THE ROLE OF INNATE LYMPHOID CELLS IN MUCOSAL IMMUNITY

EDITED BY: Jessica Borger, Joanna Kirman and Graham Le Gros
PUBLISHED IN: Frontiers in Immunology

IUIS

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-925-0
DOI 10.3389/978-2-88963-925-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

THE ROLE OF INNATE LYMPHOID CELLS IN MUCOSAL IMMUNITY

Topic Editors:
Jessica Borger, Monash University, Australia
Joanna Kirman, University of Otago, New Zealand
Graham Le Gros, Victoria University of Wellington, New Zealand

We acknowledge the initiation and support of this Research Topic by the International Union of Immunological Societies (IUIS). We hereby state publicly that the IUIS has had no editorial input in articles included in this Research Topic, thus ensuring that all aspects of this Research Topic are evaluated objectively, unbiased by any specific policy or opinion of the IUIS.

Citation: Borger, J., Kirman, J., Le Gros, G., eds. (2020). The Role of Innate Lymphoid Cells in Mucosal Immunity. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-925-0

Table of Contents

05 ***Editorial: The Role of Innate Lymphoid Cells in Mucosal Immunity***
Jessica G. Borger, Graham Le Gros and Joanna R. Kirman

09 ***Innate Lymphoid Cells in the Maternal and Fetal Compartments***
Derek Miller, Kenichiro Motomura, Valeria Garcia-Flores, Roberto Romero and Nardhy Gomez-Lopez

23 ***Type 3 ILCs in Lung Disease***
Amanda Ardain, James Zachary Porterfield, Henrik N. Kløverpris and Alasdair Leslie

31 ***ILC2 Orchestration of Local Immune Function in Adipose Tissue***
Cécile Bénézech and Lucy Helen Jackson-Jones

37 ***Heterogeneity of NK Cells and Other Innate Lymphoid Cells in Human and Murine Decidua***
Paola Vacca, Laura Chiossone, Maria Cristina Mingari and Lorenzo Moretta

45 ***Fingolimod Alters Tissue Distribution and Cytokine Production of Human and Murine Innate Lymphoid Cells***
Ahmet Eken, Mehmet Fatih Yetkin, Alperen Vural, Fatma Zehra Okus, Serife Erdem, Zehra Busra Azizoglu, Yesim Haliloglu, Mustafa Cakir, Enes Mehmet Turkoglu, Omer Kilic, Irfan Kara, Hamiyet Dönmez Altuntaş, Mohamed Oukka, Mehmet Serdar Kutuk, Meral Mirza and Halit Canatan

57 ***Differential Effects of the Absence of Nkx2-3 and MAdCAM-1 on the Distribution of Intestinal Type 3 Innate Lymphoid Cells and Postnatal SILT Formation in Mice***
Dóra Vojkovics, Zoltán Kellermayer, Fanni Gábris, Angela Schippers, Norbert Wagner, Gergely Berta, Kornélia Farkas and Péter Balogh

68 ***Physiological Regulation of Innate Lymphoid Cells***
Nicolas Jacquelot, Kylie Luong and Cyril Seillet

75 ***Commensal and Pathogenic Bacteria Indirectly Induce IL-22 but Not IFNγ Production From Human Colonic ILC3s via Multiple Mechanisms***
Moriah J. Castleman, Stephanie M. Dillon, Christine M. Purba, Andrew C. Cogswell, Jon J. Kibbie, Martin D. McCarter, Mario L. Santiago, Edward Barker and Cara C. Wilson

89 ***ILC2s—Trailblazers in the Host Response Against Intestinal Helminths***
Tiffany Bouchery, Graham Le Gros and Nicola Harris

101 ***Innate Lymphoid Cells in Helminth Infections—Obligatory or Accessory?***
Stephan Löser, Katherine A. Smith and Rick M. Maizels

112 ***Group 2 Innate Lymphoid Cells are Redundant in Experimental Renal Ischemia-Reperfusion Injury***
Guy J. M. Cameron, Kelly M. Cautivo, Svenja Loering, Simon H. Jiang, Aniruddh V. Deshpande, Paul S. Foster, Andrew N. J. McKenzie, Ari B. Molofsky, Philip M. Hansbro and Malcolm R. Starkey

121 ***Innate Lymphoid Cells in Mucosal Immunity***
Santosh K. Panda and Marco Colonna

134 ***Group 2 Innate Lymphoid Cells in Respiratory Allergic Inflammation***
Sofia Helfrich, Barbara C. Mindt, Jörg H. Fritz and Claudia U. Duerr

146 ***The Influence of Innate Lymphoid Cells and Unconventional T Cells in Chronic Inflammatory Lung Disease***
Jessica G. Borger, Maverick Lau and Margaret L. Hibbs

162 ***Invariant NKT Cell-Mediated Modulation of ILC1s as a Tool for Mucosal Immune Intervention***
Stephanie Trittel, Neha Vashist, Thomas Ebensen, Benedict J. Chambers, Carlos A. Guzmán and Peggy Riese

177 ***Peripheral Innate Lymphoid Cells are Increased in First Line Metastatic Colorectal Carcinoma Patients: A Negative Correlation With Th1 Immune Responses***
Romain Loyon, Marine Jary, Bérengère Salomé, Alejandra Gomez-Cadena, Jeanne Galaine, Marie Kroemer, Pedro Romero, Sara Trabanelli, Olivier Adotévi, Christophe Borg and Camilla Jandus

frontiers in Immunology

EDITORIAL
published: 17 June 2020
doi: 10.3389/fimmu.2020.01233

Editorial: The Role of Innate Lymphoid Cells in Mucosal Immunity

Jessica G. Borger[1*†], *Graham Le Gros*[2†] *and Joanna R. Kirman*[3†]

[1] Department of Immunology and Pathology, Central Clinical School, Monash University, Alfred Research Alliance, Melbourne, VIC, Australia, [2] Allergic & Parasitic Diseases Programme, Malaghan Institute of Medical Research, Wellington, New Zealand, [3] Department of Microbiology & Immunology, University of Otago, Dunedin, New Zealand

Keywords: ILC, NK cell, mucosal, immunity, lung, gastrointestinal tract, genitourinary tract

Editorial on the Research Topic

The Role of Innate Lymphoid Cells in Mucosal Immunity

Innate Lymphoid Cells (ILCs) are tissue-resident innate cells that are deeply integrated within mucosal tissues and play a critical role in tissue homeostasis and inflammatory processes. The collection of articles in this Frontiers Research Topic investigates and discusses the new and emerging roles of ILCs in mucosal immunity.

The field of ILC biology has increased exponentially in recent years. Although the scientific community had been aware of the existence of certain innate cells of lymphoid origin for many years, it has only been in the past decade that the full extent of the ILC family and their wide-reaching biological roles has been recognized. As the field expanded, to avoid confusion due to the different names being used in the literature for the same ILC subsets, in 2013 it was proposed that ILCs should be divided into three broad lineages based on specific transcription factor expression and the cytokines they produce. Group 1 (ILC-1) express *T-bet* and produce IFN-γ; Group 2 (ILC-2) express *GATA-3* and produce IL-5, IL-4, and IL-13; and Group 3 (ILC-3) express *RORγt* and produce IL-17 and IL-22 (1). These groups parallel the transcriptional and functional characteristics of T helper 1 (Th1), Th2, and Th17 conventional T cells, respectively. Like T helper subset plasticity, there is heterogeneity within the different ILCs groups, and some overlap between the subsets.

When comparing studies, it is important to recognize that methods to identify and categorize ILCs and their subsets vary from laboratory to laboratory. The lineage cocktail used to identify non-ILCs is the first potential source of variation between studies. This may result in contamination of ILCs and account for differences in the numbers of ILCs in different studies (2). The molecules used to categorize ILCs also vary and can greatly influence the number of cells identified in each subset; some studies use transcription factors to categorize ILCs, whilst others rely on the presence or absence of specific cell surface molecules.

Unlike their adaptive counterparts, activation of ILCs is not modulated by genetically rearranged antigen receptors. Rather, in the absence of conventional antigen recognition, ILCs respond directly or indirectly to a diverse array of pathogen- and host-derived stimuli including stress-related neuropeptides and hormones, cytokines, and metabolites (Jacquelot et al.). Due to their integration in tissues at barrier surfaces, and their ability to respond directly to signals without the requirement for antigen presentation, ILCs are poised to respond rapidly to pathogens and tissue injury. This response is facilitated by a transcriptional program that enables phenotypic and functional plasticity of ILCs such that they can mediate and regulate tissue homeostasis, morphogenesis, metabolism, repair and regeneration.

OPEN ACCESS

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

****Correspondence:***
Jessica G. Borger
jessica.borger@monash.edu

†ORCID:
Jessica G. Borger
orcid.org/0000-0003-0704-1254
Graham Le Gros
orcid.org/0000-0002-5721-0442
Joanna R. Kirman
orcid.org/0000-0002-4802-7048

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

Received: *04 May 2020*
Accepted: *18 May 2020*
Published: *17 June 2020*

Citation:
Borger JG, Le Gros G and Kirman JR (2020) Editorial: The Role of Innate Lymphoid Cells in Mucosal Immunity. Front. Immunol. 11:1233. doi: 10.3389/fimmu.2020.01233

ILCs are located throughout the body, though they are concentrated at barrier sites, including the skin and mucosal surfaces. ILCs in the skin are important for regulating and maintaining epithelial and stromal cells and maintaining skin barrier homeostasis. Although skin ILCs are not covered in this Research Topic, understanding how their unique effector functions may underlie their contribution to the pathogenesis of inflammatory skin disorders such as dermatitis, psoriasis and immunity remains an exciting area for future investigation.

This Research Topic comprehensively addresses the role of ILCs in maintaining tissue homeostasis at mucosal sites including: the maternal and fetal compartments (Miller et al.; Vacca et al.), lung (Ardain et al.; Borger et al.), gut (Vojkovics et al.), adipose tissue (Bénézech and Jackson-Jones), and kidney (Cameron et al.). ILCs function within these mucosal tissues is regulated through a delicate and complex cross-talk between the immune system and the signals received from the physiological systems of the body. This cross-talk requires an exquisite balance to maintain homeostasis and when appropriate to respond to environmental cues to control infection and restore tissue damage (Jacquelot et al.).

During infection, ILCs are sensitive to diverse environmental signals and are critical for regulating responses to commensal bacteria (Castleman et al.) and pathogens including bacteria (Jacquelot et al.), helminthic parasites (Bouchery et al.; Löser et al.), and viruses (Ardain et al.; Panda and Colonna; Trittel et al.). Indeed, understanding how the dysregulation of ILC responses can lead to chronic inflammatory diseases including: allergy (Helfrich et al.; Panda and Colonna) and asthma (Ardain et al.; Borger et al.), chronic obstructive pulmonary disease (COPD) (Ardain et al.; Borger et al.; Panda and Colonna), and pulmonary fibrosis (Ardain et al.); as well as autoimmune diseases such as inflammatory bowel disease (IBD) (Vojkovics et al.), colitis (Panda and Colonna) and Crohn's disease, is a consistent theme within this Research Topic. ILCs also influence the development of mucosal tumors, such as colorectal cancer (Loyon et al.). Some ILC subsets play a pro-tumorigenic role; others appear to have a protective role through promotion of anti-tumor responses or by limiting damage to tissue (Loyon et al.). Although many mechanisms and fine details of the interplay between ILCs and their environment remain to be elucidated, it is clear that ILCs are critical for protecting mucosal barriers against communicable and non-communicable disease.

REGULATION OF ILCS

The physiological regulation of ILCs in the maintenance of tissue homeostasis in mucosal sites is reliant on the exquisitely sensitive sensing of host-derived peptide and non-peptide signals that enable ILCs to integrate the physiological systems of the body including nervous, endocrine, digestive and reproductive systems. Jacquelot et al.. reviews recent advances in our understanding of the mechanisms by which ILCs are regulated by physiological signals. Jacquelot et al. evaluates how ILCs are able to integrate these signals to maintain homeostasis and prevent immunopathology without compromising infection control.

One such host-derived signal is S1P, a blood-borne bioactive lysosphingolipid which binds to the S1P receptor 1 (S1PR1) to promote lymphocyte egress from the secondary lymphoid organs. Until recently, the effects of S1P and pharmacological agonists of S1PR1 on ILCs was unknown. Such agonists include fingolimod, an immunomodulatory drug approved to modify the autoimmune disease multiple sclerosis (MS). In this Research Topic, Eken et al. reveals that ILCs express S1P1R and administration of S1P agonists including fingolimod, drives ILC-penia in MS patients and decreases ILC accumulation in the secondary lymphoid organs of mice. Notably, long-term administration of fingolimod in mice led to decreased ILC3s in the small intestine. This study characterizes a further mechanism by which ILCs can sense and respond to host-derived signals to maintain tissue homeostasis.

ILCS IN THE RESPIRATORY TRACT

The lung is considered a non-lymphoid mucosal organ comprising an innate cell network of epithelial cells, macrophages, and dendritic cells, all crucial for maintenance of immune homeostasis and the initiation of inflammatory processes. In addition, ILCs, which although only comprise a small proportion of the total immune cells in the lung, have been shown to promote lung homeostasis and contribute to disease processes. In mice, ILC2s are the predominant ILC subset in the lung (3) and in this Research Topic, Helfrich et al., provides a comprehensive review on the contribution of ILC2s to allergic airway inflammation including allergic asthma, obesity-induced asthma, and rhinosinusitis.

Helfrich et al. considers the effect of experimental and therapeutic strategies that are employed to ameliorate respiratory inflammation on ILC responses in the lung. Corticosteroids and β2-agonists are common treatments for asthmatic patients and β2 adrenergic receptors are expressed by both human and murine ILC2s. The ILC2s respond β2-agonists by reducing proliferation and effector function, suggesting that targeting ILC2s early in disease could also block initiation of respiratory inflammation and redirect ILC2 activity Helfrich et al..

Although the role of ILC2s in Th2 allergic airway responses is well-supported, the contributions of ILCs to chronic lung inflammation are poorly understood. In this issue Borger et al. and Panda and Colonna review the contribution of ILCs to chronic pulmonary diseases. Dysregulation of ILCs and their ability to respond to changing local tissue environmental cues by altering their traits and functional attributes, contributes to the immunopathology underlying COPD, asthma, and chronic rhinosinusitis (Borger et al.; Panda and Colonna). The dysregulation of ILCs becomes even more pronounced during disease exacerbations, which are common in COPD and asthma. The major cause of disease exacerbation is viral infection. During viral infection, ILC2-activating cytokines, IL-25 and IL-33, are released by airway epithelial cells upon viral infection, enhancing type 2 responses and the recruitment of ILC2s.

ILC1s also contribute to the clearance of murine influenza infections of the lung. Trittel et al. provide evidence that ILC1s can be modulated via activated iNKT cells, to improve functional responsiveness. Notably, heightened cytokine production appeared restricted to the ILC1s that expressing checkpoint molecules on their surface. This suggests the exciting possibility that control of influenza infection and potentially other rhinovirus infections could be modulated by immunotherapeutic interventions.

In humans, by using cell surface markers to categorize ILCs, ILC3s, rather than ILC2s, are the most prevalent subset in lung tissue (4). ILC3 secretion of IL-17A and IL-22 has prompted investigations into their involvement in inflammatory and infectious diseases. Although the importance of ILC3s as a source of GM-CSF in the lung remains unknown, its role in allergic airway disease, antimicrobial pulmonary host defense function, and surfactant homeostasis is recognized. Together, this lends ILC3s in humans to have a pronounced capacity to drive inflammation and repair in numerous infectious and autoimmune diseases including: tuberculosis, bacterial pneumonia, asthma, COPD, and pulmonary fibrosis. In their review, Ardain et al. highlight the intriguing possibility of ILC3s as a therapeutic target for human pulmonary disorders.

In addition to the critical role tissue-resident ILCs provide in lung homeostasis and during inflammation, the contribution of other innate-like unconventional αβ- and γδ-T cells including MR1-restricted MAIT cells and CD1d-restriced NKT cells is now emerging in pulmonary immunity. Borger et al. evaluate the role of these tissue-resident unconventional T cells in chronic pulmonary disorders and interrogate their interplay and functional overlap with ILCs, asking whether these cell subsets co-regulate one another or function independently. The heterogenous nature and functional plasticity of ILCs and their deep integration within the lung tissue, would suggest that unconventional T cells can influence ILC responses during inflammation and this important relationship remains to be addressed.

ILCS IN THE GASTROINTESTINAL TRACT

ILCs are involved in normal mucosal lymphoid tissue formation as well as in inflammation and epithelial regeneration in IBD. Nkx2-3 is a transcriptional regulator of MAdCAM-1, which is a susceptibility factor for IBD in humans, and is associated with neogenesis of lymphoid tissue in inflamed intestines. In this Research Topic, Vojkovics et al., explore the role of Nkx2-3 in the organogenesis of the solitary intestinal lymphoid tissues involving type 3 innate lymphoid cells (ILC3). It was found that the total absence of MAdCAM-1 partially impaired postnatal seeding of ILC3s in the intestine and caused partial blockade of solitary intestinal lymphoid tissues maturation, suggesting other adhesion molecules may compensate for the intestinal homing of ILC3s in the absence of MAdCAM-15(Vojkovics et al.).

ILCs are also important regulators of adipose tissue function, and in particular ILC2s in the adipose tissue of mucosal sites, such as the mesenteries, and play a critical role in innate B cell activation and antibody production. Bénézech and Jackson-Jones discuss the role of adipose tissue ILCs in the lean state and in obesity, including their link to metabolic dysfunction. Fat associated lymphoid clusters are found throughout the pleural and peritoneal cavities and the ILCs located within these are critical for initiation of immune responses near mucosal sites and for maintaining intestinal barrier function.

Another important means by which ILCs promote gut homeostasis is via IL-22 produced by ILC3s, which enhances epithelial barrier integrity. When the epithelial barrier is compromised, either due to inflammation or infection, microbes can translocate from the gut lumen into the lamina propria, and in mouse models this induces pro-inflammatory cytokine production from ILC3. Castleman et al. used an *in vitro* human lamina propria mononuclear cell model to investigate whether enteric commensal and pathogenic bacteria drives human ILC3 to produce pro-inflammatory cytokines such as IFN-γ. Although IFN-γ was not induced in this *in vitro* model by commensal or pathogenic bacteria, the model provided insight into the mechanisms by which commensal bacterial drive IL-22 production by ILC3. Enteric commensal bacteria cannot directly induce cytokine production by ILC3s, but rather acted on mononuclear cells to produce cytokines that in turn enhanced IL-22 production by ILC3s (Castleman et al.).

In addition to their role in lymphoid and adipose tissue formation and homeostasis in the gastrointestinal tract, ILCs are important for controlling infection. ILC2s were first discovered in experimental studies of intestinal helminth infection (Bouchery et al.). ILC2s sense a wide array of stimuli from helminths, allergens and some bacteria as well as host-derived danger signals induced by tissue damage and release of chemical messengers and other molecules from stressed cells. As helminths mature within the gut tissue, they drive epithelial and mucosal defenses from the host to activate tissue resident intestinal ILC2s, that closely associate with the sensory enteric neuron network. In this Research Topic Löser et al. and Bouchery et al. discuss how ILC2s are embedded in a network of barrier responses during helminth infection, including luminal and epithelial crosstalk, the translation of alarmin signals from host epithelium and eicosanoid mediators, as well as the essential role of ILCs in neuro-immune responses in the lung and gut respectively.

ILCs can play a dual role in the context of tumors, displaying pro- or antitumour effects dependent on the ILC subset and type of cancer. Loyon et al. reveal that treatment-free naïve metastatic colorectal cancer patients have increased peripheral ILCs skewed toward the ILC1 subset and in particular $CD56^+$ ILC1-like cells, which negatively correlate with the anti-tumor CD4 T cell response. Of interest, this study also highlights that although different chemotherapy routines did not alter ILC numbers, there was potential to modulate ILC1 subsets such that the balance of ILC1 and $CD56^+$ ILC1-like cells could be reversed, with ILC2 refractory to the effects of treatment, highlighting the importance of considering the ILC compartment in treatment and monitoring of cancer (Loyon et al.).

ILCS IN THE GENITOURINARY TRACT

In this Research Topic Cameron et al. show that in mice, a higher proportion of $CD45^+$ cells are ILC2s in the kidney than in the lungs. The kidney resident ILC2s constitutively produced IL-5 under homeostatic conditions. Others have found that artificially-induced ILC2s can reduce the severity of experimental renal injury. In this study, the impact of ILC2-deficiency on ischemia-reperfusion injury was investigated, and surprisingly the extent of renal injury was similar whether or not ILC2s were present (Cameron et al.). Thus, although ILC2s can ameliorate renal injury, other compensatory mechanisms exist in their absence.

During pregnancy there is an careful balance between maternal and fetal immune systems, with reviews by Miller et al. and Vacca et al. discussing the potential role of ILCs within the maternal and fetal compartments Miller et al. provide a broad characterization of ILC populations within the uterus, decidua, fetal tissues, and amniotic cavity and highlights the many differences between human and mouse ILC subsets in these locations. For example, although ILC1, ILC2, and ILC3 are present in the mouse uterus prior and during pregnancy, they have only been detected in the non-pregnant endometrium in humans demonstrating a vast disparity in the requirement of ILCs for shaping a successful pregnancy between humans and mice. Moreover, in the decidua which contains decidual NK cells, tissue-resident NK cells and ILC3s, Vacca et al. consider how the changing decidual microenvironment has influenced the plasticity and function of ILCs during early pregnancy, and reflects that their involvement in the establishment and maintenance of pregnancy remains unanswered.

CONCLUSION

The collection of original research articles and reviews in this Research Topic demonstrated the ability of ILCs to modulate the course of infection, inflammatory processes, and cancer across different mucosal barriers in both mice and humans. The important role ILCs have in supporting homeostasis at these barrier sites was also highlighted. Although there have been enormous advances in our understanding of ILCs over the past decade, much remains to be discovered, including: the signals that activate different ILC subsets at each site; the redundancy and plasticity of ILCs and ILC subsets at different anatomical locations; and how ILCs can be modulated in the clinic to improve outcomes.

AUTHOR CONTRIBUTIONS

JB and JK jointly recruited authors and scope for this issue. JB, GL, and JK reviewed submissions and contributed to the writing of the forward to this issue. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part with funds from the CASS Foundation Medicine/Science Grant (JB).

ACKNOWLEDGMENTS

We thank all the contributors to this volume for their thoughtful submission on this important topic. We also thank Kathy Sircombe for her comments on the manuscript.

