birth day 1. The results showed that ILC2s existed, while there were very few type 1 ILCs (including ILC1 and NK cells) and no ILC3s, in mouse heart at both E18.5 and post-birth day 1 (**Supplementary Figure S1**).

Heart ILC2s Have Unique Phenotypes Compared With Lung ILC2s

Next, we investigated whether heart ILC2s were different from lung ILC2s in terms of surface markers, transcription factor, proliferation and ability of cytokines secretion. Specifically, we gated $CD45^+Lin^-CD127^+CD90.2^+ST2^+$ for ILC2s in the heart and lung to measure the expression levels of KLRG1, ICOS, CD25, Sca-1, GATA3, and Ki-67. Besides, we gated $CD45^+Lin^-CD25^+GATA3^+$ for ILC2s to measure the expression levels of CD127, CD90.2, and ST2. Our finding clearly implied that the protein levels of CD127 (IL-7R), CD90.2 (Thy1.2), ST2 (IL-33R), and KLRG1 in heart ILC2s were similar to lung ILC2s, whereas the protein levels of ICOS and CD25 (IL-2Rα) were lower in heart ILC2s than these in lung ILC2s (**Figures 3A,B**). In contrast, the protein level of Sca-1 in heart ILC2s was higher as compared with that in lung ILC2s (∼2.2-fold). A significant increasing of GATA3 was found in heart ILC2s compared with lung ILC2s (∼1.6-fold) (**Figure 3C**). As seen in the **Figure 3D**, heart ILC2s had a weaker proliferation ability than lung ILC2s, indicated by Ki-67 positive cells (∼0.33-fold).

In respond to the cytokines IL-25, TSLP, and IL-33, and ILC2s are the potent sources to produce IL-4, IL-5, and IL-13. Both IL-4 and IL-13 could induce smooth-muscle contraction and wound repairing after infections (26, 27). We therefore stimulated isolated mouse heart and lung lymphocytes with IL-33, following determined the

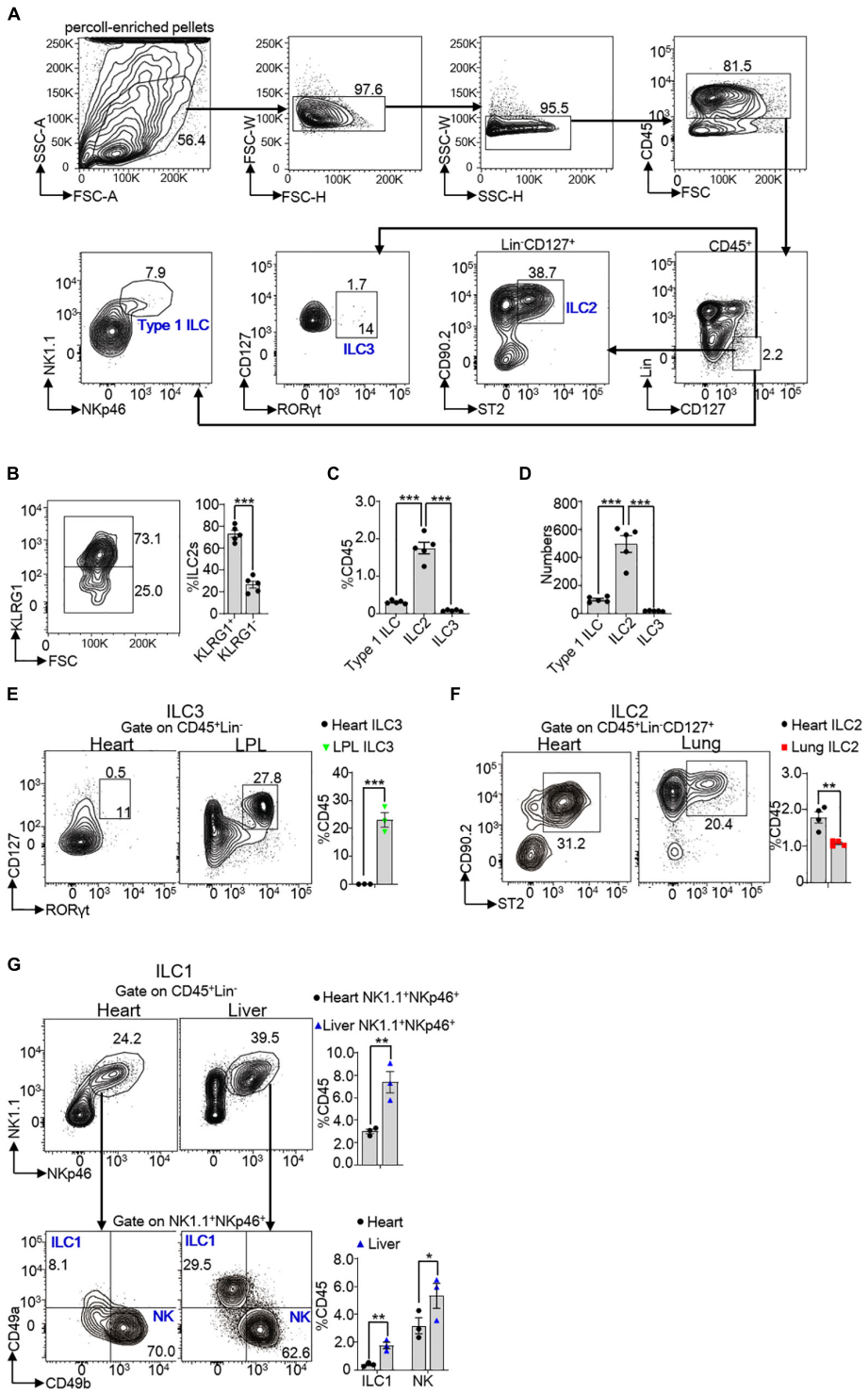

FIGURE 1 | Subsets of ILCs in mouse heart tissue. **(A)** Gate strategy of ILCs in the heart of mice. Lineage (Lin) markers included CD3e, CD19, B220, and Gr-1. The number inside of gate indicates cell events. **(B)** Expression of KLRG1 in heart ILC2s of 8 weeks old mice. **(C,D)** Cumulative frequencies **(C)** and enumeration **(D)** of Type I ILCs (including ILC1s and NK cells), ILC2s and ILC3s in $CD45^+$ lymphocyte in the heart of 8 weeks old mice. **(E)** Cumulative frequencies of ILC3s in $CD45^+$ lymphocyte in heart and LPL of 8 weeks old mice. The number inside of gate indicates cell events. **(F)** Cumulative frequencies of ILC2s among $CD45^+$ lymphocyte in the heart and lung tissue of 8 weeks old mice. **(G)** Another gate strategy of ILC1s irrespective of CD127 expression and cumulative frequencies of ILC1s ($CD45^+Lin^-NK1.1^+NKp46^+CD49a^+CD49b^-$) and NK cells ($CD45^+Lin^-NK1.1^+NKp46^+CD49a^-CD49b^+$) in heart and liver of 8 weeks old mice. Each dot represents one mouse; error bars represent SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Unpaired two-tailed Student's *t*-test **(B,E–G)**. One-way ANOVA **(C,D)**.

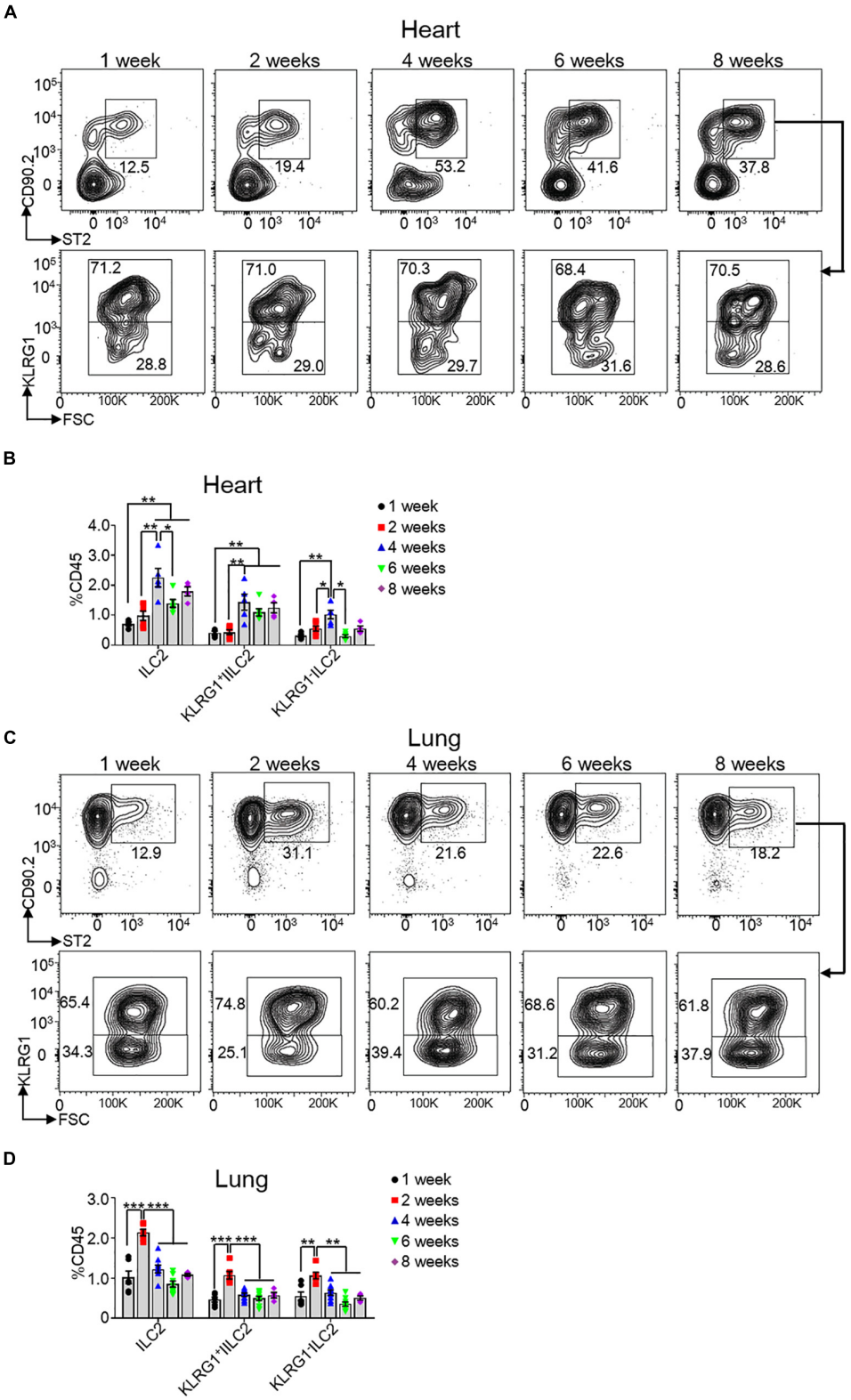

FIGURE 2 | Kinetics of heart ILC2s development after birth. **(A,C)** Flow cytometric analysis of ILC2, KLRG1^{+} ILC2 and KLRG1^{-} ILC2 in the heart **(A)** and lung **(C)**, respectively, of mice at the indicated age after birth. **(B,D)** Cumulative frequencies of ILC2s in the heart **(B)** and in the lung **(D)** of mice at the indicated age after birth. Each dot represents one mouse; error bars represent SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. One-way ANOVA **(B,D)**.

production of IL-4, IL-5, and IL-13 by ILC2s. Compared with lung ILC2s, heart ILC2s had a stronger ability to produce IL-4 and IL-13 (∼2.4-fold and ∼6.8-fold in IL-33 stimulation, respectively) (**Figure 3E**). Heart ILC2s and lung ILC2s had the similar ability to produce IL-5 (∼0.92-fold) (**Figure 3E**). Besides, compared with lung ILC2s, heart ILC2s also had a stronger ability to produce IL-4 in response to PMA/ionomycin (**Figure 3F**). These results suggest that heart ILC2s had unique phenotypes in terms of surface marker, transcription factor, proliferation and cytokine production.

Circulating Cells Minimally Replace Heart ILC2s

Consideration of affluent bloodstream in the heart, we tested directly whether hematogenous precursors continuously replenished the pool of heart ILC2s in 8 weeks old mice. For this reason, we generated parabiotic mice model, which widely used for the verification of tissue-resident cells in non-lymphoid tissues (13, 28). After 2 months of parabiosis, we analyzed the percentages of various lymphocyte subsets that derived from the donor or host parabiont. Our results clearly show that about 46.8% of $CD4^+$ T and 47.4% $CD8^+$ T cells in the peripheral blood (pBL) versus about 45.8% of $CD4^+$ T and 46.3% $CD8^+$ T cells in the spleen (SP) belonged to the parabiont donor (**Figure 4A**), suggesting that the circulatory system was balanced between the parabiotic mice. Besides, about 47.7% of $CD4^+$ T and 44.0% $CD8^+$ T cells in the heart tissue and about 44.6% of $CD4^+$ T and 45.4% $CD8^+$ T cells in the lung tissue derived from parabiont donor, which demonstrated that circulating T cells infiltrated adequately in local tissues (**Figure 4B**). Remarkably, very few heart ILC2s (∼2.0%) were derived from the blood, the same as lung ILC2s (∼1.1%) (**Figure 4C**). This indicates that heart ILC2s are initially generated and seed tissues during fetal development and regenerate predominantly through local renewal.

Heart ILC2s Rapidly Expand and Secrete IL-4 During Myocardial Necroptosis

Necroptosis and apoptosis are crucially involved in severe cardiac pathological conditions, including myocardial infarction, ischemia-reperfusion injury and heart failure (14). To investigate whether ILC2s may participate in this process, we established a mouse model of oxidative stress-induced myocardial necroptosis (14). We used the DOX, a well-evaluated chemotherapeutic agent, to establish the irreversible cardiac toxicity, including massive cardiomyocytes loss, cardiomyopathy and heart failure (29, 30). DOX-induced heart injury was firstly confirmed by hematoxylin-eosin (HE) staining. Compared to untreated mice, the DOX-treated mice had significant myocardium necrosis along with nuclear enlargement and swollen of cardiomyocytes (**Figure 5A**). Meanwhile, we found that the frequency and number of ILC2s were significant higher in DOX-treated mouse (17.68 ± 4.46) than that in untreated mouse (8.51 ± 1.87) after 24 h treatment (**Figure 5B**) as well as after 96 h treatment (**Figure 5C**). The frequency of Ki-67^+ ILC2s was increased in DOX-treated mice (16.5 ± 1.8), compared with that in untreated mice (7.1 ± 1.4) after 24 h treatment (**Figure 5D**). Interestingly, the frequency and proliferation activity of lung ILC2s were not changed after 24 h DOX treatment (**Figures 5E,F**). In addition, we also found that the frequencies of total macrophages ($CD11b^+F4/80^+$cells) and type 1 conventional dendritc cells (cDC1s) ($CD11b^-CD11c^+MHCII^+$), which were involved in heart injury (31, 32), were not noticeable changed after 24 h DOX treatment (**Supplementary Figures S2A,B**).

Because IL-33 and IL-25 are reported to promote ILC2s proliferation and activation (33, 34), we measured the *IL-33* and *IL-25* mRNA expression in the heart tissue. *IL-33* but not *IL-25* mRNA expression level increased after DOX treatment (∼3.5-fold and ∼0.87-fold, respectively) (**Figure 5G**). Compared with control mice, heart ILC2s produced more IL-4, but not IL-5 and IL-13 (∼2.6-fold, ∼1.1-fold and ∼1.2-fold, respectively) (**Figure 5H**). We also measured the $CD3^+$T cells and IL-4^+T cells and both of them were not significant changed after 24 h DOX treatment (**Supplementary Figure S2C**). Thus, the obtained data proposes that rapid IL-33 production resulted in ILC2s expansion and IL-4 secretion prior to other immune cells during DOX-induced myocardial necroptosis.

DISCUSSION

In this report, we present the detailed analysis of various subsets of ILCs and phenotypes in the heart tissue. The findings of this study demonstrates the predominant heart ILC2s subset, even greater than lung ILC2s. Our results illustrated that ILC2s at the age of 4 weeks after birth can confidential as heart tissue of mouse. Notably, the heart ILC2s were characterized by lower expression of ICOS, CD25 (IL-2Rα), Ki-67, as well as higher expression of IL-4, IL-13, Sca-1 and GATA3. Our results highlighted that heart-resident ILC2s showed tissue-specific phenotypes and rapidly responded to DOX-induced cardiotoxicity.

Almost all subsets of ILCs and ILC precursors express IL-7R (CD127) and response to IL-7 stimulation (3). We found that $CD45^+Lin^-CD127^+$ $CD90.2^+ST2^+$ ILC2s, defined in lung tissue (8), was the most dominant ILC subset in mouse heart, while $CD45^+Lin^-$ $CD127^+NK1.1^+NKp46^+$ Type I ILCs (15), and $CD45^+Lin^-CD127^+ROR\gamma t^+$ILC3s (22) was merely found in mouse heart. ILC2s were the predominant part of ILCs in human and mouse heart tissue (11), although the authors and we used different gating strategy. The authors identified that ILC2s (identified as $CD45^+Lin^-CD90^+ROR\gamma t^-$T-$bet^-ST2^+KLRG1^+$) accounted for about 20% of $CD45^+$ Lin^-CD90^+ cells in the mice heart tissue, however, our data showed that ILC2s accounted for about 40% of $CD45^+$ Lin^-CD90^+ $CD127^+$ cells. This might because that a part of $CD45^+Lin^-CD90^+$ cells did not express CD127 (Data not shown).

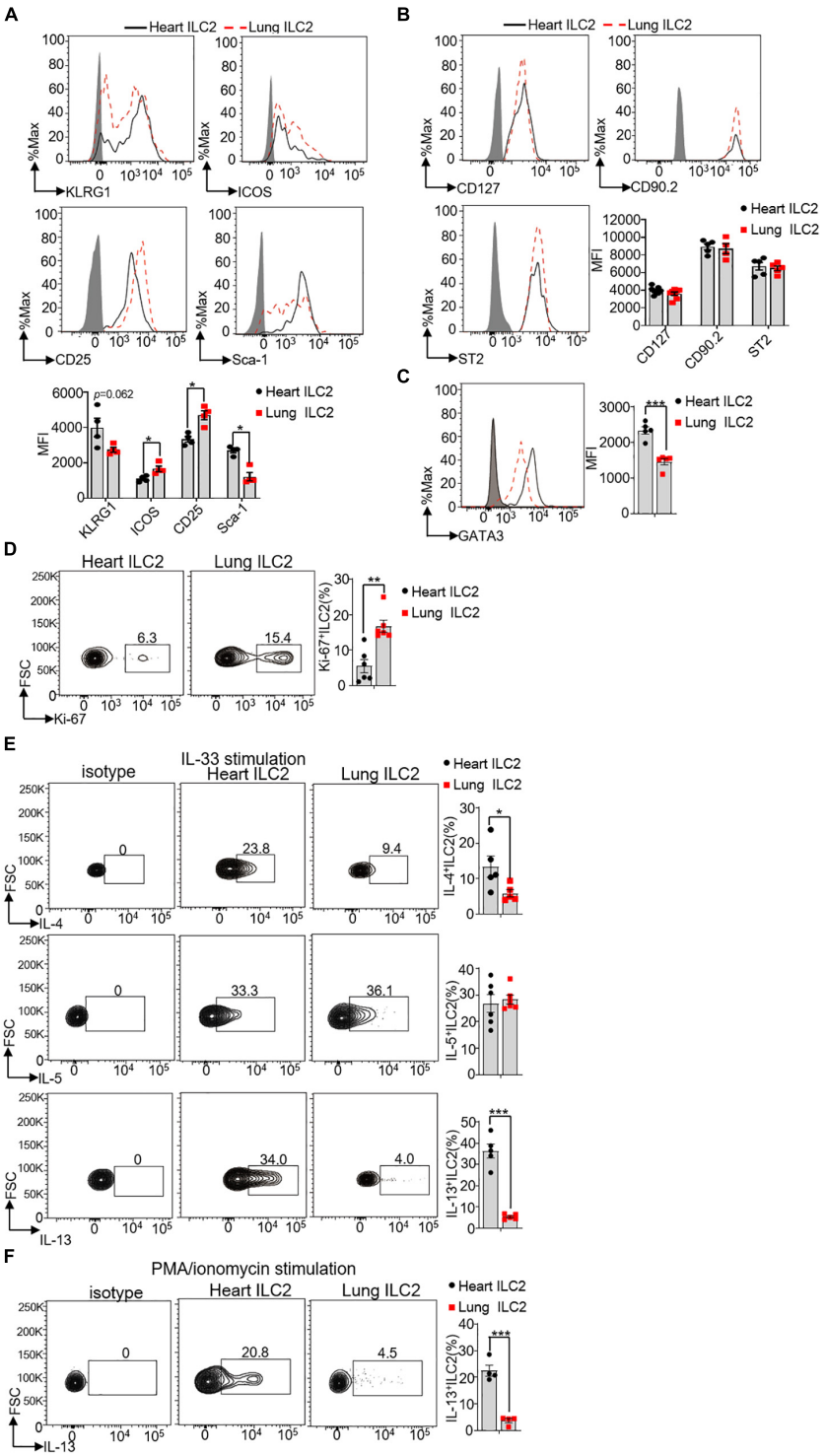

FIGURE 3 | Phenotype differences between heart and lung ILC2s. **(A)** Histograms of cell surface expression and the mean fluorescence intensity (MFI) of KLRG1, ICOS, CD25 and Sca-1 in heart and lung ILC2s (identification as CD45$^+$Lin$^-$CD127$^+$CD90.2$^+$ST2$^+$cells) of 8 weeks old mice. **(B)** CD127, CD90.2 and ST2 in heart and lung ILC2s (identification as CD45$^+$Lin$^-$CD127$^+$CD25$^+$GATA3$^+$cells) of 8 weeks old mice. **(C)** The relative expression of GATA3 in heart and lung ILC2s, respectively, of 8 weeks old mice. **(D)** Flow cytometric analysis and cumulative frequencies of Ki-67-expressing ILC2s in heart and lung, respectively, of 8 weeks old mice. **(E)** Flow cytometric analysis and cumulative frequencies of IL-4 (upper), IL-5 (middle) and IL-13 (lower) by heart and lung ILC2s, respectively, following stimulation with IL-33 in the presence of Golgi Plug for 4 h of 8 weeks old mice. **(F)** Flow cytometric analysis and cumulative frequencies of IL-13 by heart and lung ILC2s, respectively, following stimulation with PMA/ionomycin in the presence of Golgi Plug for 4 h of 8 weeks old mice. Each dot represents one mouse; error bars represent SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Unpaired two-tailed Student's *t*-test **(A–F)**.

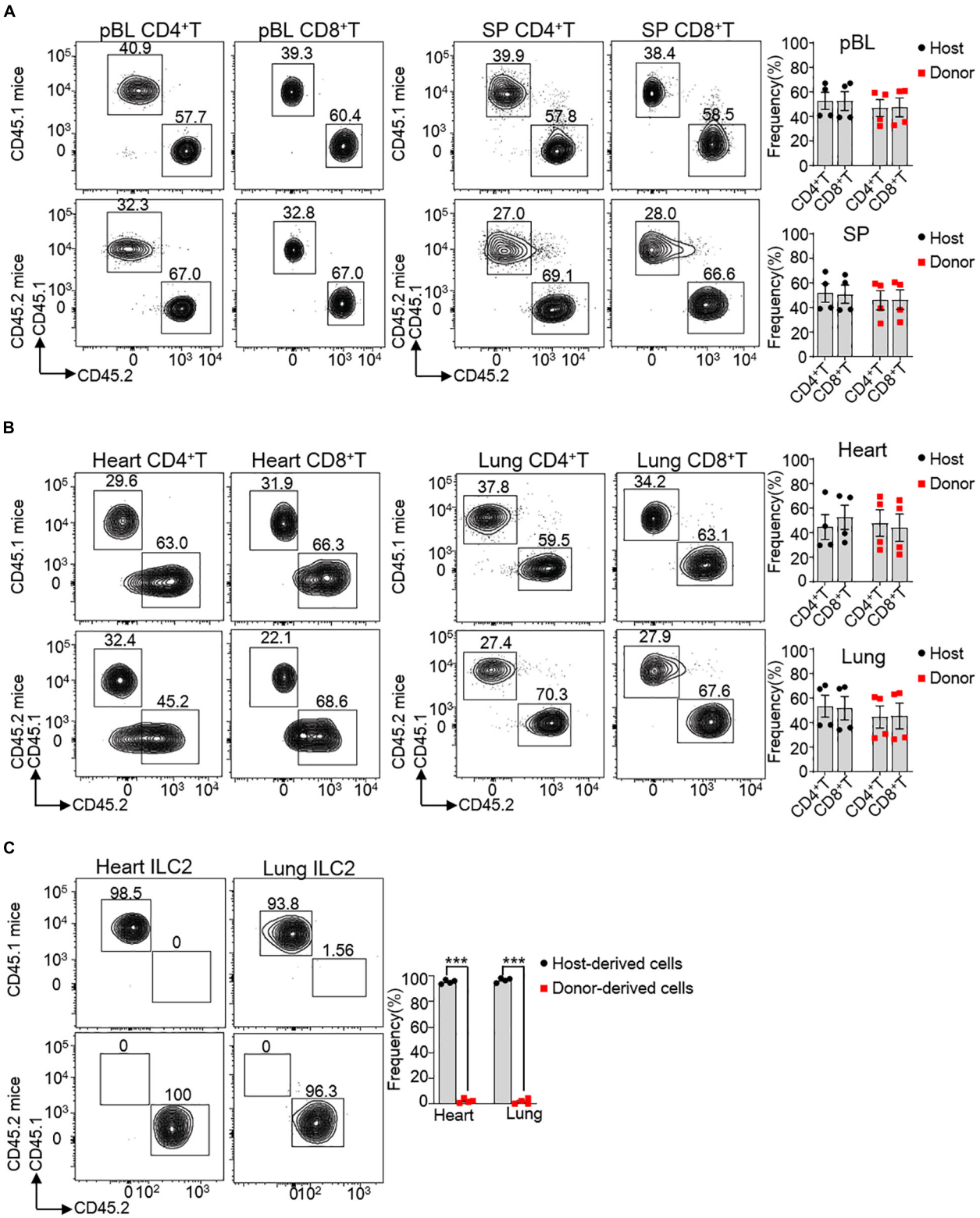

FIGURE 4 | Circulating cells minimally contribute to heart ILC2s renew. **(A,B)** Flow cytometric analysis and cumulative frequencies of $CD4^+$ T cells and $CD8^+$ T cells in peripheral blood (pBL) and spleen (SP) **(A)** as well as heart and lung **(B)** of parabiotic mice after 2 months of parabiosis between 8 weeks old WT CD45.1 and WT CD45.2 C57BL/6 mice. **(C)** Flow cytometric analysis and cumulative frequencies of ILC2s in heart and lung of parabiotic mice after 2 months of parabiosis between WT CD45.1 and WT CD45.2 C57BL/6 mice. Each dot represents one mouse; error bars represent SEM; ***$p < 0.001$. Unpaired two-tailed Student's *t*-test **(A–C)**.

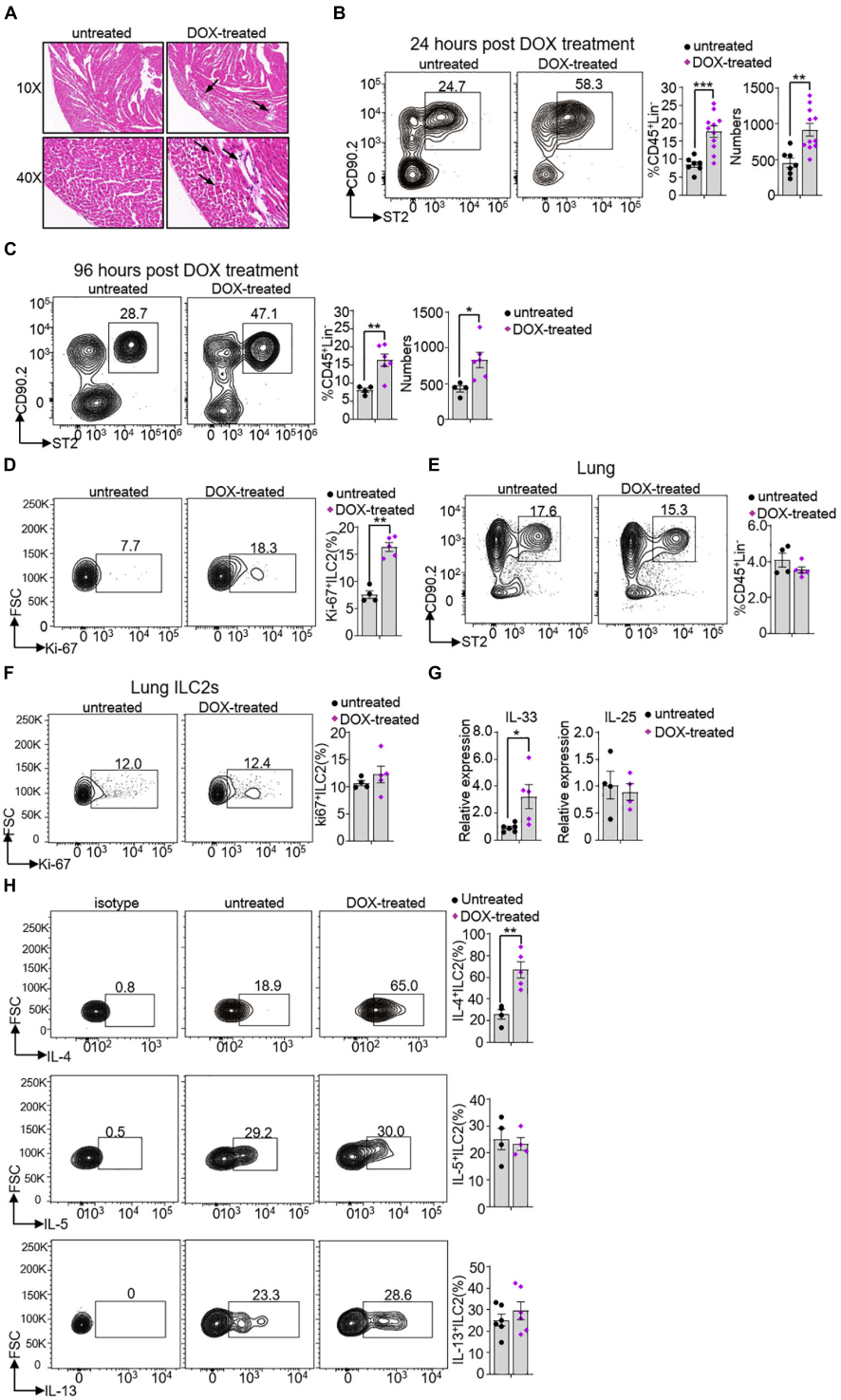

FIGURE 5 | Heart ILC2s expansion and cytokine secretion in response to Doxorubicin treatment. **(A)** Hematoxylin-eosin (HE) staining and representative pictures from heart of 8 weeks old mice after 24 h DOX treatment. The arrow direction indicates representative changes. **(B,C)** Flow cytometric analysis, cumulative frequencies and enumeration of ILC2s in the heart of 8 weeks old mice after 24 h **(B)** and 96 h **(C)** DOX treatment. **(D)** Flow cytometric analysis and cumulative frequency of Ki-67-expressing ILC2s in heart of 8 weeks old mice after 24 h DOX treatment. **(E)** Flow cytometric analysis and cumulative frequency of ILC2s in the lung of 8 weeks old mice after 24 h DOX treatment. **(F)** Flow cytometric analysis and cumulative frequency of Ki67-expressing ILC2s in lung of 8 weeks old mice after 24 h DOX treatment. **(G)** The relative mRNA expression of *IL-33* and *IL-25* in the heart tissue of 8 weeks old mice after 24 h DOX treatment. **(H)** Flow cytometric analysis and cumulative frequencies of IL-4-producing (upper), IL-5-producing (middle) and IL-13-producing ILC2s (lower) in the heart tissue of mice after 24 h DOX treatment, $n = 4–11$. Error bars represent SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Unpaired two-tailed Student's *t*-test **(B–H)**.

All ILCs generate and seed tissues during fetal development and the perinatal period is a critical window for the distribution of innate tissue-resident immune cells within developing organs (25, 35). Unlike tissue macrophages, a majority of peripheral ILC2 pools are generated *de novo* during the postnatal window (5, 8), by display little hematogenous redistribution to other tissues (28). Although a minor contribution from circulating precursors can contribute to tissue pools, ILCs regenerate predominantly through local renewal after birth in the resting state (5). Our study also suggested that circulating cells minimally replace heart ILC2s under physiological status. However, whether circulating ILC2s or interorgan migration of tissue-resident ILC2s contributes to heart ILC2s under pathophysiological status are still unknown. This is because a recent study reported that a population of inflammatory ILC2s (iILC2s), which are circulating cells and derived from intestinal ILC2s, could migrate to the lung after IL-25 stimulation or helminth infection (36). Thus, although we found obvious proliferation of ILC2s in heart tissue after DOX treatment, we still could not exclude the possibility that ILC2s migrate to heart from other organs when the heart damage occurs.