REFERENCES

1. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
2. Burkhard SH, Mair F, Nussbaum K, Hasler S, Becher B. T cell contamination in flow cytometry gating approaches for analysis of innate lymphoid cells. *PLoS ONE.* (2014) 9:e94196. doi: 10.1371/journal.pone.0094196
3. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1038/ni.2131
4. De Grove KC, Provoost S, Verhamme FM, Bracke KR, Joos GF, Maes T, et al. Characterization and quantification of innate lymphoid cell subsets in human lung. *PLoS ONE.* (2016) 11:e0145961. doi: 10.1371/journal.pone.0145961

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: 26 October 2018
doi: 10.3389/fimmu.2018.02396

Innate Lymphoid Cells in the Maternal and Fetal Compartments

Derek Miller[1,2†], *Kenichiro Motomura*[1,2†], *Valeria Garcia-Flores*[1,2], *Roberto Romero*[1,3,4,5] *and Nardhy Gomez-Lopez*[1,2,6*]

[1] Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services, Bethesda, MD and Detroit, MI, United States, [2] Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI, United States, [3] Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI, United States, [4] Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, MI, United States, [5] Center for Molecular Medicine and Genetics, Wayne State University, Detroit, MI, United States, [6] Department of Immunology, Microbiology and Biochemistry, Wayne State University School of Medicine, Detroit, MI, United States

Pregnancy success is orchestrated by the complex balance between the maternal and fetal immune systems. Herein, we summarize the potential role of innate lymphoid cells (ILCs) in the maternal and fetal compartments. We reviewed published literature describing different ILC subsets [ILC1s, ILC2s, ILC3s, and lymphoid tissue inducer (LTi) cells] in the uterus, decidua, fetal tissues [liver, secondary lymphoid organs (SLO), intestine, and lung] and amniotic cavity. ILC1s, ILC2s, and ILC3s are present in the murine uterus prior to and during pregnancy but have only been detected in the non-pregnant endometrium in humans. Specifically, ILC2s reside in the murine uterus from mid-pregnancy to term, ILC1s increase throughout gestation, and ILC3s remain constant. Yet, LTi cells have only been detected in the non-pregnant murine uterus. In the human decidua, ILC1s, ILC3s, and LTi-like cells are more abundant during early gestation, whereas ILC2s increase at the end of pregnancy. Decidual ILC1s were also detected during mid-gestation in mice. Interestingly, functional decidual ILC2s and ILC3s increased in women who underwent spontaneous preterm labor, indicating the involvement of such cells in this pregnancy complication. Fetal ILCs exist in the liver, SLO, intestine, lung, and amniotic cavity. The fetal liver is thought to be the source of ILC progenitors since the differentiation of these cells from hematopoietic stem cells occurs at this site, and mature ILC subsets can be found in this compartment as well. The interaction between LTi cells and specialized stromal cells is important during the formation of SLO. Mature ILCs are found at the mucosal surfaces of the lung and intestine, from where they can extravasate into the amniotic cavity. Amniotic fluid ILCs express high levels of RORγt, CD161, and CD103, hallmarks of ILC3s. Such cells are more abundant in the second trimester than later in gestation. Although amniotic fluid ILC3s produce IL-17A and TNFα, indicating their functionality, their numbers in patients with intra-amniotic infection/inflammation remain unchanged compared to those without this pregnancy complication. Collectively, these findings suggest that maternal (uterine and decidual) ILCs play central roles in both the initiation and maintenance of pregnancy, and fetal ILCs participate in the development of immunity.

Keywords: amniotic cavity, decidua, LTi, maternal-fetal interface, neonate, pregnancy, preterm labor, uterus

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Francesco Colucci,
University of Cambridge, United Kingdom
Rachel Golub,
Paris Diderot University, France

***Correspondence:**
Nardhy Gomez-Lopez
nardhy.gomez-lopez@wayne.edu;
ngomezlo@med.wayne.edu

†These authors have contributed equally to this work

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

Received: *30 August 2018*
Accepted: *27 September 2018*
Published: *26 October 2018*

Citation:
Miller D, Motomura K, Garcia-Flores V, Romero R and Gomez-Lopez N (2018) Innate Lymphoid Cells in the Maternal and Fetal Compartments. Front. Immunol. 9:2396. doi: 10.3389/fimmu.2018.02396

INTRODUCTION

Successful pregnancy requires the participation of numerous immune cell subsets that must be maintained at perfect equilibrium in the maternal and fetal compartments (1, 2). Both innate and adaptive immune cells have been shown to play important roles in the maintenance and completion of pregnancy (3, 4). The discovery of innate lymphoid cells (ILCs), which bridge the innate and adaptive immune systems, has opened up a new field of investigation with the potential to further uncover the complex immune state of pregnancy.

Innate lymphoid cells (ILCs) are defined by the following characteristics: (1) a lack of antigen-specific receptors, (2) the absence of the expression of known immune cell lineage markers, and (3) lymphoid cellular morphology (5–7). ILCs were divided into three primary groups based on their phenotype and functions (6). Type 1 ILCs (ILC1s) include the prototypical natural killer (NK) cells as well as non-cytotoxic IFNγ-producing ILC1s, identified by expression of the transcription factor T-bet (6, 8, 9). Type 2 ILCs (ILC2s) function through the release of type 2 cytokines such as IL-5 and IL-13 (10–15) and are thought to rely primarily on GATA-binding protein 3 (GATA3) and retinoic acid receptor-related orphan receptor-α (RORα) during their differentiation (6, 16, 17). These ILC2s participate in immune responses such as parasitic infection (12) and allergy (18) but also serve as systemic regulators of homeostasis (19–21). Type 3 ILCs (ILC3s) were divided into two main groups: lymphoid tissue inducer (LTi) cells and non-LTi ILC3s, referred to hereafter as ILC3s (6). LTi cells are critical for the formation of secondary lymphoid organs (SLO) and isolated lymphoid tissues (i.e., Peyer's patches) during fetal development (22–26). Such cells are also found in adults, where they are referred to as LTi-like cells since they do not generate new lymphoid tissue (27). LTi cells and ILC3s rely on expression of RORγt for their development (26) and can express IL-17A and/or IL-22; however, multiple ILC3 subsets with slightly different phenotypes and functional profiles have been described (6). Moreover, a degree of plasticity exists between ILCs, creating an additional layer of complexity within the ILC family (28–31). Recently, it was proposed that the classification of ILCs be expanded to five subsets in order to reflect their distinct developmental pathways: NK cells, ILC1s, ILC2s, ILC3s, and LTi cells (7).

In this review, we aimed to highlight the potential roles of ILCs in the uterus, the decidua, which is the site of direct contact between the maternal and fetal (chorion or trophoblast) tissues, the fetal organs, and the amniotic cavity. Within the field of perinatal immunology, it has been established that uterine (decidual) NK cells play important roles in the maintenance of pregnancy, and their functions are well reviewed elsewhere (32–35). Recent studies have shown that the other ILC subsets exist in the maternal and fetal tissues, suggesting that they also contribute to pregnancy maintenance and outcome. Therefore, in this review, we have focused on ILC1s, ILC2s, ILC3s, and LTi cells. Despite recent advances in the study of ILCs during pregnancy, several gaps still exist in the current knowledge. This review may provide insight into the known roles of ILCs during pregnancy and reveal new potential areas for future studies.

UTERINE INNATE LYMPHOID CELLS

Over the last decade, valuable information has been provided about the presence of ILCs in both the non-pregnant and pregnant uterus. A subset of ILC-like cells was first described in the human uterine mucosa (36). Such cells were originally considered precursors to uterine NK cells, yet showed a divergent phenotype and functionality through the expression of ILC3- and LTi-specific genes such as *RORC*, *LTA*, and *IL22* (36), indicating a different role for these cells. These results were confirmed later by the detection of ILC1s (37), ILC2s (38), and ILC3s (37, 38) in the human non-pregnant endometrium and reinforced by the demonstration that such cells are present in the murine uterus during pregnancy as well (37–41). Such studies have formed a foundation for the understanding of uterine ILCs; yet, future research is needed to further elucidate the role of these cells during pregnancy.

UTERINE ILC1s

Uterine ILC1s were first described in non-pregnant mice as a distinct subset of NK-like cells (42). This ILC1-like population was maintained in the murine uterus of *Nfil3*$^{-/-}$ and *Tbet*$^{-/-}$ mice, whereas NK cell subsets were affected (42). The transcription factors Nfil3/E4BP4 (43–45) and Tbet (6, 8, 9, 46) are both thought to be important for general NK and ILC development; thus, this study indicates that uterine ILC1s may have alternative developmental pathways. This study was confirmed by the detection of a similar ILC1 subset in the uterine mucosa of non-pregnant mice which was negative for CD127 expression (38), highlighting the variability of uterine ILCs since CD127 (IL-7Rα) is also considered to be important for ILC development (6).

In mice, uterine ILC1s are increased throughout gestation compared to the non-pregnant state (37, 39, 40). The production of IFNγ by stimulated total uterine ILCs is increased during gestation (40), which would suggest that these cells have an enhanced capacity for activation in this reproductive tissue. Indeed, uterine ILC1s were shown to contribute to IFNγ production during pregnancy, although not to the same extent as uterine NK cells (38). Consistent with previous findings (42), the uterine ILC1 population was not affected by the knockout of *Nfil3* (38); indeed, ILC1s were increased in these mice (38, 39), indicating that alternative developmental pathways exist for such cells. Since *Nfil3* is crucial for expression of *Eomes* (47), a transcription factor associated with NK cells (48), it was proposed that the uterine ILC1 population observed in *Nfil3*$^{-/-}$ mice includes developmentally arrested NK cells (38). However, these residual ILC1s may not be sufficient for mediating uterine adaptation during pregnancy since placental and fetal abnormalities are observed in *Nfil3*$^{-/-}$ mice (39).

In humans, ILC1s are found in the non-pregnant endometrium as a subset of Lin-CD56+CD127-CD117-RORγt-cells, which are further distinguished based on expression of NKp44 and CD103 (37). ILC1-like cells, which are CD103+NKp44–, are the most significant source of IFNγ (37). Expression of CD103, which facilitates the communication

between lymphocytes and epithelial cells (49), was previously described on tonsillar ILC1s (50), suggesting an epithelial association of such cells in the uterus. However, additional research is needed to determine the role of uterine ILC1s prior to pregnancy and whether such cells are present during gestation.

UTERINE ILC2s

A low frequency of ILC2s has been detected in the human non-pregnant uterine wall (38, 51). In addition, ILC2s and ILC2-like cells have been identified in the non-pregnant murine myometrium (38, 40, 41). The ILC2-like population is the most abundant ILC subset in the pregnant murine myometrium (40). The proportions of murine uterine ILC2s and ILC2-like cells are higher during pregnancy compared to the non-pregnant state, reaching their peak during mid-gestation (38, 40, 51). However, the uterine ILC2-like population (CD45+Lin-Thy1.2+RORγt-NKp46-KLRG1+ cells) identified by Li et al. may have also included other cell types since conventional ILC2 markers such as GATA3 (16) or CRTH2 (52) were not included (40). A recent study utilizing conventional ILC2 markers identified a population of CD127-ILC2s in the non-pregnant human endometrium and in both the non-pregnant and pregnant murine myometrium, confirming the presence of such cells (51). Total uterine ILCs expressing IL-5 and IL-13 were increased during gestation (40), supporting functional roles for uterine ILC2s such as promotion of homeostatic immune cell phenotypes and resolution of inflammatory responses (51).

Uterine ILC2s are almost completely ablated in *Nfil3*$^{-/-}$ mice as opposed to the other subsets (38, 39), confirming that these cells are developmentally reliant on this transcription factor. It is possible that the placental and fetal changes observed in *Nfil3*$^{-/-}$ mice are due to the loss of ILC2-dependent regulatory mechanisms in the myometrium (39); however, since conventional NK cells were also greatly impacted in such mice (38, 39), this finding will require further studies to confirm.

It was recently shown that murine uterine ILC2s express the IL-33 receptor, ST2 (IL-1RL1) (41). A previous report highlighted the importance of IL-33/ST2 signaling for homeostatic immune responses such as those mediated by T helper 2 cells, regulatory T cells, M2-polarized macrophages, and ILC2s, among others (53). In line with these findings, uterine ILC2s were increased in proportion after *in vitro* stimulation with IL-33 (41). ILC2 activity was also increased by *in vitro* IL-33 stimulation as indicated by enhanced release of IL-5 and IL-13 (41). Moreover, an IL-5 reporter mouse (54) was used to verify that *in vivo* administration of IL-33 increased uterine ILC2 proportions and expression of IL-5 (41). Interestingly, the original research describing the IL-5 reporter mouse model demonstrated that the majority of IL-5+ cells in different murine tissues had an ILC2 phenotype, including expression of CD127 and ST2 (54), providing further evidence that IL-33-receptive ILC2s are important for the production of IL-5. Pups born to *ST2*$^{-/-}$ dams had significantly reduced viability (41), suggesting that this pathway may be beneficial for fetal development; however, *IL33*$^{-/-}$ mice do not experience any fertility or pregnancy complications (55). Additionally, IL-33 is important for type 2 mucosal immune responses (55). Together, these observations support pregnancy-specific functions for IL-33-receptive ILC2s in the murine uterus.

Murine uterine ILC2s can also express the estrogen receptor α (41). The proportion of these cells is increased in response to *in vitro* stimulation with 17β-estradiol; however, such a response is not seen in ILC2s from the murine lung (41), providing evidence for specific female sex hormone-driven regulation of uterine ILC2s during pregnancy. Yet, whether female sex hormones specifically target ILC2s, or the observed ILC2 proliferation was a secondary response due to signaling within the uterine tissues, has not been shown (41).

Collectively, these findings provide firm evidence of ILC2s in the non-pregnant uterine tissues from humans and mice, and that such cells are enhanced in number and function during murine gestation. Further studies are required to uncover the specific mechanisms and cellular interactions of uterine ILC2s.

UTERINE ILC3s

ILC3s were first described in the human non-pregnant endometrium as a distinct subset of NK precursor-like cells expressing ILC-associated markers such as CD127 and CD161 (36). Further analysis of these cells revealed expression of the *RORC* and *IL22* genes, indicative of an ILC3 phenotype (36). Later studies confirmed the presence of ILC3s in the human endometrium (37, 38) and indicated that these cells could be divided into two main subsets: NCR– (human NKp44-; mouse NKp46-) and NCR+ (human NKp44+; mouse NKp46+) ILC3s (7), with the NCR– ILC3s being the dominant population in mice and the NCR+ ILC3s in humans (38). During murine pregnancy, uterine ILC3s are elevated compared to non-pregnant mice (38) with the highest proportions occurring in early- and mid-gestation (40). Uterine ILC3s from both pregnant and non-pregnant mice constitutively produce IL-17A and IL-22, which is further upregulated in response to *in vitro* stimulation with IL-1β and IL-23 (38). Yet, production of IL-17A and IL-22 by uterine ILC3s from pregnant mice is not significantly elevated in mid-gestation compared to that of non-pregnant mice (38, 40), suggesting that either an increase in ILC3-specific functionality is not required for successful pregnancy, or that such an increase may only occur in late gestation/prior to parturition. Further studies are required to pursue this concept.

In contrast with ILC2s, the uterine ILC3 population is not affected in non-pregnant *Nfil3*$^{-/-}$ mice (38, 39); however, such cells fail to undergo the pregnancy-specific expansion observed in wildtype mice (39). This lack of ILC3 expansion is associated with fetal growth compromise and defective placentation (39), indicating that uterine ILC3s may be important for the physiological progression of pregnancy, e.g., decidualization (see decidual ILC section for more information).

UTERINE LTi-LIKE CELLS

Information regarding LTi-like cells in the human and murine uterus is scarce. One potential explanation is that LTi-like cells have been identified as ILC3s due to the shared expression of markers such as RORγt (6). LTi-like cells were reported in

the non-pregnant murine uterus in similar proportions to the closely-related ILC3s (38). Moreover, similar to ILC3s, LTi-like cells were not affected in the uterus of non-pregnant *Nfil3*$^{-/-}$ mice (38), suggesting a distinct developmental pathway for these cells. It will be interesting for future studies to uncover the functions of LTi-like cells in the human uterus.

In conclusion (**Figure 1A**), ILC1s, ILC2s, and ILC3s are present in the murine uterus prior to and during pregnancy, but have only been detected in the non-pregnant endometrium in humans. Specifically, ILC2s reside in the murine uterus from mid-pregnancy to term, ILC1s increase throughout gestation, and ILC3s remain constant. Yet, LTi cells have only been detected in the non-pregnant murine uterus. Further studies are needed to confirm the presence and functions of uterine ILCs during human pregnancy.

DECIDUAL INNATE LYMPHOID CELLS

Upon implantation, endometrial stromal cells undergo a specialized transformation that includes significant morphological and functional changes to the endometrium, a phenomenon termed as "decidualization" (56, 57). This process facilitates invasion of the fetal trophoblast (56) and leads to formation of the area of contact between the endometrium and the placenta (decidua basalis) or fetal membranes (decidua parietalis). The decidua is therefore an interface in which maternal and fetal cells converge and unique immune interactions take place.

ILCs have been identified in the decidua as early as 9–12 weeks of gestation (58). The origin of decidual ILCs is unclear. Several sources have been proposed for the prototypical ILC1s, NK cells, in the reproductive tissues including derivation from hematopoietic precursors (59), maturation from already-present endometrial NK cells (36), or migration from the periphery (60). Since hematopoietic precursor cells can express the ID2 transcription factor [required for ILC differentiation (61)], this is one plausible explanation for the source of decidual ILCs (59). The origin and developmental timeline of decidual ILCs requires further investigation. Importantly, phenotypic (58) and functional (62) evidence suggests that decidual ILC subsets have unique profiles that are not found in other non-reproductive tissues.

DECIDUAL ILC1s

ILCs expressing an ILC1-like phenotype distinct from NK cells have been detected in the human decidua during the first trimester (37, 58). Two ILC1 subsets were detected. The first was identified within the CD56+ population (Lin-CD56+CD94-CD127-CD117-) and expressed the ILC1-associated Tbet as well as Eomes (37, 58). Interestingly, this ILC1 subset also expressed CD103 (36, 37), indicating a possible epithelial association (50). The other subset fell within the CD56- population (Lin-CD56-CD127-CD117-Tbet+Eomes-) and was therefore more distinguishable from decidual NK cells (36). In line with defined ILC1 phenotypes, both described subsets expressed IFNγ (37, 58). In mice, ILC1s were also described in the decidua during mid-gestation, where they produced IFNγ (38).

At the end of pregnancy, ILC1s are the rarest ILC subset in the human decidua and were not altered with the presence of spontaneous labor, suggesting that such cells may have only a minor role in late gestation that may be shared by other decidual ILC subsets due to the unique cytokine profile observed in such cells (62).

DECIDUAL ILC2s

The first-trimester human decidua has been reported to contain a small proportion of ILC2s (38, 51, 58). The expression of the ILC2 marker CRTH2 (52) was only minimally detected on decidual ILCs (38, 58); however, CD161 (52) was expressed by two of the potential ILC subsets described, indicating possible plasticity or shared expression of ILC2 markers by other subsets (58). ILC2s were found in the murine uterus, but not the decidua, in mid-gestation (38, 51) and at term (51). In contrast, ILC2s were the most abundant decidual ILC subset in the third trimester (62) where they may play a role in maintaining the homeostatic environment at the maternal-fetal interface. ILC2s are considered to have a homeostatic phenotype (19, 20), rendering them unnecessary in early pregnancy when the inflammatory mechanisms of implantation and tissue remodeling occur within the endometrium. Interestingly, ILC2s were increased in the decidua basalis of women with spontaneous preterm labor compared to those who delivered preterm without labor (62), suggesting that this ILC subset may participate in the chronic inflammatory process that occurs during pathological pregnancy. Moreover, ILC2s from the third-trimester human decidua seemed to share the expression of cytokines such as IL-13 and IL-22 with ILC3s, suggesting that decidual ILC subsets may have shared functionality toward the end of pregnancy (62).

DECIDUAL ILC3s

Among the described ILC subsets in the human decidua, ILC3s have been the most extensively studied (37, 38, 58, 62, 63). During the first trimester, a subset of ILCs expressing the traditional ILC3-associated transcription factor RORγt (26) is found in the human decidua (37, 38, 58). Decidual ILC3s expressed GM-CSF (63), IL-22 (58), and IL-8 (58). Notably, GM-CSF and IL-8 released by decidual ILC3s were shown to promote neutrophil migration and survival in the first-trimester decidua (63). This finding is consistent with a previous study that showed the participation of neutrophils in spiral artery remodeling during pregnancy (64), and adds a new layer of complexity to the role of decidual ILC3s in the successful establishment of pregnancy (63). Interestingly, the murine decidua did not contain ILC3s during mid-gestation (38).

During the third trimester, human decidual ILC3s express a unique cytokine profile that includes IFNγ, IL-13, IL-17A, and IL-22 (62). Previous findings have suggested that some degree of plasticity exists between ILC subsets (28, 29, 65), which would explain the expression of the ILC1-associated cytokine IFNγ by

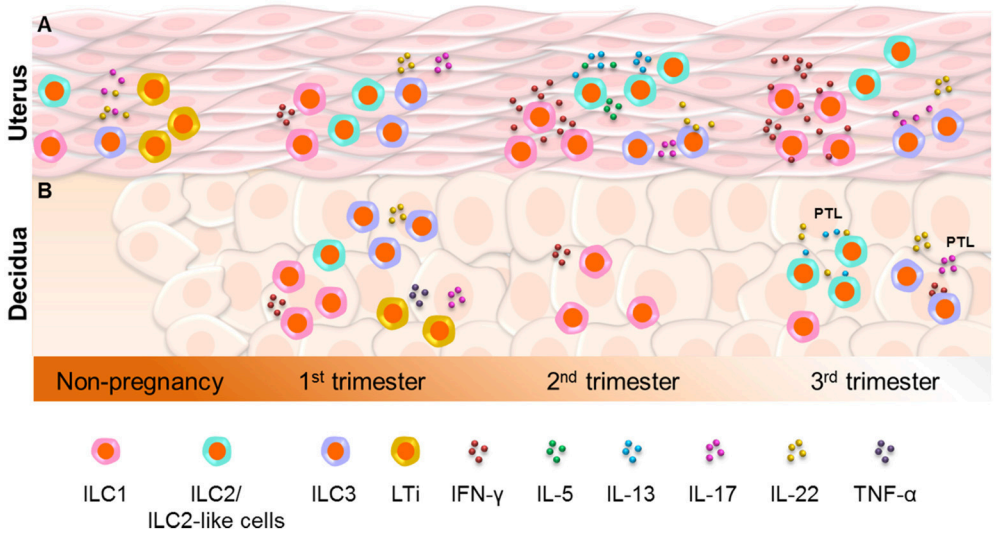

FIGURE 1 | Innate lymphoid cells in the uterus and decidua. **(A)** ILC1s, ILC2s, and ILC3s are present in the murine uterus prior to and during pregnancy, but have only been detected in the non-pregnant endometrium in humans. ILC2s reside in the murine uterus from mid-pregnancy to term, whereas ILC1s increase throughout gestation and ILC3s remain constant. However, lymphoid tissue inducer (LTi) cells have only been detected in the non-pregnant murine uterus. Further studies are needed to confirm the presence and functions of uterine ILCs during human pregnancy. **(B)** In the human decidua, ILC1s, ILC3s, and LTi-like cells are more abundant during early gestation, whereas ILC2s are increased at the end of pregnancy. Decidual ILC1s were also detected during mid-gestation in mice. Interestingly, functional decidual ILC2s and ILC3s are increased in women who underwent spontaneous preterm labor (PTL), indicating the involvement of such cells in this pregnancy complication.

decidual ILC3s. This may also explain the low proportions of decidual ILC1s in late gestation (62), since their presence may be redundant.

Interestingly, increased proportions of ILC3s in the decidua parietalis are found in women who undergo spontaneous preterm labor (62), suggesting that a local dysregulation of such cells may occur in these patients. Whether decidual ILC3s directly participate in the inflammation associated with spontaneous preterm labor or are increased as a consequence of such a process remains to be determined.

DECIDUAL LTi-LIKE CELLS

Human LTi cells have important functions in the formation of fetal SLO (66), a process described in more detail below; however, their role at the maternal-fetal interface is less understood. A population of LTi-like cells has been described in the human first-trimester decidua where they express IL-17A and TNFα (58). These decidual LTi-like cells are closely related to ILC3s as evidenced by the shared expression of RORγt and production of IL-17A (58). Moreover, both decidual LTi-like cells and ILC3s display lymphoid tissue inducer-like functions (22–25, 67–69) through the upregulation of ICAM-1 and VCAM-1 on decidual stromal cells (58), further indicating a degree of redundancy between these two cell types (27, 70). However, the developmental pathways of LTi cells and ILC3s are different and, unlike other ILC subsets, no LTi plasticity has been reported (27). It is possible that the initiation of a lymphoid tissue induction-like process in the decidua is necessary for recruitment of other immune cells and pregnancy maintenance. Therefore, it is important for future studies to investigate the purpose of LTi-like activity at the maternal-fetal interface.

In conclusion (**Figure 1B**), the human decidua contains ILC1s, ILC3s, and LTi-like cells, which are more abundant during early gestation. In contrast, decidual ILC2s are increased at the end of pregnancy. Decidual ILC1s were also detected during mid-gestation in mice. Functional decidual ILC2s and ILC3s are increased in women who underwent spontaneous preterm labor, indicating the involvement of such cells in this pregnancy complication.

FETAL INNATE LYMPHOID CELLS

Fetal ILCs are reported to exist in the human liver, SLO, intestine, and lung, which are described in detail below. The fetal liver is a center of hematopoiesis (71, 72), and it has been shown that ILC progenitors (ILCP) originate from this compartment (65, 73–76). Indeed, ILCPs can be detected in the cord blood as well, indicating that such cells may migrate to other sites of organogenesis (76). It has been proposed that the differentiation of ILCPs to mature ILC subsets primarily takes place after such cells have migrated to their sites of residence (76). In the fetus, the presence of specialized ILCs (i.e., LTi cells) is important for the successful formation of SLO such as the spleen, mesenteric lymph nodes (mLN), and Peyer's patches (23, 26, 77, 78). At non-lymphoid sites such as the intestine and lung, mature ILC subsets may participate in mucosal immunity after birth by regulating inflammation during colonization with commensal bacteria (79, 80).

In mice, development of the fetal lymphatic system is described as early as gestational day 10.5 (81). The murine

fetal lymph nodes follow a staggered developmental timeline beginning with the mLN at gestational day 10.5, closely followed by the sacral and cervical lymph nodes and ending with the complete formation of Peyer's patches in the intestine [for more information about fetal lymphogenesis, please see (82, 83)]. The mesenteric and peripheral lymph nodes are present in the fetus by gestational day 16.5 (68). ILCPs are detected in the murine fetal liver at day 12.5 (84–88), and in both the fetal liver and intestine at day 13.5–14.5 (74, 89). Information regarding the presence of ILCPs in the fetal tissues in early pregnancy is lacking; therefore, further studies are required to determine the complete timeline for the generation of ILCPs and mature ILC subsets during fetal development.

ILCs IN THE FETAL LIVER

A subset of ILCPs was described in the human fetal liver during the second trimester (76). It was shown that these ILCPs were generated from the CD34+ hematopoietic stem cells (76) also found in this compartment (71, 72, 76). These ILCPs primarily express RORγt and, after *in vitro* expansion, mainly produce IL-17A, indicating an ILC3 phenotype (76). However, subsets of fetal liver ILCPs also produce IFNγ or IL-13, suggesting that such cells have differentiation potential for ILC1s and ILC2s as well (76). The murine fetal liver also contains an ILCP subset with potential for differentiation into ILC1s, ILC2s, or ILC3s (75). Ablation of *Zbtb16*, which encodes the ILCP-associated transcription factor PLZF [reviewed in (88)], affected fetal ILC1s and ILC2s but not ILC3s or NK cells (75), supporting the existence of alternative progenitors or developmental pathways for these ILC subsets.

The human fetal liver also contains mature ILC populations during the first and second trimester (90). ILC1s, ILC2s, and both NCR+ and NCR– ILC3s are detected (90). Prior to 15 weeks of gestation, only NCR– ILC3s can be distinguished, whereas the remaining subsets appear later (90). A population of fetal liver ILC3s express neuropilin-1 (NRP-1) (90, 91), suggesting an LTi phenotype (92). Together, these findings indicate that the fetal liver is the primary site of ILC progenitors. Mature ILC subsets also exist within the fetal liver, yet their role is currently unknown.

ILCs IN THE FETAL LYMPHOID TISSUES

Murine experiments have shown that the interaction between LTi cells and mesenchymal stromal cells is fundamental for the formation of SLO (93). It has been observed that during murine embryogenesis a subset of stromal cells interacts with LTi cells at the site of LN formation (25, 26, 68). LTi cells express ligands such as lymphotoxins α and β (LTA and LTB) that activate specific stromal cells (25, 94–96). Such activated stromal cells will upregulate expression of the adhesion molecules ICAM-1 and VCAM-1 (25, 69, 78, 97) and begin the process of forming SLO (26).

A subset of Lin-CD127+ ILCs was originally described in human fetal mesenteric tissue (97). It was shown that these ILCs were localized at the same locations at which the mLN developed (91, 97), indicating that lymph node-specific ILCs are present in the fetal mesentery even prior to the complete formation of the mLN. Moreover, stromal organizer cells form a niche for LTi cells in the human fetal spleen and LN between 8 and 15 weeks of gestation (78), providing further evidence of an important role for fetal LTi cells in tissue neogenesis. The mLN from first- and second-trimester human fetuses have been shown to contain an ILC subset that expressed RORγt and had increased gene expression of *IL17A* and *IL22* (98), and a similar subset was described in the fetal spleen that also expressed NRP-1 (91, 92). *NRP1*$^{-/-}$ knockout mice have severely affected yolk sac and embryonic development (99), suggesting that the expression of this receptor is required for organogenesis. Additionally, RORγt+ ILCs are found in specific physiological locations in the human fetal LN and spleen in the second trimester, where they are co-localized with specialized stromal cells (78). This interaction leads to induced expression of ICAM-1 and VCAM-1 on the stromal cells (78, 97), indicating that these ILCs have LTi functions. LTi cells in the human fetal LN express *IL17A* and *IL22* and participate in LN formation (97).

ILCs IN THE FETAL INTESTINE

LTi cells cluster at the site where Peyer's patches are formed in the developing murine fetal intestine (22, 89). The development of intestinal lymphoid tissues such as the Peyer's patches is imperative for regulation of mucosal immunity in the intestine (100, 101), and fetal LTi cells have been shown to be crucial to this process (23, 26). Importantly, a subset of transitional ILCPs exists in the fetal intestine that can further differentiate into other ILC subsets, indicating that some of these ILCs are not terminally differentiated and can provide other functions even after SLO formation is complete (89).

Recently, the presence of mature ILC subsets in the fetal intestine was confirmed using mass cytometry (102). All known ILC subsets were detected together with several novel intermediates that included a subset with potential to differentiate into ILC3s or NK cells (102). These findings confirmed previous studies that indicated the presence of ILC2s (52) and ILC3s (52, 98, 103) in the second-trimester human fetal intestine. ILC2s in the fetal intestine produce IL-13 (52), whereas ILC3s and LTi-like cells produce IL-17A and IL-22 (98). ILC3s are increased in the fetal intestine during the second trimester compared to the first (98, 103). Importantly, fetal CD103+ ILC3s can be found in the amniotic fluid during the first and second trimesters (see amniotic cavity section for more information) (103), suggesting that these cells can migrate from the fetal tissues into the amniotic cavity.

ILCs are increased in the intestinal tissues from neonates with gastroschisis compared to those from healthy controls (104). This finding was corroborated using a murine model with gastroschisis-like symptoms showing that ILC2s and ILC3s are increased in the intestines of affected mice compared to littermate controls (104). Neutralization of IL-5 [a primary ILC2 cytokine (13)] during late gestation results in a dramatic decrease in eosinophil and ILC2 infiltration in the fetal intestine (104),

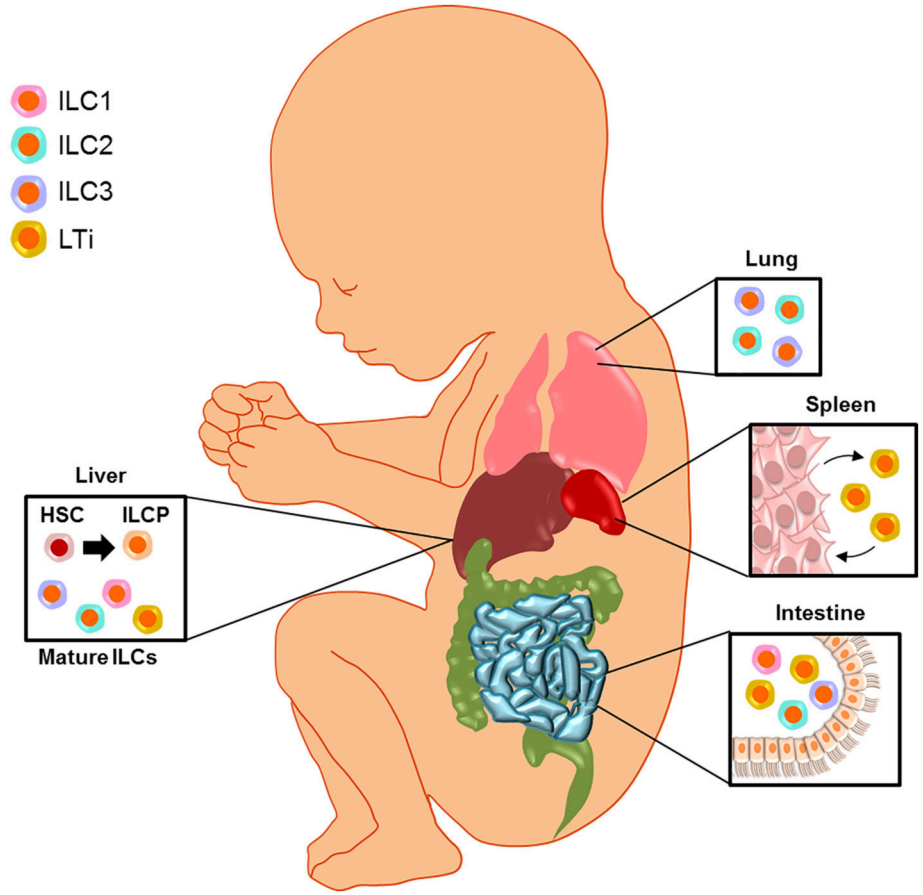

FIGURE 2 | Fetal innate lymphoid cells. Fetal ILCs exist in the liver, secondary lymphoid organs (SLO), intestine, lung, and amniotic cavity. The fetal liver is thought to be the source of ILC progenitors (ILCP) since the differentiation of these cells from hematopoietic stem cells (HSC) occurs at this site, and mature ILC subsets can be found in this compartment as well. The interaction between lymphoid tissue inducer (LTi) cells and specialized stromal cells is important during the formation of SLO. Mature ILCs are found at the mucosal surfaces of the lung and intestine, from where they can extravasate into the amniotic cavity. These findings support a role for ILCs as central regulators in fetal development and immunity.

implicating ILC2s in the chronic inflammatory process that accompanies this condition.

Collectively, these data confirm the requirement for LTi cells during the formation of fetal SLO, and indicate that mature ILC1s, ILC2s, and ILC3s are found in the intestinal mucosa where they may participate in inflammatory processes; however, their specific role during fetal life is unclear.

ILCs IN THE FETAL LUNG

A single report established the presence of ILC2s (identified by CRTH2 and CD161 expression) in the human fetal lung mucosa during the second trimester (52). In the neonatal and adult lungs, the primary function of ILC2s is to protect against threats such as helminth infection (12, 13, 105). ILC2s are also implicated in asthma and its complications as well as allergy (18, 106, 107). A subset of CD103+ ILC3s is also detected in the human fetal lung, which is increased in the second trimester compared to the first (103). Recently, a population of ILC2s was described in the murine fetal lung just prior to birth (gestational day 19), which rapidly expanded during the first 2 weeks of life (108). It was suggested that these homeostatic cells may help prevent hyper-inflammation resulting from exposure of the newborn lungs to airborne particles (108). Future studies may further reveal the specific role of ILC2s and ILC3s in this fetal compartment.

In conclusion (**Figure 2**), fetal ILCs exist in the liver, SLO, intestine, lung, and amniotic cavity. The fetal liver is thought to be the source of ILC progenitors since the differentiation of these cells from hematopoietic stem cells occurs at this site, and mature ILC subsets can be found in this compartment as well. The interaction between LTi cells and specialized stromal cells is important during the formation of SLO. Mature ILCs are found at the mucosal surfaces of the lung and intestine, from where they can extravasate into the amniotic cavity. These findings support a role for ILCs as central regulators in fetal development and immunity.

INNATE LYMPHOID CELLS IN THE AMNIOTIC CAVITY

The amniotic cavity serves as the fetal habitat, which is surrounded by the protective liquid termed amniotic fluid

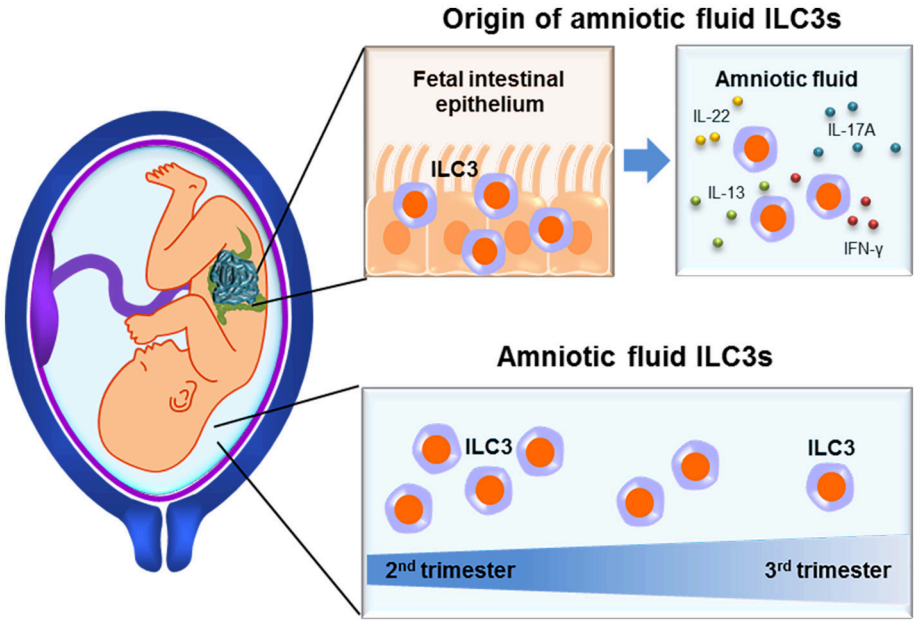

FIGURE 3 | Innate lymphoid cells in the amniotic cavity. Functional ILC3s are found in the mucosal epithelium of fetal tissues such as the intestines, from where they may extravasate into the amniotic cavity. Amniotic fluid ILCs reach their highest proportions during the second trimester, yet are still present at the end of gestation.

(109). Besides providing mechanical cushioning, the amniotic fluid contains nutrients as well as other factors required for fetal growth and represents an immunological barrier against invading pathogens (109, 110). In clinical medicine, the amniotic fluid is used to assess fetal well-being (111–114), lung maturity (115–117), karyotype (118, 119), and intra-amniotic inflammation associated with bacteria [intra-amniotic infection (120–132)] or danger signals [sterile intra-amniotic inflammation (133–138)]. In the context of intra-amniotic inflammation, the most abundant leukocytes in the amniotic fluid are neutrophils (139, 140), which can be of fetal and/or maternal origin (141, 142). These innate immune cells actively participate in the mechanisms of host defense against microbial invasion of the amniotic cavity by releasing cytokines (140) and anti-microbial molecules (143–145), performing phagocytosis (146), and forming neutrophil extracellular traps or NETs (147, 148). Therefore, it was thought that, in the absence of intra-amniotic inflammation, the cellular component of the amniotic fluid was of limited research value. Recent studies have shown that, indeed, the opposite is true (103, 149). The amniotic fluid contains both innate (monocyte/macrophages, neutrophils, NK cells, and ILCs), and adaptive (T cells and B cells) immune cell populations, each of which fluctuates independently throughout gestation (149).

Amniotic fluid ILCs are abundant during the second trimester (103) and their numbers decay as gestation progresses (149) (**Figure 3**). In this compartment, ILCs express high levels of RORγt (103, 149), a hallmark of ILC3s (6, 26). Amniotic fluid ILC3s also express CD127, CD117, CD161, and CD56 but not NK cell-markers such as Eomes, T-bet, CD94/NKG2A, and CD16 (103). Such ILCs are functional since they produce high levels of IL-17A and TNFα upon PMA/ionomycin stimulation (103). The fetal origin of amniotic fluid ILC3s was demonstrated by the expression of HLA class I molecules, which were not expressed on maternal peripheral blood mononuclear cells (103). Interestingly, amniotic fluid ILC3s seem to originate in the fetal lungs and intestine since a similar ILC subpopulation was identified in these organs (103). Amniotic fluid ILC3s expressed CD103, indicating an epithelial association (49, 50) that was confirmed by detection of these cells in the fetal intestinal epithelium (103). Moreover, CD103+ ILC3s were not detected in the amnion or chorion (chorioamniotic membranes), eliminating those tissues as a source of such cells in the amniotic fluid (103). Together with the observation that immune cells in the amniotic fluid during preterm gestation can be predominantly of fetal origin (142), evidence points to the fetus as a likely source of CD103+ ILC3s (**Figure 3**). It was proposed that these cells participate in regulating intra-amniotic infection (103); yet, their numbers remain constant between patients with and without this clinical condition (149). This finding does not discard the possibility that amniotic fluid ILC3s can acquire a regulatory phenotype, which can then participate in controlling the inflammatory response induced by microbes or danger signals in the amniotic cavity.

Together, these studies demonstrate the presence of functional ILC3s in the amniotic cavity, which are likely derived from the fetal tissues. Such cells reach their highest proportions in the second trimester yet are still present at the end of gestation. Moreover, the detection of ILC3s in the amniotic cavity of patients with intra-amniotic inflammation suggests the participation of these cells in such a clinical condition.

Molecular studies have suggested that there is a placental microbiome (150–161). Nonetheless, recent publications have

not confirmed that the placenta harbors a unique microbiome [(162); Theis et al., Am J Obstet Gynecol; in press], which supports the ongoing controversy [(162–166); Theis et al., Am J Obstet Gynecol; in press]. The absence of a placental microbiota, however, does not exclude the possibility that the fetus is exposed to microbial products from the mother. This concept is supported by another recent study showing that transient microbial colonization of the maternal gut during pregnancy induces short- and long-term innate immune changes in the offspring (167). Of interest to the ILC field, neonates born to mothers transiently microbial-colonized displayed an increased number of ILC3s in their mucosal tissues (167). The proposed mechanism for fetal exposure to maternal gut microbiota was mediated by transmission of microbial-derived metabolites via antibodies (167). Such education of the neonatal immune system was enhanced by breastfeeding (167). Therefore, microbial-derived metabolites, rather than viable bacteria, may be required for fetal and neonatal development of the ILC system.

CONCLUSION

The discovery of ILCs in the reproductive and fetal tissues has led to new knowledge of the immune cellular processes required for successful pregnancy and fetal development. At the same time, new questions have arisen as to the functions and interactions of ILCs in the maternal and fetal compartments. The studies reviewed herein have provided evidence that ILCs fill an important role during pregnancy, especially in mucosal defenses and fetal development, yet also share certain functions with other innate and adaptive immune cell subsets. In the mother, uterine ILCs may participate in mucosal immunity and help facilitate tissue remodeling and homeostasis during and after implantation, while decidual ILCs take part in the immune interactions required for pregnancy maintenance and maternal-fetal tolerance. Meanwhile, fetal ILCs mediate the formation of lymphoid tissues during organogenesis and reside at key mucosal surfaces, such as the intestine and lung, in preparation for fetal exposure to both commensal and pathogenic microbes. Importantly, such fetal ILCs may migrate to the amniotic fluid during intra-amniotic infection/inflammation to further participate in host defense. Collectively, the presented findings paint a complex picture of the ILC network during pregnancy, and future studies will be required in order to reveal the complete story of these unique immune cells.

AUTHOR CONTRIBUTIONS

DM, KM, VG-F, RR, and NG-L participated in the conception, interpretation, and writing of the manuscript.

FUNDING

The authors are supported by the Perinatology Research Branch (PRB), Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services (NICHD/NIH/DHHS), and with federal funds from the NICHD/NIH/DHHS under Contract No. HHSN275201300006C. NG-L is also supported by the Wayne State University Perinatal Initiative in Maternal, Perinatal and Child Health.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Marcia Arenas-Hernandez, MSc., for her participation in critical discussions.