During the alveolar phase of lung development, the increasing production of IL-33 accumulates ILC2 cells and the frequency of ILC2s in the mouse lung reached the peak at the age of 2 weeks after birth (8). But, heart resident ILC2s peaks at the age of 4 weeks, which may be due to the lower IL-33 production by cardiac fibroblasts during heart development (37) or less antigens exposure delays the development of ILC2s in the heart (35). In our study, increased IL-33 expression are parallel with increased Ki-67$^+$ ILC2s after DOX treatment indicated that IL-33 signal pathway in the heart is important for maintenance of ILC2s. In addition, previous study indicated that IL-4 can activate STAT6 and then induce the expression of GATA3, which forms a positive feedback loop to reinforce Th2 differentiation (38, 39). Thus, we assumed that this IL-4/STAT6/GATA3 axis maybe also take effect in heart ILC2s development.

Compared with lung ILC2s in the lung, heart ILC2s have unique features in the terms of surface marker, such as lower expression level of ICOS and CD25 and higher expression level of Sca-1. ICOS is an important molecule in T cell signal transduction (40) and deficiency of ICOS showed decreased ratio of ILC2s and cytokine production (41, 42). CD25 is a key receptor of IL-2 signaling, which regulates cells survival (43). Besides, Sca-1 is surface molecule stem cell antigen-1, representing the differentiation potential (44). Consistent with these, heart ILC2s showed lower expression of Ki-67. As compared with lung tissue, heart ILC2s might be with a lower proliferation capacity and a more immature phenotype, which might because of the relative sterile micro-environment. Lung ILC2s must maintain a higher proliferative level to expand rapidly in response to various stimulations, such as antigens, virus and worm (8, 45). Interestingly, compared to lung ILC2s (46), heart ILC2s have a stronger ability to produce IL-4 and IL-13 in response to IL-33 or PMA/ionomycin stimulation. Previous study have demonstrated that GATA3 together with STAT6 promotes the expression of IL-4 and IL-13 (47–49). So, these evidence suggest that higher GATA3 expression level of heart ILC2s might be responsible for the higher capacity of IL-4 and IL-13 production. These difference between heart ILC2s and lung ILC2s further demonstrates that the local tissue microenvironment had a profound influence on cells phenotype and function.

Myocardial damage causes sterile inflammation, by recruitment and activation of innate and adaptive immune system cells (31, 50). In this study, we found that ILC2s expanded and produced IL-4 immediately after DOX-induced myocardial necroptosis prior to macrophage, dendrtic cells and IL-4$^+$T activation. IL-4 is well-known to regulate a variety of immune responses, including T-cell differentiation and macrophage M2 polarization (51, 52). Previous studies showed that IL-4 serves as an early endogenous neuroprotective mechanism soon after stroke onset and is important in the acute stages of stroke (53, 54). Thus, we speculate that in response to myocardial damage, heart ILC2s act as the first line of responder and produce IL-4 to promote the response during inflammation and cardiac tissue repair. However, production of IL-4 by ILC2s and T cells persistent in the end of recovery stage may also promote myocardial fibrosis (55). In the line with previous study, IL-4 could upregulated the expression of procollagen genes and stimulates collagen production in mouse cardiac fibroblasts (56).

Overall, ILC2s with unique phenotypes are the major subset of ILCs in the heart and different from lung ILC2s in mouse model. Importantly, ILC2s could expand and activate immediately in response to heart damage. Our finding raises the potential for IL-33-elicited ILC2s response as therapeutics for attenuating heart damage.

LIMITATION

A tissue-specific knock-out mouse model of ILCs and acquirement enough amount of ILCs to transplant are some significant limitations in the current work. Undoubtedly, future well-accepted studies would be needed to provide the localization of ILC2s within the heart and more direct evidence of a functional requirement for ILC2s in this cardiac injury model.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

All animal experiments were carried out in accordance with the recommendations of the Animal Ethics Committee of the Army Medical University. The animal experiment protocols

were approved by the Animal Ethics Committee of the Army Medical University.

AUTHOR CONTRIBUTIONS

The work presented was performed in collaboration with all authors. YaD designed and performed the experiments, analyzed the data, and wrote the manuscript. SW, YY, MM, XC, and SC performed the experiments. LL, YG, YC, and SI designed the experiments and edited the manuscript. BC, SL, and XL designed the research and supervised the study. YoD devised the concept, designed the research, supervised the study, and wrote the manuscript.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (No. 81703521 to YaD; Nos. 81874313 and 81922068 to YoD; and Nos. 81520108029 and 81773742 to XL). This study was also supported by grants from the Army Laboratory animal administration office [SYDW(2017)01 to BC].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00802/full#supplementary-material

FIGURE S1 | Heart ILC2s exist before birth. Flow cytometric analysis as described in **Figure 1A** and cumulative frequencies of type I ILCs (including ILC1 and NK cells), ILC2s and ILC3s in the heart of mice at the E18.5 and post-birth day 1. The number inside of gate indicates cell events. Each dot represents one mouse; error bars represent SEM; $^{**}p < 0.01$, $^{***}p < 0.001$. One-way ANOVA.

FIGURE S2 | Macrophages, dendritic cells (DCs) and IL-4^+ $CD4^+$ T cells in the heart are not increased after 24 h of DOX treatment. **(A,B)** Flow cytometric analysis and cumulative frequencies of macrophages ($CD11b^+F4/80^+$ cells) **(A)** and type 1 conventional dendritc cells (cDC1s) ($CD11b^-CD11c^+$ $MHCII^+$ cells) **(B)** in the heart of mice after 24 h DOX treatment. **(C)** Flow cytometric analysis and cumulative frequencies of $CD3^+$ T and IL-4^+ $CD4^+$ T cells in the heart following stimulation with PMA/ionomycin in the presence of Golgi Plug for 4 h of mice after 24 h DOX treatment, $n = 3$–4. Error bars represent SEM. Unpaired two-tailed Student's *t*-test **(A–C)**.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 21 April 2021
doi: 10.3389/fimmu.2021.619465

The Immunoproteasome Subunits LMP2, LMP7 and MECL-1 Are Crucial Along the Induction of Cerebral Toxoplasmosis

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Cell survival and function critically relies on the fine-tuned balance of protein synthesis and degradation. In the steady state, the standard proteasome is sufficient to maintain this proteostasis. However, upon inflammation, the sharp increase in protein production requires additional mechanisms to limit protein-associated cellular stress. Under inflammatory conditions and the release of interferons, the immunoproteasome (IP) is induced to support protein processing and recycling. In antigen-presenting cells constitutively expressing IPs, inflammation-related mechanisms contribute to the formation of MHC class I/II-peptide complexes, which are required for the induction of T cell responses. The control of *Toxoplasma gondii* infection relies on Interferon-γ (IFNγ)-related T cell responses. Whether and how the IP affects the course of anti-parasitic T cell responses along the infection as well as inflammation of the central nervous system is still unknown. To answer this question we used triple knockout (TKO) mice lacking the 3 catalytic subunits of the immunoproteasome (β1i/LMP2, β2i/MECL-1 and β5i/LMP7). Here we show that the numbers of dendritic cells, monocytes and $CD8^+$ T cells were reduced in *Toxoplasma gondii*-infected TKO mice. Furthermore, impaired IFNγ, TNF and iNOS production was accompanied by dysregulated chemokine expression and altered immune cell recruitment to the brain. T cell differentiation was altered, apoptosis rates of microglia and monocytes were elevated and STAT3 downstream signaling was diminished. Consequently, anti-parasitic immune responses were impaired in TKO mice leading to elevated *T. gondii* burden and prolonged neuroinflammation. In summary we provide evidence for a critical role of the IP subunits β1i/LMP2, β2i/MECL-1 and β5i/LMP7 for the control of cerebral *Toxoplasma gondii* infection and subsequent neuroinflammation.

Keywords: Toxoplasma, immunoproteasome, neuroinflammation, cerebral toxoplasmosis, LMP

OPEN ACCESS

Edited by:
Claudia U. Duerr, McGill University, Canada

Reviewed by:
Peter Michael Kloetzel, Charité–Universitätsmedizin Berlin, Germany
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Specialty section:
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology

Received: *20 October 2020*
Accepted: *16 March 2021*
Published: *21 April 2021*

Citation:
French T, Israel N, Düsedau HP, Tersteegen A, Steffen J, Cammann C, Topfstedt E, Dieterich D, Schüler T, Seifert U and Dunay IR (2021) The Immunoproteasome Subunits LMP2, LMP7 and MECL-1 Are Crucial Along the Induction of Cerebral Toxoplasmosis. Front. Immunol. 12:619465. doi: 10.3389/fimmu.2021.619465

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a highly successful intracellular parasite capable of infecting all mammals including around 30-70% of all humans (1). In humans, *T. gondii* infection is usually asymptomatic and resolves with minimal pathology. However, if infected individuals acquire an immunodeficiency with impaired T cell function later in life, they are at risk for reactivation of latent toxoplasmosis (2). Early control of *T. gondii* is dominated by innate immune cells such as macrophages, dendritic cells (DCs) and circulating monocytes as well as their secreted proinflammatory cytokines, e.g. tumor necrosis factor (TNF) and interleukin (IL)-12 (3–5). Interferon-γ (IFNγ) is essential for the cell-mediated control of *T. gondii*. Its production by natural killer (NK) cells and T cells is induced by TNF and IL-12 (6). Moreoever, two major mechanisms involved in parasite control are the IFNγ-induced activation of myeloid cells and cytotoxic activity of $CD8^+$ T cells (7). IFNγ induces inducible nitric oxide synthase (iNOS) expression by myeloid cells which in turn promotes the production of nitric oxide (NO) thereby inhibiting parasite growth (8). $CD8^+$ T cells are known to be crucial for long-term control and containment of *T. gondii*. They prevent the transformation of cyst-forming bradyzoites into fast-replicating tachyzoites thereby achieving both, a restriction of parasite burden as well as the establishment of chronic infection (9, 10). $CD8^+$ T cell-derived IFNγ is crucial for long term disease control and relies on $CD4^+$ T cell help to facilitate antigen-presentation and upregulate co-stimulatory molecule expression on antigen-presenting cells (APCs). In order to maintain a stable anti-parasite $CD8^+$ T cell response, APCs must present parasite-derived peptides *via* major histocompatibility complex class I (MHC I) (11, 12). This requires intracellular processing of parasite proteins, a mechanism which is mainly mediated by the immunoproteasome (IP), a proteolytic protein complex which is induced upon inflammation, e.g. by IFNγ (7, 13).

Upon IFNγ stimulation, standard proteasomes are replaced by *de-novo* synthesized IPs, harboring the three catalytically active subunits β1i/LMP2, β2i/MECL-1 and β5i/LMP7 instead of β1/delta, β2/zeta and β5/MB1. In cells of hematopoietic origin IPs are constitutively expressed (14). In APCs IP expression results in the generation of an altered peptide repertoire and increased number of MHC I ligands due to enhanced protein substrate turnover and changed cleavage specificities (15–17). Whether and how the simultaneous absence of the inducible catalytic subunits β1i/LMP2 (*Psmb8*), β2i/MECL-1 (*Psmb9*) and β5i/LMP7 (*Psmb10*) alters the course of infections remains unclarified.

Research exploring IP function in inflammatory diseases of the central nervous system (CNS) has largely focused on stroke and Alzheimer's disease (18, 19), where a marked upregulation of IP in reactive glia has been described. The IP is also associated with an increase in phagocytosis and iNOS production in microglia, a common feature of many neurodegenerative diseases (20–22). To better understand how the IP functions in the CNS and especially during neuroinflammation, infection models are sorely needed. Upon LCMV infection in the CNS, LMP7 was vital for the $CD8^+$ T cell-induced pathogenesis of LCMV-induced meningitis as $LMP7^{-/-}$ mice exhibited a reduced and delayed disease outcome with fewer infiltrating immune cells (23). Interestingly, this seemed to be LMP7 specific, as $LMP2^{-/-}$ and $MECL\text{-}1^{-/-}$ mice had no change in disease compared to WT mice.

In regards to the IP's role during *T. gondii* infection, previous work from Tu et al., described that mice absent of the single subunits LMP2 or LMP7 were more susceptible to acute *T. gondii* infection (24). Primarily investigating the effect of the IP on the induction of a Th1 immune response, they observed that the acute stage of the infection with fast replicating tachyzoites strongly upregulated the expression of both IP subunits, LMP2 and LMP7, in APCs collected from peritoneal exudate cells (PEC). Further, $LMP7^{-/-}$ mice exhibited strong DC dysfunction as their ability to present immunogenic peptides was impaired and the subseqeunt $CD8^+$ T cell IFNγ and Granzyme B response was significanlty reduced compared to WT counterparts. Of note, there was little observable change in these cell types in $LMP2^{-/-}$ mice in the periphery, however, these mice were still susceptible to *T. gondii* infection.

In order to investigate the role of the IP through the course of CNS infection-induced inflammation, we assessed how the absence of all three catalytic IP subunits in TKO mice affects the course of infection-induced inflammation using the neurotropic parasite *T. gondii*. Hereby, we investigated IP deficiency over the course of *T. gondii* infection, focusing on its role in the chronic phase of infection, where the encysted parasite resides primarily in the CNS. This study shows for the first time a prolonged neuroinflammation that is maintained by perturbed cytokine release due to chronic *T. gondii* infection. In addition, we demonstrate increased production of iNOS in microglia and myeloid subsets in brain tissue of infected TKO animals as well as reduced numbers of regulatory T cells, reduced STAT3 phosphorylation but increased induction of apoptosis in myeloid cells. This study demonstrates that IP deficiency results in a lack of parasite control by ultimately increasing susceptibility of these animals to *T. gondii*, highlighting the importance of the IP in terms of induction, maintenance and resolution of *T. gondii*-induced neuroinflammation.

METHODS

Animals

Conventional immunoproteasome Triple KO (TKO) mice C57BL/6J-LMP2/$Psmb9^{-/-}$MECL-1/$Psmb10^{-/-}$LMP7/$Psmb8^{-/-}$ were kindly provided by Prof. Kenneth L. Rock and Regeneron Pharmaceuticals, Inc. (VG MAID number VG1230 + *Psmb10*) (15). 8 to 12 week-old C57BL/6J mice were bred in the same animal facility. Mice were age and sex matched between the wild type (WT) and deficient mice. All mice were group-housed in 12-h day/night cycles at 22 °C with free access to food and water. All animal experiments were approved by local authorities according to German and European legislation.

Toxoplasma gondii Infection

T. gondii cysts of type II strain ME49 were harvested from brains of female NMRI mice chronically infected with *T. gondii* cysts

6-10 months earlier, as described previously (25). In short, isolated brains were mechanically homogenized in 1 ml sterile phosphate-buffered saline (PBS), and the number of cysts in the homogenate was determined using a light microscope. Mice were infected with two cysts *via* oral gavage.

Organ Collection

First, mice were deeply anaesthetized by isoflurane inhalation (Baxter). Subsequently, mice were transcardially perfused with 60 ml sterile PBS. Single-cell suspension of mesenteric lymph nodes and spleen were generated by mechanically passing tissue through a 40 µm strainer in PBS complemented with 2% fetal calf serum (FCS). Brains were removed and stored in RPMI medium (life technologies) or RNA*later* (Qiagen) for additional analysis. Samples stored in RNA*later* were kept at 4 °C overnight and then transferred to -20°C. Samples in RPMI medium were stored on ice until further experimental procedures.

Cell Isolation

To isolate brain immune cells, brains were homogenized in a buffer containing 1 M HEPES (pH 7.3) and 45 % glucose and then filtered through a 70 µm strainer. Leukocytes were separated *via* Percoll density gradient centrifugation (GE Healthcare) as we described previously (26). Living cells were counted using a Neubauer counting chamber and trypan blue staining.

Flow Cytometric Analysis

Single cell suspensions were incubated with an anti-FcγIII/II receptor antibody (clone 93, eBioscience) to block unspecific binding and Zombie NIR™ (BioLegend), a fixable viability dye. Thereafter, cells were stained with fluorochrome-conjugated antibodies against cell surface markers: CD45 (30-F11), CD11b (M1/70), Ly6C (HK1.4), CD45.2 (104), CD40 (3/23), MHCI (28-14-8) and MHCII (M5/114.15.2) all purchased from eBioscience; CD3 (17A2), CD4 (RM4-5), CD8α (53-6.7), CD80 (16-10A1), CD44 (IM7), CD62-L (MEL-14), PD-1 (29F.1A12) and NK1.1 (PK136) all purchased from BioLegend; and Ly6G (1A8) purchased from BD Biosciences in FACS buffer (with 2% FBS, 0.1% NaN3) at 4 °C for 30 min and then fixed in 4% paraformaldehyde (PFA, Affymetrix) for 15 min. Matched FMO controls were used to assess the level of background fluorescence in the respective detection channel.

Intracellular staining was performed on $5x10^5$ cells/well after *ex vivo* stimulation with *Toxoplasma* lysate antigen (200 µg/mL) in the presence of brefeldin A (10 µg/mL, BioLegend) and monensin (10 µg/mL, BioLegend) at 37 °C for 6 h. Afterwards, cells were incubated with anti-FcγIII/II receptor antibody (clone 93, eBioscience) and Zombie NIR™ (BioLegend). Surface epitopes were then stained with CD45 (30-F11), CD11b (M1/70), Ly6C (HK1.4), Ly6G (1A8), CD3 (17A2), CD4 (RM4-5) and CD8α (53-6.7) for 30 min at 4 °C. Stained cells were fixed in 4% PFA and permeabilized using Perm/Wash Buffer (BioLegend). To measure cytokine expression, cells were stained with the flourochrome-conjugated antibodies against intracellular proteins TNF (MP6-XT22), FoxP3 (FJK.16s) and IL-12p40 (C17.8) purchased from eBioscience; iNOS (clone 6, BD Biosciences), Granzyme B (QA16AO2, BioLegend), and IFNγ (XMG1.2, BioLegend) in permeabilization buffer (Invitrogen) for 45 min. Matched isotype controls were used to assess the level of non-specific binding. Flow cytometric analysis was performed on BD LSRFortessa (BD Bioscience) and on Attune NxT Flow Cytometer (Thermo Fisher) and analyzed with FlowJo (version 10, Flowjo LLC).

Calculation of absolute cell count was performed by multiplying the viable population frequencies derived from flow cytometry analysis with the hemocytometer cell count of the respective sample.

Apoptosis Assay

Cellular apoptosis was quantified using a FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend) following the manufacturer's instructions. $5x10^5$ splenocytes were isolated, as described above, rinsed with staining buffer and resuspended in Annexin V Binding Buffer (BioLegend). The cells were then incubated with 5 µL of FITC Annexin V and 10 µL of 7-AAD solution for 20 min at room temperature light protected. Fluorescence was measured on Attune NxT Flow Cytometer (Thermo Fisher) and analyzed with FlowJo (version 10, Flowjo LLC).

Transwell $CD8^+$ T Cell Migration Assay

Naïve $CD8^+$ T cells were purified using CD8α T Cell Isolation Kit mouse (Miltenyi Biotec) following the manufacturer's instruction. Chemokines CXCL12 and CCL21 (Peprotech) were used at 250 ng/mL each in 500 µL of Assay Medium containing RPMI 1640, 10mM HEPES and 0.1% BSA (Applichem). Migration assay was performed by seeding $2x10^6$ cells in 200 µL Assay Medium into the upper chamber of 48-well transwell plates (Corning) with a pore size of 5 µm. Strainer was pre-coated with poly-L-lysine (1:100 in PBS) for 20 min at 37 °C prior to the experiment. Following 2.5 h of incubation at 37 °C and 5% CO_2, cells were collected from the lower chamber and analyzed using MACSQuant® Analyzer (Miltenyi Biotec). Total migrated cells of control mice were set to 100% and relative migration of $CD8^+$ T cells from TKO mice was calculated.

Western Blot

Proteins of whole brain lysates were analyzed by immunoblotting against β1i/LMP2 gp, β5i/LMP7 rb (both custom-generated), β2i/MECL-1 [K65 rb; (27)] and β-Actin (#A1978, Sigma-Aldrich).

Tibias and femurs of 10-14 weeks-old WT and TKO mice were aseptically removed, and bone marrow cells were flushed out with sterile PBS and centrifuged at 150 ×g for 10 min. Cells were resuspended in RPMI medium containing 10% FCS (Capricorn), recombinant murine granulocyte-macrophage colony-stimulating factor (2 ng/ml; Cell Signaling Technology) and 50 µM mercaptoethanol (Sigma-Aldrich) and cultivated for at least 10 days at 37 °C and 5% CO_2. Twenty-four hours prior to experiments, cells were harvested by scraping and seeded into 6-well plates. For investigation of signaling events cells were treated for the depicted time points with 30 µg/ml *Toxoplasma* lysate Antigen (TLA) and harvested using Trizol reagent (Invitrogen). Proteins were quantified *via* Bradford assay and subsequently

analyzed by immunoblotting against pStat3 (Tyr705) (D3A7; XP® Rabbit mAb #9145 CST), Stat3, pMEK (Ser217/221) (41G9; Rabbit mAb #9154 CST), pErk (Thr202/Tyr204) (20G11; Rabbit mAb #4376 CST), Erk and GAPDH (all Cell Signaling Technology) antibodies.

DNA and RNA Isolation

Samples stored in RNA*later* were homogenized in BashingBeads tubes (Zymo Research, Freiburg, Germany). AllPrep DNA/RNA Mini Kit (Qiagen) was used to isolate DNA and the peqGOLD total RNA kit (Peqlab, Erlangen, Germany) was used to isolate total RNA from the homogenate following the manufacturer's instructions.

Semiquantitative RT-qPCR

T. gondii burden was determined using the FastStart Essential DNA Green Master kit (Roche). The target *T. gondii* gene used was *Tgb1*, and *Mm. Asl* (TIBMolbiol, Berlin, Germany) was used as a reference gene. The stage of parasite burden was quantified using the Power SYBR® Green RNA-to-CT™ 1-Step Kit (Thermo Fisher) for bradyzoite-specific Bag1 and tachyzoite-specific Sag1 using *Gapdh* as reference gene. All genes were purchased from TIBMolbiol, Berlin, Germany.

Relative gene expression was determined similar to previous descriptions (28, 29) using the TaqMan® RNA-to-CT™ 1-Step Kit (life technologies). TaqMan® Gene Expression Assays (life technologies) were used for mRNA amplification of *Psmb8* (Mm00 440207_m1), *Psmb9* (Mm00479004_m1), *Psmb10* (Mm00 479052_g1), *Ccl2* (Mm00441242_m1), *Ccl3* (Mm00441259_g1), *Cxcl2* (Mm00436450_m1), *Cxcl10* (Mm00445235_m1), *Ifng* (Mm00801778_m1), *Tnf* (Mm00443258_m1), *Il12a* (Mm00 434165_m1), *Nos2* (Mm00440485_m1). Expression of *Hprt* (Mm01 545399_m1) was chosen as reference and target/reference ratios were calculated with the LightCycler® 96 software version 1.1 (Roche). All results were further normalized to the mean of the WT infected group.

Cytokine and Chemokine Assessment

Cytokine and chemokine profile was characterized using the LEGENDplex™ system (BioLegend). A more detailed protocol is published (30). Briefly, we used the Mouse Inflammation Panel (13-plex) system. Serum from WT and TKO mice was collected and incubated with fluorescence-encoded capture beads to cytokine and chemokine targets including CCL2, TNF and IFNγ. The fluorescent signals of analyte-specific bead regions were quantified using flow cytometry, and the concentrations of particular analytes were determined using provided data analysis software (BioLegend, LegendPlex™ software v8.0).

Statistical Analysis

Datasets were analyzed statistically using GraphPad Prism 7.02 (Graphpad software). To test for significance, we used a Mann-Whitney test for comparing two groups and a 2way ANOVA with uncorrected Fischer's LSD test for multiple comparisons. Owing to the small sample sizes, unequal variances were assumed in all *t*-tests. The significance level was set to $P < 0.05$ for all statistical comparisons. Symbols represent individual animals, columns represent mean values and error bars represent ± SEM.

RESULTS

TKO Mice Show Increased Susceptibility to *T. gondii* Infection

The 20S catalytic core particle of the IP consists of multiple subunits, three subunits harbor the six active sites that differ from those in the standard proteasomes. The relative contribution of immunoproteasomes to immune responses against *T. gondii* is unclear. To determine the relative expression of the three IP catalytic-subunits LMP2 (*Psmb9*), LMP7 (*Psmb8*) and MECL-1 (*Psmb10*) during the acute and chronic neuroinflammatory stage of infection, mRNA and protein was isolated from brain homogenates of *T. gondii* infected wild type (WT) mice at day 28 post-infection (*p.i.*). As compared to uninfected controls, the expression of all three IP subunits LMP2 (*Psmb9*), LMP7 (*Psmb8*) and MECL-1 (*Psmb10*) was significantly increased in *T. gondii* infected WT mice both on the RNA and protein level (**Figure 1A** and **Supplementary Figure 1**). To investigate the functional significance of these IP subunits we used mice with a combined deficiency of LMP2, MECL-1 and LMP7. These triple-knockout (TKO) mice and WT controls were infected with *T. gondii* orally (*p.o.*) and body weight was monitored daily throughout the course of the infection (**Figure 1B**). During the acute phase of infection, from day 10 to 14 *p.i.*, WT mice showed a higher weight loss when compared to TKO mice. Starting around day 13 *p.i.*, however, this effect was reversed and bodyweight loss was significantly more pronounced in TKO mice from day 21 to 28 *p.i.* Parasite burden was significantly increased in the spleen of TKO mice already at day 10 *p.i.*, an effect that was not observed at day 28 *p.i.* (**Figure 1C**). This might be due to the fact that *T. gondii* invades deeper tissues including the brain to evade the hosts' immune system (31).

Consequently, we analyzed parasite burden in the brain. To assess differences in stage conversion of the fast replicating tachyzoite and slow replicating bradyzoite stages of *T. gondii*, we utilized *T. gondii*-specific genes (TgSAG1 and TgBAG1, respectively). We detected a reduced mRNA expression of both tachyzoites and bradyzoites genes in brains of infected TKO mice in the acute phase of infection (**Figure 1D**), but increased mRNA expression in the chronic phase of infection (**Figure 1E**). Hence, altered tissue distribution of *T. gondii* in TKO mice argues for impaired peripheral immune responses in the absence of a functional IP.

Reduced/Delayed Type 1 Immune Response to *T. gondii* in TKO Mice

Early immune responses against *T. gondii* strongly depend on the pathogen-associated molecular pattern (PAMP)-dependent activation of APCs. They produce TNF and IL-12, promote the activation of NK and T cells, which produce anti-parasitic IFNγ (6). To determine if the IP affects early parasite recognition in the

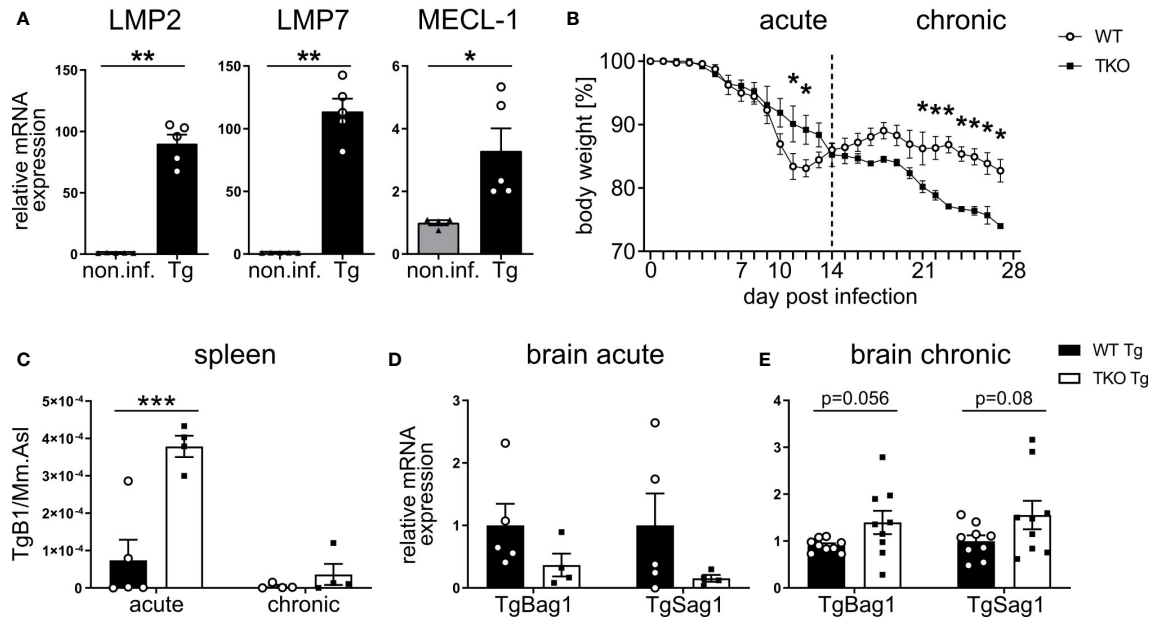

FIGURE 1 | Increased susceptibility of TKO mice in the chronic, but not acute, phase of *T. gondii* infection. Wild type (WT) mice were orally infected with a low dose (2 cysts) of *T. gondii* (ME49) for 28 days. Brains were collected from WT non-infected (non.inf., n ≥ 4) and *T. gondii* infected (Tg, n ≥ 4) animals on day 28 *p.i.* and following homogenization, mRNA was extracted for RT-qPCR analysis. **(A)** mRNA expression of the immunoproteasome subunits (LMP7/*Psmb8*, LMP2/*Psmb9*, MECL-1/*Psmb10*) were normalized to the non-infected group. Data is representative of four independent experiments. **(B)** WT mice and triple-knocked out (TKO) for the immunoproteasome subunits (LMP7/Psmb8$^{-/-}$LMP2/Psmb9$^{-/-}$MECL-1/Psmb10$^{-/-}$) mice were orally infected with a low dose (2 cysts) of *T. gondii* and weighed daily. Day 10 and 28 *p.i.* were chosen as time points for the acute and chronic immune response. The spleens and brains were taken from acute (d10 *p.i.*) and chronic (d28 *p.i.*) *T. gondii*-infected WT (WT Tg, n=4) and triple-knockout (TKO Tg, n=4) mice. Organs were homogenized and DNA/RNA was isolated from each for qPCR and RT-qPCR analysis. **(C)** qPCR analysis from DNA extracted from spleens of *T. gondii* infected WT and TKO mice. Relative quantification of *T. gondii* gene *TgB1* in spleen from acute (d10 *p.i.*) and chronic (d28 *p.i.*) *T. gondii* infected WT and TKO mice. *TgB1* gene expression was normalized to the gene expression of the reference gene *Mm.Asl*. **(D, E)** RT-qPCR analysis from RNA extracted from brain homogenates of mice from the acute (d10 *p.i.*) and chronic (d28 *p.i.*) phase of infection. Relative mRNA levels were normalized to the mean expression of the infected WT group. Data shown in **(A)** represents three independent experiments and data shown in **(B–E)** represent four independent experiments. In **(A, C–E)** symbols represent individual animals, columns represent mean values and error bars represent ± SEM. In **(B)**, data points represent mean values and error bars represent ± SEM. In **(A)**, a Mann-Whitney test for two groups and in **(B–E)** a 2way ANOVA following Fisher's LSD test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001.

periphery, splenic Ly6C^{hi} inflammatory monocytes and DCs from WT and TKO mice were analyzed in the acute phase of infection. As shown in **Figures 2A–C**, numbers and MHC I levels of Ly6C^{hi} inflammatory monocytes and DCs were significantly reduced in the spleen of infected TKO compared to WT mice. In contrast, MHC II expression proved to be independent of the IP which is consistent with previously published data (15).

Next, we investigated whether IP deficiency affects IL-12 and TNF production by Ly6C^{hi} monocytes and DCs. Upon *ex vivo* restimulation with Toxoplasma lysate antigen (TLA), we observed a significantly higher percentage of Ly6C^{hi} monocytes producing TNF with increased TNF production and non-significant change in frequencies of TNF producing DCs (**Figure 3A**, **A'**) in TKO mice in the acute phase. We detected no difference in the percentage of IL-12-producing DCs and Ly6C^{hi} monocytes or in the IL-12 produced (**Figure 3B**, **B'**). TNF and IL-12 production lead to the expression of IFNγ, a key molecule for *T. gondii* elimination (4, 6). IFNγ induces cell-autonomous immune responses (32), such as induction of inducible nitric oxide synthase (iNOS) which produces nitric oxide (NO) thereby promoting parasite clearance (33, 34). As shown in **Figure 3C**, **C'**, iNOS production by DCs and Ly6C^{hi} monocytes was also indistinguishable between infected WT and TKO animals. These results indicate that the IP has only a minor impact on early innate immune responses against the parasite but may be required for IFNγ-related adaptive immune responses.