REFERENCES

1. Gomez-Lopez N, Stlouis D, Lehr MA, Sanchez-Rodriguez EN, Arenas-Hernandez M. Immune cells in term and preterm labor. *Cell Mol Immunol.* (2014) 11:571–81. doi: 10.1038/cmi.2014.46
2. Bonney EA. Immune regulation in pregnancy: a matter of perspective? *Obstet Gynecol Clin.* (2016) 43:679–98. doi: 10.1016/j.ogc.2016.07.004
3. Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol.* (2013) 31:387–411. doi: 10.1146/annurev-immunol-032712-100003
4. Bonney EA. Alternative theories: pregnancy and immune tolerance. *J Reprod Immunol.* (2017) 123:65–71. doi: 10.1016/j.jri.2017.09.005
5. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol.* (2012) 30:647–75. doi: 10.1146/annurev-immunol-020711-075053
6. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
7. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid *cells*: 10 Years On. *Cell* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
8. Powell N, Walker AW, Stolarczyk E, Canavan JB, Gokmen MR, Marks E, et al. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. *Immunity* (2012) 37:674–84. doi: 10.1016/j.immuni.2012.09.008
9. Bernink JH, Peters CP, Munneke M, Te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol.* (2013) 14:221–9. doi: 10.1038/ni.2534
10. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity* (2001) 15:985–95. doi: 10.1016/S1074-7613(01)00243-6
11. Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, et al. New IL-17 family members promote Th1 or Th2 responses in the lung: *in vivo* function of the novel cytokine IL-25. *J Immunol.* (2002) 169:443–53. doi: 10.4049/jimmunol.169.1.443
12. Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, et al. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med.* (2006) 203:1105–16. doi: 10.1084/jem.20051615
13. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* (2010) 463:540–4. doi: 10.1038/nature08636
14. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* (2010) 464:1367–70. doi: 10.1038/nature08900
15. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA.* (2010) 107:11489–94. doi: 10.1073/pnas.1003988107

16. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity* (2012) 37:649–59. doi: 10.1016/j.immuni.2012.08.015
17. Wong SH, Walker JA, Jolin HE, Drynan LF, Hams E, Camelo A, et al. Transcription factor RORalpha is critical for nuocyte development. *Nat Immunol.* (2012) 13:229–36. doi: 10.1038/ni.2208
18. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* (2012) 36:451–63. doi: 10.1016/j.immuni.2011.12.020
19. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1038/ni.2131
20. Molofsky AB, Nussbaum JC, Liang HE, Van Dyken SJ, Cheng LE, Mohapatra A, et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J Exp Med.* (2013) 210:535–49. doi: 10.1084/jem.20121964
21. Nussbaum JC, Van Dyken SJ, Von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* (2013) 502:245–8. doi: 10.1038/nature12526
22. Adachi S, Yoshida H, Kataoka H, Nishikawa S. Three distinctive steps in Peyer's patch formation of murine embryo. *Int Immunol.* (1997) 9:507–14. doi: 10.1093/intimm/9.4.507
23. Yoshida H, Honda K, Shinkura R, Adachi S, Nishikawa S, Maki K, et al. IL-7 receptor alpha+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int Immunol.* (1999) 11:643–55. doi: 10.1093/intimm/11.5.643
24. Hashi H, Yoshida H, Honda K, Fraser S, Kubo H, Awane M, et al. Compartmentalization of Peyer's patch anlagen before lymphocyte entry. *J Immunol.* (2001) 166:3702–9. doi: 10.4049/jimmunol.166.6.3702
25. Honda K, Nakano H, Yoshida H, Nishikawa S, Rennert P, Ikuta K, et al. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *J Exp Med.* (2001) 193:621–30. doi: 10.1084/jem.193.5.621
26. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol.* (2004) 5:64–73. doi: 10.1038/ni1022
27. Zhong C, Zheng M, Zhu J. Lymphoid tissue inducer-a divergent member of the ILC family. *Cytokine Growth Factor Rev.* (2018) 42:5–12. doi: 10.1016/j.cytogfr.2018.02.004
28. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proc Natl Acad Sci USA.* (2010) 107:10961–6. doi: 10.1073/pnas.1005641107
29. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, et al. Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity* (2010) 33:736–51. doi: 10.1016/j.immuni.2010.10.017
30. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, D'hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature* (2013) 494:261–5. doi: 10.1038/nature11813
31. Bernink JH, Krabbendam L, Germar K, De Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and-23 control plasticity of CD127(+) group 1 and group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity* (2015) 43:146–60. doi: 10.1016/j.immuni.2015.06.019
32. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol.* (2002) 2:656–63. doi: 10.1038/nri886
33. Vacca P, Mingari MC, Moretta L. Natural killer cells in human pregnancy. *J Reprod Immunol.* (2013) 97:14–9. doi: 10.1016/j.jri.2012.10.008
34. Moffett A, Colucci F. Uterine NK cells: active regulators at the maternal-fetal interface. *J Clin Invest.* (2014) 124:1872–9. doi: 10.1172/JCI68107
35. Gaynor LM, Colucci F. Uterine natural killer cells: functional distinctions and influence on pregnancy in humans and mice. *Front Immunol.* (2017) 8:467. doi: 10.3389/fimmu.2017.00467
36. Male V, Hughes T, Mcclory S, Colucci F, Caligiuri MA, Moffett A. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *J Immunol.* (2010) 185:3913–8. doi: 10.4049/jimmunol.1001637
37. Montaldo E, Vacca P, Chiossone L, Croxatto D, Loiacono F, Martini S, et al. Unique eomes(+) NK cell subsets are present in uterus and decidua during early pregnancy. *Front Immunol.* (2015) 6:646. doi: 10.3389/fimmu.2015.00646
38. Doisne JM, Balmas E, Boulenouar S, Gaynor LM, Kieckbusch J, Gardner L, et al. Composition, development, and function of uterine innate lymphoid cells. *J Immunol.* (2015) 195:3937–45. doi: 10.4049/jimmunol.1500689
39. Boulenouar S, Doisne JM, Sferruzzi-Perri A, Gaynor LM, Kieckbusch J, Balmas E, et al. The residual innate lymphoid cells in NFIL3-deficient mice support suboptimal maternal adaptations to pregnancy. *Front Immunol.* (2016) 7:43. doi: 10.3389/fimmu.2016.00043
40. Li M, Gao Y, Yong L, Huang D, Shen J, Liu M, et al. Molecular signature and functional analysis of uterine ILCs in mouse pregnancy. *J Reprod Immunol.* (2017) 123:48–57. doi: 10.1016/j.jri.2017.09.003
41. Bartemes K, Chen CC, Iijima K, Drake L, Kita H. IL-33-Responsive group 2 innate lymphoid cells are regulated by female sex hormones in the uterus. *J Immunol.* (2018) 200:229–36. doi: 10.4049/jimmunol.1602085
42. Sojka DK, Plougastel-Douglas B, Yang L, Pak-Wittel MA, Artyomov MN, Ivanova Y, et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *eLife* (2014) 3:e01659. doi: 10.7554/eLife.01659
43. Geiger TL, Abt MC, Gasteiger G, Firth MA, O'connor MH, Geary CD, et al. Nfil3 is crucial for development of innate lymphoid cells and host protection against intestinal pathogens. *J Exp Med.* (2014) 211:1723–31. doi: 10.1084/jem.20140212
44. Seillet C, Rankin LC, Groom JR, Mielke LA, Tellier J, Chopin M, et al. Nfil3 is required for the development of all innate lymphoid cell subsets. *J Exp Med.* (2014) 211:1733–40. doi: 10.1084/jem.20140145
45. Yu X, Wang Y, Deng M, Li Y, Ruhn KA, Zhang CC, et al. The basic leucine zipper transcription factor NFIL3 directs the development of a common innate lymphoid cell precursor. *eLife* (2014) 3:e04406. doi: 10.7554/eLife.04406
46. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *J Exp Med.* (2014) 211:563–77. doi: 10.1084/jem.20131560
47. Male V, Nisoli I, Kostrzewski T, Allan DS, Carlyle JR, Lord GM, et al. The transcription factor E4bp4/Nfil3 controls commitment to the NK lineage and directly regulates Eomes and Id2 expression. *J Exp Med.* (2014) 211:635–42. doi: 10.1084/jem.20132398
48. Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* (2012) 36:55–67. doi: 10.1016/j.immuni.2011.11.016
49. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, et al. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* (1994) 372:190–3. doi: 10.1038/372190a0
50. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity* (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
51. Balmas E, Rana BM, Hamilton RS, Shreeve N, Kieckbusch J, Aye I, et al. Maternal group 2 innate lymphoid cells control fetal growth and protect from endotoxin-induced abortion in mice. *bioRxiv [Preprint]. bioRxiv:348755* (2018). doi: 10.1101/348755
52. Mjosberg JM, Trifari S, Crellin NK, Peters CP, Van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol.* (2011) 12:1055–62. doi: 10.1038/ni.2104
53. Griesenauer B, Paczesny S. The ST2/IL-33 axis in immune cells during inflammatory diseases. *Front Immunol.* (2017) 8:475. doi: 10.3389/fimmu.2017.00475
54. Ikutani M, Yanagibashi T, Ogasawara M, Tsuneyama K, Yamamoto S, Hattori Y, et al. Identification of innate IL-5-producing cells and their role in lung

eosinophil regulation and antitumor immunity. *J Immunol.* (2012) 188:703–13. doi: 10.4049/jimmunol.1101270
55. Oboki K, Ohno T, Kajiwara N, Arae K, Morita H, Ishii A, et al. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci USA*. (2010) 107:18581–6. doi: 10.1073/pnas.1003059107
56. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med.* (2007) 25:445–53. doi: 10.1055/s-2007-991042
57. Mori M, Bogdan A, Balassa T, Csabai T, Szekeres-Bartho J. The decidua-the maternal bed embracing the embryo-maintains the pregnancy. *Semin Immunopathol.* (2016) 38:635–49. doi: 10.1007/s00281-016-0574-0
58. Vacca P, Montaldo E, Croxatto D, Loiacono F, Canegallo F, Venturini PL, et al. Identification of diverse innate lymphoid cells in human decidua. *Mucosal Immunol.* (2015) 8:254–64. doi: 10.1038/mi.2014.63
59. Vacca P, Vitale C, Montaldo E, Conte R, Cantoni C, Fulcheri E, et al. CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proc Natl Acad Sci USA*. (2011) 108:2402–7. doi: 10.1073/pnas.1016257108
60. Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, et al. Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood* (2008) 111:3108–15. doi: 10.1182/blood-2007-08-105965
61. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* (1999) 397:702–6. doi: 10.1038/17812
62. Xu Y, Romero R, Miller D, Silva P, Panaitescu B, Theis KR, et al. Innate lymphoid cells at the human maternal-fetal interface in spontaneous preterm labor. *Am J Reprod Immunol.* (2018) 79:e12820. doi: 10.1111/aji.12820
63. Croxatto D, Micheletti A, Montaldo E, Orecchia P, Loiacono F, Canegallo F, et al. Group 3 innate lymphoid cells regulate neutrophil migration and function in human decidua. *Mucosal Immunol.* (2016) 9:1372–83. doi: 10.1038/mi.2016.10
64. Amsalem H, Kwan M, Hazan A, Zhang J, Jones RL, Whittle W, et al. Identification of a novel neutrophil population: proangiogenic granulocytes in second-trimester human decidua. *J Immunol.* (2014) 193:3070–9. doi: 10.4049/jimmunol.1303117
65. Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
66. Cherrier M, Eberl G. The development of LTi cells. *Curr Opin Immunol.* (2012) 24:178–83. doi: 10.1016/j.coi.2012.02.003
67. Adachi S, Yoshida H, Honda K, Maki K, Saijo K, Ikuta K, et al. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. *Int Immunol.* (1998) 10:1–6. doi: 10.1093/intimm/10.1.1
68. Cupedo T, Vondenhoff MF, Heeregrave EJ, De Weerd AE, Jansen W, Jackson DG, et al. Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. *J Immunol.* (2004) 173:2968–75. doi: 10.4049/jimmunol.173.5.2968
69. Okuda M, Togawa A, Wada H, Nishikawa S. Distinct activities of stromal cells involved in the organogenesis of lymph nodes and Peyer's patches. *J Immunol.* (2007) 179:804–11. doi: 10.4049/jimmunol.179.2.804
70. Song C, Lee JS, Gilfillan S, Robinette ML, Newberry RD, Stappenbeck TS, et al. Unique and redundant functions of NKp46+ ILC3s in models of intestinal inflammation. *J Exp Med.* (2015) 212:1869–82. doi: 10.1084/jem.20151403
71. Pahal GS, Jauniaux E, Kinnon C, Thrasher AJ, Rodeck CH. Normal development of human fetal hematopoiesis between eight and seventeen weeks' gestation. *Am J Obstet Gynecol.* (2000) 183:1029–34. doi: 10.1067/mob.2000.106976
72. Rollini P, Faes-Van't Hull E, Kaiser S, Kapp U, Leyvraz S. Phenotypic and functional analysis of human fetal liver hematopoietic stem cells in culture. *Stem Cells Dev.* (2007) 16:281–96. doi: 10.1089/scd.2006.0096
73. Mebius RE, Miyamoto T, Christensen J, Domen J, Cupedo T, Weissman IL, et al. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. *J Immunol.* (2001) 166:6593–601. doi: 10.4049/jimmunol.166.11.6593
74. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORgammat sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *J Exp Med.* (2012) 209:729–40. doi: 10.1084/jem.20111594
75. Constantinides MG, Mcdonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature* (2014) 508:397–401. doi: 10.1038/nature13047
76. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell* (2017) 168:1086–1100.E10. doi: 10.1016/j.cell.2017.02.021
77. Tan JK, Watanabe T. Murine spleen tissue regeneration from neonatal spleen capsule requires lymphotoxin priming of stromal cells. *J Immunol.* (2014) 193:1194–203. doi: 10.4049/jimmunol.1302115
78. Hoorweg K, Narang P, Li Z, Thuery A, Papazian N, Withers DR, et al. A stromal cell niche for human and mouse type 3 innate lymphoid cells. *J Immunol.* (2015) 195:4257–63. doi: 10.4049/jimmunol.1402584
79. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* (2015) 517:293–301. doi: 10.1038/nature14189
80. Yu JC, Khodadadi H, Malik A, Davidson B, Salles E, Bhatia J, et al. Innate immunity of neonates and infants. *Front Immunol.* (2018) 9:1759. doi: 10.3389/fimmu.2018.01759
81. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell* (1999) 98:769–78. doi: 10.1016/S0092-8674(00)81511-1
82. Mebius RE. Organogenesis of lymphoid tissues. *Nat Rev Immunol.* (2003) 3:292–303. doi: 10.1038/nri1054
83. Van De Pavert SA, Mebius RE. New insights into the development of lymphoid tissues. *Nat Rev Immunol.* (2010) 10:664–74. doi: 10.1038/nri2832
84. Yoshida H, Kawamoto H, Santee SM, Hashi H, Honda K, Nishikawa S, et al. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J Immunol.* (2001) 167:2511–21. doi: 10.4049/jimmunol.167.5.2511
85. Possot C, Schmutz S, Chea S, Boucontet L, Louise A, Cumano A, et al. Notch signaling is necessary for adult, but not fetal, development of RORgammat(+) innate lymphoid cells. *Nat Immunol.* (2011) 12:949–58. doi: 10.1038/ni.2105
86. Tachibana M, Tenno M, Tezuka C, Sugiyama M, Yoshida H, Taniuchi I. Runx1/Cbfbeta2 complexes are required for lymphoid tissue inducer cell differentiation at two developmental stages. *J Immunol.* (2011) 186:1450–7. doi: 10.4049/jimmunol.1000162
87. Serafini N, Klein Wolterink RG, Satoh-Takayama N, Xu W, Vosshenrich CA, Hendriks RW, et al. Gata3 drives development of RORgammat+ group 3 innate lymphoid cells. *J Exp Med.* (2014) 211:199–208. doi: 10.1084/jem.20131038
88. Ishizuka IE, Constantinides MG, Gudjonson H, Bendelac A. The innate lymphoid cell precursor. *Annu Rev Immunol.* (2016) 34:299–316. doi: 10.1146/annurev-immunol-041015-055549
89. Bando JK, Liang HE, Locksley RM. Identification and distribution of developing innate lymphoid cells in the fetal mouse intestine. *Nat Immunol.* (2015) 16:153–60. doi: 10.1038/ni.3057
90. Forkel M, Berglin L, Kekalainen E, Carlsson A, Svedin E, Michaelsson J, et al. Composition and functionality of the intrahepatic innate lymphoid cell-compartment in human nonfibrotic and fibrotic livers. *Eur J Immunol.* (2017) 47:1280–94. doi: 10.1002/eji.201646890
91. Shikhagaie MM, Bjorklund AK, Mjosberg J, Erjefalt JS, Cornelissen AS, Ros XR, et al. Neuropilin-1 is expressed on lymphoid tissue residing LTi-like group 3 innate lymphoid cells and associated with ectopic lymphoid aggregates. *Cell Rep.* (2017) 18:1761–73. doi: 10.1016/j.celrep.2017.01.063
92. Robinette ML, Fuchs A, Cortez VS, Lee JS, Wang Y, Durum SK, et al. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nat Immunol.* (2015) 16:306–17. doi: 10.1038/ni.3094
93. Roozendaal R, Mebius RE. Stromal cell-immune cell interactions. *Annu Rev Immunol.* (2011) 29:23–43. doi: 10.1146/annurev-immunol-031210-101357

94. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* (1994) 264:703–7. doi: 10.1126/science.8171322
95. Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity* (1997) 6:491–500. doi: 10.1016/S1074-7613(00)80292-7
96. Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* (1998) 9:59–70. doi: 10.1016/S1074-7613(00)80588-9
97. Cupedo T, Crellin NK, Papazian N, Rombouts EJ, Weijer K, Grogan JL, et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol.* (2009) 10:66–74. doi: 10.1038/ni.1668
98. Hoorweg K, Peters CP, Cornelissen F, Aparicio-Domingo P, Papazian N, Kazemier G, et al. Functional differences between human NKp44(-) and NKp44(+) RORC(+) innate lymphoid cells. *Front Immunol.* (2012) 3:72. doi: 10.3389/fimmu.2012.00072
99. Takashima S, Kitakaze M, Asakura M, Asanuma H, Sanada S, Tashiro F, et al. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc Natl Acad Sci USA*. (2002) 99:3657–62. doi: 10.1073/pnas.022017899
100. Sonnenberg GF, Monticelli LA, Elloso MM, Fouser LA, Artis D. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity* (2011) 34:122–34. doi: 10.1016/j.immuni.2010.12.009
101. Hashiguchi M, Kashiwakura Y, Kojima H, Kobayashi A, Kanno Y, Kobata T. Peyer's patch innate lymphoid cells regulate commensal bacteria expansion. *Immunol Lett.* (2015) 165:1–9. doi: 10.1016/j.imlet.2015.03.002
102. Li N, Van Unen V, Hollt T, Thompson A, Van Bergen J, Pezzotti N, et al. Mass cytometry reveals innate lymphoid cell differentiation pathways in the human fetal intestine. *J Exp Med.* (2018) 215:1383–96. doi: 10.1084/jem.20171934
103. Marquardt N, Ivarsson MA, Sundstrom E, Akesson E, Martini E, Eidsmo L, et al. Fetal CD103+ IL-17-producing group 3 innate lymphoid cells represent the dominant lymphocyte subset in human amniotic fluid. *J Immunol.* (2016) 197:3069–75. doi: 10.4049/jimmunol.1502204
104. Frascoli M, Jeanty C, Fleck S, Moradi PW, Keating S, Mattis AN, et al. Heightened immune activation in fetuses with gastroschisis may be blocked by targeting IL-5. *J Immunol.* (2016) 196:4957–66. doi: 10.4049/jimmunol.1502587
105. Koyasu S, Moro K. Type 2 innate immune responses and the natural helper cell. *Immunology* (2011) 132:475–81. doi: 10.1111/j.1365-2567.2011.03413.x
106. Chang YJ, Kim HY, Albacker LA, Baumgarth N, Mckenzie AN, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol.* (2011) 12:631–8. doi: 10.1038/ni.2045
107. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* (2015) 16:45–56. doi: 10.1038/ni.3049
108. Saluzzo S, Gorki AD, Rana BMJ, Martins R, Scanlon S, Starkl P, et al. First-Breath-induced type 2 pathways shape the lung immune environment. *Cell Rep.* (2017) 18:1893–905. doi: 10.1016/j.celrep.2017.01.071
109. Schmidt W. The amniotic fluid compartment: the fetal habitat. *Adv Anat Embryol Cell Biol.* (1992) 127:1–100.
110. Davis LE, Mclaren LC, Stewart JA, James CG, Levine MD, Skipper BJ. Immunological and microbiological studies of midtrimester amniotic fluid. *Gynecol Obstet Invest.* (1983) 16:261–8. doi: 10.1159/000299275
111. Cherry SH. Amniotic fluid analysis as an index of fetal health *in utero*. *Med Times* (1967) 95:713–7.
112. Barham KA. Amnioscopy, meconium and fetal well-being. *J Obstet Gynaecol Br Commonw.* (1969) 76:412–8. doi: 10.1111/j.1471-0528.1969.tb05856.x
113. Clark SL, Romero R, Dildy GA, Callaghan WM, Smiley RM, Bracey AW, et al. Proposed diagnostic criteria for the case definition of amniotic fluid embolism in research studies. *Am J Obstet Gynecol.* (2016) 215:408–12. doi: 10.1016/j.ajog.2016.06.037
114. Tarui T, Kim A, Flake A, Mcclain L, Stratigis JD, Fried I, et al. Amniotic fluid transcriptomics reflects novel disease mechanisms in fetuses with myelomeningocele. *Am J Obstet Gynecol.* (2017) 217:587. e1–10. doi: 10.1016/j.ajog.2017.07.022
115. Gluck L. The evaluation of fetal lung maturity. *Calif Med.* (1972) 116:58–9.
116. Hobbins JC, Brock W, Speroff L, Anderson GG, Caldwell B. L-S ratio in predicting pulmonary maturity in utero. *Obstet Gynecol.* (1972) 39:660–4.
117. Palacio M, Bonet-Carne E, Cobo T, Perez-Moreno A, Sabria J, Richter J, et al. Prediction of neonatal respiratory morbidity by quantitative ultrasound lung texture analysis: a multicenter study. *Am J Obstet Gynecol.* (2017) 217:196. e1–14. doi: 10.1016/j.ajog.2017.03.016
118. Jacobson CB, Barter RH. Intrauterine diagnosis and management of genetic defects. *Am J Obstet Gynecol.* (1967) 99:796–807. doi: 10.1016/0002-9378(67)90395-X
119. Lisgar F, Gertner M, Cherry S, Hsu LY, Hirschhorn K. Prenatal chromosome analysis. *Nature* (1970) 225:280–1. doi: 10.1038/225280a0
120. Romero R, Emamian M, Quintero R, Wan M, Hobbins JC, Mitchell MD. Amniotic fluid prostaglandin levels and intra-amniotic infections. *Lancet* (1986) 1:1380. doi: 10.1016/S0140-6736(86)91685-5
121. Romero R, Emamian M, Quintero R, Wan M, Hobbins JC, Mazor M, et al. The value and limitations of the Gram stain examination in the diagnosis of intraamniotic infection. *Am J Obstet Gynecol.* (1988) 159:114–9. doi: 10.1016/0002-9378(88)90503-0
122. Romero R, Sirtori M, Oyarzun E, Avila C, Mazor M, Callahan R, et al. Infection and labor. V Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes *Am J Obstet Gynecol.* (1989) 161:817–24. doi: 10.1016/0002-9378(89)90409-2
123. Romero R, Avila C, Santhanam U, Sehgal PB. Amniotic fluid interleukin 6 in preterm labor. Association with infection. *J Clin Invest.* (1990) 85:1392–400. doi: 10.1172/JCI114583
124. Romero R, Jimenez C, Lohda AK, Nores J, Hanaoka S, Avila C, et al. Amniotic fluid glucose concentration: a rapid and simple method for the detection of intraamniotic infection in preterm labor. *Am J Obstet Gynecol.* (1990) 163:968–74. doi: 10.1016/0002-9378(90)91106-M
125. Hsu CD, Meaddough E, Aversa K, Copel JA. The role of amniotic fluid L-selectin, GRO-alpha, and interleukin-8 in the pathogenesis of intraamniotic infection. *Am J Obstet Gynecol.* (1998) 178:428–32. doi: 10.1016/S0002-9378(98)70414-4
126. Yoon BH, Romero R, Moon JB, Shim SS, Kim M, Kim G, et al. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Obstet Gynecol.* (2001) 185:1130–6. doi: 10.1067/mob.2001.117680
127. Keelan JA, Yang J, Romero RJ, Chaiworapongsa T, Marvin KW, Sato TA, et al. Epithelial cell-derived neutrophil-activating peptide-78 is present in fetal membranes and amniotic fluid at increased concentrations with intra-amniotic infection and preterm delivery. *Biol Reprod.* (2004) 70:253–9. doi: 10.1095/biolreprod.103.016204
128. Kusanovic JP, Espinoza J, Romero R, Goncalves LF, Nien JK, Soto E, et al. Clinical significance of the presence of amniotic fluid 'sludge' in asymptomatic patients at high risk for spontaneous preterm delivery. *Ultrasound Obstet Gynecol.* (2007) 30:706–14. doi: 10.1002/uog.4081
129. Chaemsaithong P, Romero R, Korzeniewski SJ, Martinez-Varea A, Dong Z, Yoon BH, et al. A rapid interleukin-6 bedside test for the identification of intra-amniotic inflammation in preterm labor with intact membranes. *J Matern Fetal Neonatal Med.* (2016) 29:349–59. doi: 10.3109/14767058.2015.1006620
130. Yoneda N, Yoneda S, Niimi H, Ueno T, Hayashi S, Ito M, et al. Polymicrobial amniotic fluid infection with mycoplasma/ureaplasma and other bacteria induces severe intra-amniotic inflammation associated with poor perinatal prognosis in preterm labor. *Am J Reprod Immunol.* (2016) 75:112–25. doi: 10.1111/aji.12456
131. Yoneda S, Shiozaki A, Yoneda N, Ito M, Shima T, Fukuda K, et al. Antibiotic therapy increases the risk of preterm birth in preterm labor without intra-amniotic microbes, but may prolong the gestation period in preterm labor with microbes, evaluated by rapid and high-sensitive PCR system. *Am J Reprod Immunol.* (2016) 75:440–50. doi: 10.1111/aji.12484
132. Rowlands S, Danielewski JA, Tabrizi SN, Walker SP, Garland SM. Microbial invasion of the amniotic cavity in midtrimester pregnancies using molecular microbiology. *Am J Obstet Gynecol.* (2017) 217:71. e1–5. doi: 10.1016/j.ajog.2017.02.051

133. Combs CA, Gravett M, Garite TJ, Hickok DE, Lapidus J, Porreco R, et al. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. *Am J Obstet Gynecol.* (2014) 210:125. e1–15. doi: 10.1016/j.ajog.2013.11.032
134. Romero R, Miranda J, Chaiworapongsa T, Chaemsaithong P, Gotsch F, Dong Z, et al. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. *Am J Reprod Immunol.* (2014) 71:330–58. doi: 10.1111/aji.12189
135. Romero R, Miranda J, Chaiworapongsa T, Chaemsaithong P, Gotsch F, Dong Z, et al. Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: prevalence and clinical significance. *J Matern Fetal Neonatal Med.* (2015) 28:1343–59. doi: 10.3109/14767058.2014.954243
136. Romero R, Miranda J, Chaiworapongsa T, Korzeniewski SJ, Chaemsaithong P, Gotsch F, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Reprod Immunol.* (2014) 72:458–74. doi: 10.1111/aji.12296
137. Romero R, Miranda J, Chaemsaithong P, Chaiworapongsa T, Kusanovic JP, Dong Z, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. *J Matern Fetal Neonatal Med.* (2015) 28:1394–409. doi: 10.3109/14767058.2014.958463
138. Gomez-Lopez N, Romero R, Panaitescu B, Leng Y, Xu Y, Tarca AL, et al. Inflammasome activation during spontaneous preterm labor with intra-amniotic infection or sterile intra-amniotic inflammation. *Am J Reprod Immunol.* (2018) 18:e13049. doi: 10.1111/aji.13049
139. Romero R, Quintero R, Nores J, Avila C, Mazor M, Hanaoka S, et al. Amniotic fluid white blood cell count: a rapid and simple test to diagnose microbial invasion of the amniotic cavity and predict preterm delivery. *Am J Obstet Gynecol.* (1991) 165:821–30. doi: 10.1016/0002-9378(91)90423-O
140. Martinez-Varea A, Romero R, Xu Y, Miller D, Ahmed AI, Chaemsaithong P, et al. Clinical chorioamnionitis at term VII: the amniotic fluid cellular immune response. *J Perinat Med.* (2017) 45:523–38. doi: 10.1515/jpm-2016-0225
141. Sampson JE, Theve RP, Blatman RN, Shipp TD, Bianchi DW, Ward BE, et al. Fetal origin of amniotic fluid polymorphonuclear leukocytes. *Am J Obstet Gynecol.* (1997) 176:77–81. doi: 10.1016/S0002-9378(97)80015-4
142. Gomez-Lopez N, Romero R, Xu Y, Leng Y, Garcia-Flores V, Miller D, et al. Are amniotic fluid neutrophils in women with intraamniotic infection and/or inflammation of fetal or maternal origin? *Am J Obstet Gynecol.* (2017) 217:693. e1–e16. doi: 10.1016/j.ajog.2017.09.013
143. Espinoza J, Chaiworapongsa T, Romero R, Edwin S, Rathnasabapathy C, Gomez R, et al. Antimicrobial peptides in amniotic fluid: defensins, calprotectin and bacterial/permeability-increasing protein in patients with microbial invasion of the amniotic cavity, intra-amniotic inflammation, preterm labor and premature rupture of membranes. *J Matern Fetal Neonatal Med.* (2003) 13:2–21. doi: 10.1080/jmf.13.1.2.21
144. Soto E, Espinoza J, Nien JK, Kusanovic JP, Erez O, Richani K, et al. Human beta-defensin-2: a natural antimicrobial peptide present in amniotic fluid participates in the host response to microbial invasion of the amniotic cavity. *J Matern Fetal Neonatal Med.* (2007) 20:15–22. doi: 10.1080/14767050601036212
145. Varrey A, Romero R, Panaitescu B, Miller D, Chaiworapongsa T, Patwardhan M, et al. Human beta-defensin-1: a natural antimicrobial peptide present in amniotic fluid that is increased in spontaneous preterm labor with intra-amniotic infection. *Am J Reprod Immunol.* (2018) 80:e13031. doi: 10.1111/aji.13031
146. Gomez-Lopez N, Romero R, Garcia-Flores V, Xu Y, Leng Y, Alhousseini A, et al. Amniotic fluid neutrophils can phagocytize bacteria: a mechanism for microbial killing in the amniotic cavity. *Am J Reprod Immunol.* (2017) 78:e12723. doi: 10.1111/aji.12723
147. Gomez-Lopez N, Romero R, Leng Y, Garcia-Flores V, Xu Y, Miller D, et al. Neutrophil extracellular traps in acute chorioamnionitis: a mechanism of host defense. *Am J Reprod Immunol.* (2017) 77:e12617. doi: 10.1111/aji.12617
148. Gomez-Lopez N, Romero R, Xu Y, Miller D, Unkel R, Shaman M, et al. Neutrophil extracellular traps in the amniotic cavity of women with intra-amniotic infection: a new mechanism of host defense. *Reprod Sci.* (2017) 24:1139–53. doi: 10.1177/1933719116678690
149. Gomez-Lopez N, Romero R, Xu Y, Miller D, Leng Y, Panaitescu B, et al. The immunophenotype of amniotic fluid leukocytes in normal and complicated pregnancies. *Am J Reprod Immunol.* (2018) 79:e12827. doi: 10.1111/aji.12827
150. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med.* (2014) 6:237ra265. doi: 10.1126/scitranslmed.3008599
151. Doyle RM, Alber DG, Jones HE, Harris K, Fitzgerald F, Peebles D, et al. Term and preterm labour are associated with distinct microbial community structures in placental membranes which are independent of mode of delivery. *Placenta* (2014) 35:1099–101. doi: 10.1016/j.placenta.2014.10.007
152. Antony KM, Ma J, Mitchell KB, Racusin DA, Versalovic J, Aagaard K. The preterm placental microbiome varies in association with excess maternal gestational weight gain. *Am J Obstet Gynecol.* (2015) 212:653.e1-16. doi: 10.1016/j.ajog.2014.12.041
153. Zheng J, Xiao X, Zhang Q, Mao L, Yu M, Xu J. The placental microbiome varies in association with low birth weight in full-term neonates. *Nutrients* (2015) 7:6924–37. doi: 10.3390/nu7085315
154. Bassols J, Serino M, Carreras-Badosa G, Burcelin R, Blasco-Baque V, Lopez-Bermejo A, et al. Gestational diabetes is associated with changes in placental microbiota and microbiome. *Pediatr Res.* (2016) 80:777–84. doi: 10.1038/pr.2016.155
155. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci Rep.* (2016) 6:23129. doi: 10.1038/srep23129
156. Prince AL, Ma J, Kannan PS, Alvarez M, Gisslen T, Harris RA, et al. The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. *Am J Obstet Gynecol.* (2016) 214:627.e1–16. doi: 10.1016/j.ajog.2016.01.193
157. Doyle RM, Harris K, Kamiza S, Harjunmaa U, Ashorn U, Nkhoma M, et al. Bacterial communities found in placental tissues are associated with severe chorioamnionitis and adverse birth outcomes. *PLoS ONE* (2017) 12:e0180167. doi: 10.1371/journal.pone.0180167
158. Gomez-Arango LF, Barrett HL, Mcintyre HD, Callaway LK, Morrison M, Nitert MD. Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. *Sci Rep.* (2017) 7:2860. doi: 10.1038/s41598-017-03066-4
159. Parnell LA, Briggs CM, Cao B, Delannoy-Bruno O, Schrieffer AE, Mysorekar IU. Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. *Sci Rep.* (2017) 7:11200. doi: 10.1038/s41598-017-11514-4
160. Zheng J, Xiao XH, Zhang Q, Mao LL, Yu M, Xu JP, et al. Correlation of placental microbiota with fetal macrosomia and clinical characteristics in mothers and newborns. *Oncotarget* (2017) 8:82314–25. doi: 10.18632/oncotarget.19319
161. Leon LJ, Doyle R, Diez-Benavente E, Clark TG, Klein N, Stanier P, et al. Enrichment of clinically relevant organisms in spontaneous preterm delivered placenta and reagent contamination across all clinical groups in a large pregnancy cohort in the United Kingdom. *Appl Environ Microbiol.* (2018) 84:e00483–18. doi: 10.1128/AEM.00483-18
162. Lauder AP, Roche AM, Sherrill-Mix S, Bailey A, Laughlin AL, Bittinger K, et al. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. *Microbiome* (2016) 4:29. doi: 10.1186/s40168-016-0172-3
163. Hornef M, Penders J. Does a prenatal bacterial microbiota exist? *Mucosal Immunol.* (2017) 10:598–601. doi: 10.1038/mi.2016.141
164. Perez-Munoz ME, Arrieta MC, Ramer-Tait AE, Walter J. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses:

implications for research on the pioneer infant microbiome. *Microbiome* (2017) 5:48. doi: 10.1186/s40168-017-0268-4

165. de Goffau MC, Lager S, Salter SJ, Wagner J, Kronbichler A, Charnock-Jones DS, et al. Recognizing the reagent microbiome. *Nat Microbiol.* (2018) 3:851–3. doi: 10.1038/s41564-018-0202-y
166. Willyard C. Could baby's first bacteria take root before birth? *Nature* (2018) 553:264–6. doi: 10.1038/d41586-018-00664-8
167. Gomez De Aguero M, Ganal-Vonarburg SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The maternal microbiota drives early postnatal innate immune development. *Science* (2016) 351:1296–302. doi: 10.1126/science.aad2571

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: 29 January 2019
doi: 10.3389/fimmu.2019.00092

Type 3 ILCs in Lung Disease

Amanda Ardain[1,2], *James Zachary Porterfield*[1,2,3], *Henrik N. Kløverpris*[1,4,5] *and Alasdair Leslie*[1,5*]

[1] *Africa Health Research Institute, Durban, South Africa,* [2] *College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa,* [3] *Yale School of Public Health, Yale University, New Haven, CT, United States,* [4] *Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark,* [5] *Department of Infection and Immunity, University College London, London, United Kingdom*

The lungs represent a complex immune setting, balancing external environmental signals with a poised immune response that must protect from infection, mediate tissue repair, and maintain lung function. Innate lymphoid cells (ILCs) play a central role in tissue repair and homeostasis, and mediate protective immunity in a variety of mucosal tissues, including the lung. All three ILC subsets are present in the airways of both mice and humans; and ILC2s shown to have pivotal roles in asthma, airway hyper-responsiveness, and parasitic worm infection. The involvement of ILC3s in respiratory diseases is less well-defined, but they are known to be critical in homeostasis, infection and inflammation at other mucosal barriers, such as the gut. Moreover, they are important players in the IL17/IL22 axis, which is key to lung health. In this review, we discuss the emerging role of ILC3s in the context of infectious and inflammatory lung diseases, with a focus on data from human subjects.

Keywords: ILC3, lung disease, pnemonia, tuberulosis, COPD, airway hypersensitivity, pulmonary fibrosis

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
William Garrow Kerr,
Upstate Medical University, United States
Avinash Bhandoola,
National Institutes of Health (NIH), United States

****Correspondence:***
Alasdair Leslie
al.leslie@ahri.org

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

Received: *26 November 2018*
Accepted: *14 January 2019*
Published: *29 January 2019*

Citation:
Ardain A, Porterfield JZ, Kløverpris HN and Leslie A (2019) Type 3 ILCs in Lung Disease. Front. Immunol. 10:92. doi: 10.3389/fimmu.2019.00092

INTRODUCTION

The lungs are complex organs, the proper functioning of which is essential for good health, but whose exposure to the external environment renders them susceptible to infections and environmental toxins. The average person will breathe 11,500 L of air into their lungs in a given day. This air is full of small particulate matter that includes more than just the bacteria and viruses most typically associated with a lung immune response, and also includes dust, dirt, smoke, pollen, aerosols, and liquid droplets. The lung immune environment must therefore strike a delicate balance between dealing with this constant assault, whilst preventing immune mediated damage. This results in an intricate immune setting that is still not completely understood (1), but which growing evidence suggests includes an important role for innate lymphoid cells (ILCs).

Innate lymphoid cells (ILCs) are a family of innate immune cells that lack specific antigen receptors but produce a broad range of effector cytokines and have diverse roles at barrier surfaces. ILCs mediate protective immunity from pathogens and promote tissue repair and homeostasis following infection, but may also play a role in pathogenesis when their functions become dysregulated (2–4). Broadly, ILCs are classified into three subsets based on surface markers, cytokine production, and transcription factor requirements (5). ILC1s are classically associated with the immune response to viral infection and tumor cells, while ILC2s are often compared to $CD4^+$ Th2 cells and mediate the response to helminth infections and allergies. ILC3s are the most heterogeneous subset of ILCs, defined by expression of the Retinoid-related orphan receptor γt (RORγt), and produce IL-17, IL-22, granulocyte-macrophage colony stimulating factor (GM-CSF) and/or tumor necrosis factor α (TNFα) in response to IL-23, IL1-β and aryl hydrocarbon receptor (AHR) ligands (2, 6) (**Figure 1**). ILC3s can be broadly categorized into two lineages: the lymphoid tissue inducer cells (LTi cells) including fetal and adult-equivalent LTi-like cells

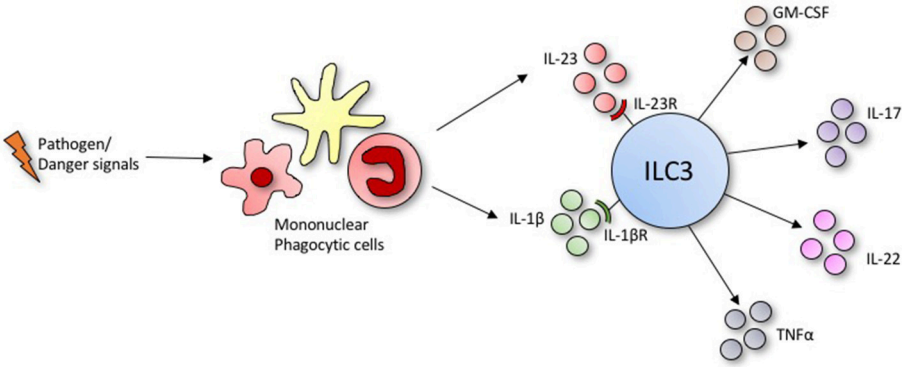

FIGURE 1 | Under conditions of stress or infection, ILC3s produce an array of effector cytokines in response to activating ligands secreted by phagocytic mononuclear cells.

(7), that have established roles in lymphoid organogenesis and secondary lymphoid tissue development, respectively; and non-LTi ILC3s which may or may not express natural cytotoxicity receptors Nkp44 and/or Nkp46 (8). ILC3s are widely distributed throughout the body and are constitutively present at mucosal barrier sites such as the lung, liver, gut, spleen, skin and secondary lymphoid tissues (9), where they play crucial roles in immunity and tissue homeostasis through the production of cytokines and interaction with the adaptive immune system (10).

While all three ILC subsets have been identified in the airways, the majority of studies to date have focused on ILC2s, highlighting their importance in tissue repair, protection from helminth infections, and involvement in multiple allergic diseases (3, 11, 12). However, ILC3s are in fact the most prevalent group in human lung tissues (13), and their rapid secretion of IL-17A and IL-22 has prompted investigations into their involvement in inflammatory and infectious diseases in the lung, as the IL-17/IL-22 axis is so important to lung health (14) (Overview in **Figure 2**). Moreover, GM-CSF also plays an important role in allergic airway disease, antimicrobial pulmonary host defense function, and is essential for surfactant homeostasis (15). Although the importance of ILCs as a source of GM-CSF in the lung remains untested, ILC3s in the gut are known to orchestrate inflammation through its activity (6).

INFECTIOUS DISEASES

The constitutive presence of ILC3s at mucosal barriers, including the lung, and their rapid production of cytokines allows them to efficiently orchestrate inflammation and antimicrobial peptide production, providing essential protection at these mucosal sites (4, 16, 17). Here we discuss several important human lung infections in which ILC3s may play an important role.

Viral Infections

The influenza viruses are some of the most important human respiratory pathogens, causing substantial seasonal, and pandemic morbidity and mortality (18). Following infection, the innate immune system provides the first line of defense, with various innate cell types playing important roles in killing infected cells and/or limiting viral replication. However, an excessive innate immune response correlates with poor outcomes and can lead to pathology (19, 20), and lung regeneration following viral-induced injury is vital (18). ILC2s have been best studied in relation to influenza, due to their ability to mediate tissue repair following Influenza infection via the production of amphiregulin (3). However, both IL-22 and IL-17 are implicated in the immune response to pulmonary viral infection, suggesting a potential role for ILC3s, particularly prior to the onset of the adaptive immune response or in primary infection. Mice infected with Influenza A/PR/8, for example, produced IL-17A as early as 2 days post-infection, resulting in lung injury associated with excessive neutrophil recruitment (21), and IL-17 deficiency or treatment with anti-IL-17 antibodies is able to ameliorate IL-17 associated lung injury in infected mice (22). However, as with many things in immunology, this is a double edged sword, as IL-17 also plays an important protective role in preventing secondary bacterial infections. Two separate studies have shown in co-infection experiments with influenza and *Staphylococcus aureus*, that the induction of type I interferon from the virus increased susceptibility to secondary bacterial pneumonia by directly inhibiting IL-17 production (23, 24). Irrespective, IL-17 is likely to be relevant in human influenza infection, as elevated levels of IL-17 and IL-17 associated cytokines are detected in serum from humans infected with swine-origin Influenza virus (22). As antigen-specific $CD4^+$ T cells do not appear in the lung earlier than 4 days post-infection (25), innate sources of IL-17 are likely to play an important role. γδ T cells have been implicated as being the early source of IL-17 (21, 26) however, although not directly linked as yet, it is likely that ILC3s represent an additional source of IL-17 during the first week of an influenza infection, particularly in primary infections.

IL-22, also has critical functions in host defense and in maintaining epithelial integrity during influenza infection (10, 27). In mice infected with a sub-lethal dose of H3N2 Influenza A, ILCs had enhanced expression of *IL-22* transcripts as early as 2 days post-infection, and mice able to produce IL-22 had reduced lung injury and better protection from post-influenza

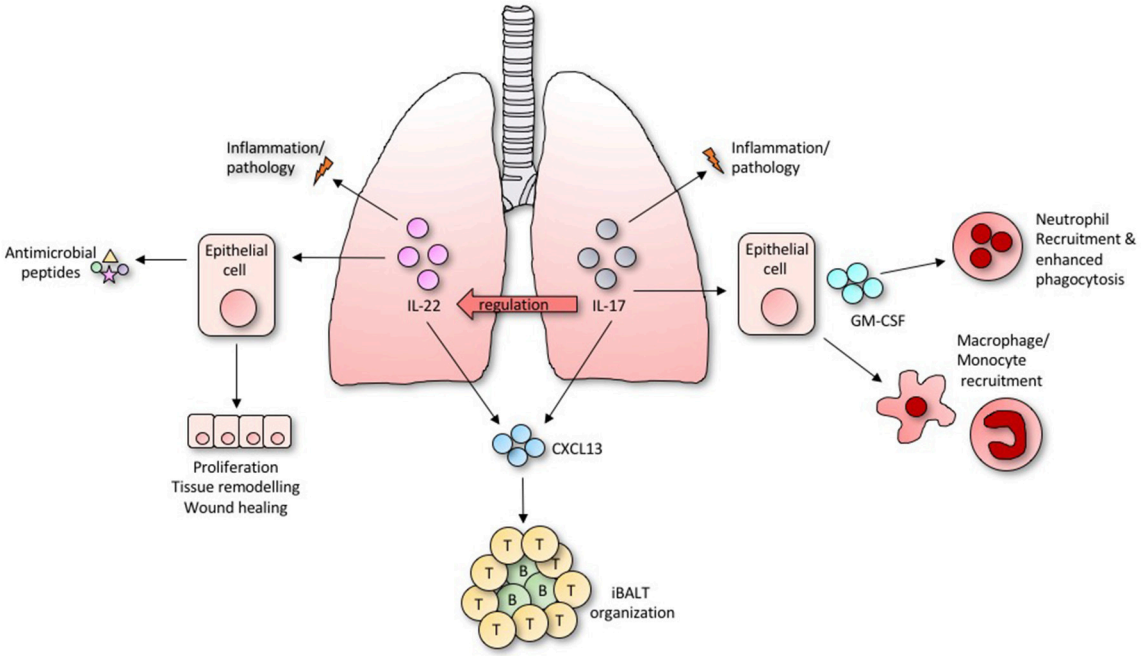

FIGURE 2 | Diverse and overlapping roles of IL-22 and IL-17 within the lung.

Streptococcus pneumoniae superinfection, in comparison to IL-22-deficient animals (28). In another study, IL-22 knock out mice were found to have exacerbated lung injury compared with wild-type mice, correlating with decreased lung function 21 days post-infection with a PR8/34 H1N1 virus (29). Similarly, Kumar et al. (30) found that IL-22$^{-/-}$ mice infected with a PR8 Influenza virus had severely impaired epithelial regeneration and continuous loss of body weight, which was restored by adoptive transfer of IL-22 sufficient, "ILC3-like" CD3^{-}NCR1^{+}NK1.1^{+} cells. Guo and Topham (31), also identified a CD3-NCR1+NK1.1+ subset as the predominant producers of IL-22, although the protective role of IL-22 was less clear in this instance. Subsequently, transcription factor staining and transcriptomics of mucosal barrier surfaces have demonstrated that ILC3s are a major source of IL-22 (32).

Bacterial Pneumonia

Pneumonia remains one of the largest infectious causes of death globally, accounting for the deaths of 16% of children under 5 years old (33).

The severity of pneumonia depends on the interplay between the ability of the host to control infection and the extent to which the stress of microbial challenge can be tolerated (34). IL-17 is important in the control of several key bacterial causes of pneumonia in humans, including *Klebsiella pneumoniae, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa* (23, 35–37). Humans with inherited complete autosomal recessive IL-17RA deficiency, for example, have increased susceptibility to pneumonia (38). In mechanistic studies, IL-17R knockout mice were found to be particularly susceptible to *K. pneumonia*, demonstrating 100% mortality after just 48 h in comparison to 40% mortality amongst controls (39). This was attributed to a considerable delay in alveolar neutrophil recruitment via IL-17. Similarly, IL-17R deficient mice, or mice with depleted neutrophil populations had significantly diminished protection from pneumococcal infection in the lung, and IL-17 is known to enhance the phagocytic activity of neutrophils *in vitro* (40). These observations are supported by recent work showing that IL-17 signaling in the lung epithelium plays a critical role in establishing the chemokine gradients that are essential for mucosal immunity against pulmonary bacterial pathogens. Here, mice that lacked IL-17R expression specifically on lung epithelia lost CXCR5 signaling and failed to recruit sufficient neutrophils to clear *K. pneumoniae* (36). The protective effect of IL-17 is not only limited to the activity neutrophils. Clearance of primary infection with *S. pneumoniae,* for example, requires the recruitment of monocyte/macrophages, and this protective response is blocked by the depletion of IL-17 (41). Again, while Th17 and γδ T-cells are important sources of IL-17 in the recall response to bacterial infection (37, 42), in primary infections, other sources, including ILC3s are likely to be crucial. This is highlighted by the recent work of Xiong et al. (43) demonstrating the importance of ILCs in primary infection with *K. pneumoniae*. This study described a positive feedback loop in which TNF-producing monocytes recruited to lungs drove an increase in IL-17-secreting ILCs, which in turn enhanced monocyte-mediated bacterial uptake and killing. Importantly, bacterial clearance was similar in T-cell deficient and WT mice, but depletion of ILC3s led to increased bacterial burden and mortality (43). In experimental infection with *P. aeruginosa,* for which IL-17 is also

essential, 90% of early IL-17 production appears to come from ILC3 rather than CD3+ lineages, including γδ T-cells (35).