IFNγ produced in the course of *T. gondii* infection facilitates IL-12 production by DCs and monocytes (35). With an increased parasite burden in spleens of TKO mice, one would expect increased expression of IL-12. However, we detected no change in IL-12 production (**Figure 3B**, **B'**). In order to characterize IFNγ production by immune cells, CD8$^+$ and CD4$^+$ T cells, NK1.1$^+$ cells and neutrophils were restimulated with TLA *ex vivo* and analyzed by flow cytometry. Fewer CD8$^+$ T cells were isolated from the spleens of infected TKO animals compared to WT mice during the acute phase of infection (**Figure 4A**). This, together with the observed reduced MHC I expression on APCs (**Figure 2**) is in line with previously reported results (15, 24, 36) describing reduced CD8$^+$ T cell numbers when MHC I/peptide presentation is impaired (24). In addition, we observed slightly elevated numbers of CD4$^+$ T cells in spleens of infected

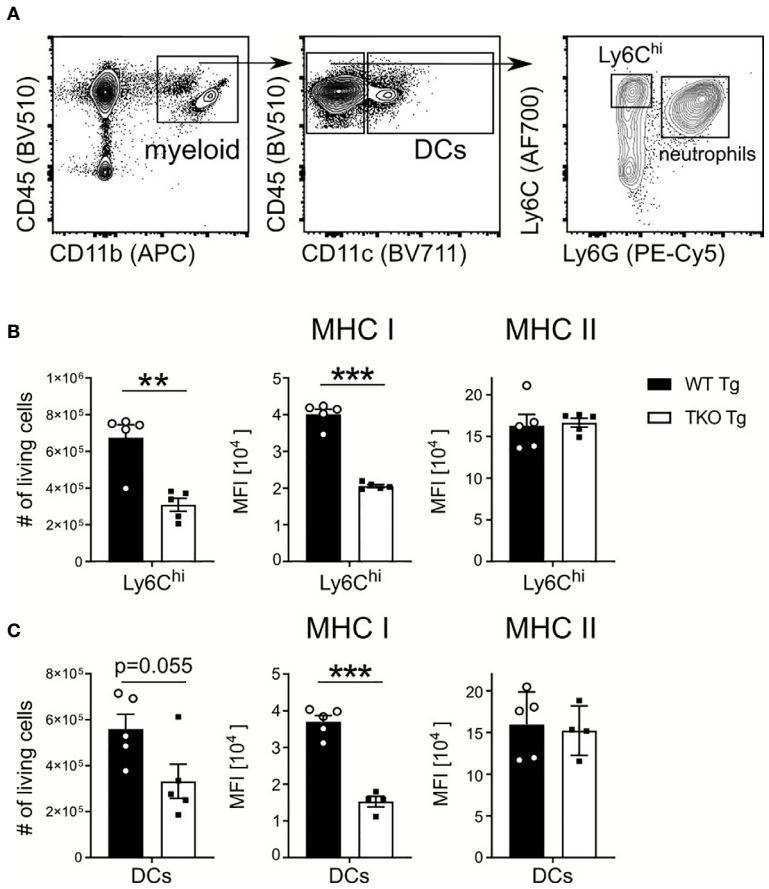

FIGURE 2 | Reduced numbers of Ly6C^{hi} monocytes and DCs in spleen of infected TKO mice. Immune cells were isolated from the spleens of *T. gondii* infected WT (WT Tg, n=5) and TKO (TKO Tg, n ≥ 4) mice on day 10 *p.i.* and analyzed by flow cytometry. Following viability staining and the basic FSC/SSC gating, viable single cells were chosen for further characterization. **(A)** Splenocytes were first gated based on surface expression of CD45, a hematopoietic marker, and CD11b, a myeloid cell marker (left plot). CD11b$^+$CD45$^+$ cells were further gated for CD11c and CD11c$^+$ cells identified as dendritic cells (DCs) (center plot). CD11c$^-$ cells were further divided into inflammatory monocytes (Ly6G$^-$Ly6C^{hi}) and neutrophils (Ly6G$^+$) (right plot). The total number of living cells and surface expression of MHC I and MHC II were assessed for Ly6C^{hi} monocytes **(B)** and DCs **(C)**. Expression of MHC I and MHC II was quantified using the mean fluorescence intensity (MFI) of their respective fluorochrome. Data shown in **(A)** is a representative of three independent experiments. Data shown in **(B, C)** represent three independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. A Mann-Whitney test was used for statistical analysis. $^{**}P < 0.01$, $^{***}P < 0.001$.

TKO mice, a finding that was already visible in naïve TKO mice (**Figure 4B** and **Supplementary Figures 2A–C, E, F**). Consistent with previous findings (37), significantly reduced frequencies in the IFNγ$^+$CD4$^+$ T cells were detected in spleens of infected TKO mice, whereas steady state analyses revealed no difference in the circulating IFNγ in WT and TKO mice (**Figure 4B** and **Supplementary Figure 2D**). Neither numbers, nor frequencies of IFNγ producing NK1.1$^+$ cells or neutrophils (Ly6G$^+$) differed significantly between TKO and WT mice (**Figures 4C, D**).

Parasite Dissemination Into the Brain of WT and TKO Mice in the Acute Phase of *T. gondii* Infection

To establish the chronic phase of infection in the CNS, *T. gondii* has to cross the blood-brain barrier (BBB) and enter the brain. When *T. gondii* infects DCs or monocytes, they induce a hypermotility phenotype and enhanced transmigration capacity, effectively shuttling the parasite into the brain, thereby functioning as a Trojan horse to cross the BBB (38). In TKO animals, we observed an increased parasite burden in the periphery but the opposite in the brain on d10 *p.i.* (**Figure 1**). This is also associated with a dysregulated DC and CD8$^+$ T cell recruitment to the spleen (**Figures 2** and **4**). To investigate whether and how impaired immune pressure in the periphery corresponds to altered immune cell composition in the brain, we analyzed different immune cell populations in brains of *T. gondii* infected mice on day 10 *p.i.* Using flow cytometry analysis, we assessed recruited myeloid and lymphoid cells into the CNS along with the resident microglia (**Figure 5A**). We observed fewer numbers of myeloid cells recruited into the brain of TKO mice, though not significant (**Figure 5B**). Interestingly, these myeloid cells exhibited a similar phenotype to the peripheral cells (**Figures 2B, C** and **3A**) as they had reduced MHC I expression

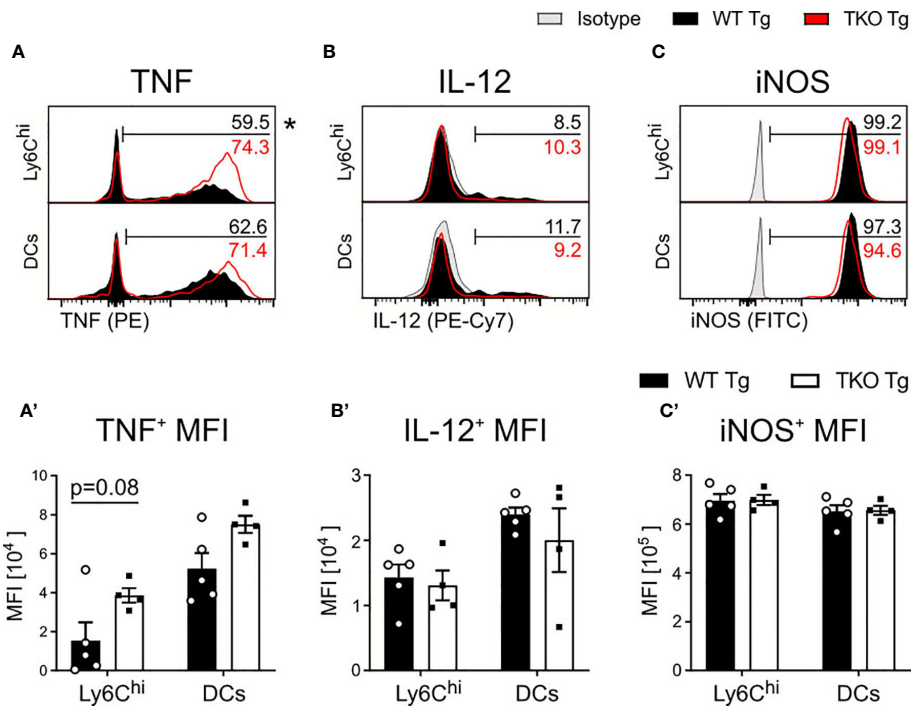

FIGURE 3 | Cytokine production by APCs in spleens of *T. gondii* infected mice. Immune cells were isolated from the spleens of *T. gondii* infected WT (WT Tg, n=5) and TKO (TKO Tg, n=4) mice on day 10 *p.i.* Isolated cells were then restimulated with *T. gondii*-lysate antigen (TLA) for 6 hours, stained and analyzed by flow cytometry. **(A–C)** Histograms of Ly6C^{hi} monocytes and DCs intracellular production of **(A)** TNF **(B)** IL-12 and **(C)** iNOS and their resulting MFI expression **(A'–C')**. The histogram values (right side) represent the average percentage of positively expressing cells (determined by isotype control; in gray) for each respective immune marker and group (WT in black; TKO in red outline). The bar **(A–C)** outlines where positive expression begins for each respective cell and marker. Data shown in **(A–C)** are representatives of three individual experiments. Data shown in **(A'–C')** represent three independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. 2way ANOVA following Fisher's LSD test was used for statistical analysis. *P < 0.05.

and normal TNF production (**Figures 5B**, **2B, C**, and **3A**). Nonetheless, myeloid cells in the TKO-brain displayed slightly reduced MHC II expression (**Figure 5B**), which is expected to be due to the reduced presence of parasites in the brain in the acute phase of infection and was not observed on Ly6C^{hi} inflammatory monocytes and DCs obtained from the spleens of TKO mice (**Figures 2B, C**).

T. gondii activates resident microglia, which induces the recruitment of immune cells into the brain (39). In infected TKO mice, MHC I and TNF expression by microglia was significantly reduced compared to WT mice (**Figure 5C**). In contrast, circulating TNF was not altered in non-infected TKO mice (**Supplementary Figure 2D**). Furthermore, in the brain of infected TKO mice the size of CD8$^+$ T cell pool and availability to produce IFNγ were slightly reduced (**Figure 5D**). The number and IFNγ production of brain CD4$^+$ T cells was unchanged whereas the number of NK1.1$^+$ cells as well as IFNγ production, were slightly increased in acutely infected TKO mice (**Figures 5E, F**). To directly assess the ability of CD8$^+$ T cells to migrate to sites of *T. gondii* infection, we used a transwell migration assay. CD8$^+$ T cells were isolated from *T. gondii*-infected WT and TKO mice and stimulated using CCL21 or CXCL12. Interestingly, CD8$^+$ T cells from spleens of infected TKO mice showed significantly reduced migration upon both CCL21 and CXCL12 *ex vivo* stimulation compared to WT mice (**Supplementary Figure 3A**). This indicates that CD8$^+$ T cells from TKO mice possess a reduced capacity to migrate to the site of infection in the acute phase that suggests a failure of the immune system to limit infection by inducing tachyzoite differentiation into bradyzoites.

An alternative explanation for the reduced pathogen burden in brains of acutely infected TKO mice could be reduced parasite shuttling by myeloid cells, a process which is CCL2-dependent (3, 39). In the serum of infected TKO mice CCL2 levels were slightly, (albeit non-significantly) reduced (**Supplementary Figure 3B**) which aligns with the number of myeloid cells in the brain (**Figure 5B**). Correspondingly, mRNA levels of CCL2 and other myeloid-associated chemokines such as CCL3, CXCL2 and CXCL10 were reduced in brains of infected TKO mice at day 10 *p.i.* (**Supplementary Figure 3C**). This was also the case for IFNγ (**Supplementary Figure 3D**), which is known to induce chemokine gene activity (40). Overall, an absent IP correlates

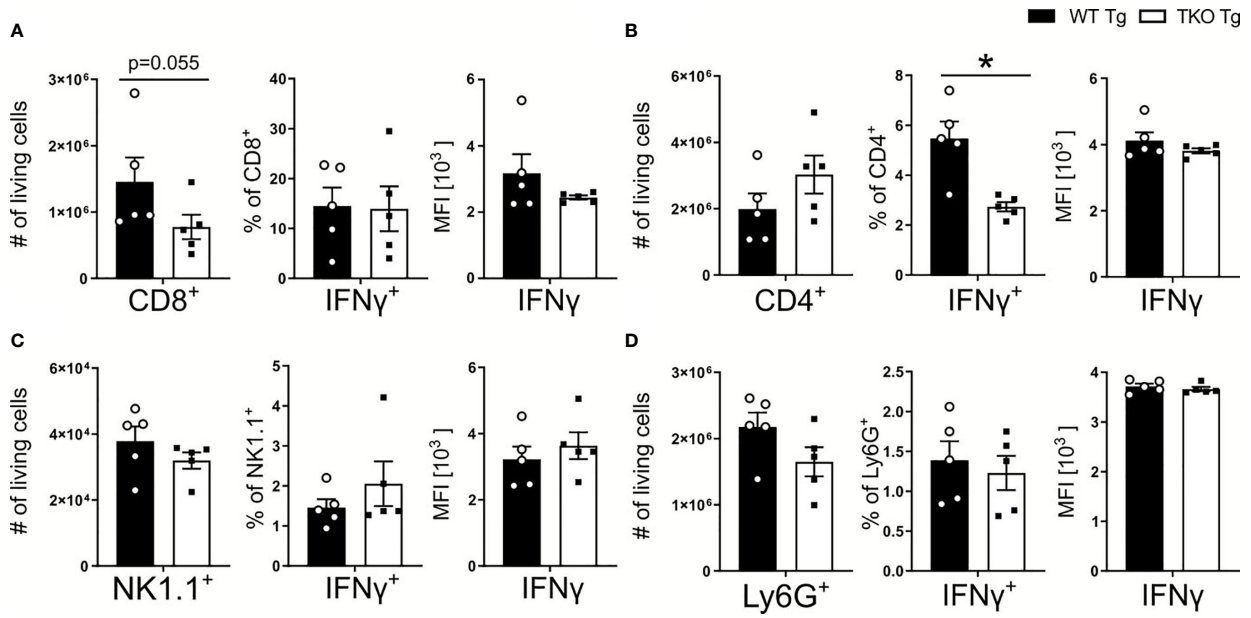

FIGURE 4 | Reduced $CD8^+$ T cell numbers and impaired Th1 responses in *T. gondii* infected TKO mice. Immune cells were isolated from the spleens of *T. gondii* infected WT (WT Tg, n=5) and TKO (TKO Tg, n=5) mice on day 10 *p.i.* and analyzed by flow cytometry. Following viability staining and the basic FSC/SSC gating, viable single cells were determined by first removing $CD11b^+$ and $CD3^-$ immune cells. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were identified for further analysis. $CD45^+NK1.1^+$ cells were determined after gating out $CD3^+$, $CD8^+$, $Ly6C^+$ and $Ly6G^+$ cells. The total cell number of **(A)** $CD8^+$ T cells, **(B)** $CD4^+$ T cells, **(C)** $NK1.1^+$ cells and **(D)** neutrophils, the percentage of IFNγ producing cells and their respective IFNγ production were measured. Data shown represents three independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. A Mann-Whitney test was used for statistical analysis. *$P < 0.05$.

with impaired early induction of adaptive immune responses, leading to a loss of parasite control in the acute phase of infection, subsequently resulting in an increased peripheral parasite burden.

WT and TKO Mice During Chronic *T. gondii* Infection

Parasite control during chronic neuroinflammation requires persistent, basal levels of inflammation involving resident microglia and recruited immune cells such as monocytes and T cells. Upon chronic infection, we observed an increased parasite burden in combination with a more severe weight loss in TKO compared to WT mice (**Figure 1E**) that resembled reactivated toxoplasmosis. To further investigate this phenotype, immune cells were isolated from brains of chronically infected mice and analyzed *via* flow cytometry. $Ly6C^{hi}$ inflammatory monocytes and DCs exhibited comparable total numbers in the brains of infected TKO mice (**Figure 6A**). Next, we determined the influence of the IP on the functional capacity of resident microglia and recruited immune cells in chronic inflammation. Again, expression of MHC I continued to be impaired as all cell types exhibited significant reduced expression (**Figure 6B**). Microglia showed a slight increased expression of MHC II in the chronic stage of infection, which is expected with an increased parasite burden (**Figure 6C**). To investigate the effector function of these cells in the chronic stage of infection, we then analyzed their production of TNF, IL-12 and iNOS. $Ly6C^{hi}$ monocytes recruited into the brains of TKO mice showed a trend of increased TNF expression whereas significantly fewer microglia were producing TNF when compared to WT mice (**Figure 6D**, **D'**). Fewer DCs produced IL-12 while no differences in producing microglia or $Ly6C^{hi}$ monocytes could be detected between WT and TKO mice in the chronic stage of infection (**Figure 6E**, **E'**). Interestingly, when assessing iNOS expression in these cell types, they all, especially microglia, showed significantly increased iNOS production in brains of TKO compared to WT mice (**Figure 6F**, **F'**). These results show that in the chronic stage of infection, TKO mice are able to induce IFNγ-driven anti-parasitic immune responses such as the expression of iNOS. Although in TKO mice expression of cell autonomous anti-parasitic effector molecules was induced, they regardless were not able to sufficiently control parasite proliferation in the brain. It is crucial to have *T. gondii* specific T cells that can recognize active, ongoing parasite infection and then prime the local cells to adequately defend and prevent further parasite spread. Thus, we hypothesized that T cells are responsible for the lack of parasite control in the chronic stage of infection and we analyzed T cell responses in chronic inflammation in more detail.

When assessing $CD4^+$ and $CD8^+$ T cell recruitment into the brain, TKO mice compared to WT mice showed comparable $CD4^+$ T cell numbers, but a trend for fewer $CD8^+$ T cells

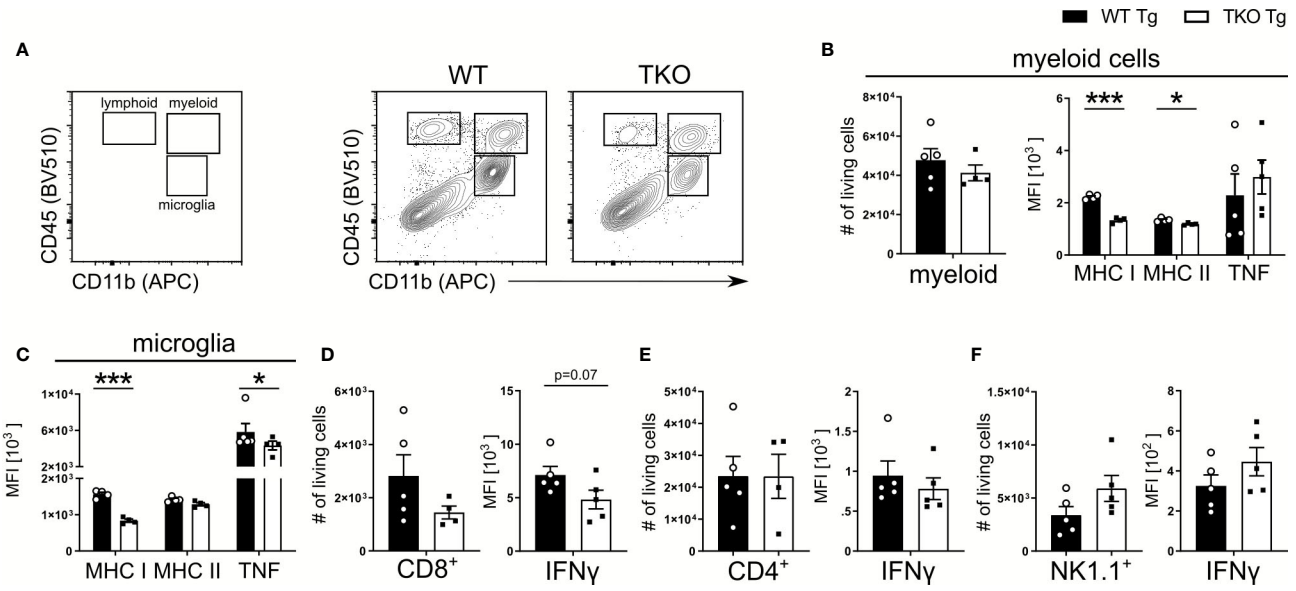

FIGURE 5 | Impaired recruitment and IFNγ-dependent activation of proinflammatory myeloid cells in TKO mice. Immune cells were isolated from brains of *T. gondii* infected WT (WT Tg, n=5) and TKO (TKO Tg, n ≥ 4) mice on day 10 *p.i.* For the measurement of TNF and IFNγ, brain cells were restimulated with TLA for 6 hours and then stained and analyzed by flow cytometry. Following viability staining and the basic FSC/SSC gating, single cells were chosen for further characterization. **(A)** Representative gating strategy using CD45 and CD11b to distinguish between microglia, myeloid cells and lymphoid cells (left plot) and representative plots from from brain tissue of infected WT (center panel) and TKO (right plot) mice. $CD11b^+CD45^{int}$ cells were identified as microglia and $CD11b^+CD45^{hi}$ were identified as recruited myeloid cells then divided into $Ly6G^-$ monocytes as depicted in Fig 2A. $CD45^+CD11b^-$ cells were divided into $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells. $CD45^+NK1.1^+$ cells were determined after removing $CD3^+$, $CD8^+$, $Ly6C^+$ and $Ly6G^+$ cells. Total number of cells was assessed for recruited **(B)** myeloid cells, **(D)** $CD8^+$ T cells, **(E)** $CD4^+$ T cells and **(F)** $NK1.1^+$ cells. Recruited **(B)** myeloid cells and **(C)** microglia were measured for their surface expression of MHC I and MHC II as well as their production of TNF. Recruited **(D)** $CD8^+$, **(E)** $CD4^+$ and **(F)** $NK1.1^+$ cells had their IFNγ production quantified. The expression or production of each immune marker was quantified using the MFI of their respective fluorochromes. Data shown in A is a representative of three independent experiments. Data shown in **(B–F)** represents three independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. A Mann-Whitney test for comparing two groups and a 2way ANOVA followed by Fisher's LSD test for comparing multiple groups was used for statistical analysis. *P < 0.05; ***P < 0.001.

(**Figure 7A**). To further assess T cell functionality in response to *T. gondii*, we analyzed IFNγ and TNF production of $CD4^+$ T cells as well as IFNγ and Granzyme B secretion by $CD8^+$ T cells following *ex vivo* TLA stimulation. Granzyme B is a cytotoxic protein contained in granules of cytotoxic $CD8^+$ T cells that is able to induce apoptosis in neighboring infected cells after release. Interestingly, we observed significantly increased frequencies of IFNγ and TNF secreting $CD4^+$ T cells in TKO mice compared to WT mice (**Figure 7B**) which is in concordance with our finding that in whole TKO-brains significantly enhanced TNF and non-significantly increased IFNγ mRNA levels can be found (**Supplementary Figure 3E**). Similar to the immune response in the acute phase of infection, TKO mice compared to WT mice showed a lower frequency of IFNγ producing $CD8^+$ T cells (**Figure 7C**). Surprisingly, no differences of granzyme B containing $CD8^+$ T cells could be detected between TKO and WT mice in brain tissue in the chronic stage of infection (**Figure 7C**). Since it is described that regulatory T cells (Tregs) mediate T cell suppression during the acute phase of *T. gondii* infection, we next analyzed whether TKO mice have changes in the recruitment of Tregs into the CNS. And indeed, we found significantly reduced frequencies of $CD4^+$ Tregs in brains of TKO mice compared to WT mice in the chronic phase of infection (**Figures 7D, E**). These results show that the absence of the IP leads to reduced Treg frequencies in the *T. gondii* infection model and subsequent reduced T cell suppression, resulting in increased cytokine production by $CD4^+$ T cells (**Figure 7B**).

The immunoproteasome is crucial to induce T cell maturation (41). Thus, we further analyzed different T cells subtypes in respect to their surface expression of CD62L and CD44, allowing us to distinguish between naïve ($CD44^-CD62L^+$), central memory ($CD44^+CD62L^+$) and effector memory ($CD44^+CD62L^-$) T cells. First, we investigated the number of T cell subtypes recruited into the CNS and observed a significant reduction of $CD8^+$ T effector memory (T_{em}) cells but not $CD4^+$ T effector cells in brains of TKO mice in the chronic phase of infection (**Figures 7F, F'**). To assess if this significant difference in T cell differentiation is restricted to the chronic infection, we investigated different T cell subtypes of splenocytes in uninfected mice as well as infected mice in the acute and chronic phase of infection (**Figures 7G, H**). Already uninfected TKO mice showed a significant reduction of naïve $CD8^+$ T cells and vice versa a significant increase of naïve $CD4^+$ T cells in spleen tissue

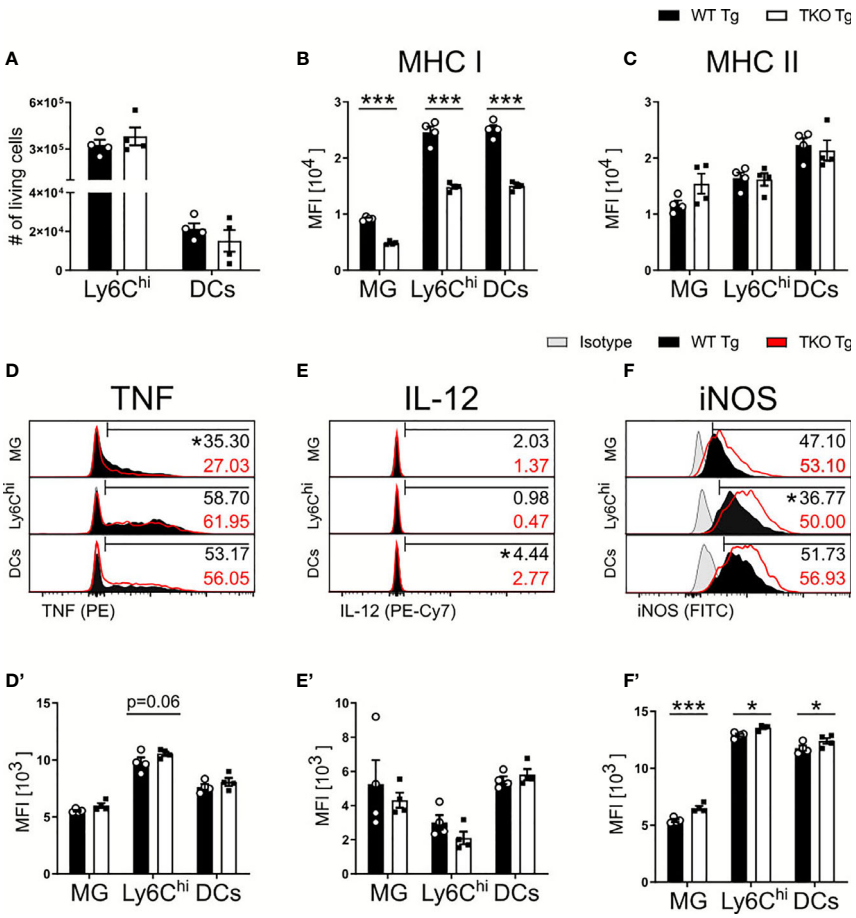

FIGURE 6 | Increased anti-parasitic immune response in brains of TKO mice in chronic stage of infection. Immune cells were isolated from brain homogenate of *T. gondii* infected WT (WT Tg, n=4) and TKO (TKO Tg, n=4) mice on day 28 *p.i.* For the measurement of TNF, IL-12 and iNOS brain cells were restimulated with TLA for 6 hours, stained and analyzed by flow cytometry. Following viability staining and the basic FSC/SSC gating, single cells were chosen for further characterization. Using the same gating strategy as described for Fig 2A and 5A, $CD11b^{+}CD45^{int}$ microglia (MG), $CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{hi}$ inflammatory monocytes and $CD11b^{+}CD45^{+}CD11c^{+}$ DCs were analyzed. **(A)** Total cell numbers were calculated as a percentage of live cells found in the brain for $Ly6C^{hi}$ monocytes and DCs. The surface expression of **(B)** MHC I and **(C)** MHC II expression was determined on MG, DCs and $Ly6C^{hi}$ monocytes. Histograms of the intracellular production of **(D)** TNF, **(E)** IL-12 and **(F)** iNOS and their resulting MFI **(D'–F')**. The histogram values (right side) represent the percentage of positively expressing cells (determined by isotype control; in gray) for each respective immune marker and group (WT in black; TKO in red outline). The bar **(D–F)** outlines where positive expression begins for each respective cell and marker. Data shown represent four independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. 2way ANOVA followed by Fisher's LSD test was performed for statistical analysis. *P < 0.05, ***P < 0.001.

compared to WT mice (**Figures 7G', H'** and **Supplementary Figures 2B, C**), which is consistent with previous findings (15). We found that TKO mice compared to WT mice had significantly increased numbers of naïve $CD4^{+}$ T cells as well as comparable numbers of central memory T cells (T_{cm}) and T_{em} cells throughout the infection (**Figure 7G, G", G'"**).

Splenocytes of TKO mice compared to WT mice possessed significantly fewer naïve $CD8^{+}$ T cells in uninfected mice (**Figure 7H'**). However, during the course of infection WT and TKO mice had comparable numbers of naïve $CD8^{+}$ T cells (**Figure 7H"**, **H'"**), but TKO mice exhibited reduced T_{em} cells in the chronic stage of infection (**Figure 7H'"**). These data describe that the absence of the IP hampers the ability to induce effector T cells and affect $CD8^{+}$ T cell differentiation into memory/effector T cells, since an increased proportion of T cells were differentiated into central memory cells (**Figure 7H**).

IP Deficiency Affects Apoptosis and Signaling *via* STAT3 in TKO Mice in Chronic *T. gondii* Infection

Since *T. gondii* is known to infect APCs, DCs in particular, as well as the IP primarily seems to affect $CD8^{+}$ T cell numbers by altered MHC I/peptide presentation, this suggests an important role for APCs in the brain in the chronic stage of infection. To further investigate this hypothesis, we determined the frequencies of apoptotic APCs in brain (**Figure 8A**) and spleen (**Figure 8B**) tissue of WT and TKO mice in the chronic stage of infection. Using Annexin V and 7AAD, we assessed early and late apoptotic APCs in infected animals in the chronic stage of

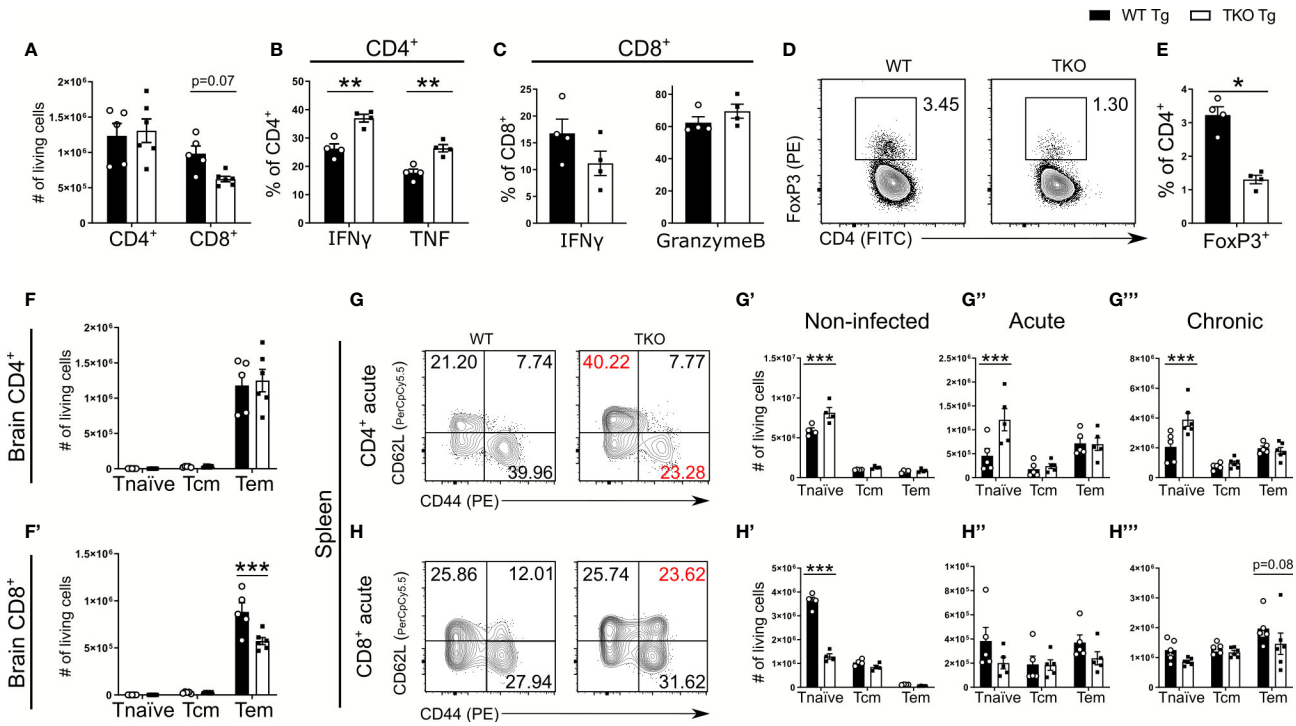

FIGURE 7 | Altered T cell differentiation in infected TKO mice. Immune cells were isolated from brain homogenate of *T. gondii* infected WT (WT Tg, n=4) and TKO (TKO Tg, n=4) mice on day 28 *p.i.* For the measurement of IFNγ, TNF and Granzyme B, cells were restimulated with TLA for 6 hours, stained and analyzed by flow cytometry. **(A)** Total number of $CD4^+$ and $CD8^+$ T cells recruited to the brain. **(B, C)** Intracellular production of proteins in T cells was characterized by the percentage of cells positive for IFNγ, Granzyme B or TNF. **(D)** Representative gating for regulatory T cells after selecting $CD11b^-CD3^+$ cells. Tregs were determined by gating for $CD4^+FoxP3^+$ cells. **(E)** The frequency of recruited $FoxP3^+$ cells was calculated as a percentage of $CD4^+$ T cells in the brain. Using CD62L and CD44, $CD4^+$ and $CD8^+$ T cells were divided into naïve ($CD62L^+CD44^-$), T central memory (T_{cm}, $CD62L^+CD44^+$) and T effector memory (T_{eff}, $CD62L^-CD44^+$) populations. Total number of differentiated **(F)** $CD4^+$ and **(F')** $CD8^+$ T cells from the brains of WT and TKO mice from day 28 *p.i.* **(G, H)** Immune cells were isolated from spleens at steady state, day 10 *p.i.* (acute) and day 28 *p.i.* (chronic) from WT and TKO mice and analyzed by flow cytometry. **(G, H)** Representative gating strategies of these T cell subpopulations for both $CD4^+$ and $CD8^+$ T cells (acute stage shown). The absolute number of $CD4^+$ and $CD8^+$ T cells for the respective subpopulations from WT and TKO mice **(G', H')** non-infected, **(G'', H'')** acute stage infection, **(G''', H''')** chronic stage infection. Data shown in D, G & H are representatives of three independent experiments. Data shown in **A–C, E, F, F', G'-G'''** and **H'–H'''** represent four independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. In C&E a Mann-Whitney test for comparing two groups and in **A, B, F, F', G–G'''** and **H–H'''** a 2way ANOVA followed by Fisher's LSD test were used for statistical analysis. *P < 0.05; **P < 0.01, ***P < 0.001.

infection. First analyzing $CD11b^+$ cells (to include microglia) in brains from infected animals on day 28 *p.i.*, we detected comparable early apoptotic, but significantly increased frequencies of late apoptotic cells in TKO mice compared to WT mice (**Figure 8A**). Splenocytes were isolated from infected animals on day 28 *p.i.* and all $CD11b^+$ splenocytes were further divided into $Ly6C^{hi}$ and $Ly6C^{lo}$ cells. We observed significantly increased frequencies of early apoptotic $Ly6C^{lo}$ cells, whereas significantly increased frequencies of late apoptotic $Ly6C^{hi}$ and $Ly6C^{lo}$ cells were found (**Figure 8B**). Thus, with the absence of the IP, APCs in brain and spleen tissue of chronically infected animals have increased rates of apoptosis (**Figures 8A, B**). It is conceivable that this is a potential mechanism, explaining the observed reduced numbers of $CD8^+$ T_{em} cells in brains of TKO mice (**Figures 7F'**).

During inflammation, the IP is a crucial component needed for cell signaling and protein degradation. Studies have hypothesized that the IP plays a role in regulating pro-inflammatory cytokines (42, 43). Thus, we aimed to determine if deficiency of the IP affects any major cytokine signaling pathways found in APCs such as MAPK/NF-κB or STAT pathways. These signaling pathways are known to be essentially involved in *T. gondii* containment (35, 44, 45) and further can be manipulated by the parasite itself thereby using them to evade the host immune system (46, 47). Bone marrow derived macrophages (BMDMs) were stimulated with TLA *ex vivo* and protein expression was analyzed *via* immunoblot (**Figures 8C**). We analyzed different key proteins from different stages of the MAPK/NF-κB pathway. No differences in the phosphorylation of MEK and ERK could be detected between WT and TKO mice following stimulation. We further analyzed STAT3 and its phosphorylated variant (pSTAT3) as a key component of the STAT pathway. BMDMs of TKO mice compared to WT mice showed a marked reduction in STAT3 phosphorylation. It is described that STAT3, and subsequent pSTAT3, are crucial components for cell survival and IL-6/10/12

signaling (48–50). This finding fits to our observation of increased apoptosis in brains of TKO mice in the chronic stage of infection. These data highlight that the absence of the IP impairs STAT3 signaling *via* dysregulated phosphorylation (**Figure 8C** and **Supplementary Figures 5A, B**), correlating with the observed reduced myeloid cell survival (**Figures 8A, B**) and altered T cell differentiation (**Figures 7F, F'**) in infected TKO mice in the chronic stage of infection.

DISCUSSION

The results presented in the current study demonstrate that the IP is a crucial component of the immune system for the transition between innate and adaptive immune responses against *T. gondii*. The absence of the IP subunits LMP2, MECL-1 and LMP7 indirectly showed a reduced ability of APCs to present peptides to T cells by displaying decreased MHC I cell surface level, thereby reducing the pool of the available $CD8^+$ T cells, all crucial steps for *T. gondii* containment and clearance. Furthermore, these APCs were more prone to apoptosis and lacked STAT3 phosphorylation. Ultimately, this impaired immune response lead to an inability of TKO mice to control parasite proliferation, causing reactivation of toxoplasmosis resulting in an increased susceptibility of TKO mice in a *T. gondii* infection model.

TKO mice showed an increased weight loss during the chronic course of *T. gondii* infection that is often associated with an enhanced immune response. And in fact, brain tissue of chronically infected TKO mice showed increased TNF and IFNγ as well as increased production of these cytokines released by $CD4^+$ T cells in the chronic phase of infection. Nevertheless, *T. gondii* infected TKO mice showed an inability to control the parasite burden, particularly, in the acute phase but also in the chronic phase of infection. This inability for early parasite containment is presumably caused by a delayed antigen presentation by APCs. Dysregulated antigen presentation by APCs can delay parasite specific T cell activation and proliferation thereby delaying expression of IFNγ induced anti-parasitic effector molecules. This mechanism aligns with other infection models using TKO animals. Infection with *Brucella abortus* in TKO mice led to an increased bacterial burden. This was associated with an impaired MHC I presentation of $CD11c^+$ cells and a reduced percentage of both $CD4^+$ and $CD8^+$ IFNγ

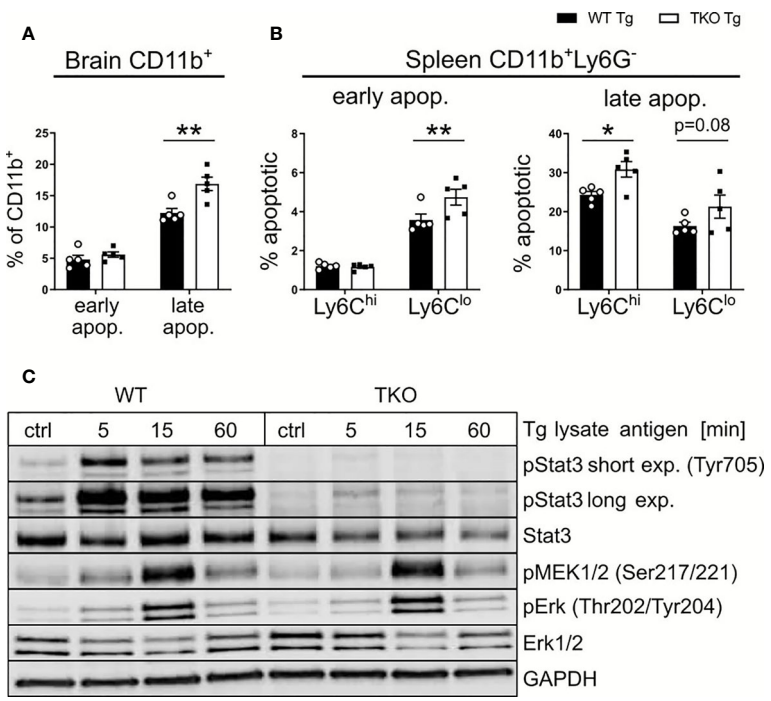

FIGURE 8 | Altered STAT3 signaling in TKO APCs. Immune cells were isolated from the brain and spleen tissue of *T. gondii*-infected WT (WT Tg, n=5) and TKO (TKO Tg, n=5) mice on day 28 *p.i.* and analyzed by flow cytometry. **(A, B)** Isolated cells were stained with Annexin V and 7AAD to determine early apoptotic ($7AAD^-$ $AnnexinV^+$) and late apoptotic ($7AAD^+AnnexinV^+$) cells. **(A)** Percentage of early and late apoptotic $CD11b^+$ cells isolated from brain tissue. **(B)** Percentage of early and late apoptotic $Ly6C^{hi}$ and $Ly6C^{lo}$ mononuclear cells isolated from spleen. **(C)** Bone marrow derived macrophages from WT and TKO mice were treated with 30µg/ml *toxoplasma* lysate for the depicted time. Proteins were isolated and quantified *via* Bradford assay and immunoblotted using pMEK (Ser217/221), Erk, pErk (Thr202/Tyr204), Stat3, pStat3 (Tyr705) and GAPDH antibodies. For apoptosis assay, n=5. Data shown in **(A, B)** represent three independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. Data shown in **(C)** represents a representative of three independent experiments. 2way ANOVA followed by Fisher's LSD test was used for statistical analysis. *P < 0.05, **P < 0.01.

producing T cells as well as fewer Granzyme B producing $CD8^+$ T cells (37). Similarly, infection with the protozoan *Trypanosoma cruzi* in TKO mice resulted in reduced MHC I expression and altered $CD8^+$ effector T cell function, in both quantity and quality as there were fewer overall $CD8^+$ effector cells and fewer IFNγ producers (36). However, depending on the pathogen type, its organ specificity and impaired IP subunit expression as well as the duration of the challenge, the IP's contribution varies.

In a *Leishmania major* infection model, the absence of the subunit LMP7 had no effect on the ability of DCs to stimulate $CD8^+$ T cells in both WT and $LMP7^{-/-}$ mice, as well as the authors showed similar IFNγ production and T cell proliferation (51). The role of LMP7 was further highlighted in a malaria infection model, since the absence of LMP7 resulted in lower parasite growth, reduced parasite burden but an enhanced immune response with increased phagocytosis activity (52). $LMP7^{-/-}$ mice displayed reduced MHC I expression on APCs (53) and infected $LMP2^{-/-}$ mice showed a strong reduction (~70%) of $CD8^+$ lymphocytes compared to WT mice (54). In addition, $MECL\text{-}1^{-/-}$ mice similar to $LMP2^{-/-}$ mice, showed a reduction of $CD8^+$ T cells in the spleen compared to WT mice (55), at which MECL-1 contributes to T cell homeostatic expansion (56). Notably, using the LCMV infection model, Nussbaum et al., observed that although $LMP2^{-/-}$ or $LMP7^{-/-}$ mice had fewer $CD8^+$ T cells, these animals were able to mount strong $CD8^+$ anti-viral immune responses demonstrated by similar kinetics of viral clearance compared to WT mice (57). In addition, analyzing the role of mouse adenovirus type 1 infection in pathogenesis of TKO mice the authors detected age-dependent differing effects (58). All these studies demonstrate that the role of the IP during infection is multifaceted and most likely pathogen specific.

DCs and $Ly6C^{hi}$ monocytes in spleens of acutely infected TKO mice possessed a slightly increased production of TNF but not IL-12, indicating that parasite detection was still intact. However, $Ly6C^{hi}$ monocytes and DCs from TKO mice showed reduced cell numbers with impaired MHC I expression in spleen and brain tissue during both the acute and the chronic stage of infection. This reduced recruitment of APCs to the sites of infection not only delays IFNγ induced T cell priming, but also leads to a delayed initiation of the adaptive immune response as fewer APCs are able to present parasite specific antigens. Thus, in the acute phase of infection an attenuated inflammation can be detected which is similar to the phenotype observed in models of autoimmune-related myocarditis and experimental autoimmune encephalomyelitis due to immunoproteasome inhibition (59, 60). In contrast, an opposite scenario could be observed during the chronic stage of infection where $Ly6C^{hi}$ monocytes and DCs could be found in the brain of TKO mice which released higher levels of TNF and iNOS. In addition, proinflammatory cytokines were increased in whole brain homogenates of chronically infected TKO mice. These results indicate a dysregulated immune response to *T. gondii*. In the absence of the immunoproteasome, an efficient immune response cannot be initiated during the acute phase of infection. Further, the resulting excessive inflammatory response in the chronic phase is insufficient to efficiently control the infection. This is in concordance with previously published data showing that IP-formation is crucial for protection from virus-induced inflammatory tissue damage as observed in coxsackievirus B3 myocarditis (27). Notably, enhanced NF-κB activity and TNF production can be mediated even in the absence of immunoproteasomes as observed in our study e.g. by increasing the degradation of the NF-κB inhibitor IκBα through 20S proteasome complexes associated with the proteasome activator PA28 that is constitutively expressed in various tissues (61, 62).

An impaired MHC I-antigen peptide activation of $CD8^+$ T cells is in line with previous results illustrating the pivotal role of the IP subunit LMP7 during *T. gondii* infection in regard to induction of DC driven activation of cytotoxic $CD8^+$ T cells (24). Furthermore, mice deficient for the single IP subunits LMP2 or LMP7 showed increased susceptibility to *T. gondii* infection and displayed less IFNγ-secreting $CD8^+$ T cells following infection although they had similar numbers of activated $CD8^+$ T cells compared to WT mice (24). It should be noted that in our study a lower dose of *T. gondii* as well as a different infection route was used, thus reducing inflammation that resulted in reduced susceptibility of TKO mice compared to single subunit knock out mice in *T. gondii* infection (24).

As described above, *T. gondii* infected TKO mice showed a clearly reduced capability of APCs for antigen presentation, further suggesting a delayed induction of a Th1 adaptive immune response to *T. gondii* in TKO mice. And in indeed, we observed reduced numbers of $CD8^+$ T cells as well as IFNγ producing $CD4^+$ T cells in spleens of infected TKO mice in the acute phase of *T. gondii* infection, whereby parasite proliferation is not restricted properly. In addition, we detected increased numbers of $NK1.1^+$ cells in brains of infected TKO mice which could possibly compensate for the absence of activated $CD8^+$ T cells.

Similar to the $NK1.1^+$ cells in brains of TKO mice in the acute phase of infection, it seems that $CD4^+$ T cells in the brain of TKO mice in the chronic phase of infection could compensate for the reduced $CD8^+$ T cell response. We found significantly more IFNγ and TNF producing $CD4^+$ T cells in brains of infected TKO mice in the chronic stage of infection. This correlates with an increase in iNOS production in mononuclear cells. Given the fact that iNOS is a crucial anti-parasitic effector molecule during chronic infection (63), it could compensate in part for the lack of CD8-mediated intracellular parasite clearance in the brain. In contrast, TKO mice exhibited reduced $CD8^+$ Tem cells in the chronic stage of infection suggesting that the absence of the IP hampers the ability to induce effector T cells timely after infectious challenge.

Regulatory T cells (Tregs), as a subpopulation of T cells, are important to suppress T cell function to regulate self-tolerance thereby preventing autoimmunity (64). We hypothesized that fewer Tregs would affect the contraction phase of the T cell response. Usually, the contraction phase begins once the pathogen has been cleared. This in turn leads to the upregulation of exhaustion markers resulting in apoptosis (65–67). Although parasites are still present, it is possible that the reduced MHC I/TCR signaling leads to reduced $CD8^+$ T cell interaction with their associated antigen, thus behaving as if there is no pathogen present, ultimately starting exhaustion earlier than anticipated. Infected TKO mice, however, showed comparable expression of T cell exhaustion and apoptosis markers in $CD8^+$ and $CD4^+$ T cells (**Supplementary Figure 4**).

Further, we found increased numbers of apoptotic monocytes in spleens of TKO mice in the chronic phase of infection. This could be explained by the inability of TKO derived myeloid cells to induce STAT3-signaling by its phosphorylation, a mechanism which has also been described in Th17 cells after IP inhibition (68). Consistent with this finding, STAT3-deficiency in B lymphocytes has been shown to induce apoptosis in a model of experimental autoimmune uveitis (69). However, it still has to be investigated whether the observed apoptosis is caused by direct parasite invasion or by the absence of the IP itself.

In summary, our results established the importance of the IP in infection-induced neuroinflammation with *T. gondii*. Without the IP, animals were impeded in developing an efficient *T. gondii* specific Th1 immune response. With reduced MHC I expression, $CD8^+$ T cell numbers and IFNγ in the acute phase, TKO mice were not able to control parasite proliferation, especially by their inability to promote the transition of the acute phase to an efficient long lasting immune response during the chronic stage of *T. gondii* infection.

We described an enhanced compensatory $CD4^+$ T cell effector function in TKO mice with increased IFNγ release during the course of infection. In addition, we detected increased production of iNOS in microglia and myeloid subsets and overall enhanced TNF level in brain tissue of chronically infected TKO animals as well as reduced numbers of regulatory T cells, reduced STAT3 phosphorylation but increased induction of apoptosis in myeloid cells. This study demonstrates that IP deficiency leads to impaired parasite control and thus increased susceptibility of these animals to *T. gondii*, highlighting the importance of the IP in terms of induction and maintenance of *T. gondii*-induced neuroinflammation.

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 study was performed in accordance with the German National Guidelines for the Use of Experimental Animals and the protocol was approved by the Landesverwaltungsamt Sachsen-Anhalt. Food and water were available *ad libitum*. All efforts were done to minimize the suffering of mice used in this investigation. The animal study was reviewed and approved by German and European legislation.

AUTHOR CONTRIBUTIONS

TF and IRD designed and organized the experiments. TF, NI, HD, CC, and ET conducted the experiments. TF and NI analyzed data. TF, AT, JS, DD, TS, US, and IRD interpreted data. TF, TS, US and IRD wrote the paper. US and IRD supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the European Structural and Investment Funds (ESF, 2014-2020; project number ZS/2016/08/80645 to IRD and the DFG (Deutsche Forschungsgemeinschaft) within the CRC854 to DD, US and IRD. We acknowledge support for the Article Processing Charge from the DFG (German Research Foundation, 393148499) and the Open Access Publication Fund of the University of Greifswald.

ACKNOWLEDGMENTS

We thank Nadine Riemann, Andrea Kröber, Petra Grüneberg and Dr. Abidat Schneider for excellent technical assistance. Prof. Kenneth L. Rock and Regeneron Pharmaceuticals, Inc. (VG MAID number VG1230 + *Psmb10*) for kindly providing us the Triple Knock-out (i20S KO) mice.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.619465/full#supplementary-material

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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.

frontiers in Immunology

REVIEW
published: 10 March 2022
doi: 10.3389/fimmu.2022.836999

Tissue-Dependent Adaptations and Functions of Innate Lymphoid Cells

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *16 December 2021*
Accepted: *11 February 2022*
Published: *10 March 2022*

Citation:
Murphy JM, Ngai L, Mortha A and Crome SQ (2022) Tissue-Dependent Adaptations and Functions of Innate Lymphoid Cells. Front. Immunol. 13:836999. doi: 10.3389/fimmu.2022.836999

Tissue-resident immune cells reside in distinct niches across organs, where they contribute to tissue homeostasis and rapidly respond to perturbations in the local microenvironment. Innate lymphoid cells (ILCs) are a family of innate immune cells that regulate immune and tissue homeostasis. Across anatomical locations throughout the body, ILCs adopt tissue-specific fates, differing from circulating ILC populations. Adaptations of ILCs to microenvironmental changes have been documented in several inflammatory contexts, including obesity, asthma, and inflammatory bowel disease. While our understanding of ILC functions within tissues have predominantly been based on mouse studies, development of advanced single cell platforms to study tissue-resident ILCs in humans and emerging patient-based data is providing new insights into this lymphocyte family. Within this review, we discuss current concepts of ILC fate and function, exploring tissue-specific functions of ILCs and their contribution to health and disease across organ systems.

Keywords: innate lymphoid cell (ILC), NK cell, tissue-resident immune cells, tissue homeostasis, autoimmunity, inflammation, immune tolerance

INTRODUCTION

Innate lymphoid cells (ILCs) orchestrate immune responses to signals such as cytokines, alarmins, neuropeptides and hormones, interacting with hematopoietic and non-hematopoietic cells alike. ILCs lack rearranged antigen receptors and while predominantly tissue-resident, are also observed in circulation and secondary lymphoid tissues where they exhibit distinct spatial and temporal functions (1). Outside of roles in immunity, ILCs have key roles in maintaining tissue homeostasis, promoting tissue repair, and regulating inflammation. *Via* crosstalk with parenchymal cells, ILCs are also involved in processes previously thought to lack immune system influence, such as thermal regulation, neuronal signal transduction, circadian rhythms, and tissue remodeling (2–6). The regulation of both immune functions and tissue-specific processes by ILCs highlights the importance of understanding how they respond and function within tissue niches, and conversely how ILC biology is controlled by the microenvironment in which they reside.

Development of ILCs in non-lymphoid tissues occurs when circulating ILC progenitors seed tissue niches, and requires the expression of local survival factors including IL-7 and thymic stromal lymphopoietin (TSLP) (7, 8). Differentiated ILCs express signature cytokines and transcription factors that parallel $CD4^+$ and $CD8^+$ T cells in both humans and mice (**Figure 1**) (6, 8), and can be

broadly categorized as cytotoxic (NK cells) or non-cytotoxic 'helper' ILCs. Human NK cells express TBET and Eomesodermin (EOMES), release IFN-γ and TNF-α and are grouped into $CD56^{dim}CD16^{+}$ or $CD56^{bright}CD16^{-}$ NK cells. $CD56^{dim}CD16^{+}$ NK cells express killer cell immunoglobulin-like receptors (KIRs) and exhibit profound cytotoxic potential (6, 8). $CD56^{bright}CD16^{-}$ NK cells lack KIR expression but are superior producers of IFN-γ and TNF-α (9, 10). NK cells discriminate between self and non-self or altered-self and function in anti-viral and anti-tumor immunity similar to $CD8^{+}$ cytotoxic T cells (6, 8). 'Helper' ILC (hILCs) are non-cytotoxic and are classified based on function and development into Group 1 (ILC1s), Group 2 (ILC2s), Group 3 (ILC3s) as well as Lymphoid Tissue inducer LTi cells (6). ILC1s produce IFN-γ in a TBET-dependent but EOMES-independent manner (6). ILC2s express GATA-3 and RORα and secrete interleukin (IL)-4, IL-5, IL-9, IL-13 and Amphiregulin (AREG), aiding in anti-parasite immunity or the promotion of allergic responses (6). ILC3s rely on the transcription factor RORC and produce IL-22, IL-17, and GM-CSF (6). ILC3s include subsets which express natural cytotoxicity receptors (NCRs) NKp44 (human) and NKp46 (mouse and human). LTi cells express ILC3-associated transcription factors and cytokines but also express surface Lymphotoxin (sLT) (11). Further, ILCs with immunosuppressive activity have been identified in cancer, intestinal inflammation, allergy, autoimmunity and ischemia reperfusion injury (12–18). These include both NK-like ILCs, IL-10 producing ILC2s ($ILC2_{10}$) and $ID3^{+}$ regulatory ILCs [reviewed in Jegatheeswaran et al. (19)]. Despite growing appreciation of ILCs with regulatory functions, their development and function are poorly characterized, particularly in humans.

Mouse studies identified central roles for ILCs in regulating tissue homeostasis, repair and remodeling, transforming our understanding of cellular interactions between immune cells and the tissues in which they reside. Across tissue microenvironments, ILCs adapt and acquire distinct phenotypes and functional properties (**Figure 2**). While ILC subsets have important functions within these tissues, dysregulation of ILC numbers and functions is associated with diverse human pathologies including arthritis, diabetes, psoriasis, asthma, and inflammatory bowel disease [reviewed in (20)], highlighting the need to identify how local tissue factors promote or inhibit inflammatory ILC responses. Within this review, we explore NK cell and hILC biology across different tissues in health and disease, highlighting evidence of similarities between human and mouse ILC function where data is available. We summarize current understanding of organ-specific functions of ILCs, focusing on their contributions to tissue homeostasis, host-defense, and inflammatory disease progression across the body.

ILCS IN THE NERVOUS SYSTEM

While the central nervous system (CNS) is considered an immune-privileged site with minimal immune infiltrate, ILCs have been identified in the CNS of healthy humans and mice, accounting for ~2.5% of leukocytes by sequencing (21–25). CNS-resident NK cells are present in low proportions in the naïve mouse brain and enriched in a $IL\text{-}2R^{+}$ $CD27^{+}$ $CD62L^{high}$ subset, suggesting a more mature phenotype compared to infiltrating NK cells (22). CNS ILC2s accumulate with age and reside in the healthy murine meninges, localizing within dural sinuses and surrounding blood vessels (23–25). Interestingly, the transcriptional profile of meningeal ILC2s showed downregulation of genes related to metabolism, signal transduction, and inflammation compared to lung-derived ILC2s, suggesting a tissue-specific quiescent adaptation to the CNS environment (23). Upon spinal cord injury in mice, ILC2s migrate to the injured site independently of IL-33 and upregulate *Calca* (CGRP) and its receptor *Ramp3*, associated with nerve regeneration (23), yet the regenerative activity of ILC2s in the spinal cord remains to be demonstrated experimentally.

ILCs in Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating and neurodegenerative autoimmune disease that is one of the most common neurological disabilities in young adults (26). NK cells mediate several treatment-related effects in MS patients (**Figure 3A**). For example, Daclizumab targets the high affinity IL-2 receptor (CD25), inhibiting activated T cells and resulting in greater availability of IL-2, which expands $CD56^{bright}$ NK cells expressing high levels of the medium-affinity IL-2 receptor chain (CD122) (18, 27). This expansion of $CD56^{bright}$ NK cells or elevated baseline expression of CD122 in patients correlated with lower inflammation and fewer inflammatory lesions (18, 28). While T cells are only modestly depleted by Daclizumab directly, induction of T cell apoptosis by $CD56^{bright}$ NK cells is supported by findings of Granzyme K^{+} NK cell co-localization with T cells in active MS lesions (18, 29, 30). Takahashi et al. further found that during the remission phase of MS, CD95 expression increased on NK cells alongside decreased response of memory T cells, suggesting that $CD95^{+}$ NK cells regulate autoimmune memory T cell responses during remission (31). In autologous hematopoietic stem cell transplantation, another MS treatment modality, NK cells reconstitute faster than $CD4^{+}$ T cells and regulate disease-promoting Th17 cells *via* NKG2D-mediated cytotoxicity, preventing lesion formation and relapse (32).

A higher ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells is observed in the cerebrospinal fluid of patients with MS compared to those with other inflammatory and non-inflammatory neurological diseases, suggesting an MS-specific alteration in resident NK cells with controversial effects on the abundance of NK cells in circulation (30, 33, 34). Despite conflicting findings regarding abundance, circulating $CD56^{bright}$ NK cells from MS patients have reduced IFN-γ production in response to IL-12 and an impaired ability to regulate autologous $CD4^{+}$ T cells compared to healthy controls (33, 35). This impaired regulatory capacity was due to HLA-E upregulation on autologous T cells engaging the inhibitory receptor NKG2A on NK cells (35). Further, DNAM-1 and 2B4 were reduced on NK cells alongside reduced expression of the DNAM-1 ligand CD155 on $CD4^{+}$ T cells, while Daclizumab treatment induced CD155 upregulation on T cells to partially rescue the impaired ability of NK cells to regulate autologous T cells (30). A genome-wide association study of MS patients demonstrated lower expression of *TBX21* and *EOMES* in

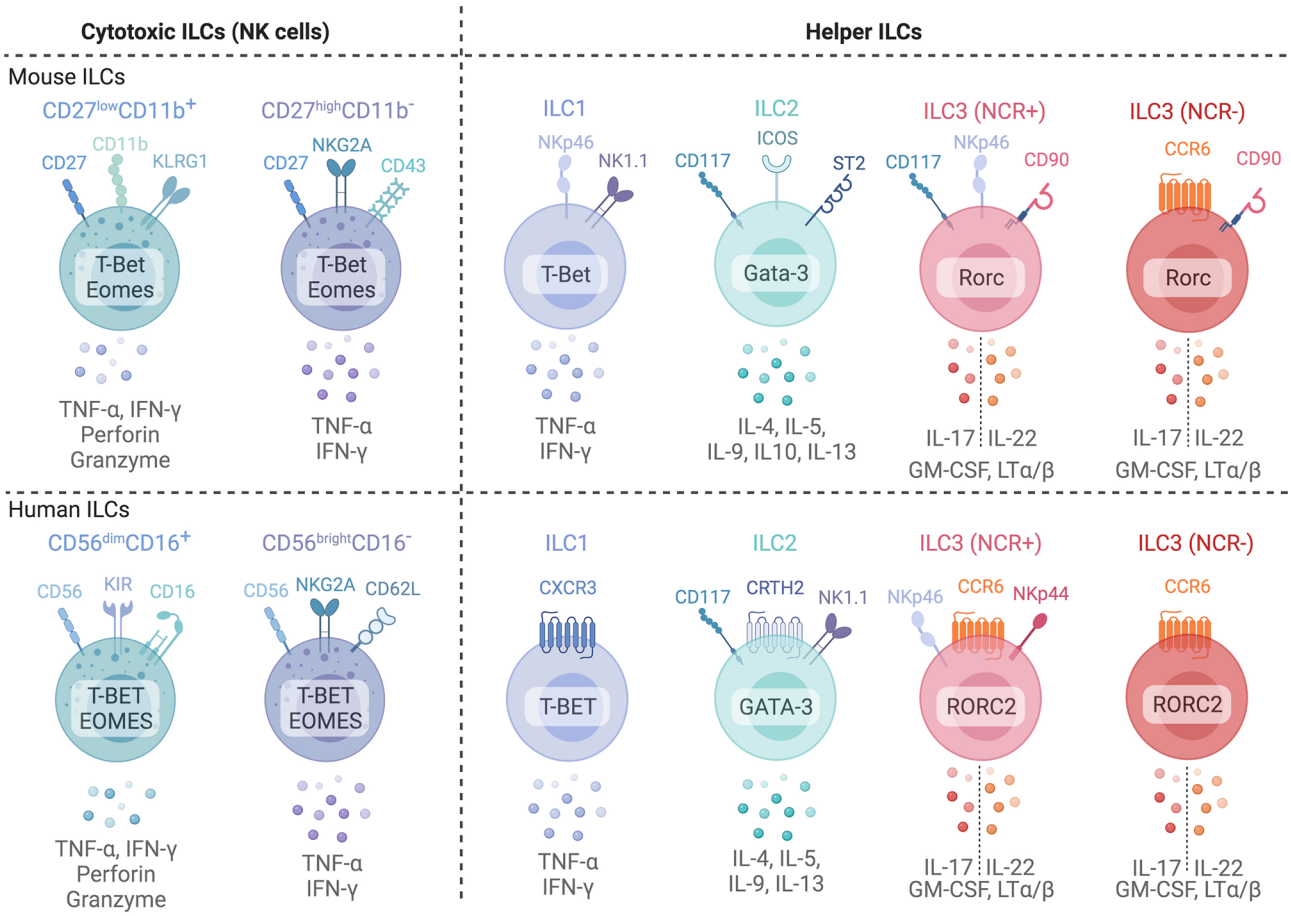

FIGURE 1 | Common phenotypic markers of mice (top) and human (bottom) ILCs and their common cytokine expression profiles. Cytotoxic Natural Killer cells can be subdivided into two major subsets based on surface marker expression in both mice and humans. In mice, NK cells are subdivided into two subsets based on CD27 and CD11b expression: $CD27^{high}CD11b^{-}$ immature NK cells and mature $CD27^{low}CD11b^{+}$ NK cells. In humans, $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ are generally used to identify immature and mature NK cells in blood. However, tissue NK cells often display a $CD56^{bright}CD16^{-}$ phenotype. ILC1s, ILC2s, and ILC3s are classified based on surface marker and transcription factor expression profiles that parallel $CD4^{+}$ T helper subsets. ILC3s are further subdivided into natural cytotoxicity receptor $(NCR)^{+}$ and NCR^{-} subsets. Created with Biorender.org.

NK cells, supporting that impairment of NK cells may be a driver of MS (36).