As with viral infection, IL-22 also plays key roles in host response to bacterial pneumonia. In mice, IL-22 is detected in lungs as early as 6 h post-infection, where it increases lung epithelial cell proliferation and significantly augments killing of *K. pneumoniae in vitro* by upregulating the expression of lipocalin-2 (44). The early expression of IL-22 implicates an innate source. Following on from this Van Maele et al. (32), using $Rag^{-/-}$ and $Rag^{-/-}/Il2rg^{-/-}$ mice, demonstrated IL-22 production by CCR6+ ILC3s during *S. pneumoniae* infection. Interestingly, this ILC3 response could be boosted by exogenous administration of the TLR ligand flagellin, and was sufficient to control and protect from an otherwise lethal infection (32). This raises the intriguing possibility that modulation of ILC3s may represent a novel treatment modality for some lung infections. The potential interplay between IL-22 and IL-17 in bacterial pneumonia is highlighted by recent work showing that, whilst IL-17 driven neutrophil recruitment maybe required for bacterial clearance, infection with *P. aeruginosa* in the absence of IL-22 is associated with pathogenesis and increased susceptibility driven by an excessive neutrophil response (45). Finally, IL-22 production by ILC3s was recently found to be vital in neonates, being the primary source of this cytokine in both mice and humans. In this study, recruitment of IL-22-producing ILC3s to the lung, driven by commensal microbes in the gut, was required for protection from *S. pneumoniae* infection (46). This unexpected link between the gut microbiome and ILC3-mediated immunity in the lung again raises the possibility of manipulating ILC3s to improve lung health. It also demonstrates, as with primary infection, that the importance of ILC3 produced cytokines may vary depending on the context.

Tuberculosis

Tuberculosis (TB), despite typically being a fully treatable disease, continues to contribute to more deaths worldwide each year than any other infectious disease (47), pulmonary or otherwise. While the immune response to TB is complex and not fully understood, human and mouse models have progressively elucidated roles for many different immune cells including alveolar macrophages, epithelial cells, and CD4+ T cells (48, 49). Studies of ILCs in this mix are extremely limited to date. However, ILCs are activated and accumulate in the lungs and lymph nodes of mice following intranasal vaccination with *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) (50). Moreover, their roles in lung tissue repair, hepatic granuloma formation (3, 51), and pro-inflammatory cytokine production support their involvement. Indeed, prior to the description of ILCs, Feng et al. (52) found that $Rag^{-/-}\gamma c^{-/-}$ mice, which lack T-cells and all ILC subsets, had significantly poorer survival than T-cell deficient $Rag2^{-/-}$ mice or WT controls, reflected in a more than 2-fold increase in lung bacterial burden. Consistent with this, whilst Th1-associated molecules, such as TNFα and IFNγ are crucial to mycobacterial resistance (53–55), recent studies have extended this to include IL-17 and IL-22. BCG-infected mice, for example, fail to develop mature granulomas in the absence of IL-17 (56), and both IL-17 and IL-22 protect mice from the hypervirulent strain *Mtb* HN878 (57, 58). Using cells sorted from human lung tissue explants, ILC3s have recently been show to upregulate GM-CSF and IL-22 transcripts when stimulated with *Mtb ex vivo* (48), and IL-22-producing NK cells, now recognized as ILC3s, inhibit *Mtb* growth by enhanced phagolysosomal fusion (59). Additionally, humans with inherited bi-allelic RORC mutations, which lead to compromised IL-17A/F mediated immunity, have markedly increased susceptibility to mycobacterial disease (60).

One potential mechanism by which ILCs may contribute to the immune response to TB infection is through the formation of inducible bronchus-associate lymphoid tissue (iBALT). These are tertiary lymphoid structures that develop following pulmonary inflammation, at least in part through the IL-17/22 axis, and which appear to orchestrate protective immune responses (57, 61–63). Several independent studies have highlighted the importance of iBALT formation in the immunopathology of TB. Mice deficient in IL-17 had reduced B cell follicle formations during early stages of *Mtb* challenge, while IL-22-deficient mice had impaired follicle formation at an intermediate stage, although neither of these deficiencies impacted the mouse's ability to control the infection. A lack of IL-23, which induces cytokine production by ILC3s, led to both a sustained follicle deficiency and increased bacterial growth (64). Separately, IL-17, and not IFNγ, was found to be required for mucosal vaccine-induced immunity against *Mtb*, and was associated with the induction of organized iBALT structures in the lung (65). Thus, early and/or sustained production of IL17/IL22 by ILC3s may play and important role in iBALT formation during *Mtb* infection. It is important to note, however, that iBALT formation can be observed in neonatal RORγt KO mice (66), showing that ILC3s are not absolutely required for this process.

AUTOIMMUNE DISEASE

ILC3s have been associated with several autoimmune diseases, including inflammatory bowel disease, multiple sclerosis, psoriatic arthritis, and ankylosing spondylitis, either by direct evidence or by involvement of the IL17/IL22 axis (67–70). ILCs are also implicated in numerous autoimmune diseases which can have lung associated pathology, including systemic sclerosis, systemic lupus erythromatosus, and the ANCA-associated vasculitides, however, the relationship is most clearly worked out in asthma, COPD, and pulmonary fibrosis.

Asthma and Airway Hyper-Reactivity

Asthma is a chronic respiratory disease characterized by airflow obstruction, excessive inflammation and airway remodeling (71). IL-17 appears to be an important cytokine driving this this condition. IL-17 expressing cells, for example, are elevated in both sputum and Bronchoalveolar lavage fluid (BALF) from asthmatic patients, (72) and more recently, IL-17+ ILC3s in particular were found to be increased in BALF from human patients with severe asthma (73). Consistent with this, ILC3 gene signatures are enriched in total RNA from patients with adult onset asthma (74). In mice, ILC3s facilitate obesity-linked asthma via IL-17 production. Here, in both wild-type mice fed a high fat diet, and mice that are obese due to a mutation in the leptin gene, airway hyperresponsiveness was found to mediated by IL-17+ ILC3s (73). Importantly, this was observed in T-cell deficient

mice and was induced by adoptive transfer of ILC3s into mice lacking all lymphoid cells. Subsequently, IL-17-producing ILC3s were similarly implicated in allergic asthma. Here, IL-17+ ILC3s and ILC2s were increased in mice fed a high fat diet following house dust mite challenge, and this was ameliorated by antibody depletion of ILCs using anti-CD90 (75).

IL-22 expression is associated with increased eosinophil recruitment to lungs and airway hyper-responsiveness, and can be detected in mice with ovalbumin induced allergic asthma (76), and in serum from human patients (77). The major source of IL-22 in mouse models is yet to be confirmed, as one study identified the origin to be CD4+ T cells (78), while another study found it was predominantly produced by lineage negative, $CD90^+$, $Sca\text{-}1^+$ ILCs (79). However, in both studies, as well as others, neutralization of IL-22 led to increased eosinophil infiltration and allergic inflammation, while treatment with recombinant IL-22 ameliorated this response (76–79). Here, the implication that IL-17 is detrimental and IL-22 protective in these situations is likely to be a simplistic one due to the cross talk between these cytokines. Besnard et al. (77), for example found that IL-22 played a protective role in the initial stages following antigen challenge, but exacerbated inflammation in the later stages.

COPD

Chronic obstructive pulmonary disease (COPD) is largely caused by smoking, and is associated with an aberrant inflammatory lung environment (80), characterized by lung destruction and increased neutrophilic infiltration (81). Consistent with their roles in tissue immunity and homeostasis, recent studies have identified roles for ILCs in COPD, particularly ILC1s (82). However, ILC3s may also be involved, as IL-17 is recognized as a key driver of neutrophilic inflammation in COPD (83). In mice, cigarette smoke exposure leads to an increase of all ILC subsets in BALF, particularly IFNγ+ and IL-17+ ILCs (84). In line with this, human patients with COPD have higher sputum IL-17 than non-smoking controls (85), and IL-17-, IL-22-, and IL-23-expressing cells are increased in bronchial biopsies from COPD patients (81). While direct mechanistic evidence linking ILC3s to COPD is limited, one human study showed that a subset of ILC3s was notably increased in lung tissue from COPD patients, and an increase in IL-17 and IL-22 production by ILC3s (13). In separate work, gene set enrichment analysis revealed LTi ILC3s and NK genes are enriched in lymphocytes sorted from human lungs tissues of patients with severe COPD (86).

Pulmonary Fibrosis

Pulmonary fibrosis occurs as a result of damage and scarring of the lung parenchyma that can have numerous causes including, environmental toxins, medication side effects, radiation therapy, and autoimmune conditions. In fibrotic disease, chronic inflammation and persistent extracellular matrix deposition can lead to remodeling and progressive tissue destruction (87). This remodeling and extracellular matrix deposition is largely driven by connective tissue cells, such as fibroblasts, whose functions are directly affected by pro-fibrotic cytokines produced by adaptive and innate immune cells (88). The types of inflammatory processes facilitated by ILCs, and their cytokine expression suggests they are substantial contributors to fibrosis (87). While all three ILC subsets secrete fibroblast activating cytokines, ILC3s have capacity to regulate synovial fibroblasts and connective lung tissue cells via IL-17 (89, 90), and elevated levels of IL-17 have been observed in patients with cystic and pulmonary fibrosis (91, 92). In a mouse model of fibrosis, IL-17 was produced early on (between days 2 and 7), and associated with neutrophil influx into the lungs. Mice deficient in IL-17 had lower neutrophil frequencies and better disease outcomes (92). It seems that timing is important, however, as treatment with IL-17 has no effect on collagen deposition during chronic disease (93).

IL-22 is also involved in fibrosis, again in a largely protective role. In a mouse model of hypersensitivity pneumonia, for example, blockade of IL-22 signaling caused accelerated lung fibrosis and enhanced collagen deposition in the lung, which was inhibited by the administration of recombinant IL-22 (94). Similarly, bleomycin treatment in mice, which induces fibrosis, significantly reduced IL-22 in their lungs, and human epithelial cell lines treated with IL-22 had a slower progression to fibrosis (95). However, again this protective view of IL-22 is probably simplistic as one study found that bleomycin induced IL-22 production was only protective in IL-17 deficient mice (96). Conversely, in the presence of IL-17, IL-22 was proinflammatory and promoted airway pathology, which was blocked by the administration of anti-IL-22 antibody. Clearly then, the involvement of IL-17 and IL-22 in the lung should not be considered separately. As important innate sources of both cytokines in the lung, it appears likely that ILC3s would have a role in pulmonary fibrosis and they produce represent promising new targets for molecular therapies (92, 94).

CONCLUDING REMARKS

As we have highlighted here, there are many studies showing the importance of ILC3 cytokines in lung immunity. In many cases the early source of cytokines such as IL-17 and IL-22 has been assumed to be γδ T cells, as antigen-specific T cells only accumulate after several days (25). However, we now know that ILC3s, though fewer in number, are prolific producers of IL-17 and/or IL-22, as well as other important lung cytokines such as GM-CSF, and thus have the capacity to drive inflammation or repair in a number of infectious and autoimmune diseases (97–100). This is certainly supported by the relatively few studies to date that have directly sought to investigate their importance as a source of these cytokines. Clearly much remains to be done, but the possibility that lung ILC3s may be manipulated to influence lung health is intriguing and should encourage these further studies.

AUTHOR CONTRIBUTIONS

AA and JP: Literature research and writing; HK and AL: Literature research and editing.

FUNDING

This work was supported by Wellcome Trust by AL Grant No. 210662/Z/18/Z; HK Grant No. 202485/Z/16/Z.

REFERENCES

1. Saluzzo S, Gorki AD, Rana BMJ, Martins R, Scanlon S, Starkl P, et al. First-breath-induced type 2 pathways shape the lung immune environment. *Cell Rep.* (2017) 18:1893–905. doi: 10.1016/j.celrep.2017.01.071
2. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive IL-23 dependent innate intestinal pathology. *Nature* (2010) 464:1371–5. doi: 10.1038/nature08949
3. Monticelli La, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CGK, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1038/ni.2131
4. Satoh-Takayama N, Vosshenrich CAJ, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* (2008) 29:958–70. doi: 10.1016/j.immuni.2008.11.001
5. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells - a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–49. doi: 10.1038/nri3365
6. Pearson C, Thornton EE, McKenzie B, Schaupp AL, Huskens N, Griseri T, et al. ILC3 GM-CSF production and mobilisation orchestrate acute intestinal inflammation. *eLife* (2016) 5:1–21. doi: 10.7554/eLife.10066
7. Kim MY, Rossi S, Withers D, McConnell F, Toellner KM, Gaspal F, et al. Heterogeneity of lymphoid tissue inducer cell populations present in embryonic and adult mouse lymphoid tissues. *Immunology* (2008) 124:166–74. doi: 10.1111/j.1365-2567.2007.02750.x
8. Montaldo E, Juelke K, Romagnani C. Group 3 innate lymphoid cells (ILC3s): origin, differentiation, and plasticity in humans and mice. *Eur J Immunol.* (2015) 45:2171–82. doi: 10.1002/eji.201545598
9. Kim CH, Hashimoto-hill S, Kim M. Migration and tissue tropism of innate lymphoid cells. *Trends Immunol.* (2016) 37:68–79. doi: 10.1016/j.it.2015.11.003
10. Sonnenberg GF, Fouser La, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol.* (2011) 12:383–90. doi: 10.1038/ni.2025
11. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol.* (2012) 129:191–8.e1–4. doi: 10.1016/j.jaci.2011.09.041
12. Turner J-E, Morrison PJ, Wilhelm C, Wilson M, Ahlfors H, Renauld J-C, et al. IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J Exp Med.* (2013) 210:2951–65. doi: 10.1084/jem.20130071
13. De Grove KC, Provoost S, Verhamme FM, Bracke KR, Joos GF, Maes T, et al. Characterization and quantification of innate lymphoid cell subsets in human lung. *PLoS ONE* (2016) 11:e0145961. doi: 10.1371/journal.pone.0145961
14. Gurczynski SJ, Moore BB. IL-17 in the lung: the good, the bad, and the ugly. *Am J Physiol.*(2017) 314:L6–16. doi: 10.1152/ajplung.00344.2017
15. Rösler B, Herold S. Lung epithelial GM-CSF improves host defense function and epithelial repair in influenza virus pneumonia—a new therapeutic strategy? *Mol Cell Pediatr.* (2016) 3:29. doi: 10.1186/s40348-016-0055-5
16. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JKM, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* (2009) 457:722–5. doi: 10.1038/nature07537
17. Cupedo T, Crellin NK, Papazian N, Rombouts EJ, Weijer K, Grogan JL, et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol.* (2009) 10:66–74. doi: 10.1038/ni.1668
18. Herold S, Becker C, Ridge KM, Budinger GRS. Influenza virus-induced lung injury: pathogenesis and implications for treatment. *Eur Respir J.* (2015) 45:1463–78. doi: 10.1183/09031936.00186214
19. Cole SL, Ho L-P. Contribution of innate immune cells to pathogenesis of severe influenza virus infection. *Clin Sci.* (2017) 131:269–83. doi: 10.1042/CS20160484
20. Wang Z, Zhang A, Wan Y, Liu X, Qiu C, Xi X, et al. Early hypercytokinemia is associated with interferon- induced transmembrane protein-3 dysfunction and predictive of fatal H7N9 infection. *Proc Natl Acad Sci USA.* (2014) 111:769–74. doi: 10.1073/pnas.1321748111
21. Crowe CR, Chen K, Pociask DA, Alcorn JF, Krivich C, Enelow RI, et al. Critical role of IL-17RA in immunopathology of inflenza infection. *J Immunol.* (2013) 183:5301–10. doi: 10.4049/jimmunol.0900995.
22. Li C, Yang P, Sun Y, Li T, Wang C, Wang Z, et al. IL-17 response mediates acute lung injury induced by the 2009 pandemic influenza A (H1N1) virus. *Cell Res.* (2011) 22:528–38. doi: 10.1038/cr.2011.165
23. Kudva A, Scheller EV, Robinson KM, Crowe CR, Mi Choi S, Slight SR, et al. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J Immunol.* (2011) 186:166–1674. doi: 10.1021/nl061786n.Core-Shell
24. Robinson KM, Choi SM, McHugh KJ, Mandalapu S, Enelow RI, Kolls JK, et al. Influenza A exacerbates *Staphylococcus aureus* pneumonia by attenuating IL-1 production in mice. *J Immunol.* (2013) 191:5153–9. doi: 10.4049/jimmunol.1301237
25. Doherty PC, Christensen JP. ACCESSING COMPLEXITY : the dynamics of virus-specific T cell Responses. *Annu Rev Immunol.* (2000) 18:561–92. doi: 10.1146/annurev.immunol.18.1.561
26. Xue C, Wen M, Bao L, Li H, Li F, Liu M, et al. Vγ4+ γδT Cells Aggravate severe H1N1 inuenza virus infection-induced acute pulmonary immunopathological injury via secreting interleukin-17A. *Front. Immunol.* (2017) 8:1054. doi: 10.3389/?mmu.2017.01054
27. Rutz S. IL-22, not simply a Th17 cytokine. *Immunol Rev.* (2013) 252:116–32. doi: 10.1111/imr.12027
28. Ivanov S, Renneson J, Fontaine J, Barthelemy A, Paget C, Fernandez EM, et al. Interleukin-22 reduces lung inflammation during influenza A virus infection and protects against secondary bacterial infection. *J Virol.* (2013) 87:6911–24. doi: 10.1128/JVI.02943-12
29. Pociask DA, Scheller EV, Mandalapu S, McHugh KJ, Enelow RI, Fattman CL, et al. IL-22 is essential for lung epithelial repair following influenza infection. *Am J Pathol.* (2013) 182:1286–96. doi: 10.1016/j.ajpath.2012.12.007
30. Kumar P, Thankar MS, Ouyang W, Malarkannan S. IL-22 from conventional NK cells is epithelial regenerative and inflammation protective during influenza infection. *Mucosal Immunol.* (2013) 6:69–82. doi: 10.3109/02656736.2015.1007538.Histotripsy
31. Guo H, Topham DJ. Interleukin-22 (IL-22) production by pulmonary natural killer cells and the potential role of IL-22 during primary influenza virus infection. *J. Virol.* (2010) 84:7750–9. doi: 10.1128/JVI.00187-10
32. Van Maele L, Carnoy C, Cayet D, Ivanov S, Porte R, Deruy E, et al. Activation of type 3 innate lymphoid cells and interleukin 22 secretion in the lungs during *Streptococcus pneumoniae* infection. *J Infect Dis.* (2014) 210:493–503. doi: 10.1093/infdis/jiu106
33. World Health Organization. *Pneumonia Fact Sheet* (2016). Available online at: http://www.who.int/news-room/fact-sheets/detail/pneumonia (Accessed July 30, 2018).
34. Mizgerd JP. Pathogenesis of severe pneumonia - advances and knowledge gaps. *Curr Opin Pulm Med.* (2017) 23:193–7. doi: 10.1097/MCP.0000000000000365
35. Bayes HK, Ritchie ND, Evans TJ. IL-17 is required for control of chronic lung infection caused by *Pseudomonas aeruginosa*. *Infect Immun.* (2016) 84:3507–16. doi: 10.1128/IAI.00717-16
36. Chen K, Eddens T, Trevejo-nunez G, Way EE, Elsegeiny W, Ricks DM, et al. IL-17 receptor signalling in the lung epithelium is required for mucosal chemokine gradients and pulmonary defence against *K. pneumoniae. Cell Host Microb.* (2017) 20:596–605. doi: 10.1016/j.chom.2016.10.003.IL-17
37. Murakami T, Hatano S, Yamada H, Iwakura Y, Yoshikai Y. Two types of interleukin 17A-producing γδ T cells in protection against pulmonary infection with *Klebsiella pneumoniae*. *J Infect Dis.* (2016) 214:1752–61. doi: 10.1093/infdis/jiw443
38. Lévy R, Okada S, Béziat V, Moriya K, Liu C, Chai LYA, et al. Genetic, immunological, and clinical features of patients with bacterial and fungal infections due to inherited IL-17RA deficiency. *Proc Natl Acad Sci USA.* (2016) 8277–85. doi: 10.1073/pnas.1618300114
39. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung

Cxc chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med.* (2001) 194:519–28. doi: 10.1084/jem.194.4.519
40. Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, Zhang Q, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.* (2008) 4:e1000159. doi: 10.1371/journal.ppat.1000159
41. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Investig.* (2009) 119:1899–909. doi: 10.1172/JCI36731
42. Chen K, McAleer JP, Lin Y, Paterson DL, Zheng M, Alcorn JF, et al. Th17 cells mediate clade specific, serotype-independent mucosal immunity. *Immunity* (2011) 35:997–1009. doi: 10.1016/j.dci.2009.07.003
43. Xiong H, Keith JW, Samilo DW, Carter RA, Leiner IM, Pamer EG. Innate lymphocte/Ly6Chi monocyte crosstalk promotes *Klebsiella pneumoniae* clearance. *Cell* (2017) 165:679–89. doi: 10.1016/j.cell.2016.03.017.Innate
44. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med.* (2008) 14:275–81. doi: 10.1038/nm1710
45. Broquet A, Jacqueline C, Davieau M, Besbes A, Roquilly A, Martin J, et al. Interleukin-22 level is negatively correlated with neutrophil recruitment in the lungs in a *Pseudomonas aeruginosa* pneumonia model. *Sci Rep.* (2017) 7:1–11. doi: 10.1038/s41598-017-11518-0
46. Gray J, Oehrle K, Worthen G, Alenghat T, Whitsett J, Deshmukh H. Intestinal commensal bacteria mediate lung mucosal immunity and promote resistance of newborn mice to infection. *Sci Transl Med.* (2017) 9:eaaf9412. doi: 10.1126/scitranslmed.aaf9412
47. WHO. *Global Tuberculosis Report 2016. Cdc 2016, (Global TB Report 2016)* (2016). 214 p.
48. Maertzdorf J, Tonnies M, Lozza L, Schommer-Leitner S, Mollenkopf H, Bauer TT, Kaufmann SHE. *Mycobacterium tuberculosis* invasion of the human lung : first contact. *Front Immunol.* (2018) 9:1346. doi: 10.3389/fimmu.2018.01346
49. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MPR. The immune response in tuberculosis. *Annu Rev Immunol.* (2013) 31:475–527. doi: 10.1146/annurev-immunol-032712-095939
50. Steigler P, Daniels NJ, McCulloch TR, Ryder BM, Sandford SK, Kirman JR. BCG vaccination drives accumulation and effector function of innate lymphoid cells in murine lungs. *Immunol Cell Biol.* (2018) 96:379–89. doi: 10.1111/imcb.12007
51. McHedlidze T, Waldner M, Zopf S, Walker J, Rankin AL, Schuchmann M, et al. Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity* (2013) 39:357–71. doi: 10.1016/j.immuni.2013.07.018
52. Feng CG, Kaviratne M, Rothfuchs AG, Cheever A, Hieny S, Young HA, et al. NK Cell-Derived IFN- differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with *Mycobacterium tuberculosis*. *J Immunol.* (2006) 177:7086–93. doi: 10.4049/jimmunol.177.10.7086
53. Cooper BAM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med.* (1993) 178:2243–7.
54. Flynn JAL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor-α is required in the protective immune response against mycobacterium tuberculosis in mice. *Immunity* (1995) 2:561–72. doi: 10.1016/1074-7613(95)90001-2
55. Keane J, Gershon S, Wise RP, Mirable-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *NEngl J Med.* (2001) 345:1098–104. doi: 10.2165/00128415-200108740-00003
56. Yoshida YO, Umemura M, Brien RLO, Ikuta K, Kishihara K. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *J Immunol.* (2010) 184:4414–22. doi: 10.4049/jimmunol.0903332
57. Gopal R, Monin L, Slight S, Uche U, Blanchard E, Fallert Junecko BA, et al. Unexpected role for IL-17 in protective immunity against hypervirulent *Mycobacterium tuberculosis* HN878 infection. *PLoS Pathog.* (2014) 10:e1004099. doi: 10.1371/journal.ppat.1004099
58. Treerat P, Prince O, Cruz-Lagunas A, Munoz-Torrico M, Salazar-Lezama MA, Selman M, et al. Novel role for IL-22 in protection during chronic *Mycobacterium tuberculosis* HN878 infection. *Mucosal Immunol.* (2017) 10:1069–81. doi: 10.1159/000450855.Gene-environment
59. Dhiman R, Indramohan M, Barnes PF, Nayak RC, Paidipally P, Rao LVM, et al. IL-22 Produced by human NK cells inhibits growth of *Mycobacterium tuberculosis* by enhancing phagolysosomal fusion. *J Immunol.* (2009) 183:6639–45. doi: 10.4049/jimmunol.0902587
60. Okada S, Markle JG, Deenick EK, Mele F, Averbuch D, Lagos M, et al. Impairment of immunity to Candida and Mycobacterium in humans with bi-allelic RORC mutations. *Science* (2016) 349:606–13. doi: 10.1111/acem.12550.Hot
61. Day TA, Koch M, Nouailles G, Jacobsen M, Kosmiadi GA, Miekley D, et al. Secondary lymphoid organs are dispensable for the development of T-cell-mediated immunity during tuberculosis. *Eur J Immunol.* (2010) 40:1663–73. doi: 10.1002/eji.201040299
62. Slight SR, Rangel-moreno J, Gopal R, Lin Y, Junecko BAF, Mehra S, et al. CXCR5 + T helper cells mediate protective immunity against tuberculosis. *J Clin Investig.* (2013) 123:1–15. doi: 10.1172/JCI65728DS1
63. Ulrichs T, Kosmiadi GA, Trusov V, Jörg S, Pradl L, Titukhina M, et al. Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. *J Pathol.* (2004) 204:217–28. doi: 10.1002/path.1628
64. Khader SA, Guglani L, Rangel-moreno J, Gopal R, Beth A, Junecko F, Fountain JJ, et al. IL-23 is required for long-term control of *Mycobacterium tuberculosis* and B cell follicle formation in the infected lung. *J Immunol.* (2012) 187:5402–7. doi: 10.4049/jimmunol.1101377.IL-23
65. Gopal R, Rangel-Moreno J, Slight S, Lin Y, Nawar HF, Fallert Junecko BA, et al. Interleukin-17-dependent CXCL13 mediates mucosal vaccine–induced immunity against tuberculosis. *Mucosal Immunol.* (2013) 6:972–84. doi: 10.1038/mi.2012.135
66. Rangel-Moreno J, Carragher DM, de la Lu Garcia-Hernandez M, Hwang Y, Kusser K, Hartson L, et al.. The development of inducible Bronchus-associated lymphoid tissue (iBALT) is dependent on IL-17. *Nat Immunol.* (2012) 12:639–46. doi: 10.1038/ni.2053.The
67. Ciccia F, Accardo-Palumbo A, Alessandro R, Rizzo A, Principe S, Peralta S, et al. Interleukin-22 and interleukin-22-producing NKp44+ natural killer cells in subclinical gut inflammation in ankylosing spondylitis. *Arthritis Rheum.* (2012) 64:1869–78. doi: 10.1002/art.34355
68. Roan F, Stoklasek TA, Whalen E, Molitor JA, Bluestone JA, Buckner JH, et al. CD4 $^{+}$ Group 1 Innate Lymphoid Cells (ILC) form a functionally distinct ILC subset that is increased in systemic sclerosis. *J Immunol.* (2016) 196:2051–62. doi: 10.4049/jimmunol.1501491
69. Shikhagaie MM, Germar K, Bal SM, Ros XR, Spits H. Innate lymphoid cells in autoimmunity: emerging regulators in rheumatic diseases. *Nat Rev Rheumatol.* (2017) 13:164–73. doi: 10.1038/nrrheum.2016.218
70. Teunissen MBM, Yeremenko NG, Baeten DLP, Chielie S, Spuls PI, De Rie MA, et al. The IL-17A-producing CD8 + T-cell population in psoriatic lesional skin comprises mucosa-associated invariant t cells and conventional t cells. *J Investig Dermatol.* (2014) 134:2898–907. doi: 10.1038/jid.2014.261
71. Saha S, Doe C, Mistry V, Siddiqui S, Parker D, Sleeman M, et al. Granulocyte-macrophage colony-stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD. *Thorax* (2009) 64:671–6. doi: 10.1136/thx.2008.108290
72. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol.* (2001) 108:430–8. doi: 10.1067/mai.2001.117929
73. Kim HY, Lee HJ, Chang Y-J, Pichavant M, Shore Sa, Fitzgerald KA, et al. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med.* (2014) 20:54–61. doi: 10.1038/nm.3423
74. Hekking PP, Loza MJ, Pavlidis S, de Meulder B, Lefaudeux D, Baribaud F, et al. Pathway discovery using transcriptomic profiles in adult-onset severe asthma. *J Allergy Clin Immunol.* (2018) 141:1280–90. doi: 10.1016/j.jaci.2017.06.037
75. Everaere L, Ait-Yahia S, Molendi-Coste O, Vorng H, Quemener S, LeVu P, et al. Innate lymphoid cells contribute to allergic airway disease

exacerbation by obesity. *J Allergy Clin Immunol.* (2016) 138:1309–1318.e11. doi: 10.1016/j.jaci.2016.03.019
76. Schnyder B, Lima C, Schnyder-Candrian S. Interleukin-22 is a negative regulator of the allergic response. *Cytokine* (2010) 50:220–7. doi: 10.1016/j.cyto.2010.02.003
77. Besnard AG, Sabat R, Dumoutier L, Renauld JC, Willart M, Lambrecht B, et al. Dual role of IL-22 in allergic airway inflammation and its cross-talk with IL-17A. *Am J Respir Crit Care Med.* (2011) 183:1153–63. doi: 10.1164/rccm.201008-1383OC
78. Takahashi K, Hirose K, Kawashima S, Niwa Y, Wakashin H, Iwata A, et al. IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation. *J Allergy Clin Immunol.* (2011) 128:1067–1076.e6. doi: 10.1016/j.jaci.2011.06.018
79. Taube C, Tertilt C, Gyülveszi G, Dehzad N, Kreymborg K, Schneeweiss K, et al. IL-22 is produced by innate lymphoid cells and limits inflammation in allergic airway disease. *PLoS ONE* (2011) 6:e21799. doi: 10.1371/journal.pone.0021799
80. World Health Organization. *Global Surveilance, Prevention and Control of Chronic Respiratory Diseases: A Comprehensive Approach*. Geneva: WHO Press (2007).
81. Stefano ADi, Caramori G, Gnemmi I, Contoli M, Vicari C. T helper type 17 -related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol.* (2009) 157:316–24. doi: 10.1111/j.1365-2249.2009.03965.x
82. Silver JS, Kearley J, Copenhaver AM, Sanden C, Mori M, Yu L, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat Immunol.* (2016) 17:626–35. doi: 10.1038/ni.3443
83. Lindén A, Hoshino H, Laan M. Airway neutrophils and interleukin-17. *Eur Respir J.* (2000) 15:973–7. doi: 10.1034/j.1399-3003.2000.15e28.x
84. Blomme E, Provoost S, Bracke KR, Joos GF, Brusselle GG, Maes T. Quantification of innate lymphoid cell subsets in a mouse model of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* (2018) 197:A4757. Available online at: https://www.atsjournals.org/doi/abs/10.1164/ajrccm-conference.2018.197.1_MeetingAbstracts.A4757
85. Doe C, Bafadhel M, Siddiqui S, Mccormick M, Woods J, May R. Expression of the T Helper 17-associated cytokines IL-17A and IL-17F in asthma. *Chest* (2010) 138:1140–7. doi: 10.1378/chest.09-3058
86. Suzuki M, Sze MA, Campbell JD, Brothers JF, Lenburg ME, McDonough JE, et al. The cellular and molecular determinants of emphysematous destruction in COPD. *Sci Rep.* (2017) 7:9562. doi: 10.1038/s41598-017-10126-2
87. Zhang Y, Tang J, Tian Z, van Velkinburgh JC, Song J, Wu Y, et al. Innate lymphoid cells: a promising new regulator in fibrotic diseases. *Int Rev Immunol.* (2016) 35:399–414. doi: 10.3109/08830185.2015.1068304
88. Richards CD. Innate immune cytokines, fibroblast phenotypes, and regulation of extracellular matrix in lung. *J Interferon Cytok Res.* (2017) 37:52–61. doi: 10.1089/jir.2016.0112
89. Kehlen A, Pachnio A, Thiele K, Langner J. Gene expression induced by interleukin-17 in fibroblast-like synoviocytes of patients with rheumatoid arthritis: upregulation of hyaluronan-binding protein TSG-6. *Arthritis Res Ther.* (2003) 5:R186–92. doi: 10.1186/ar762
90. Saleh A, Shan L, Halayko AJ, Kung S, Gounni AS. Critical role for STAT3 in IL-17A-mediated CCL11 expression in human airway smooth muscle cells. *J Immunol.* (2009) 182:3357–65. doi: 10.4049/jimmunol.0801882
91. Brodlie M, McKean MC, Johnson GE, Anderson AE, Hilkens CMU, Fisher AJ, et al. Raised interleukin-17 is immunolocalised to neutrophils in cystic fibrosis lung disease. *Eur Respir J.* (2011) 37:1378–85. doi: 10.1183/09031936.00067110
92. Wilson MS, Madala SK, Ramalingam TR, Gochuico R, Rosas IO, Cheever AW, et al. Bleomycin and IL-1B – mediated pulmonary fibrosis is IL-17A dependent. *J Exp Med.* (2006) 207:535–52. doi: 10.1084/jem.20092121
93. Lo Re S, Dumoutier L, Couillin I, Van Vyve C, Yakoub Y, Uwambayinema F, et al. IL-17A-Producing T and Th17 lymphocytes mediate lung inflammation but not fibrosis in experimental silicosis. *J Immunol.* (2010) 184:6367–77. doi: 10.4049/jimmunol.0900459
94. Simonian PL, Wehrmann F, Roark CL, Born WK, O'Brien RL, Fontenot AP. γδ T cells protect against lung fibrosis via IL-22. *J Exp Med.* (2010) 207:2239–53. doi: 10.1084/jem.20100061
95. Liang M, Wang J, Chu H, Zhu X, He H, Liu Q, et al. Interleukin-22 inhibits bleomycin-induced pulmonary fibrosis. *Mediat Inflamm.* (2013) 2013:209179. doi: 10.1155/2013/209179
96. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser La, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *J Exp Med.* (2010) 207:1293–305. doi: 10.1084/jem.20092054
97. Carrega P, Loiacono F, Di Carlo E, Scaramuccia A, Mora M, Conte R, et al. NCR(+)ILC3 concentrate in human lung cancer and associate with intratumoral lymphoid structures. *Nat Commun.* (2015) 6:8280. doi: 10.1038/ncomms9280
98. Hagau N, Slavcovici A, Gonganau DN, Oltean S, Dirzu DS, Brezoszki ES, et al. Clinical aspects and cytokine response in severe H1N1 influenza A virus infection. *Crit Care* (2010) 14:R203. doi: 10.1186/cc9324
99. Sendo S, Saegusa J, Okano T, Takahashi S, Akashi K, Morinobu A. CD11b+Gr-1dim tolerogenic dendritic cell–like cells are expanded in interstitial lung disease in SKG Mice. *Arthritis Rheumatol.* (2017) 69:2314–27. doi: 10.1002/art.40231
100. Tan H, Regamey N, Brown S, Bush A, Lloyd CM, Davies JC. The Th17 pathway in cystic fibrosis lung disease. *Am J Respir Crit Care Med.* (2011) 184:252–8. doi: 10.1164/rccm.201102-0236OC

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

MINI REVIEW
published: 07 February 2019
doi: 10.3389/fimmu.2019.00171

ILC2 Orchestration of Local Immune Function in Adipose Tissue

Cécile Bénézech[1]* *and Lucy Helen Jackson-Jones*[2]*

[1] BHF Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom, [2] Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, United Kingdom

ILC2s were originally identified as IL-5 and IL-13 secreting "natural helper cells" present within the fat-associated lymphoid clusters of the mesenteries in both mouse and man. The presence of ILCs in adipose tissue has more recently expanded to include all ILC groups. Since their initial discovery, our knowledge of these cells and their role in adipose immune responses has expanded significantly. In this review we summarize the current literature on the role that ILC2s play in orchestrating adipose tissue function in both lean and obese states. We go on to address new data detailing interactions of adipose ILCs with innate like B-cells (IBC) and discuss how this interaction results in localized protection of mucosal sites during infection and inflammation via the production of innate antibodies.

Keywords: adipose, ILC2, FALCs, innate, antibodies, thermogenesis, mucosal, atherosclerosis

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Timotheus You Fu Halim,
University of Cambridge, United Kingdom
Massimo Vitale,
Azienda Ospedaliera Universitaria San Martino (IRCCS), Italy

***Correspondence:**
Cécile Bénézech
cbenezec@exseed.ed.ac.uk
Lucy Helen Jackson-Jones
l.jackson-jones@lancaster.ac.uk

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

Received: *28 November 2018*
Accepted: *21 January 2019*
Published: *07 February 2019*

Citation:
Bénézech C and Jackson-Jones LH (2019) ILC2 Orchestration of Local Immune Function in Adipose Tissue. Front. Immunol. 10:171. doi: 10.3389/fimmu.2019.00171

INTRODUCTION

Innate lymphoid cells are the newest kids on the block in terms of innate immune cell function, however the previous 8 years have revealed a wealth of information on these previously enigmatic lymphocyte like cells. In a non-activated state, ILCs possess lymphocyte morphology but lack the expression of surface markers used to define other immune cell populations. ILCs are thus described as "lineage negative." Non-cytotoxic ILCs are currently segregated into three transcriptionally defined groups that mirror the four major T-helper cell subsets. Tbet dependent ILC1s which secrete IFNγ and TNFα, GATA3 dependent ILC2s which secrete IL-5/IL-13 [and can secrete IL-10 (1)], RORγt dependent ILC3s which secrete IL-17A/IL-22, and include a population of Lymphoid tissue inducer (LTi) cells which are critical for secondary lymphoid organ development (2) and finally, Id3 dependent ILCregs which produce IL-10 and require autocrine TGF-β1 (3). In addition to these non-cytotoxic cell types, are the classical cytotoxic NK cells that are important for protection against viruses and cancer. Although ILCs were first described as natural helper cells (ILC2) in the fat-associated lymphoid clusters (FALCs) of the mesenteries where they support antibody responses, their presence and importance has since been extended to the whole adipose organ with ILCs having been reported in most fat depots. ILCs are now considered as key regulators of adipose tissue function. IBCs are B cells with "innate like" properties; they have a poly-specific B-cell receptor repertoire and rapidly produce polyclonal IgM in response to both self and microbial antigens (4). Here we will discuss (1) the central regulatory role of ILC2 in the regulation of adipose tissue homeostasis and (2) the key role of ILCs in activation of the IBC compartment during infection at mucosal sites.

ILC2s ARE CRITICAL REGULATORS OF TYPE 2 IMMUNE CELLS TO MAINTAIN WHITE ADIPOSE TISSUE HOMEOSTASIS

Recently, it has become apparent that type 2 immune cells play a critical role in the maintenance of homeostasis in lean, healthy adipose tissue and that ILC2 are central regulators of this function.

Type 2 immune cells including ILC2, T regulatory cells (Treg), T-helper type 2 cells (Th2), Eosinophils, mast cells, and M2 macrophages are prevalent in healthy adipose tissue where they contribute to adipose tissue remodeling, counteracting the inflammatory effect of obesity and inducing browning of white adipose tissue (5, 6). Here, we will concentrate on the role of ILC2 in orchestrating the function of type 2 immune cells in adipose tissue.

ILC2s and Immune Homeostasis in White Adipose Tissue

ILC2s are present within visceral adipose tissue (VAT), where they are the predominant producers of IL-5 and IL-13 at homeostasis and following prolonged exposure to IL-33 or helminth infection (7, 8). Th2 cells remain a minor population of IL-5 and IL-13 producing cells within the VAT even during helminth infection (8). In lean adipose tissue, IL-33 drives the recruitment and/or proliferation of ILC2 but the cellular origin of IL-33 and the mechanisms leading to its secretion at homeostasis remains poorly understood. While we reported that Gp38$^+$ stromal cells of fat-associated lymphoid clusters express high levels of IL-33, others showed that IL-33 is also expressed by Gp38$^+$ fibroblasts, Cadherin-11$^+$ mesenchymal cells, or endothelial cells of the stromal vascular fraction of adipose tissue (9–12). It is likely that the relevant source of IL-33 in adipose tissue is context dependent and further work is needed to elucidate the mechanism of IL-33 action in adipose tissue. Tissue ILC2s are key producers of systemic IL-5 required for homeostatic eosinophil maintenance (13). In adipose tissue, secretion of IL-5 by ILC2 is essential for the recruitment and maintenance of eosinophils (8) and is dependent on IL-33 (8) (**Figure 1**). Secretion of IL-13 and IL-4 by ILC2 and eosinophils is critical for the maintenance of alternatively activated or M2-like adipose tissue macrophages and glucose homeostasis (8, 14). The precise phenotype and origin of these macrophages is not known. Interestingly IL-33 has been shown to be competent to induce macrophage proliferation independently of IL-4Rα expression in other non-adipose macrophages populations (15) and whether IL-33 can directly activate adipose tissue macrophages remains to be investigated.

Pioneering work by the group of Diane Mathis demonstrated the existence of a unique subset of GATA-3$^+$ PPARγ$^+$ regulatory T cells in adipose tissue important for preventing insulin resistance (16, 17). Regulatory T cells in adipose tissue express the IL-33 receptor ST2 and require IL-33 for their maintenance (18). Additionally, expression of ICOSL by adipose tissue ILC2 provides additional signaling through ICOS in regulatory T cells for their accumulation within VAT (10). Halim et al. elegantly advance these finding by showing that in the absence of ILC2s or specifically the absence of OX40L expression by ILC2s there is a significant deficit in the number of GATA3$^+$ T-regulatory cells within the perigonadal adipose tissue following IL-33 delivery (19).

ILC2s and Adipose Tissue Browning

Brown and beige adipose tissue are fat depots specialized in the dissipation of energy for the production of heat. While brown adipose tissue is mostly found in infants and regresses with age, white adipose tissue can undergo "browning" to form beige adipose tissue, expressing the thermogenic protein Ucp1 during exposure to cold (20). Two distinct mechanisms involving ILC2s have been implicated in the browning of adipose tissue. Mechanism one relies on the IL-33 dependent induction of methionine-enkephalin peptide release from ILC2s that acts directly on adipocytes to upregulate UCP-1 and induce beiging (21). The second published mechanism involves pharmacologic expansion and activation of ILC2 with IL-33 in thermoneutral mice which induces the proliferation of adipocytes and their differentiation into beige adipocytes (22). This is dependent on the release of IL-4 and IL-13 by ILC2 and the direct activation of adipocyte precursor cells via the IL-4Rα (22). ILC2 may also be important for the activation of eosinophils during acute cold exposure and the secretion of IL-4/13, which have been reported to induce browning through activation of alternatively activated macrophage production of catecholamines (23). However, the mechanisms leading to secretion of IL-33 upon cold exposure were not elucidated. The production of catecholamines by alternatively activated macrophages is controversial with a recent report stating that alternatively activated macrophages do not produce catecholamines and are thus unlikely to have a direct role in adipocyte metabolism or adaptive thermogenesis (24).

Is There a Link Between the Gut Mucosa and the Metabolic Regulatory Function of ILC2 in Adipose Tissue?

In the small intestine, the release of IL-5 and IL-13 by ILC2 is increased by food intake, leading to fluctuation in the levels of circulating eosinophils during the day (13). It would be interesting to know if the secretion of IL-5 and IL-13 or other important mediators such as methionine-enkephalin peptides by adipose tissue ILC2s fluctuates with food intake, thus allowing the synchronization of adipose tissue function with food intake via immune regulation.