Using the experimental autoimmune encephalomyelitis (EAE) model of MS, Hao et al. demonstrated the importance of CX3CR1-mediated recruitment in generating disease-ameliorating CNS-resident mouse NK cells (37). Transmigration of NK cells into the CNS partially depends on VLA-4 binding to endothelial VCAM-1, as antibody blockade of VLA-4 reduces NK cell recruitment by 40-70% (30, 38). Absence from or blocked transmigration results in excessive proliferation of myelin-reactive CD4+ T helper 17 (Th17) cells, indicating that NK cells must be within the CNS to limit myelin-specific T cell activity and disease progression (37). Mouse NK cell-mediated disease amelioration required an NCR- and perforin-dependent lysis of microglia to abrogate Th17 expansion (37). The tight proximity of microglia and NK cells requires reciprocal chemoattraction through secretion of MIP-1α and MCP-1 by NK cells and microglia, respectively (37, 39). Additionally, NK cells dampen EAE pathogenesis by directly modulating infiltrating $CCR2^{+}Ly6C^{hi}$ monocytes in an acetylcholine-dependent fashion. Adoptive-transfer of choline acetyltransferase (ChAT)-expressing NK cells into the CNS of *Cx3cr1*$^{-/-}$ mice reduced the abundance of infiltrating monocytes (40). $ChAT^{+}$ NK cells dampened TNF-α, IL-1β, IL-12 and Qa-1 expression by monocytes through engaging the α7-nicotinic acetylcholine receptor, rendering myeloid cells more susceptible to lysis (40). $ChAT^{+}$ NK cells preferentially localize to active demyelinated lesions in the human brain, suggesting this mechanism of microglial regulation may translate to human MS as well (40). While dampening myeloid and T cell activity reduces disease severity, murine NK cells negatively impact regeneration through lysis of Qa-1^{low} neuronal stem cells in the sub-ventricular zone, altering neuronal repair and impairing recovery in later disease (41). Of note, NK cell activation *via* NKG2D triggered motor neuron destruction in models of amyotrophic lateral

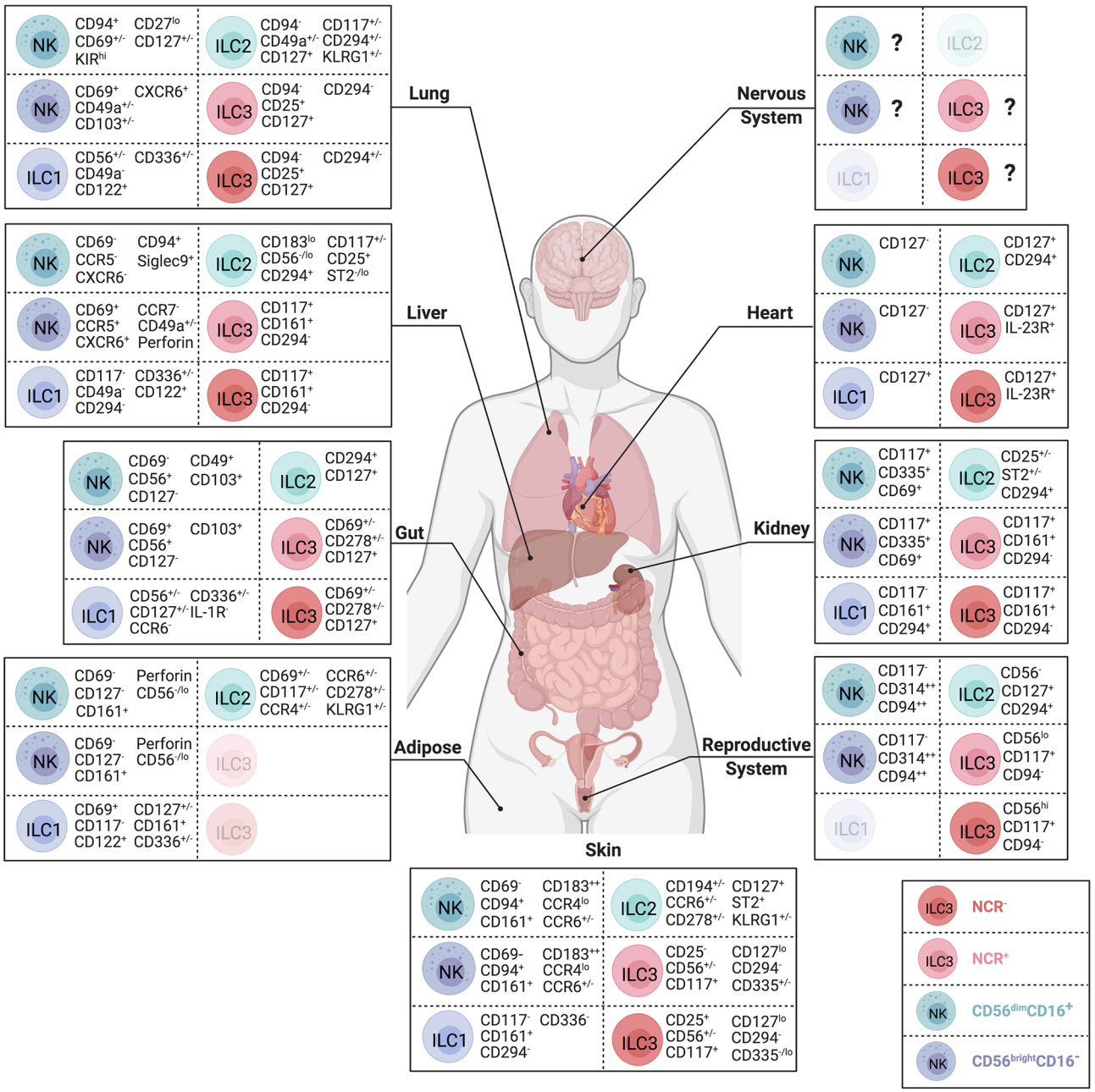

FIGURE 2 | Body-wide distribution and surface phenotypes of human ILCs. Surface marker expression of $CD56^{dim}$ NK cells (teal), $CD56^{bright}$NK cells (dark blue), ILC1s (purple), ILC2s (green), NCR^- ILC3s (red) and NCR^+ ILC3s (pink) in nervous system, lung, heart, liver, kidney, gut, reproductive system, adipose tissue, and skin. ILC subsets that have not yet been identified during steady state visualized in lighter color. Represented selection of ILC markers is based on the consistent use of these markers across multiple independent studies. Created with Biorender.org.

sclerosis, suggesting pathological NK cell-mediated lysis of neurons is not specific to MS/EAE (42).

Other ILCs have been identified in MS too, although inconsistent phenotyping has hindered identification of these ILCs. A sizable fraction of $CD3^-$ $IL\text{-}17^+$ $ROR\gamma t^+$ cells associate with newly formed meningeal lymphoid follicles of MS patients, suggestive of ILC3 involvement (43). In mice, $CD3^-ROR\gamma t^+$ populations in the cerebellum after EAE induction were predominantly $CD4^-$, consistent with ILC3 identity (44). Hatfield et al. reported both NCR^+ and NCR^- ILC3s and $CD4^+$ $CD3^-$ LTi-like ILC3s within the meninges of healthy mice which proliferated and accumulated downstream of c-kit signaling during EAE induction (45). Meningeal ILC3s produce IL-17 and GM-CSF, and express co-stimulatory molecules OX40L

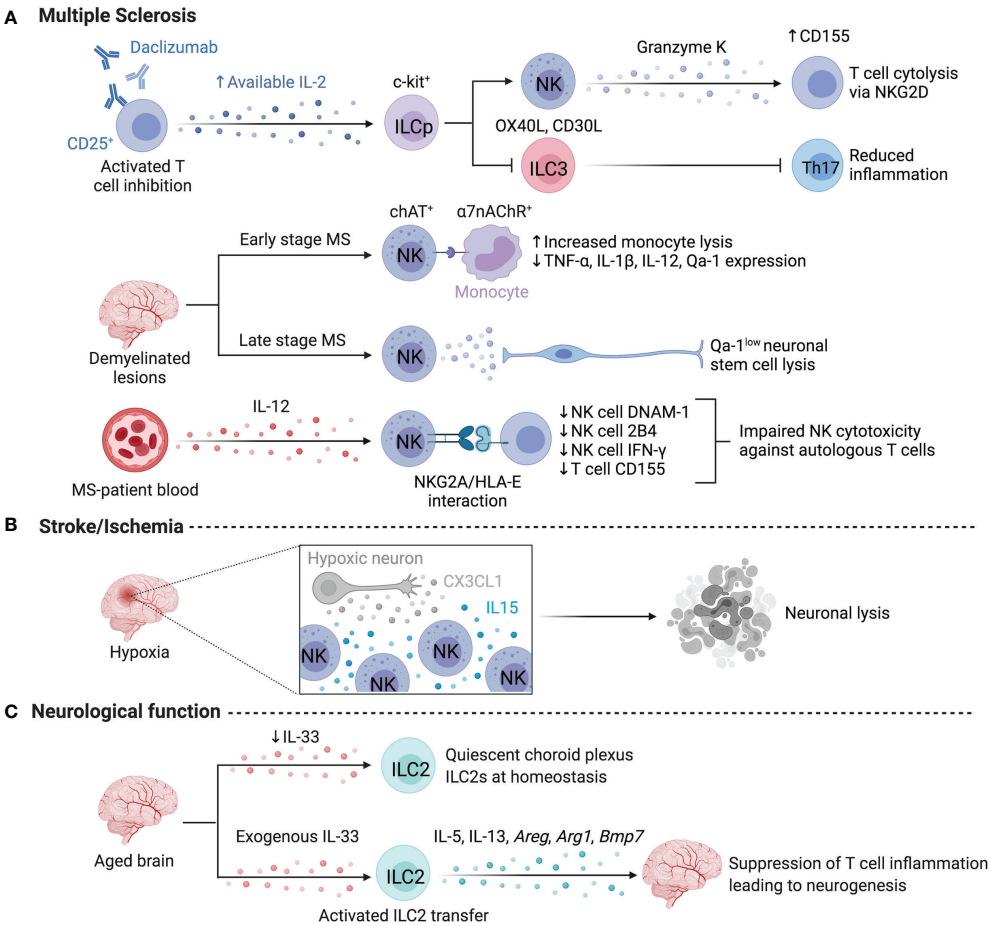

FIGURE 3 | ILCs in the nervous system. Limited information exists on human ILCs in the nervous system at steady state due to challenges in obtaining samples, however, several studies focus on ILC activity in multiple sclerosis or stroke. **(A)** Daclizumab-driven inhibition of T cells resulted in the expansion of NK cells and the elevated lysis of T cells. Daclizumab treatment lowers the abundance of Lin$^-$c-kit$^+$RORC2$^+$ ILCs and dampens Th17-associated inflammation by lowering IL-17 and GM-CSF. ChAT$^+$ NK cells preferentially localize to demyelinated lesions in the human brain to dampen monocyte-driven inflammation *via* the a7-nicotinic acetylcholine receptor (a7nAChR), rendering myeloid cells more susceptible to lysis at early stages of MS. Conversely, NK cell-mediated lysis negatively impacts regeneration during later stages of MS by targeting, Qa-1^{low} neuronal stem cells. NK cell activation may be impaired by NKG2A/HLA-E interactions with autologous CD4$^+$ T cells. **(B)** After a stroke, CX3CL1 from hypoxic neurons recruits NK cells, while local IL-15 levels facilitate NK cell enrichment and promote high NKG2D expression and neuronal lysis. **(C)** ILC2s impact neurological functions in murine brain and are supported by exogenous IL-33 to suppress T cell inflammation and enhance neurogenesis. Created with Biorender.org.

and CD30L. They accumulated near Th17 cells and antigen presenting cells (APCs) and facilitated T cell activation and entry into the brain parenchyma in a T-bet-dependent fashion, highlighting a role for ILC3s in establishing a microenvironment that sustains Th17 responses in EAE (45, 46).

Helper ILCs (hILCs) are also affected by Daclizumab treatment and appear to play a sex-biased role in MS/EAE. Untreated MS patients presenting with elevated white blood cell counts displayed higher levels of RORγt$^+$ ILCs in their cerebrospinal fluid (47). Daclizumab treatment lowered CXCL13 levels and the abundance of Lin$^-$c-kit$^+$RORγt$^+$ ILCs, suggesting that ILC3 inhibition may be another beneficial effect of Daclizumab treatment (48). *In vitro* differentiation of c-kit$^+$ ILC precursors and CD34$^+$ hematopoietic progenitor cells under high IL-2 conditions favored the development of CD56^{bright} NK cells and restrained ILC3 differentiation, implying that greater *in vivo* IL-2 availability affects the development of ILCs by altering subset composition (48). MS has a higher prevalence in females and is correlated with reduced accumulation of ILC2s in EAE models (49). Interestingly, male mice that have reduced c-kit signaling (Kit$^{W/Wv}$) failed to accumulate ILC2s and adopted a female disease phenotype suggesting a sex-dependent role for ILC2s in protection from EAE pathogenesis (49). *Il33* expression is only upregulated in male mice after myelin peptide immunization, and IL-33 administration in female mice expands ILC2s and provides protection from EAE, while anti-IL-33 treatment abrogates protection in male mice, further supporting sex effects on ILC2 function, dependent on differential IL-33 availability (50).

ILCs in Cerebral Ischemia (Stroke)

After a stroke, human peripheral blood NK cells are reduced early (< 72h) and the degree of reduction as well as expression of activation markers positively correlates with infarct volume (51, 52). Within 12 hours of intracerebral hemorrhage, $CD69^+Perforin^+$ NK cells become the dominant immune cell type in perihematomal regions (21). 24h following a stroke, $CD69^+NKp46^+$ cell numbers peaked in the brain and remained elevated (52). In mice, the accumulation of NK cells during the acute phase of stroke is mediated by the release of CX3CL1 by hypoxic neurons (53). Recruited NK cells accumulate in an IL-15-rich environment, adopt an activated phenotype, and mediate neuronal lysis through missing-self activation (**Figure 3B**) (53). Ischemia-reperfusion injury (IRI) induces IL-15 production by neurons, astrocytes and microglia, blockade of which reduced IFN-γ^+ NK cells in the murine brain (54). Liu et al. reported that cholinergic signaling in the brain and catecholaminergic signaling in the periphery suppressed NK cell function after cerebral ischemia, contributing to post-stroke susceptibility to infection (52). While adrenergic activation suppressed NK cell abundance and function in the periphery, cholinergic signaling reduces *Runx3* expression in CNS NK cells, leading to a decline in NK cell responsiveness and demonstrating the involvement of distinct neural pathways in regulating the spatial activation of NK cells in mice and humans (52). In humans, the microRNA (miRNA) profile of peripheral NK cells is altered after stroke and inhibition of miRNA-451a and miRNA-122-5p partially restored CD69 and NKG2D expression, suggesting that targeting miRNAs may alleviate immunosuppression observed after a stroke (51). Although data supporting a role for helper ILCs in response to stroke is scarce, early after an acute cerebral infarction circulating ILC1s increased and ILC2s decreased, correlating to serum ox-LDL levels, suggesting lipid-mediated regulation of ILC1 and ILC2 abundance (55).

ILCs in Neurological Function

Murine studies support a role for ILCs in regulating neurological function. Depletion of NK cells using anti-NK1.1 improved cognitive function, enhanced neurogenesis, and reduced microglial inflammation but did not affect β-amyloid concentration in a mouse model of Alzheimer's disease (56). NK cells exhibited altered expression profiles in the disease model, with higher expression of *Icam1*, *Ctsb*, *Ctsc*, *Ccl3* and *Ccl4* (56). Following NK cell depletion, microglia exhibited a return to homeostatic morphology, reduced proliferation, and reduced expression of pro-inflammatory mediators including *Il18*, *Il1a*, *Il1b* and *Tnf*, suggesting that NK cells and type I immunity contribute to cognitive decline by promoting microglial inflammation (56). In line with these findings, choroid plexus ILC2s accumulated and displayed a quiescent state in the aged brain, which was reversed with IL-33 stimulation (57). In comparison to meningeal ILC2s, choroid plexus ILC2s were resistant to senescence and exhibited higher expression of *Arg1* and genes associated with glycolysis that may underlie their enhanced proliferative and cytokine-producing capacity, and suggest niche-specific functionality (57). Intriguingly, activation of ILC2s in aged mice or transfer of activated ILC2s to the aged brain increased cognitive function, potentially through IL-5-mediated suppression of T cell inflammation leading to enhanced neurogenesis (**Figure 3C**) (57). After traumatic brain injury, ILCs are increased in frequency in human meninges and cerebrospinal fluid, and treatment with AMPK-activating metformin in a murine model specifically enhanced IL-10-producing ILC2s and improved neurological outcomes (58). Together, this suggests that ILC2s support neurological function and resolution of inflammation while NK cells exacerbate cognitive decline.

ILCs in Peripheral Nervous System

Nervous system signaling in the periphery is also impacted by ILC activity. Specialized pro-resolving mediators (SPMs) such as PCTR1 are important for resolving inflammation and promoting tissue repair (59). Acetylcholine promotes the enzymatic activity of ILC3-derived 15-LOX-1, the initiating enzyme in PCTR1 biosynthesis (60). Production of SPMs is regulated by the vagus nerve, and loss of vagus nerve signaling reduced peritoneal ILC3s in mice resulting in poor resolution of *Escherichia coli* infection (60). The circuit between ILC3s, SPMs, and macrophages is key for resolving infection and inflammation in the peritoneum (60).

ILCS IN THE LUNG

NK cells account for 10-20% of all lymphocytes in human and murine lungs (61–63). Lung NK cells are marked by higher CD57 and KIR expression, and lower CD27, indicative of a mature phenotype (64). Despite their high KIR expression, human lung $CD56^{dim}CD16^+$ NK cells are hypofunctional and some $CD56^{bright}$ subsets are characterized by the expression of markers associated with tissue-residency (e.g., *CD69*, *ITGA1* (CD49a), *ITGAE* (CD103), and *CXCR6*) (61, 64). A review by Hervier et al. nicely summarizes the development and function of NK cell subsets in the human lung (65). In addition to NK cells, all other helper ILC subsets have been observed in human lung, albeit with conflicting reports on the relative abundance of ILC1s, ILC2s and ILC3s that may reflect small sample sizes, sampling location, or inter-donor heterogeneity (66, 67).

Recruitment as well as local proliferation of ILC precursors in the lung during development shape the pool of tissue-resident ILC subsets (**Figure 4A**). Oherle et al. identified that murine pulmonary ILC3s develop from a local precursor pool sustained by insulin-like growth factor 1 provided by alveolar fibroblasts (68). Early-life seeding of ILC3s was protective against pneumonia in a CCR4-dependent fashion, driven by a gut commensal microbiota – dendritic cell (DC) axis (69). Similar interactions between adventitial stromal cells and mouse ILC2s were reported to sustain and regulate ILC2s homeostasis and function (24, 70). Adventitial stromal cells release TSLP, promoting basal IL-13 release by ILC2s, which in turn activates adventitial stromal cells to produce IL-33 in a homeostatic circuit (24). Interestingly, ILC2s localize around

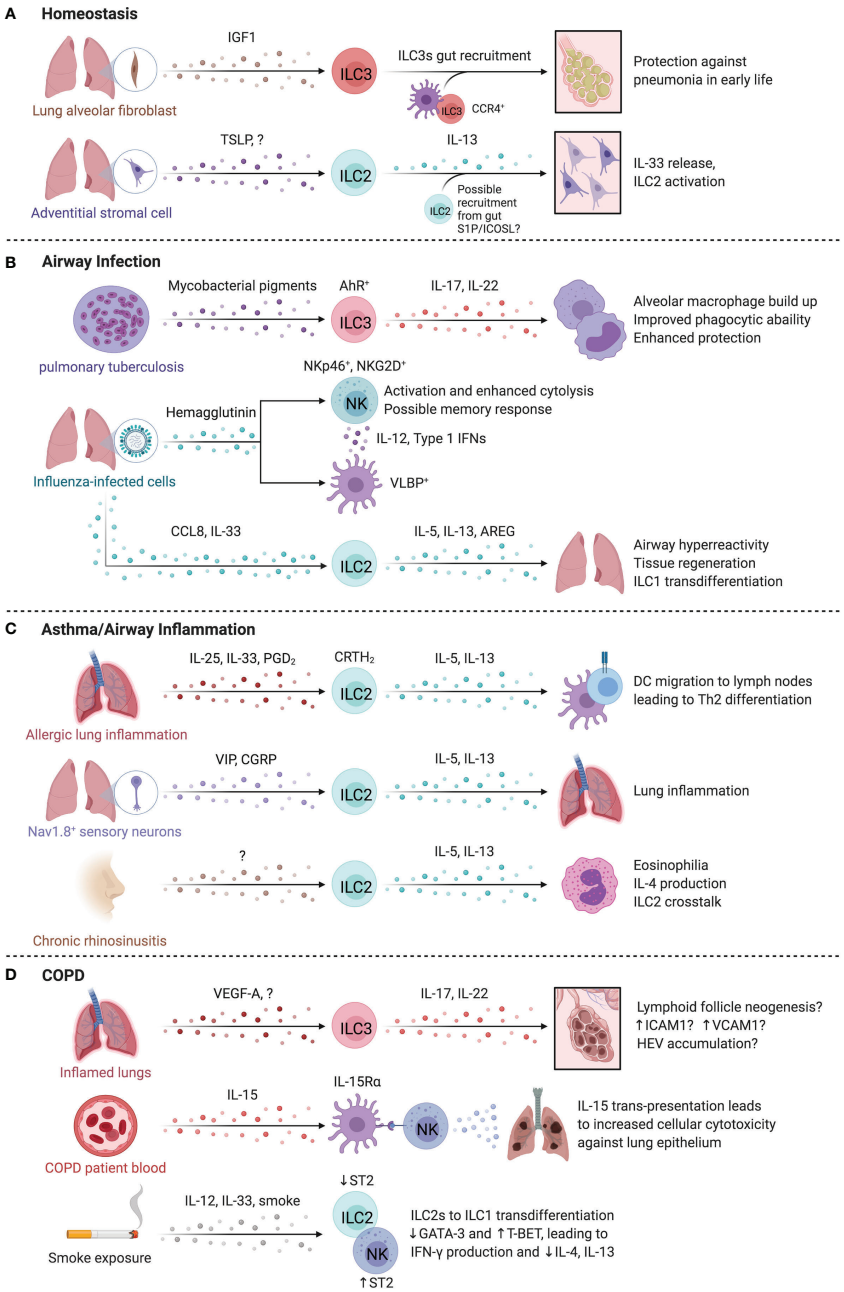

FIGURE 4 | ILCs in the lung. At homeostasis **(A)**, IL-22 producing ILC3s are required for protection against pneumonia and require commensal gut bacteria for their recruitment to the lungs. Once in the lung, ILC3s are sustained locally through insulin-like growth factor 1 (IGF1) from alveolar fibroblasts. In the steady state, Adventitial stromal cell-derived TSLP promotes IL-13 production by ILC2s that drives stromal cells to produce IL-33. **(B)** During pulmonary tuberculosis (PTB), ILC3 accumulation in the lung is regulated by pathogen-derived AhR-ligands that in turn promote phagocyte function, formation of tertiary lymphoid structures and enhanced protection. Similarly, infection by influenza virus triggers NKp46-dependent activation of NK cells resulting in IL-12 and type 1 interferon secretion by DCs that promote NK cell activation. The release of CCL8 and IL-33 during respiratory viral infection facilitated ILC2 activation and AREG-dependent epithelial repair. **(C)** In allergic lung inflammation, IL-13 from ILC2s induced Th2 cell differentiation by promoting migration of activated DCs to the draining lymph nodes. In mice, administration of the CRTH2 ligand, prostaglandin D2, promotes ILC2 accumulation. Nociceptor Nav1.8+ sensory neurons activated lung ILC2s through vasoactive intestinal peptide (VIP), while pulmonary neuroendocrine cells produced calcitonin gene related-peptide (CGRP) collectively promoting allergic inflammation in the murine lung. Nasal polyps accumulate ILC2s in chronic rhinosinusitis, which supports eosinophils and promotes chronic airway inflammation. **(D)** IL-17A^{+}IL-22^{+} ILCs and NCR^{-} ILC3s are increased in COPD. The lungs of COPD patients and smokers contain Neuropilin 1 (NRP1)-expressing ILC3s surrounding high endothelial venules. NK cells may contribute to COPD, with higher CD57 expression, IL-15-dependent activation, and greater cytotoxicity against lung epithelial cells. Smoke exposure may lead to a sustained loss of ST2 expression on ILC2s, reducing their responsiveness to IL-33 while paradoxically promoting ST2 expression on NK cells that supports a type 1 response. Created with Biorender.org.

the peribronchial and perivascular adventitial cuff regions independent of microbial signals, IL-25, IL-33 or TSLP, indicating that additional unknown signals regulate pulmonary ILC2 development and recruitment (24, 70, 71). Whether pulmonary ILC2s in mice and humans originate from other tissues at steady state remains unclear, however mouse intestinal ILC2s were demonstrated to traffic to the lungs in an S1P-dependent manner after intraperitoneal IL-25 administration or helminth infection, demonstrating coordination between tissue sites to resolve multi-organ infections (72). Additional niche-signals may be delivered through the ICOS : ICOSL axis that has been demonstrated to sustain the pool of pulmonary ILC2s by elevating anti-apoptotic genes and IL-2 responsiveness (73). Intriguingly, ILC2s express both ICOS and ICOS-L, suggesting that both self-sustaining and helper cell-dependent interactions promote ILC2 homeostasis (73).

ILCs in Airway Infections

Airborne pathogens are a constant challenge within the lung, and ILCs have a key role in anti-bacterial and anti-viral host defense (**Figure 4B**). Helper ILCs accumulate in the lungs of patients with pulmonary tuberculosis (PTB), while circulating ILCs are reduced (74), suggesting trafficking of ILCs to the lung. ILC3s are critical for host defense in PTB, as specific deletion of ILC3s ($Ahr^{fl/fl}Ror\gamma t^{Cre}$) increased mycobacterial burden, and impaired the accumulation of alveolar macrophages and formation of protective lymphoid follicles in granulomas (74). Mycobacterial pigments serve as ligands for Aryl hydrocarbon receptor (AhR), a key transcription factor for ILC3 development and function, suggesting an alternative mechanism of ILC3 activation in tuberculosis infection (75). In addition, ILC3s recruited to murine lungs produced IL-17A and IL-22 to enhance protection and support phagocytic functions of inflammatory monocytes to mediate clearance of bacterial infections (76, 77).

NK cells are critical in controlling viral infections in the lung. Indeed, influenza infection is lethal in *Ncr1*$^{-/-}$ mice (78). However, adoptive transfer and antibody-depletion experiments showed that NK cells exacerbated influenza morbidity and mortality in a manner dependent on virus titer (79). Differences in mouse genetic backgrounds, influenza strains, and infectious dosage complicate the interpretation and translation of these findings. In humans, viral hemagglutinin on infected cells triggered NKp46-dependent activation of NK cells, and upregulation of the NKG2D ligand ULBP on infected DCs and elevated secretion of IL-12 and type 1 interferon facilitated NK cell activation and cytolysis in response to influenza (80, 81). In a human lung tissue explant model, $CD56^{bright}CD49a^{+}$ NK cells robustly responded to influenza A infection, hinting at an NK cell subset-specific memory response (82). Dou et al. found that seasonal influenza vaccination induced a short-term (6 month) memory response in NK cells, correlating with downregulation of surface NKp46 and a concomitant increase in intracellular NKp46 expression (83). This memory response to re-challenge was not strain-specific, suggesting broader protection to influenza after seasonal strain-specific vaccination (83). While the role of ILC1s separate from NK cells is less clear, murine ILC1s promote antiviral defense and DC maturation, potentially through the glucocorticoid-induced TNFR-related protein (GITR):GITR-L axis (84). GITR upregulation on ILC1s resulted in stronger IFN-γ and TNF-α responses to influenza A, supporting host defense against alveolar viral infections (84).

ILC2s have conflicting roles in influenza infection response, promoting airway hyperreactivity in an IL-13-dependent manner while supporting epithelial cell integrity and tissue repair *via* the secretion of AREG following viral infections (85, 86). In response to CCL8, IL-33-activated ILC2s produce more IL-5 and IL-13, and exhibit ameboid-like movements to traffic to peribronchial and perivascular sites in mice, particularly at locations of increased collagen-I deposition (71). Human ILC2s also exhibited a chemotactic response to CCL8, suggesting shared lung recruitment responses across species (71). Infections with respiratory syncytial virus (RSV) leads to a viral titer-independent increase in respiratory disease severity in young infants driven by elevated ILC2 cytokine release (87, 88). Interestingly, patients older than 3 months had fewer ILC2s in their lungs, greater IFN-γ levels and experienced less severe disease, suggesting that the immunological changes occurring with age and development confer protection to RSV infections by balancing type 1 and type 2 immunity (88). The plasticity of ILC2s may also play a role in promoting type 1 immunity to viral infections. Silver et al. found that adoptively transferred murine ILC2s trans-differentiate into ILC1s near IL-12- and IL-18-expressing myeloid cells during influenza A infection (89). Overall, this suggests that age and plasticity shape ILC2 responses to viral infections.

ILCs in Asthma and Allergic Airway Inflammation

Asthma is a chronic inflammatory disease of the airways marked by elevated type 2 inflammation (90, 91). ILC2 activity is implicated in airway inflammatory diseases (**Figure 4C**). ILC2-derived IL-13 is critical for inducing Th2 cell differentiation in response to allergic lung inflammation by promoting the migration of activated DCs to the draining lymph nodes, supporting the development of allergic adaptive immune responses (92). Circulating ILC2s from asthmatic patients produced more IL-5 and IL-13 in response to IL-25 and IL-33 stimulation relative to controls, and administration of prostaglandin D_2, the ligand for CRTh2, promoted ILC2 accumulation in murine lungs (93, 94). A single nucleotide polymorphism resulting in elevated CRTh2 expression positively associates with asthma development in humans, although whether this corresponds directly to increased ILC2 presence is unknown (95). Interestingly, the prevalence of asthma is lower in adult males versus females, indicating sex-specific differences in type 2 immunity (96). Several animal studies recapitulated these sex-dependent changes in abundance, phenotype, and responsiveness of ILC2s and implicated the role of sex-hormones in facilitating sex-specific responses to alveolar diseases (96–100). For example, androgen-receptor signaling negatively regulated ILC2 cytokine secretion and differentiation and reduced IL-33-dependent lung inflammation in male mice (97, 100, 101).

Strikingly, neuronal and neuroendocrine-driven stimulation of ILC2s promotes allergic lung inflammation (102, 103). IL-5-stimulated nociceptor Nav1.8$^+$ sensory neurons activated ILC2s through vasoactive intestinal peptide (VIP), while pulmonary neuroendocrine cells trigger ILC2s through the calcitonin gene-related peptide (CGRP) to promote allergic inflammation in the murine lung (102, 103). CGRP-secreting pulmonary neuroendocrine cells were increased in asthmatic patients suggesting that this mechanism could also support ILC2-mediated allergic inflammation in humans, inspiring several pathways of therapeutic interventions (103). Constitutive activation of ILC2s may lead to long-lasting alterations in the lung as found in other pulmonary diseases. For example, ILC2s are enriched in nasal polyps of chronic rhinosinusitis patients along with elevated *IL5* and *IL13* transcripts, suggesting an ILC2-dependent contribution to the disease-associated eosinophilia and chronic airway inflammation (104, 105). Polyp tissues identified with eosinophilia revealed a co-localization of ILC2s and eosinophils, indicating a possible cross-talk between IL-5-producing ILC2s and IL-4-producing eosinophils to support reciprocal activation and survival (106).

Complicating our understanding of ILC2s in allergic responses are recent findings from Golebsky and colleagues that $ILC2_{10}$s are reduced in abundance in allergic individuals relative to non-allergic controls, while sublingual immunotherapy for grass pollen allergy restores this IL-10-producing subset which may confer protection and restoration of epithelial barrier integrity (16). Interestingly, murine lung ICOS$^+$ST2$^+$ ILC2s exhibit memory in response to allergen challenge dependent on ICOS and IL-33, marked by transcriptional and epigenetic programs involving the scaffold protein *Four And A Half LIM Domains 2* (FHL2) (107). Further, adoptive transfer of FHL2$^+$CRTh2$^+$ human ILC2s induced airway hyperreactivity in mice and were partially steroid resistant, suggesting memory ILC2s may be relevant to steroid-resistant asthma (107).

Similar to ILC2s, ILC3s have been linked to asthma pathology. IL-17 levels and IL-17$^+$ ILC3s were elevated in bronchial alveolar lavage fluid of asthmatic patients, especially in patients with severe disease (108, 109). An ILC3 gene signature was upregulated in nasal brushings of adult-onset severe asthma patients, while bronchial brushings revealed elevated type 2 related gene profiles, supporting the idea of an anatomic preference of distinct ILC responses that may selectively contribute to site-specific characteristics of disease (110).

ILCs also contribute to chronic pulmonary inflammation through regulation of adaptive immune cells. CD40L expression by human and murine T helper cells induces an IgE response by B cells, contributing to airway hyper-responsiveness (111, 112). CD40L expression on T cells is induced by cAMP only in the presence of CD56$^+$CD16$^+$ NK cells through a contact-dependent manner to drive asthmatic IgE responses (112). In patients with severe asthma, NK cells expressed higher levels of CD69 and NKG2D in line with an activated phenotype. Despite higher activation status, NK cell ability to induce eosinophil apoptosis was impaired (113). IL-13 production by ILC2s was attenuated and NK cell-induced eosinophil apoptosis was greatly increased by lipoxin A4 (LXA4), a pro-resolving mediator negatively affected during severe allergic asthma (113, 114). Lacking efficiency in resolution of eosinophilic inflammation due to a lack of LXA4 production in severe asthma suggests another axis of interaction promoting pulmonary dysfunction of NK cell and ILC2 responses to inflammation (113). Collectively, multiple layers of regulation affect the localized activity and accumulation of ILCs in asthma, emphasizing the need to understand tissue signals that control ILCs to develop more targeted therapies.

ILCs in COPD

Chronic obstructive pulmonary disease (COPD) is an inflammatory condition characterized by permanent and progressive loss of lung function, associated with smoking and exposure to noxious stimuli (115). ILC1s are increased in abundance in COPD patient lungs, correlating with smoking status and symptom severity (116). All helper ILC subsets localized with lymphoid aggregates in COPD lungs (116). IL-17 upregulation in end-stage COPD is implicated in lymphoid follicle neogenesis, and De Grove et al. found trends of elevated abundance of NCR$^-$ ILC3s and IL-17A$^+$ and IL-22$^+$ ILCs in the lungs of COPD patients (66, 117). While this seems to support the involvement of ILC3s in COPD, data supporting a specific role for ILC3-derived IL-17 is lacking. Co-culture of expanded human lung ILC3s with mesenchymal stromal cells induced upregulation of ICAM-1 and VCAM-1, suggestive of LTi activity, contrasting with observations in *Rorc*$^{-/-}$ and *Id2*$^{-/-}$ mice that develop lung lymphoid follicles even in the absence of ILC3s/LTis (118, 119). Interestingly, a subset of Neuropilin1$^+$ ILC3s were recruited to high endothelial venules in lung tissues of smokers and COPD patients in a VEGF-A-dependent manner, although the specific role for ILC3s in COPD development and pathogenesis remains unresolved (118).

Circulating NK cells from smokers and COPD patients express higher levels of CD57 and have greater cytotoxicity against autologous lung epithelium than non-smokers or smokers without COPD (120, 121). The increase in cytotoxicity was mirrored in a murine COPD model after cigarette smoke exposure, demonstrating that trans-presentation of IL-15Rα by lung DCs was required to prime high NK cell cytotoxicity against autologous epithelial cells (120). Interestingly, cigarette smoke exposure induces a sustained loss of ST2 expression on ILC2s, dramatically reducing their responsiveness to IL-33, despite increased IL-33 production in severe COPD (122). Conversely, smoke induces an upregulation of ST2 on NK cells, leading to IL-33-mediated activation of NK cells instead of ILC2s, explaining the increase in type 1 immunity despite elevation of the type 2-activating cytokine IL-33 (122). Paralleling this, elevation of circulating ILC1s with a strong inverse correlation to ILC2 abundance was observed in COPD patients, further suggesting a misguided immune activation and cytokine-driven ILC plasticity, similar to mechanisms observed in the response to murine influenza infections (89, 106). Stimulation of human blood-derived ILC2s with IL-12 promoted their trans-differentiation into ILC1-like cells accompanied by the downregulation of

GATA3 and an upregulation of T-BET, increasing IFN-γ release while dampening IL-4 and IL-13 production (89, 106). These results collectively demonstrate that persistent lung inflammation and exposure to smoke leads to changes in the local ILC composition and function (**Figure 4D**).

ILCS IN THE SKIN

The skin is a barrier organ that employs immunological, microbial, and physiochemical mechanisms to protect the body from pathogens and harmful environmental factors. The skin is composed of three distinct layers: the epidermis (mainly comprised of keratinocytes), the underlying dermis, and the innermost subcutis. Tissue-resident and long-lived ILC subsets have been identified in mice and humans with varying proportions identified across studies (123–127). A more granular analysis of skin by layer revealed a predominant accumulation of ILC3s in the epidermis, ILC2s in the subcutis and comparable abundance of both subsets within the dermis in mice (128). This distribution has been attributed to the localized release of IL-7 and TSLP by either hair follicle keratinocytes or epithelial cells (128, 129). Mirroring murine models, human skin ILC2s can be activated by IL-25, IL-33, and TSLP, with high expression of IL-33 and TSLP during chronic skin inflammation (130–132).

ILCs regulate essential homeostatic functions of the skin (**Figure 5A**). For example, murine $TNF^+LT\alpha_1\beta_2^+CCR6^+$ ILC3s negatively regulate the size of the lipid-secreting sebaceous glands, while the differentiation, proliferation, and expression of antimicrobial proteins by keratinocytes depends on IL-22 stimulation from ILC3s or epidermal T cells (128, 133). These interactions regulate the skin microbiome which can alter susceptibility to inflammatory disorders, impact repair pathways and influence host defense (128, 133, 134).

ILCs in Wound Healing

ILCs directly influence skin repair after damage. Murine skin-resident ILC2s activated by IL-33 from injured epithelial cells proliferate at sites of injury while anti-CD90 depletion of ILC2s in *Rag1*$^{-/-}$ mice delays wound healing (135). $CD4^+NKp46^{low/-}$ ILC3s are recruited by damage-induced CXCL13 and CCL20 and promote wound closure *via* IL-17A, IL-17F and IL-22 and indirectly through CCL3-mediated macrophage recruitment (136). Comparable findings were observed in *IL-22*$^{-/-}$ mice, where deficiency in IL-22 impaired keratinocyte proliferation, impeding repair (137). These results support a role for ILC2s and ILC3s in regenerative remodeling of the skin, yet research is needed to translate animal findings to humans and to define the differential impacts of ILCs and T helper cells (138).

ILCs in Psoriasis

Psoriasis is a chronic inflammatory skin disease that manifests as red scaly plaques caused by hyperproliferation of keratinocytes downstream of excessive repair pathways (139). Elevated IL-17 levels and Th17-associated gene expression signatures are found in psoriatic lesions and mouse models, implicating IL-17 and IL-22 in pathogenesis (140–142). IL-22- and IL-17-producing NCR^+ ILC3s and $CD56^+ROR\gamma t^+$ ILC3s are enriched in inflamed and non-inflamed skin of psoriasis patients (**Figure 5B**) (123, 124, 126). ILC3s in inflamed lesions express higher NKG2D, which likely interacts with elevated MICA on keratinocytes (143). Anti-TNF treatment reduced circulating ILC3s in patients, corresponding with a decrease in inflammatory lesions (124). Further, ILC3-derived IL-22 induces an upregulation of MHC-II on keratinocytes, which promotes T cell polarization and skin inflammation, demonstrating a key circuit mediating skin inflammation (144). Skin ILC2s are also capable of driving T cell activation directly by presenting lipid antigens in a CD1a-dependent manner, leading to local activation of T cells in response to dermal bacteria (145).

There is limited and sometimes conflicting evidence for the role of NK cells in psoriasis. Studies have indicated that circulating NK cells are reduced in psoriasis patients (146, 147), or that no change was observed compared to healthy controls (148, 149). Within psoriatic plaques, Ottaviani et al. observed $CD56^+CD16^-$ NK cells that co-expressed CD161, NKG2A, and CD69 (150). Supernatants from culturing these NK cells activated keratinocytes, increasing MHC-I, ICAM-1 and HLA-DR expression, along with CXCL10 and CCL5 secretion (150). These chemokines induced migration of skin-derived NK cells, supporting NK cell-keratinocyte cross-talk in psoriatic inflammation (150). NK cells appear to be hypofunctional in psoriasis, with reduced degranulation and IFN-γ potential (146, 149). The role of helper type 1 ILCs is even less defined, however expansion of ILC1s was observed in psoriatic lesions (126).

ILCs in Atopic Dermatitis

Atopic dermatitis (AD) is a common inflammatory skin disorder characterized by high levels of IL-4, IL-5 and IL-13 (151, 152). AD skin lesions are enriched for skin-resident ILC2s, which are activated by TSLP or IL-33, promoting type 2 inflammation (132, 153, 154). This is supported by murine models where anti-CD90 and anti-CD25 depletion of skin ILC2s in T and B cell deficient *Rag1*$^{-/-}$ mice attenuated dermatitis symptoms (153). Interestingly, KLRG1 ligation by E-cadherin reduces IL-5 and IL-13 production by human ILC2s, implicating dysregulation of parenchymal-ILC interactions in AD where E-cadherin levels are canonically downregulated on keratinocytes and ILC2s have elevated KLRG1 expression (132).

ILC3s have also been implicated in the pathogenesis of AD. Circulating ILC2s and ILC3s are elevated in AD patients, and increased IL-17 levels are apparent during acute disease (154, 155). Using several AD models, Kim et al. demonstrated AD lesions had increased numbers of $IL17A^+$ ILC3s, which induced IL-33 release by keratinocytes and fibroblasts, promoting type 2 responses and exacerbating disease in mice (154). Further supporting a role for ILC3s in AD, ILC2s and ILC3s were elevated in AD lesions, with AHR^+ ILC3s representing the most abundant subset. ILC3s in AD lesions were frequently surrounded by T cells, suggesting cellular interactions between ILC3s and T cells in AD (126).

NK cells are also altered in AD and are prone to apoptosis *via* a $CD14^+$ monocyte-driven, contact-dependent mechanism, aligning with observed reductions in peripheral NK cell abundance in AD

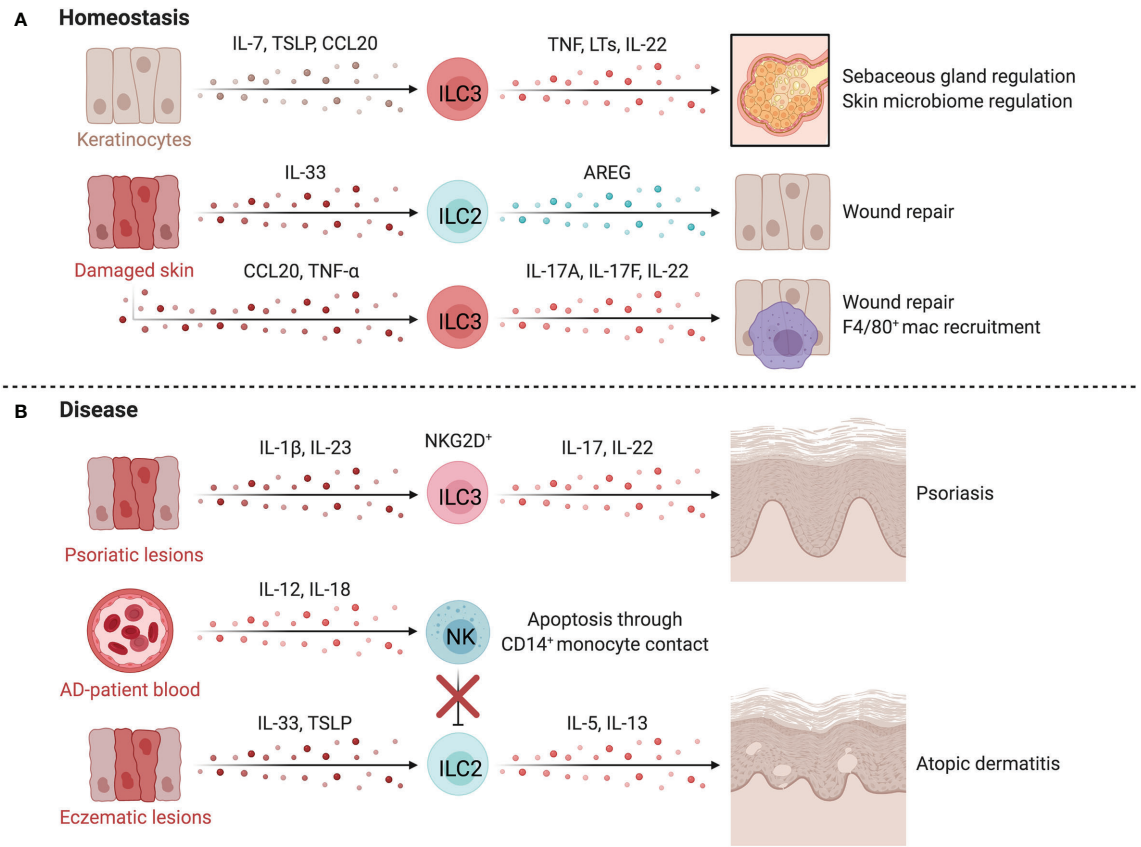

FIGURE 5 | ILCs in the skin. At homeostasis **(A)**, ILCs are retained by IL-7 and TSLP released by hair follicle keratinocytes or epithelial skin cells. TNF-α^{+}LTα1β2^{+}CCR6^{+} ILC3s negatively regulate lipid-secreting sebaceous glands, regulating the skin microbiome, which can alter susceptibility to inflammatory conditions or affect tissue repair pathways. Upon tissue damage, injured epithelial cells release IL-33, inducing proliferation of skin-resident ILC2s. In mice, anti-CD90 depletion in *Rag1*$^{-/-}$ mice delays wound healing, suggesting a role for ILC2s in promoting epithelial repair *via* AREG. The epithelium also produces TNF-α downstream of damage-induced Notch signaling in keratinocytes, recruiting CD4^{+}NKp46$^{low/-}$ ILC3s that participate in wound closure. TNF-α driven release of CCL20 and CXCL13 by keratinocytes recruit ILC3s which facilitates the recruitment of F4/80^{+} reparative macrophages. In disease **(B)**, psoriatic lesions in mice accumulate IL-17^{+} IL-22^{+} NCR^{+} ILC3s which is mirrored by the increase of CD56^{+}RORγt^{+} ILC3s in both inflamed and non-inflamed skin of psoriasis patients. ILC2s promote atopic dermatitis (AD) when activated by epithelial-derived TSLP and IL-33 in inflammatory lesions. Dermal NK cells are decreased in AD and prone to apoptosis through contact with CD14^{+} monocytes. NK cells are proposed to regulate ILC2 abundance in AD, as therapeutic expansion of NK cells lowers ILC2 counts and improves disease scores in an AD mouse model. Created with Biorender.org.

(146, 152, 156). Mack et al. found particularly reduced levels of circulating mature CD56^{dim}CD16^{+} NK cells with high expression of KIRs and CD57 in patients with moderate-to-severe AD (152). A regulatory circuit between NK cells and ILC2s is supported by three lines of evidence: NK cell recovery occurring after IL-4 blockade; ILC2 accumulation in AD lesions of NK cell-deficient mice; and NK cell recovery and activation after IL-15 superagonist treatment leading to reduced ILC2 levels and disease scores in an AD model (152). Thus, cross-talk between ILC subsets may underlie the development and severity of AD (**Figure 5B**).

ILCS IN THE INTESTINE

The intestine is the largest mucosal surface in the human body and faces unique challenges. As a barrier surface, immune function in the intestine must balance tolerance and control of commensal microbes with protection from pathogens. Among immune residents of the intestine, ILCs have key roles in sustaining gut barrier integrity, repair, immune homeostasis, and host defense (**Figure 6A**). ILC distribution along the human intestine was reported by Simoni et al. and Yudanin et al. (67, 127). In line with observations made in mice, both groups demonstrated the presence of NK cells, ILC1s, ILC2s, and ILC3s across the intestinal tract, with predominance of ILC1s and ILC3s (67, 127, 157). NK cells are low in abundance and mainly CD56^{bright} with distinct surface marker expression (64) (**Figure 2**). Intestinal ILC1s are heterogeneous, including a population of CD103^{+} ILC1s located in the epithelium, and CD127^{+} ILC1s residing in the lamina propria (LP) (158, 159). ILC3 subsets also localize within distinct microanatomic compartments of the gut epithelium/isolated lymphoid follicles (ILFs)/LP, but it remains to be shown if a similar distribution applies to humans (160, 161).

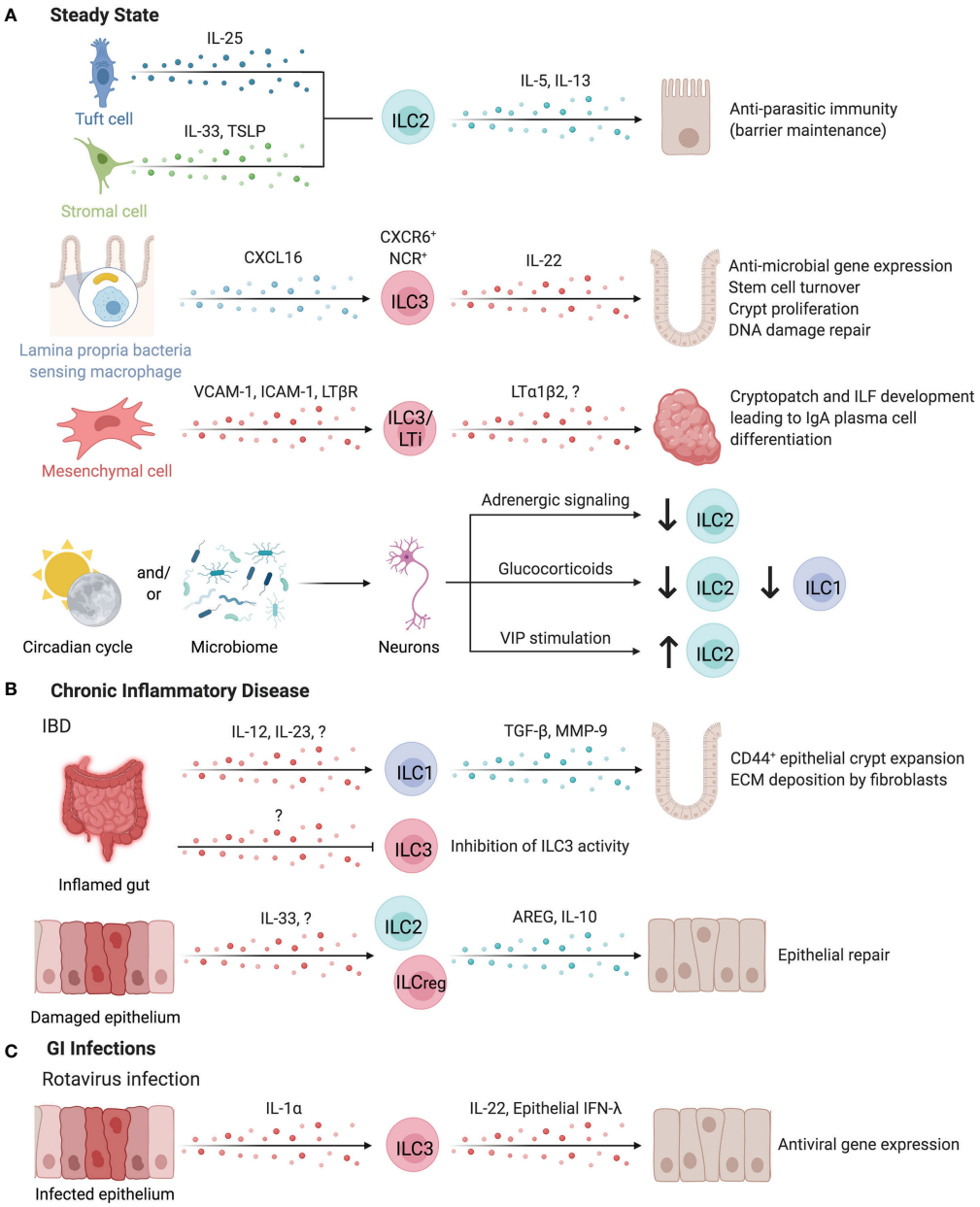

FIGURE 6 | ILCs in the intestines. ILCs have important roles in maintaining intestinal homeostasis **(A)**. ILC2s can be activated by both IL-25-producing tuft cells or IL-33 and TSLP-secreting stromal cells to promote anti-parasitic immunity in the intestines. ILC2s secrete IL-5 and IL-13 to promote host defense through parasite expulsion. Similarly, microbiota-sensing CX3CR1$^+$ macrophages position IL-22-secreting CXCR6$^+$ NCR$^+$ ILC3s in the lamina propria *via* CXCL16. IL-22 supports anti-microbial gene expression in Paneth cells and promotes stem cell turnover, crypt proliferation, and DNA damage repair. Interaction of ILC3/LTi surface LT$\alpha_1\beta_2$ and LTBR on mesenchymal cells leads to the upregulation of VCAM-1 and ICAM-1, resulting in the formation of cryptopatches and ILF. These tertiary lymphoid structures support the differentiation of IgA-producing plasma cells to promote barrier defense and host-microbiota mutualism. Clock genes and circadian cycles, modulated through feeding and the microbiota drive important homeostatic neuro-immune interactions in the gut. Disruption of circadian regulation alters ILC3 function, abundance, and trafficking into the intestines while negatively regulating ILC2s through adrenergic signaling. Glucocorticoids or vasoactive intestinal peptide further control ILC1 and ILC2 responses. **(B)** In chronic inflammatory disease such as intestinal bowel disease (IBD), the inflamed gut induced TGF-β and Matrix metalloproteinase 9 production by ILC1s leading to the expansion of epithelial crypt cells and extracellular matrix deposition by fibroblasts, exacerbating fibrosis. In contrast, pro-tolerogenic ILC3 functions including the release of GM-CSF, IL-2 or the expression of MHC-II are impaired in IBD patients, suggesting an anti-inflammatory role for ILC3s. IL-10 producing regulatory ILC2s or ILCregs may also suppress intestinal inflammation. **(C)** Enteric infection by Rotavirus induces epithelial-derived IL-1α to promote ILC3 production of IL-22, which synergizes with epithelial IFN-λ to promote the induction of antiviral responses in intestinal epithelial cells. Created with Biorender.org.

Intestinal ILCs promote host immunity against pathogenic and commensal microbes through interactions with sentinel immune and tissue cells. For example, murine ILC2s activated by IL-25-producing tuft cells or IL-33- or TSLP-secreting stromal cells promote anti-parasitic immunity, while DC-derived IL-33 promotes regulatory T cell (Treg) responses, suppressing anti-parasitic immunity (4, 24, 162–164). Myeloid cells, especially CXCL16-producing CX3CR1$^+$ macrophages are critical for sustaining lamina propria-resident CXCR6$^+$ NCR$^+$ ILC3s as a major source of IL-22 in the intestinal LP (160). These ILC3s support IL-22-dependent intestinal epithelial anti-microbial gene expression, stem cell turnover, crypt proliferation, and DNA damage repair (160, 165–168).

While ILC3-derived IL-22 protects the intestinal epithelium against genotoxic stress, risk-associated single nucleotide polymorphisms have been identified within *Il22* and the IL-23 signaling pathways as a driver of colorectal cancer in patients (165, 169, 170). Nevertheless, ILC3-derived IL-22 and LTα positively alter the glycosylation activity of epithelial cells, supporting glycan-scavenging intestinal commensal microbes and balanced host-microbe interactions and providing protection from infection (171, 172). sLT, expressed by human and mouse LTis, is essential to initiate the development of cryptopatches (CPs) and ILFs in the gut (11, 173). These tertiary lymphoid tissues support the differentiation of IgA-producing plasma cells to promote barrier defense (174, 175). Mouse CP and ILFs contain a unique subset of DCs that require LTβR signaling for their development. These DCs released IL-22 binding protein, which in turn alter intestinal epithelial IL-22R signaling and lipid transport (176).

Tregs have key functions in inducing tolerance to luminal antigens (177). IL-2 and GM-CSF-producing ILC3s directly and indirectly support the generation of Tregs in the healthy murine gastrointestinal tract, upon stimulation by microbiota-sensing IL-1β-producing macrophages. The cooperation and reciprocal crosstalk between macrophages, DCs, and ILC3s supports Treg homeostasis and T cell immunity against orally ingested antigens (177–179). MHC-II expression on murine ILC3s has been demonstrated to regulate T cell responses to microbial antigens *via* a mechanism analogous to negative selection in the thymus (180, 181). Lehmann et al. reported organ-specific expression levels of MHC-II on murine ILC3s and demonstrate that microbiota-induced IL-23 stimulation of ILC3s reversibly downregulated their MHC-II expression (182). Noteworthy, Rao et al. reported an accumulation of HLA-DR$^+$ ILC3s in T cell-rich areas of colorectal cancers suggesting antigen-presenting capacity of ILC3s in humans as well (183). Together, this suggests that ILC3s both positively and negatively regulate T cell immunity dependent on microenvironmental signals.

Several environmental factors regulate murine intestinal ILC abundance. The metabolite-sensing Ahr is highly expressed by ILCs in the gut, with an important role in sustaining ILC3s and promoting IL-22 production (165, 184, 185). In contrast to ILC3s, gut ILC2 function is suppressed by Ahr signaling, suggesting a role for Ahr ligands in regulating the balance of intestinal ILC subset abundance (186). A similar divergent stimulation between ILC2s and ILC3s has been reported for other dietary components (187, 188). Microbial short chain fatty acids (SCFAs) differentially affect mouse ILCs in a subset- and location-specific manner, generally promoting ILC3 proliferation and IL-22 production while inhibiting ILC2 expansion (189–191). Free Fatty Acid Receptor 2 (Ffar2) acts as a SCFA receptor, and agonism leads to ILC2 proliferation, yet SCFA feeding leads to contraction of ILC2 abundance, suggesting the involvement of several receptors in coordinating the response to microbial fermentation products (191). This along with reports of age and body-mass index-associated alterations in the abundance of ILC subsets suggests age and metabolism-dependent regulation of intestinal ILCs in humans (67).

Cholinergic neurons in the gut and lung of mice produce neuromedin U in response to helminth challenge, which stimulates ILC2 proliferation and production of IL-4 and IL-13 in an IL-33-independent manner (192). The neuromedin U receptor does not appear to be expressed by other hematopoietic cells besides ILC2s at significant levels (192). In humans, the *NMUR1* transcript was detected in intestinal ILC2s, yet direct evidence for this ILC2-neuronal interaction in humans is lacking (192). Other modalities where the nervous system regulates ILCs includes negative regulation of ILC2s by adrenergic signaling, glucocorticoid dampening of ILC1 and ILC2 responses, VIP stimulation of ILC2s, and ILC3 co-localization with neurons in enteric CPs, as detailed in a review by Klose and Artis (3). Interestingly, circadian light-dark cycles regulated neuron-immune interactions and intestinal ILC3-specific gene expression through diurnal oscillations of *Rorc*, *Il17a*, and *Il22*, while disruption of ILC3 circadian regulation altered their function, abundance, and trafficking in the murine intestine (2, 193–195). Interestingly, the gut microbiota contributed to control of this circuit, as antibiotic treatment partially restored ILC3 abundance and constrained cytokine production in circadian-disrupted mice (195). In mice, VIP promotes ILC3 intestinal recruitment and maintains expression of gut-homing receptor CCR9 (196). Talbot et al. reported a feeding-induced inhibition of ILC3s by VIPergic neurons, regulating mucosal immunity by dampening IL-22-induced antimicrobial peptide production in exchange for enhanced absorptive capacity of the intestinal epithelium marked by increased fatty acid transporter (*Fabp2*) expression (197). This contrasts with findings by Seillet et al. that VIP stimulation increased IL-22 production by enteric ILC3s, although the reason for these conflicting results is unclear, suggesting complex signals regulate intestinal ILC3 activity (198). Together, intestinal ILC3s are regulated by a complex circadian network involving light-dark cycles, microbial signals, and nutrient-driven neuronal regulation. Of note, the production of IL-5 by murine ILC2s was also circadian regulated (198).

Chronic Inflammatory Diseases

Chronic inflammation of the intestinal tract is a hallmark of inflammatory bowel disease (IBD) and fosters a local cytokine milieu that promotes differentiation of ILC1s (199). ILC1

expansion in inflamed intestinal tissue is location-specific, with greater expansion of LP-resident CD127$^+$ ILC1s versus intraepithelial ILC1s in Crohn's disease (CD) patients (158, 159, 200, 201). Specific expansion of CD127$^+$CD94$^+$ Granulysin$^+$ ILC1s is observed in the inflamed LP of CD patients (202). With elevated secretion of TGF-β and MMP9, mouse ILC1s facilitate the expansion of CD44$^+$ epithelial crypt cells and extracellular matrix deposition by fibroblasts, collectively supporting matrix remodeling and epithelial proliferation that may exacerbate inflammation-associated fibrosis (**Figure 6B**) (203). In contrast, ILC3 abundance and homeostatic functions in circadian oscillation, production of IL-2, and expression of MHC-II were critically impaired in IBD patients, supporting anti-inflammatory contribution of ILC3s (178, 180, 193, 195). ILC3 secretion of IL-22 is enhanced by G Protein-Coupled Receptor 34 (GPR34) recognition of lysophosphatidylserine from apoptotic neutrophils, further supporting a role of ILC3s in sensing intestinal injury and initiating repair responses (204). However, ILC3s may contribute to intestinal inflammation under permissive circumstances (205). Further, destabilizing RORγt expression promoted the differentiation of ILC3s into ILC1/ex-ILC3 in mice and humans and correlated with intestinal IBD-like inflammation (206, 207). Interestingly, this differentiation was not static, but was regulated by the myeloid cytokine milieu in the intestinal tract (159). Counterbalancing the elevated type 1 and type 3 immunity reported in IBD, ILC2-derived AREG was sufficient to reduce DSS-induced damage in mice by promoting epithelial integrity and mucus production (162). Bando et al. further identified murine ILC2s as a dominant source of IL-10 in the intestine, while Wang et al. identified a distinct subset of IL-10-producing regulatory ILCs in humans and mice, supporting that IL-10 producing ILC2s or ILCregs may suppress intestinal inflammation (**Figure 6B**) (13, 17). Targeting ILC3-to-ILC1 plasticity, ILC1 activation, and ILC3 abundance may be a promising approach to restore intestinal immune homeostasis under chronic inflammatory conditions (208–211).

Gastrointestinal Infection

ILCs play a critical role in the response to intestinal pathogens in humans, highlighted by cases of deficiency in *RORC* resulting in severe mucosal fungal and bacterial infections (212). Along this line, susceptibility to infections by enteric extracellular pathogens are increased in the absence of IL-22 or GM-CSF, highlighting a critical role for ILC3-associated cytokines in barrier defense (213–216). Mouse ILC3s and ILC1s/ex-ILC3s promote antimicrobial responses *via* surface lymphotoxin-mediated differentiation of goblet cells and IFN-γ-induced production of mucins, further emphasizing the synergistic actions of ILC1s and ILC3s that require underlying microbial recognition and activation by myeloid cells (207, 217–219). Whether this permits discrimination of commensal and pathogenic microbes requires further investigation (219). In response to mouse enteric rotavirus infections, epithelial IL-1α induced ILC3-derived IL-22 which synergized with epithelial IFN-λ, promoting the induction of antiviral gene expression in intestinal epithelial cells, limiting viral replication and tissue damage (**Figure 6C**) (220). While ILC3s can promote antiviral immunity, they experience cytokine-dependent depletion in the intestinal tract of HIV$^+$ human and SIV$^+$ non-human primates, altering epithelial permeability and homeostasis (221–223). Collectively, ILCs promote intestinal barrier defense against enteric bacterial, fungal, and viral infections by exerting cytokine or cell contact-dependent effects on intestinal epithelial cells.

Enteric parasites and worms constitute a major global health burden. Murine NK cell recruitment to the intestine early after helminth infection does not affect parasite burden but limits the tissue damage induced by infection (224). Experimental models of worm infections revealed the importance of ILC2s and ILC2-derived cytokines in intestinal host defense in mice (225–227). For example, IL-13 from murine ILC2s promoted tuft and goblet cell differentiation from crypt progenitors, contributing to epithelial remodeling and worm expulsion in the characteristic "weep and sweep" response (4, 228). ILC2s actively promoted Th2 cell responses *via* MHC-II and co-stimulatory molecules, partially acquired through trogocytosis, while T cell-derived IL-2 activated ILC2s for efficient helminth expulsion in mice (229). Further, ILC2s are activated by acetylcholine and upregulate ChAT to produce acetylcholine in response to helminth infection, supporting efficient helminth expulsion through a potential autocrine signaling mechanism (230). The activation of ILC2s following worm infection could be blunted through parasite-derived, bio-active components interfering with the IL-33-ST2 axis (231). Intriguingly, helminth infection changed the global distribution and activation of murine ILC2s through the induction of S1PR1-dependent egress of gut ILC2s and accumulation in the lungs, suggesting a coordinated response to protect distal body sites targeted by helminth infection (72, 232).

While the fetal and adult human intestine hosts a population of ILC2s capable of releasing type 2 cytokines following stimulation with IL-2, IL-25, and IL-33, their role during human parasitic infections has not been well detailed (104). Lack of sample availability has hampered investigation of intestinal ILC abundance and function of worm infected patients (233). To date only two studies analyzed ILCs in worm infected patients. Nausch et al. observed a reduced frequency of ILC2s in children infected with Schistosoma, while Boyd et al. observed an increase in circulating c-kit$^+$ ILCs and elevated IL-13 secretion in adult patients with filarial infections, suggesting heterogeneity in ILC responses dependent on age and/or helminth species (234, 235).

Collectively, intestinal ILCs support host immunity, barrier defense and tissue repair during infection and homeostasis, but also may perpetuate inflammation under permissive microenvironmental conditions.

ILCS IN THE LIVER

The liver is critical for metabolism and blood detoxification. Constant exposure to an array of antigens and microbial products within liver sinusoids promotes tolerance to predominantly harmless antigens (236, 237). The liver contains a large proportion of innate immune cells such as Kupffer cells

(specialized macrophages), inflammatory and non-inflammatory macrophages, NKT cells, NK cells and ILCs (238–240). These innate lymphocytes influence the activation and function of the various adaptive immune populations that include αβ T cells, γδ T cells and B cells, as well as parenchymal cells within the liver niche.

In humans, $CD56^{bright}CD16^{-}$ NK cells comprise 50% of all liver NK cells (241). These NK cells express CD69, CCR5 and CXCR6, but not SELL or CCR7, and are localized to sinusoids by CCL3, CCL5, and CXCL16 produced by Kupffer cells, T and NK cells, and endothelial cells, respectively (241, 242). NK cells in healthy liver of deceased donors highly express *EOMES*, *CD7*, *KLRD1*(CD94), *GZMK*, *NCR1*(NKp46) and *NCAM1*(CD56), and lowly express *FCGR3A*(CD16) and *ITGA1*(CD49a) (240). Although $CD49a^{+}$ NK cells akin to murine liver-resident NK cells have been identified in humans, they represent only a small subset of human liver-resident NK cells, while lack of CD49e protein expression differentiated human liver-resident NK cells from conventional (cNK) cells (243, 244). $CD49a^{+}CD16^{-}$ NK cells in liver have a transcriptional program consistent with cytotoxic activity and exhibited antigen-specific killing of autologous targets presenting viral or metal antigens (245). Notably, a donor-derived $EOMES^{hi}$ tissue-resident NK cell population persisted in the liver up to 13 years post-transplant in a study of HLA-mismatched liver transplants (246). This NK cell population had a phenotype consistent with those reported in transcriptomic studies of healthy human liver (240–243).

While group 1 ILCs are the most abundant hILC population in human liver, NCR^{+} and NCR^{-} ILC3s and ILC2s are also present (247). Liver ILC2s are $CRTH2^{+}CD161^{+}CD69^{+}$ and highly express fibronectin-binding VLA-5, laminin-binding VLA-6, and the chemokine receptor CCR6 (247). In contrast to mice, only 10% of intrahepatic human ILC2s express the IL-33 receptor ST2, and primarily produce IL-13 and AREG, with very little IL-5 (247).

ILCs in Viral Hepatitis

NK cells are implicated in both Hepatitis C (HCV) and Hepatitis B (HBV) infections, which are major causes of liver inflammation and cirrhosis, leading to development of hepatocellular carcinoma (248) (**Figure 7A**). Peripheral NK cell abundance is reduced in both HCV- and HBV-infected patients, with reduced IFN-γ and TNF-α potential particularly in HBV, suggesting functional dysregulation (249). Cytotoxic impairment is associated with chronic infection establishment, while acute HCV infection induces NK cell activation, including increased NKG2D expression and greater capacity for cytotoxicity and IFN-γ production (250). Despite shared dysregulation, NK cell phenotype differs between chronic HBV and HCV; an enrichment of $NKG2C^{+}$ NK cells are observed in HBV, whereas increased CD69 expression and decreased inhibitory KIR expression are observed in HCV (249). Differences in NK cell KIR and HLA allele expression may differentiate infections that are self-limited versus those that become chronic; KIR2DL3 and HLA-C1 expression is reported to be protective in HCV infection (251, 252). Weaker inhibitory signals by HLA-C1 may allow for increased NK cell activation and viral clearance (251, 252). In agreement, degranulation marker CD107a was increased on NK cells with KIR2DL2/3 and was highest in those with self-limiting infections (250). Engagement of HLA-E with elevated NKG2A and CD94 receptors on NK cells of HCV-infected individuals results in TGF-β and IL-10 production and impaired ability to activate DCs for virus-specific T cell responses, in line with findings that hepatocyte and Kupffer cell HLA-E expression correlates with HCV severity (253, 254). Of note, intrahepatic $CD56^{bright}CD16^{-}$ NK cell abundance correlates with better liver function and lower disease scores in HCV-positive patients undergoing liver transplantation (255).

In chronic HBV, circulating and intrahepatic NK cells highly express TRAIL and CD69, especially the $CD56^{bright}$ subset (256). Elevated IFN-α and IL-8 upregulate TRAIL expression on NK cells and TRAILR-2 expression on hepatocytes, respectively, suggesting TRAIL-dependent targeting of hepatocytes by $CD56^{bright}$ NK cells mediates damage during chronic HBV flares (256). Notably, HBV-specific T cells also have high expression of TRAIL-R2 and are susceptible to targeting by NK cells, supporting a role for NK cells in regulating anti-HBV T cell responses (257).

Comparatively little is known about the role of human hILCs in hepatitis infections. Increased hILCs were reported in the circulation of patients with chronic HBV (258, 259). HBV-related cirrhosis progression correlated with IL-17A and IL-22 production by ILC3s, suggesting ILC3 promotion of fibrosis, likely in part due to IL-22-mediated suppression of anti-fibrotic IFN-γ (**Figure 7A**) (259). While HCV/HBV do not infect mice, other viral hepatitis models provide some context into ILC viral responses in the liver more generally. Hepatic ILC3s produce IL-17A/F alongside γδT cells to promote antiviral T cell responses and inflammation early after infection (260). At later timepoints post-infection, ILC2s induce immunosuppressive neutrophils *via* IL-13 to limit T cell damage (261). This suggests that hepatic ILCs may have time-dependent roles to balance viral clearance and tissue protection.

ILCs in Liver Fibrosis

Liver disease is characterized by fibrogenesis of the liver, driven by type 2 immunity, with an implication for ILC2 activity (**Figure 7B**) (262). Hepatic stellate cells become activated and transdifferentiate into myofibroblasts that produce copious extracellular matrix proteins, driving fibrosis and loss of function resulting in cirrhosis (263). Patients with cirrhosis have elevated serum IL-33 and increased intrahepatic ILC2s, correlating with disease severity (247, 264, 265). Expansion of ILC2s and activation by IL-33 from damaged parenchymal cells results in IL-13 production driving fibrotic gene expression in hepatic stellate cells in fibrosis models, or IL-5 production with resultant hepatic inflammation and eosinophilia in immune-mediated hepatitis models (265, 266). While both effects are IL-13-dependent, additional signals which influence IL-5 versus IL-13 dominant responses by ILC2s are unknown. Interestingly, liver ILC2s present antigen to $CD4^{+}$ T cells which produce IL-2 to sustain ILC2 expansion (267). High levels of IL-6, linked to liver regeneration, were produced by IL-33-activated liver ILC2s,

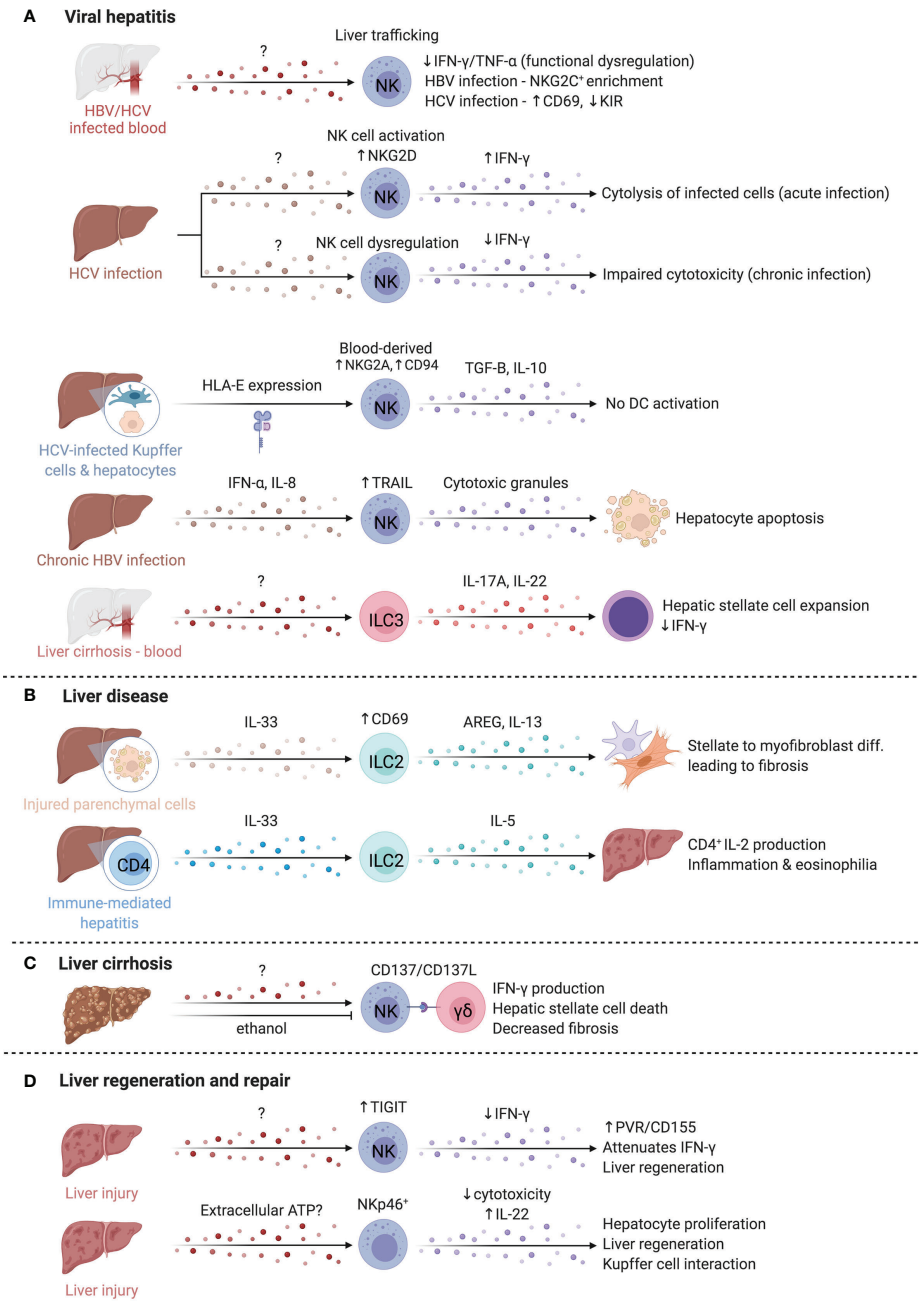

FIGURE 7 | ILCs in the liver. The liver is occupied by a variety of ILCs that play diverse roles in viral hepatitis, liver disease, liver cirrhosis, and liver regeneration and repair. **(A)** Hepatitis C (HCV) and Hepatitis B (HBV) are key inducers of liver inflammation and cirrhosis and lead to the development of hepatocellular carcinoma. The abundance of NK cells in the blood of both HBV/HCV infected patients is reduced, suggesting elevated homing to the liver where functional deficiencies like impaired IFN-γ and TNF-α production are reported. NK cells in chronic HBV and HCV infected patients adopt distinct phenotypes that manifest in an enrichment of NKG2C-expressing NK cells or altered CD69 and inhibitory KIR expression. In contrast, acute HCV infection promotes elevated NKG2D and IFN-γ expression while IFN-γ is reduced in chronic infections. HCV-infected hepatocytes and Kupffer cells express higher levels of HLA-E that boost TGF-B and IL-10 production by NK cells through NKG2A and CD94. $CD56^{bright}$ NK cells in chronic HBV infection facilitate TRAIL-dependent hepatocyte death. ILC3s separately support hepatic stellate expansion and counteract IFN-γ. **(B)** In liver disease, ILC2s are increased and activated, driving liver fibrosis *via* AREG and IL-13. Similarly, in immune-mediated hepatitis, ILC2s were expanded and produced high levels of IL-5, recruiting eosinophils, and driving inflammation. **(C)** In liver cirrhosis, crosstalk between NK cells and γδ T cells through the CD137-CD137L axis enhanced cytotoxicity of NK cells against HSCs. Alcohol exacerbates fibrosis chronically, but also attenuates NK-mediated cell killing, and reduced NKG2D, TRAIL and IFN-γ expression on NK cells. **(D)** NK cells upregulate TIGIT while hepatocytes upregulated the ligand PVR/CD155, attenuating IFN-γ production and promoting liver regeneration. Finally, extracellular ATP is elevated after liver injury and regulates regeneration in the liver *via* $NKp46^{+}$ NK cells. Created with Biorender.org.

indicating that ILC2s may have a dual roles in immune-mediated liver disease (267).

Intrahepatic human CD49a$^+$ NK cells are expanded in cirrhotic livers (268). CD49a$^+$CD25$^+$ NK cells positively correlate with serum alanine aminotransferase, linking CD49a$^+$CD25$^+$ NK cells to liver inflammation (268). Conversely, liver NK cells dampen fibrosis by killing activated hepatic stellate cells in an NKG2D- and TRAIL-dependent manner, while IFN-γ reduces hepatic stellate cell activation and matrix protein deposition (269, 270). CD137-CD137L crosstalk between NK cells and γδ T cells enhances NK cell cytotoxicity (**Figure 7C**) (271). Chronic alcohol consumption exacerbates fibrosis, and ethanol attenuates NK cell cytotoxicity towards hepatic stellate cells by reducing NKG2D, TRAIL, and IFN-γ expression, suggesting immunological and environmental mechanisms of NK cell regulation (272).

ILCs in Non-Alcoholic Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is the most common non-infectious chronic liver disease and can develop into non-alcoholic steatohepatitis (NASH) and progress to cirrhosis (273). NK cells are elevated in liver biopsies of NAFLD and NASH patients, with greater than two times increased NK cell abundance in NASH compared to NAFLD (274). *NKG2D* and *TRAIL-DR5* transcript levels also have higher expression in NASH (274). Upregulation of *MIC-A/B* positively correlates with disease score and degree of fibrosis, suggesting NK cell engagement with MIC-A/B stress ligands could be a key factor in NASH development (274). In agreement, circulating NK cells from NASH patients had higher NKG2D expression (275). Depletion of IFN-γ-producing NKp46$^+$DX5$^+$ NK cells in a NASH mouse model altered macrophage phenotype, suggesting that IFN-γ from NK cells reduces fibrosis by polarizing macrophages away from a TGF-β$^+$ pro-fibrotic phenotype (276). Additional studies are required to delineate the mechanisms that control whether NK cells limit or promote fibrosis.

While fewer studies have focused on hILCs, ILC3s appear to mitigate NAFLD. High fat diet increases ILC3 abundance in mice, while deficiency of ILC3s leads to liver fibrosis and an increase of pro-inflammatory gene expression with concomitant accumulation of saturated fatty acids (277).

ILCs in Liver Regeneration and Repair

The liver is uniquely capable of self-regeneration, including regenerating entire lobes after resection. Group 1 ILCs interact with injured tissue and influence regenerative capacity (**Figure 7D**). In models where NK cells are pre-activated by viral infection or TLR3 agonism to produce higher levels of IFN-γ, as well as in aged livers that have elevated IFN-γ signaling, regeneration is impaired (278, 279). NK cells upregulate T cell immunoreceptor with Ig and ITIM domains (TIGIT) while hepatocytes upregulate the ligand PVR/CD155, attenuating IFN-γ production and promoting liver regeneration (280). Mouse NKp46$^+$ cells co-localized with F4/80$^+$ cells in liver sinusoids, and NKG2D blockade abrogated regeneration, suggesting NKG2D-mediated crosstalk with Kupffer cells regulates regeneration (281). Extracellular ATP is elevated after injury and regulates liver regeneration (281, 282). ATP limits NK cell cytotoxicity while antagonism of ATP-receptor P2X1 reduces IL-22 production by group 1 ILCs in a murine liver resection model, resulting in dampened hepatocyte proliferation and elevated hepatocellular injury and stress (281, 283). Taken together, extracellular ATP released after resection may dampen NK cell cytotoxicity and promote IL-22 production to modulate time-dependent group 1 ILC functions supporting liver regeneration. Future studies that characterize marker expression in greater detail may clarify whether the cells identified were also inclusive of CD56$^+$ ILC3s or were NK cells or ILC1s that converted to ILC3s.

ILCS IN THE KIDNEY

Kidneys perform essential functions of filtering blood, excreting waste, and regulating the body's fluid and electrolyte balance. ILCs have been found to contribute to acute and chronic kidney diseases, with protective (**Figure 8A**) and pathological (**Figure 8B**) functions in IRI, kidney disease, and lupus nephritis, however, their role in the steady state remains poorly described.

ILCs in Chronic Kidney Disease

End-stage renal disease (ESRD) is associated with high morbidity and mortality, often associated with infections (284). Circulating ILC2 abundance, proliferation, and IL-5/IL-13 production is higher in patients with ESRD versus healthy controls, pointing to ILC responsiveness to the altered environment (285). The IL-2 rich ESRD plasma promotes STAT5 phosphorylation of ILC2s leading to expansion and activation (285). An inverse correlation between circulating ILC2 abundance and infectious complications, as well as elevated IL-33 suggest ILC2 activation as a protective mechanism in ESRD (286). These findings are supported by increased protection from chronic kidney disease by IL-33-induced ILC2 expansion and elevated eosinophil recruitment (287). In contrast, CD56^{bright} NK cells are positively correlated with loss of kidney function in chronic kidney disease and were more abundant in fibrotic biopsies, co-localizing with proximal tubular epithelial cells at sites of tubulointerstitial injury (288). In fibrotic samples, NKp46$^+$CD117$^+$CD56^{bright} NK cells were the dominant source of IFN-γ and upregulated CD69, implying a role in renal injury and fibrosis (288).

ILCs in Ischemia-Reperfusion Injury

IRI occurs when temporary disruptions in blood flow cause hypoxic stress and injury to the kidney. Several lines of evidence suggest that ILCs influence IRI severity. Anti-asialo-GM1 and anti-NK1.1 depletion, or NKG2D blockade ameliorated IRI and prevented killing of Rae-1-expressing tubular epithelial cells by NK cells in mice (289, 290). Interactions between co-stimulatory receptor 4-1BB on NK cells and its activating ligand 4-1BBL on epithelial cells activate NK cells and recruit neutrophils *via* epithelial cell-derived CXCL1 and CXCL2 (291). Together,

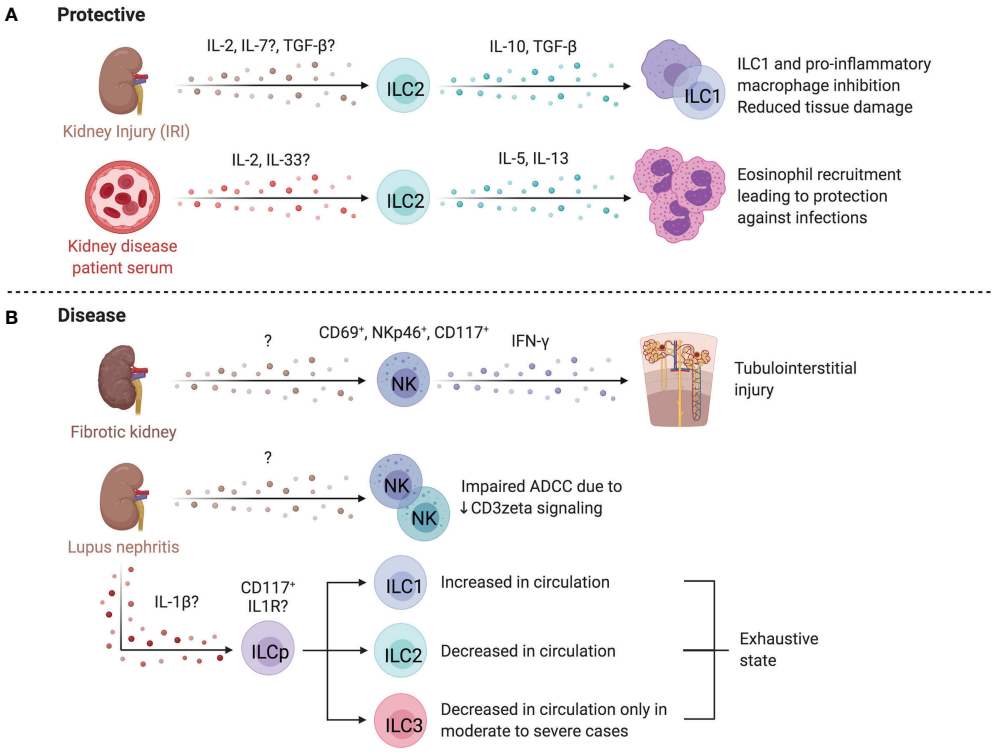

FIGURE 8 | ILCs in the kidney. **(A)** Mouse and human studies support ILC2s may limit kidney injury. Administration of IL-25 and IL-33 in a humanized mice model attenuates IRI, and in conventional mouse models promotes a Th2 response and M2 macrophage polarization resulting in decreased tissue damage post ischemic injury. ILC2s may also be protective in end-stage renal disease (ESRD), where circulating ILC2 abundance, proliferation, and cytokine release increases. IL-2 is proposed to facilitate this ILC2 expansion *via* STAT5 and protect against infections through eosinophil support. **(B)** $CD56^{bright}$ NK cells are more abundant in fibrotic kidney tissue where they upregulate CD69 and co-express NKp46 and CD117, producing the majority of IFN-γ, implying a role in driving inflammation and fibrosis. In lupus nephritis (LN), scRNAseq revealed two distinct NK cell subsets – a $CD56^{dim}CD16^{+}$ blood-derived and tissue-resident $CD56^{bright}CD16^{-}$ population. Both NK cells showed impaired antibody-dependent cell cytotoxicity because of dampened signaling efficiency by NKp30 and NKp46. LN patients further displayed elevated ILC1s and decreased ILC2s, while patients with moderate to severe disease showed an additional decrease in ILC3s. Created with Biorender.org.

these results support a role for NK cell-epithelial cell interactions in aggravating IRI.

IL-25 and IL-33 administration expands ILC2s in mice, attenuating IRI and promoting recovery post ischemic injury (292, 293). ILC2 depletion using anti-CD90 treated $Rag1^{-/-}$ mice abolished the protective effect of IL-33 administration (293). Further, adoptive transfer of either murine (into C57BL/6) or human ILC2s (into NSG (NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ) mice) conferred protection from IRI, while AREG-deficient ILC2s failed to abrogate IRI (293). In contrast, Liang et al. reported IL-33 treatment worsened disease scores and fibrosis in mice post-IRI (294). The timing and duration of IL-33 administration and experimental endpoints may account for differences in results, specifically that prolonged IL-33 administration may be detrimental, underscoring the importance of balance between tissue repair and fibrosis processes required to achieve tissue homeostasis.

Despite evidence that expansion or adoptive transfer of ILC2s is beneficial, depletion of ILC2s did not negatively impact IRI, as mice with reduced ($Rora^{fl/+}Il7r^{cre/+}$), depleted ($Icos^{dtr/+}$ $Cd4^{cre/+}$), or deficient for ILC2s ($Rora^{fl/fl}Il7r^{cre/+}$), had no effect on IRI, suggesting ILC2 functions can be compensated for by other cell types (295). A regulatory ILC population (Lin^{-} $CD127^{+}$ $CD25^{+}$ $IL\text{-}10^{+}$) was identified in mice that limited ILC1s and pro-inflammatory macrophages in an IL-10- and TGF-β-dependent manner (15). Reduced tissue damage was noted in experimental IRI when these cells were expanded in $Rag^{-/-}$ mice (15). Notably, endogenous ILCregs in IRI produced less IL-10 than expanded ILCregs, suggesting that endogenous ILCreg function is impaired in IRI. While the human counterpart of this regulatory ILC subset was identified, confirmation of their function is needed (15).

ILCs in Lupus Nephritis

ILCs have been linked to kidney autoimmune pathologies such as lupus nephritis (LN), a manifestation of systemic lupus erythematosus (SLE). Arazi et al. identified two distinct NK cell populations within LN kidney tissue by scRNAseq, annotated as $CD56^{dim}CD16^{+}$ NK cells and tissue-resident $CD56^{bright}CD16^{-}$ NK cells (296). $CD56^{+}$ NK cells from SLE patients show an impaired antibody-dependent cellular cytotoxicity due to reduced $CD3\zeta$ signaling upon NCR engagement (297). Altered abundance of

circulating ILCs in LN is associated with disease severity, with increased ILC1s across disease scores and decreased ILC3s in moderate to severe disease scores (298, 299). Further, reduced cytokine production and increased PD-1 expression suggest an exhausted ILC state in active disease (299). Specifically, $CD117^+$ ILCs, likely ILC progenitors, were markedly decreased in LN and preferentially differentiated into ILC1s when cultured in LN plasma. Blockade of IL-1R reversed this effect, suggesting IL-1β-mediated regulation of the ILC progenitor pool (299). In a murine model of LN, renal ILC3s were the dominant source of IL-22 and were increased in abundance, while IL-22 deficiency ameliorated disease, supporting a pathogenic role for ILC3s in LN, yet whether an analogous mechanism applies to humans is unknown (300). These studies collectively support dysregulation of ILCs in SLE and LN.

ILCS IN THE FEMALE REPRODUCTIVE SYSTEM

ILCs have established roles within the reproductive system and influence pregnancy outcomes (**Figure 9A**). While group 1 and group 3 ILCs are abundant in uterine tissue and participate in the dynamic regulation of reproductive health, little is known about the role of low abundance ILC2s (301).

ILCs in Pregnancy

Pregnancy is a unique case where non-self is protected from immune-mediated rejection. Dynamic changes occur in both maternal and fetal tissues as pregnancy progresses. The uterine mucosa undergoes cyclic remodeling, termed decidualization, in which the endometrium thickens in preparation for implantation (302). For implantation and placentation to occur, the decidua must be invaded by fetal tissue (trophoblast cells) (303). Together, maternal and fetal tissue interactions promote successful implantation, placentation, and arterial spiralization to facilitate blood and nutrient supply to the developing fetus (303).

Uterine NK (uNK) cells are important cellular regulators of fetal implantation and protect against maternal rejection of the fetus. These uNK cells are the most abundant leukocytes present in the uterine mucosa and exhibit differential functions and limited cytotoxicity compared to cNK cells (304). Despite the discovery of uNK cells in the early 1990's, the function of uNK cells in healthy and abnormal pregnancy is still the subject of intense research. Uterine NK cells contribute to placental remodeling, striking a balance between excessive trophoblast infiltration and defective placentation, regulated by KIR and MHC-I interactions (305). In general, activating receptor ligation improves reproductive success by promoting trophoblast invasion and vascular transformation (306). Human chorionic gonadotropin (hCG) released by the implanting fetal trophoblast induces uNK cell proliferation through hCG N-linked carbohydrate recognition by CD206 (mannose receptor) on uNK cells, establishing a pathway of uNK cell regulation by the implanting embryo (307).

ScRNAseq defined three distinct subsets of decidual NK cells (dNK cells) in humans. All subsets expressed *CD49A* and *CD9*, dNK1 cells expressed *CD39*, *CYP26A1* and *B4GALNT1*, dNK2 cells expressed *ANXA1* and *ITGB2*, and dNK3 cells expressed *ITGB2*, *CD160*, *KLRB1* and *CD103* (308). In particular, the highly granular and metabolically active dNK1 cells are hypothesized to interact with extravillous trophoblast cells because of high-level expression of KIRs and other HLA-molecule receptors (308). Unlike dNK2 and dNK3 cells, dNK1 cells were mostly IFN-γ^- in response to stimulation (309). Computational predictions suggest the mechanism behind the prevention of an inflammatory immune response relies on immune-tissue crosstalk (308). Decidual stromal cells highly express *LGALS9* and *CLEC2D*, pointing to potential NK cell inhibition *via* interaction with TIM-3 and KLRB1 (308). In line with the scRNAseq findings, Huhn et al. confirmed three subsets of dNK cells by mass cytometry with differential expression of the transcription factors TBET and EOMES (309). NK cells in the uterus or decidua acquire KIRs and CD39 along a developmental trajectory, corresponding to increased immunomodulatory and angiogenic function (310). With KIR acquisition, dNK cell expression of LILRB1, Ki-67, NKp30, and Granzyme B increased while NKG2D, CD161, and TBET decreased, unlike the relatively stable expression of these markers on cNK cells (309). Once acquired, the expression of KIRs remain remarkably stable for successive menstruation cycles (311). In another departure from cNK cells, granules were ~3 times larger in dNK cells and as KIR expression increased, degranulation and cytokine production decreased, supporting that dNK cells are phenotypically and functionally unique (309).

High NKG2C marks uNK cells that have acquired memory, or "trained immunity", contributing to improved reproductive success in subsequent pregnancies by improving vascularization and placentation (312, 313). Greater abundance of uNK cells and aberrant function resulting in higher expression of angiogenic factors in the endometrium coincides with thickening of the spiral artery walls, suggesting alteration of uNK-mediated vascular remodelling as cause for recurring miscarriage (314, 315). However, lower abundance of uNK cells is also associated with recurrent pregnancy losses through reduced decidual-uNK cell interactions. Chronic senescence of decidual cells leads to tissue dysfunction not conducive to successful pregnancies and uNK cells selectively eliminate senescent decidual cells *via* NKG2D, while differentiating decidual cells support and recruit uNK cells with *CXCL14*, *IL-15* and *TIMP3* (316, 317). Indeed, endometrial biopsies from patients with recurrent pregnancy loss exhibit excessive decidual senescence and reduced uNK cell abundance, indicating that a balanced co-operation between decidual cells and uNK cells promotes healthy pregnancies (316).

dNK cells also protect against pregnancy loss from trophoblast bacterial infection. Here, rather than forming a cytotoxic synapse, dNK cells transfer granulysin to infected trophoblasts using nanotube connections, killing the intracellular bacteria without killing the trophoblast (318).

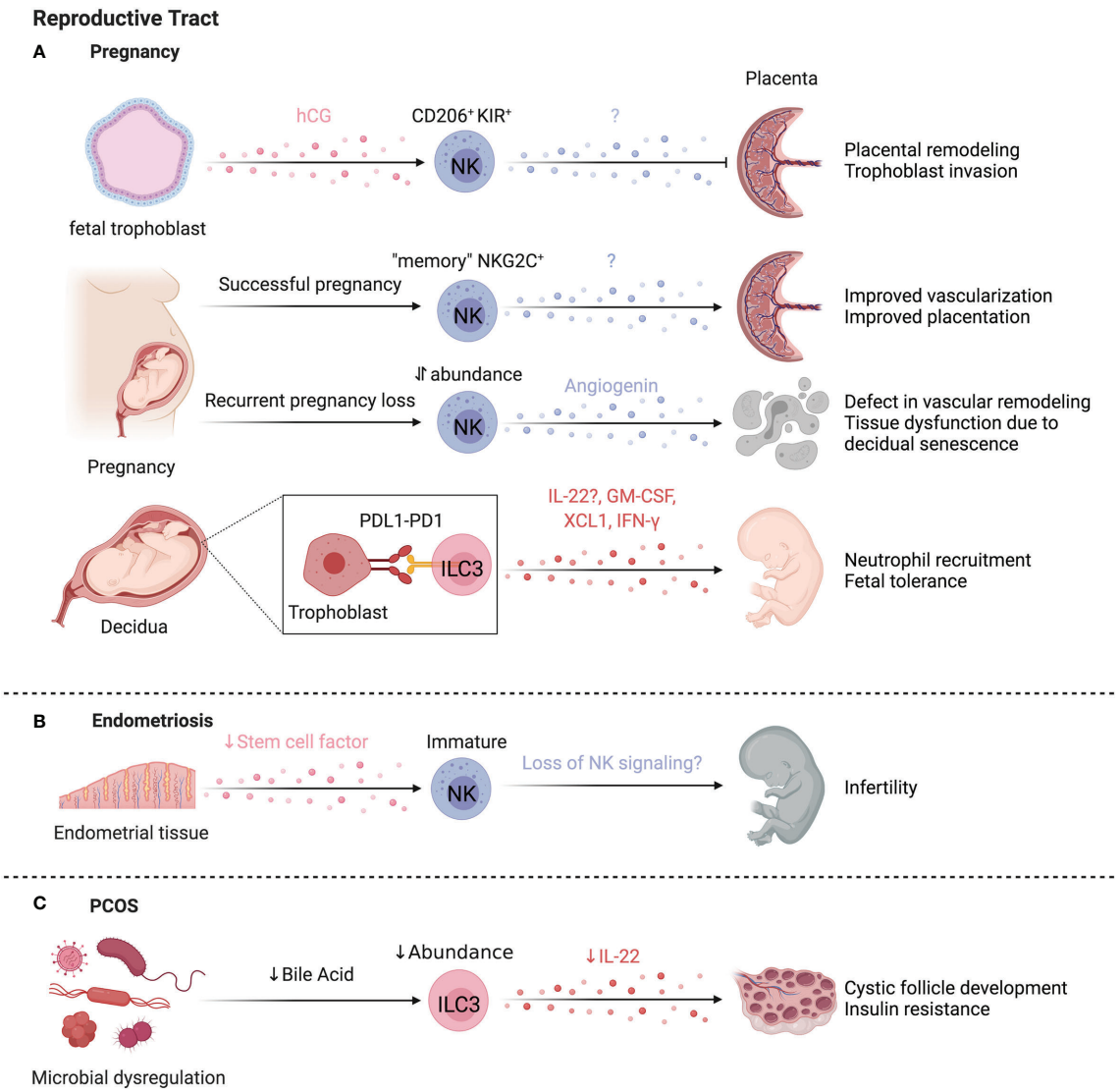

FIGURE 9 | ILCs in the reproductive system. Most ILC data focuses on the female reproductive tract, where NK cells have established roles in pregnancy **(A)**. uNK cells are regulated by human chorionic gonadotropin (hCG) released by fetal trophoblasts, signaling through CD206 (mannose receptor) to facilitate placental remodeling. Women with recurrent pregnancy losses show an increase in uNK cells in the endometrium, coinciding with thickened spiral artery walls and the resulting vascular remodeling affecting the blood flow to the fetus. In support of this, uNK cells from women with recurrent miscarriages also produced more angiogenic factors, fibroblast growth factors, and vascular endothelial growth factors. A special subset of memory uNK cells expressing higher levels of NKG2C contribute to improved reproductive success and lower incidence of pregnancy complications in subsequent pregnancies. In the human decidua, ILC3s express PD-1 and TIM-3, regulating ILC3 cytokine production, in particular IL-33. ILC3-trophoblast interactions may promote fetal tolerance during the first trimester *via* PD-1: PDL-1 interactions, supported by lower PD-L1 levels in trophoblasts of spontaneous abortions compared to healthy terminated pregnancies. **(B)** Reduced stem cell factor leads to a higher proportion of immature uNK cells in endometriosis, potentially contributing to infertility associated with endometriosis. **(C)** In a model of PCOS, microbial dysregulation led to reduced bile acids needed to support ILC3 function. The reduced ILC3 abundance and IL-22 production led to cystic follicle development and insulin resistance. Created with Biorender.org.

This pathway could account for NK-mediated host defense in a setting that aims to avoid excessive tissue damage.

Other ILCs also have defined roles in pregnancy and reproductive conditions. Vacca et al. reported ILC3s in human decidua express PD-1 and TIM-3, which regulate ILC3 cytokine production, most notably IL-22 (319). Since trophoblast cells are PD-L1^{high}, ILC3-trophoblast interactions may promote fetal tolerance during the first trimester (319). In support of this, PD-L1 levels were much lower or nonexistent in the trophoblast cells of spontaneous abortions compared to healthy terminated pregnancies (319). Subsets of CD127^{hi}CD117^{hi}AhR^{hi}CD94^{-} CD56$^{+/-}$NKp44$^{+/-}$ decidual ILC3s were capable of producing GM-CSF, XCL1 and low levels of IFN-γ upon stimulation (309). NCR^{+}ILC3s correlate with neutrophil abundance in the

human decidua and produce GM-CSF and CXCL8, supporting neutrophil recruitment and survival (320). Based on lower decidual neutrophil numbers in patients with miscarriages, Croxatto et al. hypothesize that the ILC3-neutrophil axis is beneficial, particularly in early stages of pregnancy (320).

ILCs in Endometriosis

Pathological functions of uNK cells are linked to endometriosis, a condition affecting ~10% of women where endometrial tissue grows outside of the uterus, resulting in debilitating pain and infertility. Patients with endometriosis have increased immature uNK cell counts and lower levels of stem cell factor (SCF) in the endometrial tissue, associated with infertility (**Figure 9B**) (321). Supplementing cultures of immature uNK cells with SCF supports uNK cell maturation (321). Endometrial stromal cells express high levels of SCF, suggesting stromal-uNK cell interactions influence fertility outcomes in endometriosis. SCF receptor expression is also found on helper ILCs, however, their role in endometriosis requires further investigation (322–324). While many questions remain, these reports support a critical role for uNK cell homeostasis in fertility.

Notably, IL-33 elevation is implicated in endometriosis and exogenous IL-33 exacerbates lesion severity and fibrosis dependent on ILC2s in a murine endometriosis model (325). Yet, in endometrial tissue of patients with endometriosis, ILC2s and ILC3s are reduced in abundance relative to non-endometriosis controls (326). Further work will be needed to clarify the role of hILCs in endometriosis.

ILCs in Polycystic Ovary Syndrome

Separate from protective roles in pregnancy, reduced ILC3 activity is associated with polycystic ovary syndrome (PCOS). PCOS encompasses mixed metabolic and reproductive pathologies, such as irregular ovulation, infertility, hyperandrogenism, insulin resistance, and adipose tissue inflammation associated with a complex etiology including hormonal dysregulation and heritability (327). Reductions in bile acids due to microbial dysregulation decreased IL-22 production by ILC3s and promoted insulin resistance and cystic follicle development in a murine PCOS model (328). This was reversed by supplementing missing bile acids or IL-22, supporting a link between the gut and fertility (328). However, hormonal imbalances impact ILC function in PCOS. For example, progesterone driven IL-15, IL-18 and CXCL10 expression promote uNK cell recruitment and proliferation, and are altered in PCOS endometrial tissue (**Figure 9C**) (329). These findings indicate that tissue-ILC and microbiota-ILC interactions critically regulate reproductive homeostasis on multiple levels.

ILCS IN THE HEART

In mice, NK cells account for ~3% of cardiac immune cells, ILC2s for ~1.7%, and ILC1s for 0.2%, while ILC3 abundance is negligible (330). Compared to lung ILC2s, murine cardiac ILC2s had lower expression of ICOS, CD25, and Ki-67, and higher expression of Sca-1 and GATA3 (330). Only 2% of cardiac ILC2s in mouse were donor-derived after 2 months of parabiosis, indicating that ILC2s are a stable tissue-resident population in the heart (330). Cardiac-resident ILC2s respond to IL-33 but not IL-25, and a committed cardiac ILC2 precursor (ILC2p) in mice and humans exists in a quiescent state with the capability to differentiate into ILC2s in response to myocardial infarct or myocarditis (331). The existence of undifferentiated ILC2p within tissues has been observed before and suggests a role for this pool of precursors as a reservoir for ILCs to protect from tissue damage (332). Cardiac ILC activity has been implicated in several disease models (**Figure 10**).

ILCs in Atherosclerosis and Coronary Artery Disease

Conflicting findings on ILC subset functions in atherosclerosis and coronary artery disease (CAD) have been reported. Selathurai et al. found murine NK cells were detrimental to atherosclerotic disease, increasing lesion size in a Perforin- and Granzyme B-dependent manner, while Nour-Eldine et al. found no effect of NK cells on lesion development using a distinct genetic depletion model, likely accounting for divergent findings (333, 334).

In humans, a study of acute ST-segment elevation myocardial infarction (STEMI) found elevated circulating ILC1s within 12 hours of symptom onset which produced more IL-12, IL-18, IFN-γ, and TNF-α, and were associated with a higher risk of major adverse cardiovascular events (335). In contrast, NK cells were reduced and have lower cytotoxicity in CAD patients, both in the case of stable angina and incidence of myocardial infarction or unstable disease (336–338). During follow-up, patients who failed to reconstitute their peripheral NK cells post myocardial infarction had higher levels of serum IL-6 and exhibited characteristics of metabolic syndrome, suggesting poor NK cell recovery corresponds with low-grade inflammation (336). Recovery of NK cells in CAD patients is potentially self-regulated, as apoptotic NK cells both respond to and produce FasL, which is elevated in serum and correlates with NK cell levels and apoptotic susceptibility (339). Increased proportions of $CD56^{bright}$ NK cells were identified in carotid plaques compared to autologous peripheral blood, and greater NK cell infiltration corresponded with symptomatic versus asymptomatic CAD patients (340). Soluble B7-H6 levels of 250 pg/ml were detected in symptomatic patients but not in asymptomatic patients or healthy controls (340). Notably, B7-H6 can interact with NKp30, yet further studies are needed to directly assess B7-H6 and NK cell interactions in this context. Circulating NK cells from atherosclerotic patients had higher TIM-3 expression than healthy controls, with the greatest levels in those with unstable plaques (341). TIM-3 blockade reduced the death of NK cells cultured in TNF-α, suggesting that TIM-3 promotes cytokine-induced NK cell apoptosis in atherosclerosis (341). Whether NK cells are preferentially recruited to unstable carotid plaques, or functionally contribute to plaque destabilization requires additional study (340).

ILC2s appear to have cardioprotective functions based on mouse models. ILC2s protect from cardiac fibrosis and are enhanced by exogenous IL-33, producing AREG and BMP-7 to support cardioprotective responses to injury (342). Expansion of

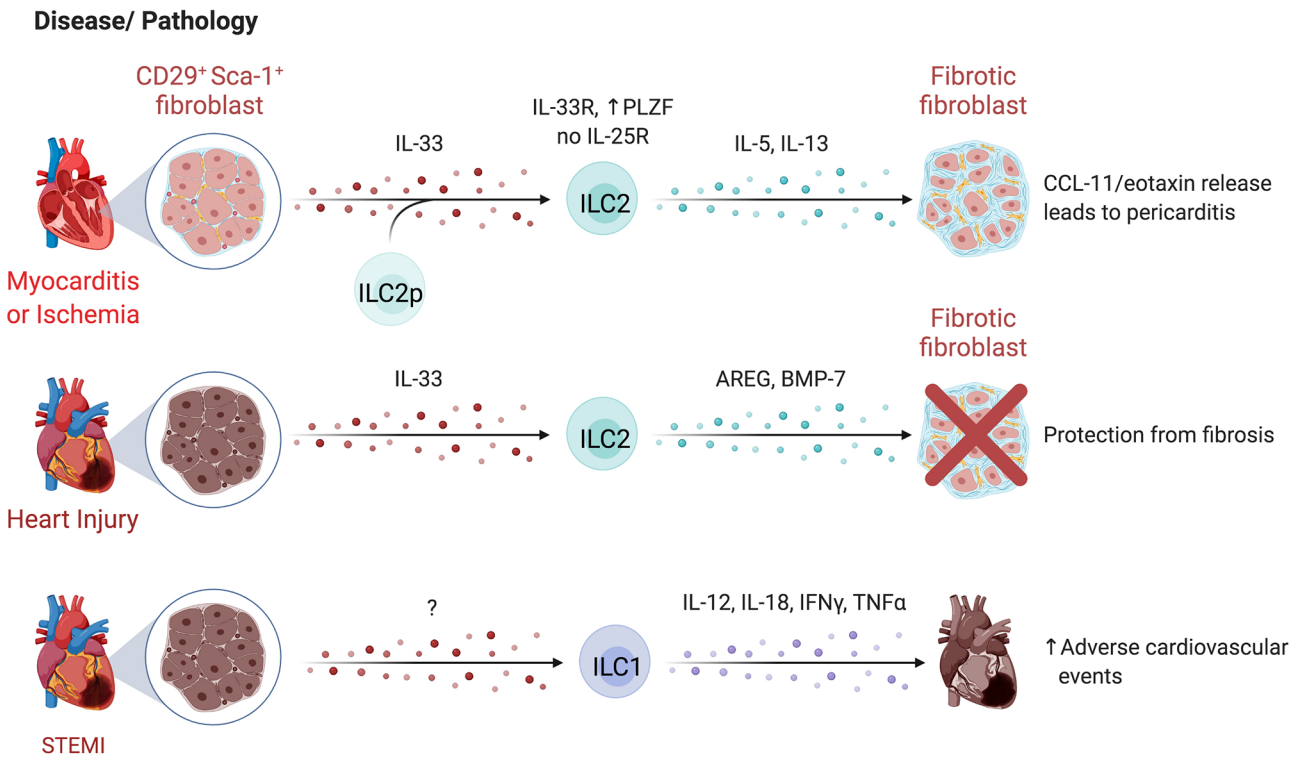

FIGURE 10 | ILCs in the heart. Limited studies have examined ILCs in the heart. In mice, IL-33 administration or CD27⁺Sca-1⁺ fibroblast-derived IL-33 expands ILC2s in the pericardium, driving cardiac fibroblasts to secrete CCL-11/eotaxin, leading to the accumulation of eosinophils. In humans, CD127⁺ ILCs increase in the pericardial fluid during in cardiac disease, particularly during pericarditis. Separately, the expansion of ILC2s by IL-33 was cardioprotective after injury, reducing fibrosis. Within 12 hours of acute ST-segment elevation myocardial infraction (STEMI), ILC1 elevation peaks. Higher ILC1-associated IL-12, IL-18, IFN-γ, and TNF-α levels lead to a higher risk of major adverse cardiovascular events. Created with Biorender.org.

ILC2s reduces atherosclerosis severity and lesion size, while genetic ablation (*Staggerer/Rora*^{Flox}*-CD127*^{Cre}) exacerbates disease (343, 344). Notably, protection is IL-5 and IL-13-dependent, recruiting eosinophils and polarizing macrophages towards an anti-inflammatory phenotype (342, 343). Further, pericardial and cardiac ILC2s expand early post-experimental myocardial infarction, peaking at day 3 before returning to homeostatic levels, while the absence of ILC2s impairs cardiac remodeling and results in larger areas of scarring (345). Together, this supports a role for cardiac-resident ILC2s in directing repair pathways in response to injury.

ILCs in Cardiac Inflammation

ILC expansion has been observed in patients with pericarditis (346). In contrast to cardioprotective findings above, ILC2s have been implicated in pericarditis pathology. Exogenous IL-33 expanded murine pericardial ILC2s, driving cardiac fibroblasts to secrete CCL11/eotaxin-1 and recruit eosinophils, initiating pericarditis (346). Pericardial fluid from humans revealed an elevated frequency of CD127⁺ ILCs in patients with cardiac disease versus controls, indicating that ILCs are also involved in human pericardial pathology (346). In an opposing role, NK cell depletion led to greater inflammation and fibrosis of the heart, dependent on NK cell-mediated prevention of eosinophilic infiltration (347). A possible cross-regulation of NK cells and ILC2s during cardiac inflammation should be investigated.

Sex differences in mortality and morbidity of Coxsackievirus B3 (CVB3) viral myocarditis may also reflect sex-based regulation of NK cell function. Male mice experience greater morbidity and mortality from myocarditis following CVB3 infection, with increased IFN-γ⁺ NK cell infiltration in cardiac tissue (348). Ovariectomized or sexually immature female mice show similar susceptibility to infection-triggered myocarditis when compared to male mice, while estrogen-treated male mice had ameliorated myocarditis (348). CVB3-stimulated NK cells cultured with estrogen down-regulated T-bet expression and consequently had reduced IFN-γ production, indicating that regulation of T-bet expression by estrogen might underlie the decreased IFN-γ⁺ NK cell infiltration in female mice and contribute to sex differences in myocarditis, in line with prior reports of hormonal regulation of other ILC subsets (100, 348).

ADIPOSE ILCS

Adipocytes are critical regulators of energy and glucose homeostasis. They are a heterogeneous population of cells, comprising energy-storing white adipocytes, thermogenic

brown adipocytes that express uncoupling protein-1 (UCP-1) to dissipate energy as heat, as well as beige adipocytes that reside within white adipose tissue (WAT) and upregulate UCP-1 in response to environmental cues (349). Beige adipocyte accumulation protects from insulin resistance, and regulation of the beiging process has become an attractive therapeutic target for metabolic dysregulation and type 2 diabetes (350).

ILC2s are the dominant hILC subtype identified in adipose tissue, with phenotypic variation across different murine adipose compartments: ILC2s in para-aortic adipose tissue have an inflammatory phenotype defined by IL-25 responsiveness and high KLRG1 expression, whereas peri-gonadal adipose ILC2s are IL-33 responsive, expressing ST2 (343, 351). Intriguingly, mouse ILC2s highly express bone morphogenetic protein (*Bmp)2* and *Bmp7*, which promote adipocyte differentiation, while ILC-deficient mice have more $CD34^+PDGFR\alpha^+$ precursor adipocytes, supporting a role for ILCs in adipogenesis (352). Under homeostatic conditions, ILC2s orchestrate immune responses in adipose tissues by recruiting eosinophils, promoting alternative activation of macrophages, and regulating beiging, glucose catabolism, and insulin sensitivity in adipose tissues (**Figure 11A**) (353, 354). In support of this, depletion of IL-5 and IL-13-producing cells (mainly ILC2s) corresponded to a reduction of eosinophils and $Arg\text{-}1^+$ adipose macrophages in visceral adipose tissue (355). ILC2s also promote Treg responses through ICOSL and OX40L co-stimulation in adipose tissues, critical for supporting insulin sensitivity (356–358).

Mouse studies support that ILC2s can directly promote adipose beiging, supporting homeostasis and preventing obesity (5, 359, 360). ILC2s are sustained by IL-33 from adipose stem and progenitor cells (ASPCs) (360). Stromal ICAM-1 interactions with LFA-1 on ILC2s promotes ILC2 activation and proliferation in adipose tissue, while IL-4/IL-13 expression by ILC2s induces eotaxin (CCL11) expression in stromal cells, supporting eosinophil recruitment (361). Peritoneal IL-33 administration expands adipocyte precursors and promotes beige lineage commitment in an ILC2-dependent manner, as effects are abolished in the absence of ILC2s and when adipocyte precursors are not receptive to IL-4/IL-13 signaling ($Il4ra^{fl/fl}Pdgfra^{Cre}$) (359). An alternative mechanism of ILC2-dependent beiging of mouse adipose tissue was proposed by Brestoff et al. who found IL-33-stimulated ILC2s produce methionine-enkephalin (MetEnk), an endogenous opioid-like peptide which induces WAT beiging (5). Overall, these studies demonstrate that ILC2-dependent eosinophil-derived IL-4 and ILC2-derived IL-13 and/or MetEnk directly promote murine adipocyte precursor proliferation and beige lineage commitment (5, 359).

Group 1 ILCs are largely resident in murine adipose tissue (362). Although their homeostatic role is poorly understood, ILC1s regulate adipose macrophage homeostasis (363). Alternatively activated macrophages scavenge potentially cytotoxic molecules released during adipose tissue remodeling and upregulate stress ligands (i.e. Rae-1) at steady state, and their selective depletion by adipose type 1 ILCs prevents stress-induced inflammation in macrophages during homeostatic tissue remodeling (363).

ILCs in Obesity

Dysregulation of the immune environment associated with obesity can lead to metabolic dysfunction and insulin resistance, driving type 2 diabetes (T2D) (364). ILC dysregulation has been implicated in obesity (**Figure 11B**). In obese humans and mice, ILC2s are reduced in WAT, possibly due to reduced IL-33 production by ASPCs in response to high fat diet (5, 360, 365). High PD-1 expression on ILC2s reduced IL-5 and IL-13 production, an effect partially rescued by macrophage depletion, suggesting PD-1/PD-L1 interactions between ILC2s and macrophages dampens ILC2 function in obese conditions in mice (365). Additionally, adipocyte-derived soluble ST2 is induced by obesity and interrupts IL-33 signaling, impairing ILC2 homeostasis (366). Infiltration of IFN-γ-producing cells also contributes to reduced ILC2 abundance and function, as IFN-γ directly represses ILC2s and counteracts IL-33 (357).

Obese mice fed a high fat diet had adipose tissue-specific IL-12-dependent accumulation of ILC1s with elevated IFN-γ production, resulting in insulin resistance and glucose intolerance (362, 367). Interestingly, $CD56^{dim}$ $CD16^-$ ILCs accumulating during obesity have reduced cytotoxicity, a potential secondary mechanism contributing to macrophage accumulation and glucose intolerance (363). Wensveen et al. demonstrated NKp46 on adipose-resident mouse NK cells may regulate this effect (368). High fat diet-induced obesity triggered the expression of NCR1 ligands on adipocytes which promoted local NK cell proliferation and production of IFN-γ, inducing the differentiation of pro-inflammatory macrophages and promoting insulin resistance (368). Wang et al. further found that the ILC1 IFN-γ-dependent expansion of pro-inflammatory macrophages exacerbated adipose fibrosis by promoting TGF-β1 and pro-fibrotic programs in macrophages, resulting in higher collagen deposition (367).

In agreement with murine models, circulating and adipose ILC1s are increased in obese patients, especially those with T2D, and the abundance of ILC1s positively correlates with measures of glucose intolerance and insulin resistance (367). A unique subpopulation of $CSF1R^+IL6R\alpha^+$ NK cells is expanded in human and murine obesity (369). Selective depletion of this subset ($Csf1r^{\text{-}loxSTOPlox\text{-}DTR}$ x Ncr^{Cre}) resulted in decreased weight gain, better glucose tolerance, and insulin responsiveness in mice fed a high fat diet (369). Further, the expression of RORγt, lymphotoxin and IL-22 all elevated weight gain and adipose tissue size, paralleling findings that IL-22 from Th17 cells exacerbates inflammation in obesity (370, 371). The regulation of metabolism by intestinal ILC3s suggests a gut-adipose axis that remains to be explored. Overall, ILC2s mediate adipose homeostasis and are dysregulated in obesity, while ILC1s and potentially ILC3s have a role in exacerbating inflammation.

CONCLUDING REMARKS

Multiple parallels and differences between murine and human ILCs exist. Their evolutionarily conserved transcriptional programs and functional similarity emphasizes their

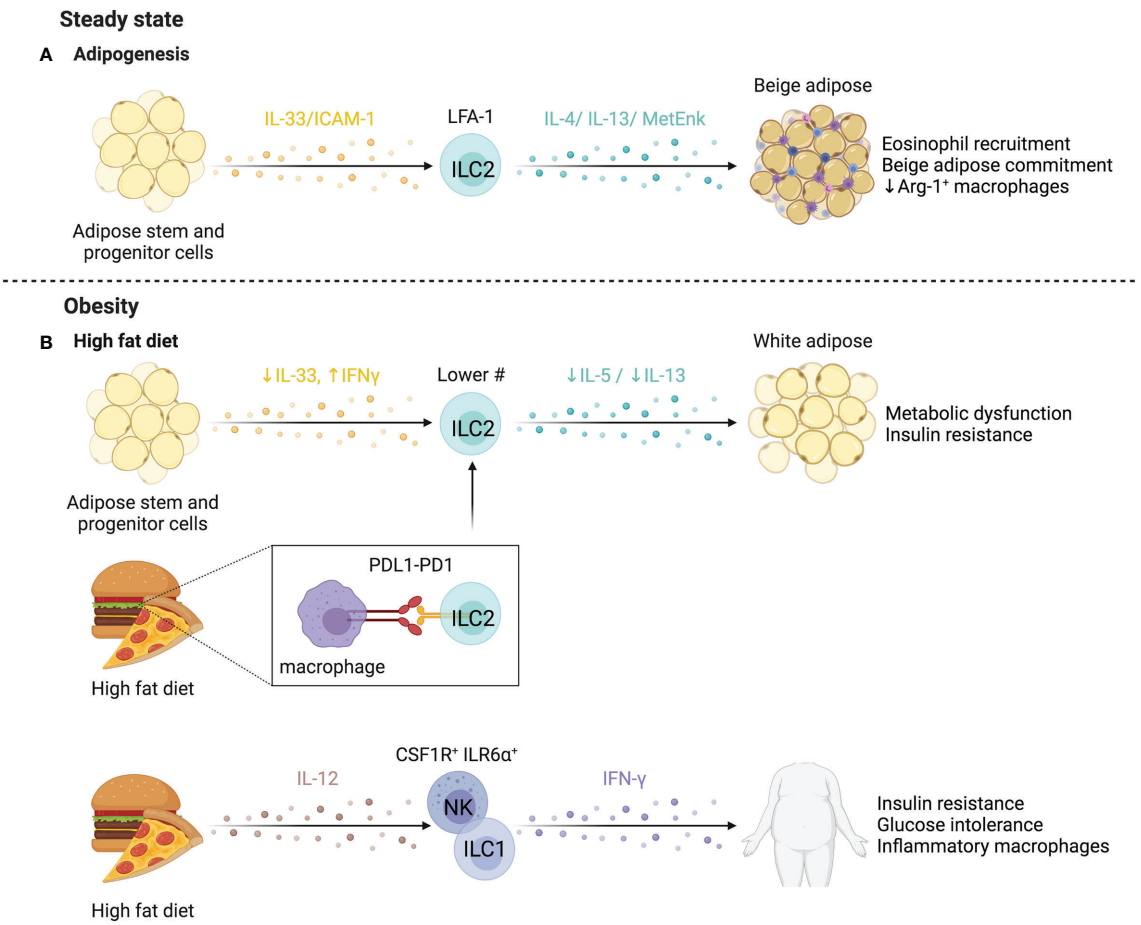

FIGURE 11 | ILCs in adipose tissue. **(A)** Using mostly mouse models, ILC2s are linked to adipogenesis, where they maintain homeostasis and prevent obesity by promoting adipose beiging. ILC2s are sustained by IL-33 from adipose stem and progenitor cells (ASPCs) and required stromal cell interactions *via* ICAM-1 and LFA-1. The resulting release of IL-4, IL-13 by ILC2s promotes eosinophil recruitment *via* stromal cell-derived eotaxin (CCL11). IL-33 stimulates ILC2-produced methionine-enkephalin (MetEnk), an endogenous opioid-like peptide that promotes adipose beiging. **(B)** Both obese humans and mice, have reduced ILC2s in the white adipose tissue due to diet-driven impairment of IL-33 production by ASPCs. The infiltration of IFN-γ-producing ILCs actively represses cytokine release by ILC2s and propagates ILC2 inhibition through *via* PD-1 and macrophage-expressed PD-L1. A unique subpopulation of CSF1R^{+}IL16Rα^{+} NK cells and increased ILC1 abundance positively correlates with glucose intolerance and insulin resistance. Overall, reduction of adipose ILC2s fosters metabolic dysfunction, insulin resistance and obesity. Created with Biorender.org.

importance across multiple distinct phylogenetic branches. However, a better understanding of homologies and analogies in their surface receptor expression and function are needed to inform conserved mechanisms underlying responses to infections, inflammation and malignancies. This will be of particular interest to enhance our understanding NK cell biology, where receptors regulating NK cells responses differ between mouse and human, but different receptors often perform similar function.

The distinct living conditions of mice and humans require conserved and specified adaptations of organs and tissues to environmental triggers. ILCs as regulators of tissue homeostasis adapt to these species-specific environments. Shared and differing microbiota within humans and mice may explain conserved and distinct functions of ILCs across these organisms. Gnotobiotic technologies, humanized mice, knock-out mouse models or adoptive transfer experiments are suitable to investigate these differences but bring their own pitfalls. Beyond these challenges, a key obstacle for the study of ILCs *in situ* is the scarcity of ILC-specific models available to tease out cell-specific or even organ-specific functions. Adding to this, ILCs typically function in cellular networks to influence the outcome of a given immune response. To properly understand their function and tease out any redundancy, more systems-based approaches are needed, particularly in humans (295, 372, 373).

Other roadblocks in understanding the role of various ILCs in homeostasis and disease are studies designed only to link the presence or absence of ILCs with disease outcome. This is especially evident in reports of NK cell function. Reporting expansion or reduction of NK cells as having a protective or detrimental effect assumes a homogeneous function of NK cells. While classically, NK cell

function has been cytotoxic and inflammatory, NK cells have also been cast in an immunoregulatory role where they dampen an immune response (19). Additionally, identifying NK cells as $CD3^-$ $CD56^+$ does not rule out other non-cytotoxic ILC1s and ILC3s that can share CD56 expression, and does not address the large degree of heterogeneity within NK cells (374). Untangling the identity and functional capacity of distinct $CD3^-$ $CD56^+$ populations may help to clarify contradictory findings.

The plasticity of ILCs makes definitively assigning them as "good" or "bad" quite problematic. Sometimes ILCs with identical or similar surface phenotype may be functionally distinct (206). While this may be context dependent, ILC plasticity may be partially responsible for conflicting reports of their function in disease. Future studies should consider assessing the functional role of ILC subsets correlated with ILC transcriptional and epigenetic profiles to identify mechanisms underlying distinct ILC functions and whether some level of 'trained' immunity contributes to differing findings. Additionally, it remains unclear if the tissue-specific functions of ILCs are due in part to the existence of specific subsets of ILCs that home to their niche, or instead these different functions are a directly due to microenvironment signals leading to niche adaptation. It is also entirely possible that both cases are true and contribute to establishing tissue-specific ILC functions. Moving forward, the characterization of tissue-specific networks and niches for ILCs will transform our understanding of ILC functions and underlying mechanisms controlling their tissue adaptations.

AUTHOR CONTRIBUTIONS

All authors contributed to conceptualization, topic curation, writing and editing of the manuscript. Figures were designed by LN, with input from JM, AM, and SC. All authors contributed to the article and approved the submitted version.

FUNDING

JMM was supported by Queen Elizabeth II, Dick and Peggy Sharpe, and Peterborough KM Hunter Scholarships. LN was supported by an Ontario Graduate Scholarship and a NSERC-PGS award. AM's research program is supported by Canadian Institutes for Health Research (CIHR, 388337) and Natural Sciences and Engineering Research Council of Canada (NSERC, RGPIN-2019-04521), and is a Tier 2 Canadian Research Chair in Mucosal Immunology. SQC's research program is supported by funding from CIHR (168960 and 169084) and a NSERC (RGPIN-2021-03672), and is a Medicine By Design Investigator (Canada First Research Excellence Fund).

ACKNOWLEDGMENTS

All figures were created with BioRender.com.

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GLOSSARY

AD	Atopic dermatitis
Ahr	Aryl hydrocarbon receptor
APC	Antigen presenting cell
AREG	Amphiregulin
ASPCs	Adipose stem and progenitor cells
CAD	Coronary artery disease
CD	Crohn's disease
ChAT	Choline acetyltransferase
COPD	Chronic obstructive pulmonary disease
cNK cells	Conventional NK cells
CNS	Central nervous system
CP	Cryptopatches
CVB3	Coxsackievirus B3
DC	Dendritic cell
dNK cells	Decidual Natural Killer cells
EAE	Experimental autoimmune encephalitis
EOMES	Eomesodermin
ESRD	End stage renal disease
FasL	Fas ligand
Ffar2	Free Fatty Acid Receptor 2
FHL2	Four And A Half LIM Domains 2
GITR	Glucocorticoid-induced TNFR-related protein
GPR34	G Protein-Coupled Receptor 34
HBV	Hepatitis B virus
hCG	Human chorionic gonadotropin
HCV	Hepatitis C virus
HDM	House dust mite
hILC	helper ILC
IBD	Inflammatory bowel disease
ID	Inhibitor of DNA binding
IFN-γ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
ILC1	Group 1 innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
$ILC2_{10}$	IL-10 producing ILC2
ILC3	Group 3 innate lymphoid cell
ILCreg	Regulatory ILC
ILF	Isolated lymphoid follicle
IRI	Ischemia reperfusion injury
KIR	Killer immunoglobulin receptor
KLRG1	Killer cell lectin like receptor G1
LN	Lupus nephritis
LP	Lamina propria
LTi	Lymphoid tissue inducer
LXA4	Lipoxin A4
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
miRNA	MicroRNA
MS	Multiple Sclerosis
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCR	Natural cytotoxicity receptor
PCOS	Polycystic ovary syndrome
RA	Retinoic acid
RORA	RAR-related orphan receptor A
RORC	RAR-related orphan receptor C
RSV	Respiratory syncytial virus
SCF	Stem cell factor
SCFA	Short chain fatty acid
scRNAseq	Single cell RNA sequencing
SLE	Systemic lupus erythematosus
sLT	Surface lymphotoxin
SPM	Specialized pro-resolving mediator
T2D	Type 2 diabetes
TBET	T-box transcription factor
Tfh	T follicular helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UCP-1	Uncoupling protein 1
uNK cells	Uterine Natural Killer cells
VIP	Vasoactive intestinal peptide
WAT	White adipose tissue

(Continued)

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AD	Atopic dermatitis
Ahr	Aryl hydrocarbon receptor
APC	Antigen presenting cell
AREG	Amphiregulin
ASPCs	Adipose stem and progenitor cells
CAD	Coronary artery disease
CD	Crohn's disease
ChAT	Choline acetyltransferase
COPD	Chronic obstructive pulmonary disease
cNK cells	Conventional NK cells
CNS	Central nervous system
CP	Cryptopatches
CVB3	Coxsackievirus B3
DC	Dendritic cell
dNK cells	Decidual Natural Killer cells
EAE	Experimental autoimmune encephalitis
EOMES	Eomesodermin
ESRD	End stage renal disease
FasL	Fas ligand
Ffar2	Free Fatty Acid Receptor 2
FHL2	Four And A Half LIM Domains 2
GITR	Glucocorticoid-induced TNFR-related protein
GPR34	G Protein-Coupled Receptor 34
HBV	Hepatitis B virus
hCG	Human chorionic gonadotropin
HCV	Hepatitis C virus
HDM	House dust mite
hILC	helper ILC
IBD	Inflammatory bowel disease
ID	Inhibitor of DNA binding
IFN-γ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
ILC1	Group 1 innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
$ILC2_{10}$	IL-10 producing ILC2
ILC3	Group 3 innate lymphoid cell
ILCreg	Regulatory ILC
ILF	Isolated lymphoid follicle
IRI	Ischemia reperfusion injury
KIR	Killer immunoglobulin receptor
KLRG1	Killer cell lectin like receptor G1
LN	Lupus nephritis
LP	Lamina propria
LTi	Lymphoid tissue inducer
LXA4	Lipoxin A4
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
miRNA	MicroRNA
MS	Multiple Sclerosis
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCR	Natural cytotoxicity receptor
PCOS	Polycystic ovary syndrome

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RA	Retinoic acid
RORA	RAR-related orphan receptor A
RORC	RAR-related orphan receptor C
RSV	Respiratory syncytial virus
SCF	Stem cell factor
SCFA	Short chain fatty acid
scRNAseq	Single cell RNA sequencing
SLE	Systemic lupus erythematosus
sLT	Surface lymphotoxin
SPM	Specialized pro-resolving mediator
T2D	Type 2 diabetes
TBET	T-box transcription factor
Tfh	T follicular helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UCP-1	Uncoupling protein 1
uNK cells	Uterine Natural Killer cells
VIP	Vasoactive intestinal peptide
WAT	White adipose tissue

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