A LINK BETWEEN ADIPOSE TISSUE ILC2s AND METABOLIC DYSFUNCTION

During obesity the number of ILC2s decreases in adipose tissue both in mouse and human, leading to decrease in overall Type-2 immunity and increased inflammation in adipose tissue. Importantly, the loss of ILC2 in obesity can be reversed by IL-33 injection in obese mice restoring glucose tolerance and insulin sensitivity. However, the mechanisms leading to the loss of ILC2 during obesity are not well-understood. Interestingly, a population of ILC1s expand in the adipose tissue during diet-induced obesity and produce IFN-γ in response to IL-12, contributing to inflammation and insulin resistance (25). IFN-γ has an antagonistic effect on ILC2 (10) which may be responsible for the loss of ILC2 during obesity. It is also possible that IFNγ and or IL-12 drives the conversion of ILC2 toward ILC1 during diet-induced obesity, as described in response to IL-12 (26). In addition, upregulation of PD-1 expression on ILC2 and its engagement via PD-L1^{hi} M1 macrophages has recently been

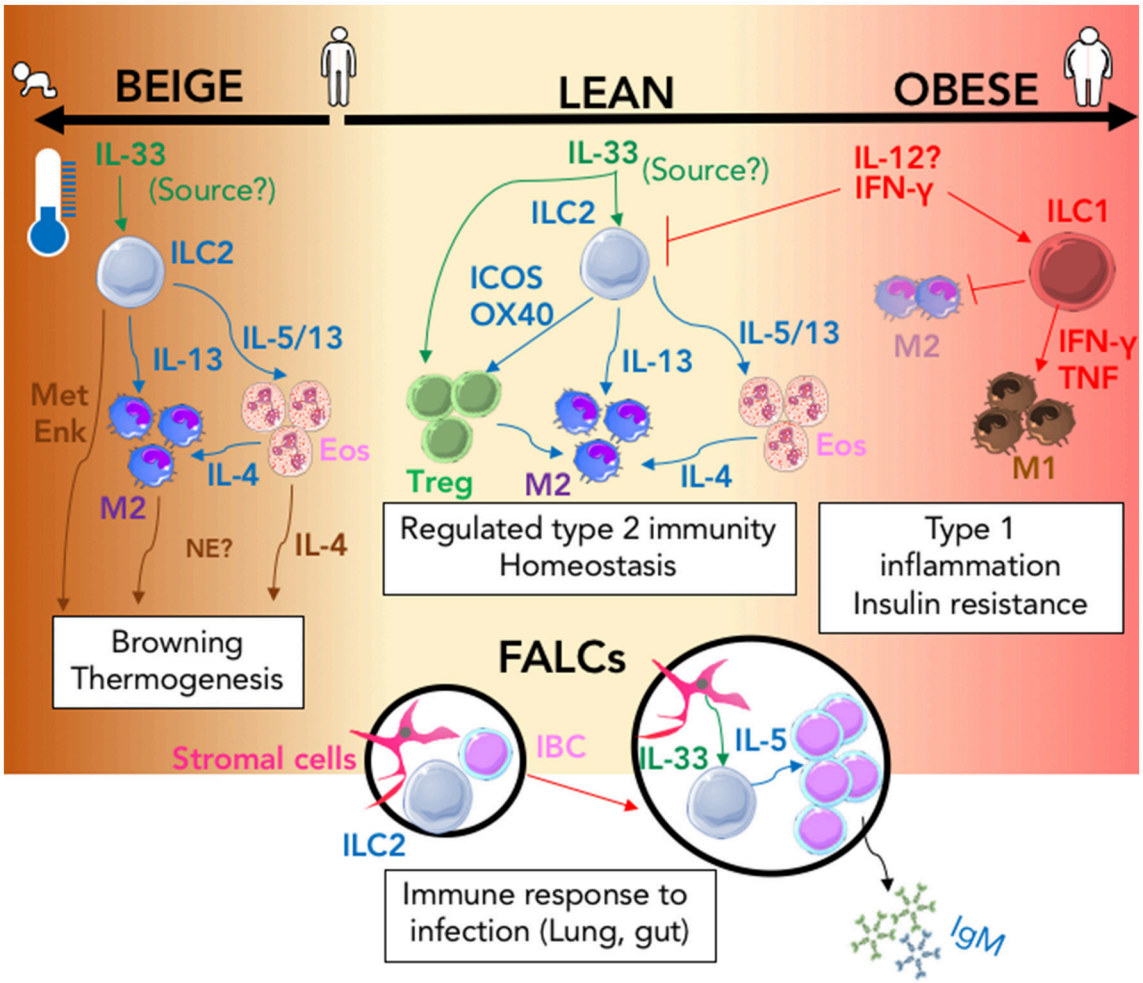

FIGURE 1 | The ILC2 driven interactions that regulate immune adipose function. In the lean state (center; cream) IL-33 action (green arrows) signals to both T-regulatory cells (Treg) and ILC2 resulting in regulated Type-2 immunity via the activity of secreted and membrane bound type-2 signals (blue arrows); this response is amplified in the presence of lower ambient living temperature and during infancy and can result in browning thermogenesis within adipose tissue (Left; brown). Type-2 signals that can control browning are shown (brown arrows). In the obese state (right; pink) Inflammation mediated by type-1 signals (red arrows) promotes the activation of ILC1 and the inhibition of ILC2 which results in inhibition of M2 and expansion of the M1 macrophage population which contribute to the development of insulin resistance. During Type-2 inflammation within the lung or gut, ILC2 containing FALCs (Black circles) expand; IL-33 produced by stromal cells (green arrow) increases IL-5 secretion (blue arrow) from ILC2 which induces innate like B cell (IBC) proliferation and secretion of IgM. (MetEnk, methionine-enkephalin peptides; NE, norepinephrine; Eos, Eosinophils; IBC, Innate Like B cell; M1/M2, M1, or M2 macrophage; FALCs, Fat-associated lymphoid clusters).

described to inhibit the protective function of ILC2s during obesity. Within obese adipose, increased PD-1 expression on ILC2s was dependent on TNFα and IL-33 (27).

In the second half of this mini-review, the original role of ILCs in the initiation of local immune function in FALCs is discussed and extended to include the newly described pleural FALCs (11, 28, 29); finally we discuss the interaction between ILC2s, IBCs, and IgM during atherosclerosis.

FAT-ASSOCIATED LYMPHOID CLUSTER FUNCTION IN MUCOSAL DEFENSE

The peritoneal and pleural cavities, primarily considered as sites of macrophage (30) and B1 cell residence represent compartments that demarcate, contain and protect the boundaries between three major mucosal sites directly exposed to environmental antigens; namely the lungs, the intestines and the reproductive tract (of females) (**Figure 2**). Immune protection within the body cavities is co-ordinated by small, inducible lymphoid clusters found within specialized small adipose tissues (the mediastinum, pericardium, mesenteries, and omentum). Initially described as "milky-spots" within the omentum (31) these inducible structures were rebranded in 2010 as Fat-Associated Lymphoid Clusters or FALCs (32). FALCs are local hubs that are important for providing a second line of defense between the mucosal surfaces and a systemic immune response, working to compartmentalize antibody mediated immune

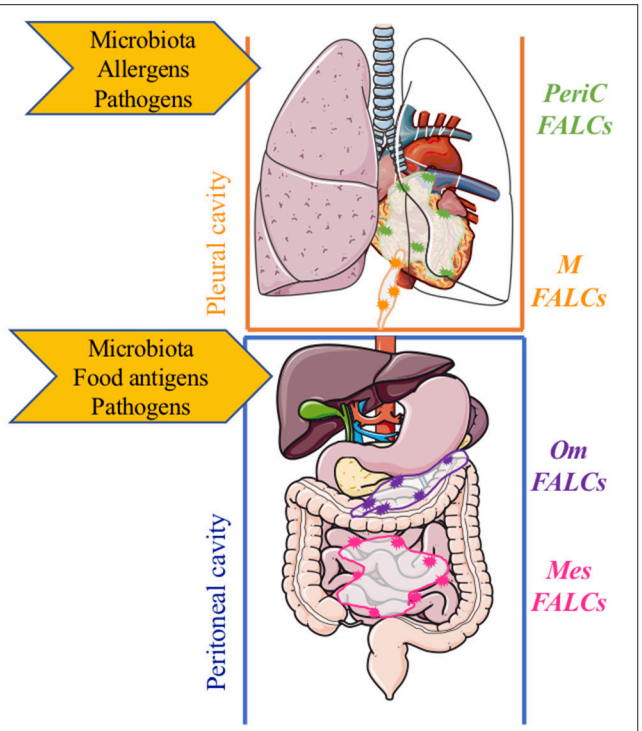

FIGURE 2 | Compartmentalized protection of mucosal sites by fat-associated lymphoid clusters within body cavities. Within the pleural cavity, protection from/regulation of, microbiota, infection, inflammation, and damage is mediated by inducible FALCs within the pericardium (green) and mediastinum (orange). Within the peritoneal cavity, protection from/regulation of microbiota, infection, inflammation, and damage is mediated by FALCs within the omentum (purple) and mesenteries (pink) m, mediastinal; PeriC, pericardial; om, omental; mes, mesenteric; FALCs, Fat-associated lymphoid clusters.

responses within body cavities. Evidence supporting FALC orchestration of antibody responses within the body cavities is mounting, with multiple reports linking FALCs to the initiation of T-independent and T-dependent immune responses (11, 29, 32–34).

FALCs, ILCs, AND THE INITIATION OF INNATE LIKE B CELL RESPONSES

Intestinal Barrier Functions

FALCs were identified as immune cell aggregates within the mesenteries, that were enriched in lineage negative, c-Kit$^+$, Sca-1$^+$ cells; these cells are now known as ILC2s (32, 35, 36). ILC2s are potent producers of IL-5 and IL-13; detectable levels of both cytokines are induced in the peritoneal lavage of *Rag2*$^{-/-}$ mice which do not have mature T or B cells, but are absent from $\gamma c^{-/-}$*Rag2*$^{-/-}$ following infection with the tissue migrating parasite *Nippostrongylus brasiliensis* (32). This result highlighted the potency of common-gamma chain receptor dependent innate immune cells for the initiation of immune responses within the peritoneal cavity in the context of intestinal worm infection. IL-5 is a critical growth factor for B1 B cells (37); Moro and colleagues showed, using elegant *in vivo* transfers and *in vitro* co-cultures of ILC2 with peritoneal B-cells in the presence or absence of a blocking antibody against IL-5, that ILC2s provide support for B1 cell self-renewal (32). ILC2s isolated from mesenteric FALCs were also shown to be competent for the induction of IgA secretion by peritoneal B cells *in vitro* (32). Peritoneal B1 cells have been shown to migrate to the intestinal lamina propria in order to secrete IgA (38, 39). In addition to the conventional "Type-2" cytokines described above, ILC2 have also been shown to secrete IL-6 (40, 41). As IL-6 has been described to induce antibody production by B-cells, as well as act as a growth factor for plasmablasts (42) and contribute to the regulation of T follicular helper cells (43), it is plausible that ILC2 secretion of this cytokine locally modifies FALC B-cell function; a hypothesis that warrants further experimental investigation to confirm. Contrary to secondary organs, the development of FALCs is not dependent on ILC3 as shown by the normal development and composition of FALCs in *Rorc*$^{-/-}$ mice (29). However, studies in germ free mice revealed that the number of FALCs forming in the mesenteries is decreased indicating that factors derived from the commensal flora are important to drive the formation of FALCs. ILC3s are an important innate source of GM-CSF, a cytokine required for the induction of IgM by innate response activator (IRA) B cells (44). Competency to support IgA secretion by B1 was also reported for peritoneal macrophages, which had been exposed to omentum culture supernatant (45). Given the almost certain presence of ILC derived factors within the omental culture supernatant, it is hard to know what component of the IgA secretion mediated by peritoneal macrophages is in part dependent upon ILCs. A thorough characterization of the ILC occupation of the murine omentum has not been carried out; however a recent report characterized the presence of ILCs in multiple human tissues including detailing the presence of ILC1 like cells within the omentum (46).

Pulmonary Barrier Functions

IgM is a large antibody and as such secretion of IgM into the circulation does not guarantee its presence at tissue sites where it is required. In the global absence of the IL-33R ST2, the secretion of IgM from FALCs within the pleural cavity is ablated (11). This is not a direct effect on the B-cells as co-transfer of IL-33R sufficient and deficient B-cells resulted in comparable induction of B-cell activation following *Alternaria alternata* delivery. Utilizing blocking antibodies against IL-5 delivered directly into the pleural space, we concluded that the IL-33 was acting via an IL-5 producing intermediate population of cells. ILC2s were the only cells found to be expressing IL-5 within FALCs of the pleural cavity during type-2 inflammation (11). Thus, the presence of IgM secreting B-cells within FALCs in the context of type-2 inflammation is assumed to depend upon IL-5 secretion from IL-33 activated ILC2s. The link between ILC2 and antibody production within the thoracic cavity was also made by Drake et al. (47) who showed that *in vitro* culture of lung derived ILCs with splenic B cells resulted in antibody production (47). However, as there are fewer B-cells within the lungs and because fluid phase B cells isolated from the pleural space do no secrete antibodies, it is likely that pleural

FALCs are the sites where the ILC/B cell interactions take place in the thoracic cavity. In support of a tight immune crosstalk between lung and pleural space is a report showing that delivery of GM-CSF secreting IRA B cells into the pleural space mediates protection from pneumonia (48). Neither the role of FALCs in the activation of the transferred IRA B cells nor the requirement for lung or FALC resident ILCs in this process was investigated. This study serves to further highlights the crosstalk which occurs between mucosal tissues and their associated serous cavities.

Is FALC Derived IgM Atheroprotective?

Innate like B-cells (IBCs) can be both protective and pathogenic in atherosclerosis. Recognition of oxidation specific epitopes on low density lipoproteins (LDL) (49) by natural IgM plays a protective role in atherosclerosis and clinical studies show that lower levels of IgM correlates with increased risk of cardiovascular diseases. The production of atheroprotective IgM by IBCs is dependent on IL-33 (50), IL-5 and IL-5 producing ILC2 (51, 52), a signaling loop that is active in FALCs (11). Importantly, it has been shown that the number of FALCs in the para-aortic adipose of ApoE$^{-/-}$ mice increases in the vicinity of atherosclerotic lesions (52) and that they contain IBC producing atheroprotective IgM (53). This suggests that ILC2 regulation of local IgM secretion by FALC IBCs could be key to IBC mediated atheroprotection and that loss of ILC2 duringthe development of obesity could contribute to accelerated atherosclerosis.

SUMMARY

Since their initial discovery 8 years ago, ILC2s have emerged as major regulators of type-2 immunity in adipose tissue where they co-ordinate eosinophil, macrophage, adipocyte and IBC function. FALCs are specialized hubs that act as a second line of immune defense sitting behind the mucosal frontline. Key to the initiation of a FALC response is the local secretion of cytokines by FALC resident ILCs, which kick-start the ensuing immune response following detection of a danger signal (e.g., IL-33).

AUTHOR CONTRIBUTIONS

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

FUNDING

Research in CBs laboratory is funded by the Medical Research Council (MRC) UK Grant (MR/M011542/1); Research in LJ-Js laboratory is funded by Lancaster University. **Figures 1**, **2** were designed using Servier Medical Art (www.servier.com) and used under a creative commons license.

REFERENCES

1. Seehus R, Kadavallore A, Torre B, Yeckes AR, Wang Y, Tang J, et al. Alternative activation generates IL-10 producing type 2 innate lymphoid cells. *Nat Commun.* (2017) 8:1900. doi: 10.1038/s41467-017-02023-z
2. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* (2015) 517:293–301. doi: 10.1038/nature14189
3. Wang S, Xia P, Chen Y, Qu Y, Xiong Z, Ye B, et al. Regulatory innate lymphoid cells control innate intestinal inflammation. *Cell* (2017) 171:201–16.e218. doi: 10.1016/j.cell.2017.07.027
4. Jackson-Jones LH, Benezech C. Control of innate-like B cell location for compartmentalised IgM production. *Curr Opin Immunol.* (2018) 50:9–13. doi: 10.1016/j.coi.2017.10.006
5. Odegaard JI, Chawla A. Type 2 responses at the interface between immunity and fat metabolism. *Curr Opin Immunol.* (2015) 36:67–72. doi: 10.1016/j.coi.2015.07.003
6. Villarroya F, Cereijo R, Villarroya J, Gavalda-Navarro A, Giralt M. Toward an understanding of how immune cells control brown and beige adipobiology. *Cell Metab.* (2018) 27:954–61. doi: 10.1016/j.cmet.2018.04.006
7. Hams E, Locksley RM, McKenzie AN, Fallon PG. Cutting edge: IL-25 elicits innate lymphoid type 2 and type II NKT cells that regulate obesity in mice. *J Immunol.* (2013) 191:5349–53. doi: 10.4049/jimmunol.1301176
8. Molofsky B, Nussbaum JC, Liang HE, Van Dyken SJ, Cheng LE, Mohapatra A, et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J Exp Med.* (2013) 210:535–49. doi: 10.1084/jem.20121964
9. Kolodin D, van Panhuys N, Li C, Magnuson AM, Cipolletta D, Miller CM, et al. Antigen- and cytokine-driven accumulation of regulatory T cells in visceral adipose tissue of lean mice. *Cell Metab.* (2015) 21:543–57. doi: 10.1016/j.cmet.2015.03.005
10. Molofsky B, Van Gool F, Liang HE, Van Dyken SJ, Nussbaum JC, Lee J, et al. Interleukin-33 and Interferon-gamma Counter-Regulate Group 2 Innate Lymphoid Cell Activation during Immune Perturbation. *Immunity* (2015) 43:161–74. doi: 10.1016/j.immuni.2015.05.019
11. Jackson-Jones LH, Duncan SM, Magalhaes MS, Campbell SM, Maizels RM, McSorley HJ, et al. Fat-associated lymphoid clusters control local IgM secretion during pleural infection and lung inflammation. *Nat Commun.* (2016) 7:12651. doi: 10.1038/ncomms12651
12. Kohlgruber C, Gal-Oz ST, LaMarche NM, Shimazaki M, Duquette D, Nguyen HN, et al. gammadelta T cells producing interleukin-17A regulate adipose regulatory T cell homeostasis and thermogenesis. *Nat Immunol.* (2018) 19:464–74. doi: 10.1038/s41590-018-0094-2
13. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* (2013) 502:245–8. doi: 10.1038/nature12526
14. Wu D, Molofsky AB, Liang HE, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, et al. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* (2011) 332:243–7. doi: 10.1126/science.1201475
15. Jackson-Jones LH, Ruckerl D, Svedberg F, Duncan S, Maizels RM, Sutherland TE, et al. IL-33 delivery induces serous cavity macrophage proliferation independent of interleukin-4 receptor alpha. *Eur J Immunol.* (2016) 46:2311–21. doi: 10.1002/eji.201646442
16. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med.* (2009) 15:930–9. doi: 10.1038/nm.2002
17. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, et al. PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* (2012) 486:549–53. doi: 10.1038/nature11132
18. Vasanthakumar A, Moro K, Xin A, Liao Y, Gloury R, Kawamoto S, et al. The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. *Nat Immunol.* (2015) 16:276–85. doi: 10.1038/ni.3085
19. Halim TYF, Rana BMJ, Walker JA, Kerscher B, Knolle MD, Jolin HE, et al. Tissue-restricted adaptive type 2 immunity is orchestrated by expression of the

costimulatory molecule OX40L on group 2 innate lymphoid cells. *Immunity* (2018) 48:1195–207.e1196. doi: 10.1016/j.immuni.2018.05.003
20. Poher L, Altirriba J, Veyrat-Durebex C, Rohner-Jeanrenaud F. Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance. *Front Physiol.* (2015) 6:4. doi: 10.3389/fphys.2015.00004
21. Brestoff JR, Kim BS, Saenz SA, Stine RR, Monticelli LA, Sonnenberg GF, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature* (2015) 519:242–6. doi: 10.1038/nature14115
22. Lee W, Odegaard JI, Mukundan L, Qiu Y, Molofsky AB, Nussbaum JC, et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. *Cell* (2015) 160:74–87. doi: 10.1016/j.cell.2014.12.011
23. Qiu Y, Nguyen KD, Odegaard JI, Cui X, Tian X, Locksley RM, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* (2014) 157:1292–308. doi: 10.1016/j.cell.2014.03.066
24. Fischer K, Ruiz HH, Jhun K, Finan B, Oberlin DJ, van der Heide V, et al. Alternatively activated macrophages do not synthesize catecholamines or contribute to adipose tissue adaptive thermogenesis. *Nat Med.* (2017) 23:623–30. doi: 10.1038/nm.4316
25. O'Sullivan TE, Rapp M, Fan X, Weizman OE, Bhardwaj P, Adams NM, et al. Adipose-resident group 1 innate lymphoid cells promote obesity-associated insulin resistance. *Immunity* (2016) 45:428–41. doi: 10.1016/j.immuni.2016.06.016
26. Lim AI, Menegatti S, Bustamante J, Le Bourhis L, Allez M, Rogge L, et al. IL-12 drives functional plasticity of human group 2 innate lymphoid cells. *J Exp Med.* (2016) 213:569–83. doi: 10.1084/jem.20151750
27. Oldenhove G, Boucquey E, Taquin A, Acolty V, Bonetti L, Ryffel B, et al. PD-1 is involved in the dysregulation of type 2 innate lymphoid cells in a murine model of obesity. *Cell Rep.* (2018) 25:2053–2060.e2054. doi: 10.1016/j.celrep.2018.10.091
28. Elewa YH, Ichii O, Otsuka S, Hashimoto Y, Kon Y. Characterization of mouse mediastinal fat-associated lymphoid clusters. *Cell Tissue Res.* (2014) 357:731–41. doi: 10.1007/s00441-014-1889-6
29. Benezech C, Luu NT, Walker JA, Kruglov AA, Loo Y, Nakamura K, et al. Inflammation-induced formation of fat-associated lymphoid clusters. *Nat Immunol.* (2015) 16:819–28. doi: 10.1038/ni.3215
30. Bain CC, Jenkins SJ. The biology of serous cavity macrophages. *Cell Immunol.* (2018) 330:126–35. doi: 10.1016/j.cellimm.2018.01.003
31. Dickinson GK. II. The omentum and its functions. *Ann Surg.* (1906) 44:652–65. doi: 10.1097/00000658-190611000-00002
32. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit^{+}Sca-1^{+} lymphoid cells. *Nature* (2010) 463:540–4. doi: 10.1038/nature08636
33. Rangel-Moreno J, Moyron-Quiroz JE, Carragher DM, Kusser K, Hartson L, Moquin A, et al. Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. *Immunity* (2009) 30:731–43. doi: 10.1016/j.immuni.2009.03.014
34. Jones DD, Racine R, Wittmer ST, Harston L, Papillion AM, Dishaw LM, et al. The omentum is a site of protective IgM production during intracellular bacterial infection. *Infect Immun.* (2015) 83:2139–47. doi: 10.1128/IAI.00295-15
35. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* (2010) 464:1367–70. doi: 10.1038/nature08900
36. Price E, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA.* (2010) 107:11489–94. doi: 10.1073/pnas.1003988107
37. Erickson LD, Foy TM, Waldschmidt TJ. Murine B1 B cells require IL-5 for optimal T cell-dependent activation. *J Immunol.* (2001) 166:1531–9. doi: 10.4049/jimmunol.166.3.1531
38. Fagarasan S, Kawamoto S, Kanagawa O, Suzuki K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. *Annu Rev Immunol.* (2010) 28:243–73. doi: 10.1146/annurev-immunol-030409-101314
39. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol.* (2011) 11:34–46. doi: 10.1038/nri2901
40. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity* (2012) 37:649–59. doi: 10.1016/j.immuni.2012.08.015
41. Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med.* (2013) 210:2939–50. doi: 10.1084/jem.20130351
42. Jego G, Bataille R, Pellat-Deceunynck C. Interleukin-6 is a growth factor for nonmalignant human plasmablasts. *Blood* (2001) 97:1817–22. doi: 10.1182/blood.V97.6.1817
43. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS ONE* (2011) 6:e17739. doi: 10.1371/journal.pone.0017739
44. Rauch PJ, Chudnovskiy A, Robbins CS, Weber GF, Etzrodt M, Hilgendorf I, et al. Innate response activator B cells protect against microbial sepsis. *Science* (2012) 335:597–601. doi: 10.1126/science.1215173
45. Okabe Y, Medzhitov R. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell* (2014) 157:832–44. doi: 10.1016/j.cell.2014.04.016
46. Simoni Y, Fehlings M, Kloverpris HN, McGovern N, Koo SL, Loh CY, et al. Human innate lymphoid cell subsets possess tissue-type based heterogeneity in phenotype and frequency. *Immunity* (2017) 46:148–61. doi: 10.1016/j.immuni.2016.11.005
47. Drake LY, Iijima K, Bartemes K, Kita H. Group 2 innate lymphoid cells promote an early antibody response to a respiratory antigen in mice. *J Immunol.* (2016) 197:1335–42. doi: 10.4049/jimmunol.1502669
48. Weber GF, Chousterman BG, Hilgendorf I, Robbins CS, Theurl I, Gerhardt LM, et al. Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis. *J Exp Med.* (2014) 211:1243–56. doi: 10.1084/jem.20131471
49. Binder CJ, Hartvigsen K, Chang MK, Miller M, Broide D, Palinski W, et al. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Invest.* (2004) 114:427–37. doi: 10.1172/JCI200420479
50. Miller AM, Xu D, Asquith DL, Denby L, Li Y, Sattar N, et al. IL-33 reduces the development of atherosclerosis. *J Exp Med.* (2008) 205:339–46. doi: 10.1084/jem.20071868
51. Perry HM, Oldham SN, Fahl SP, Que X, Gonen A, Harmon DB, et al. Helix-loop-helix factor inhibitor of differentiation 3 regulates interleukin-5 expression and B-1a B cell proliferation. *Arterioscler Thromb Vasc Biol.* (2013) 33:2771–9. doi: 10.1161/ATVBAHA.113.302571
52. Newland SA, Mohanta S, Clement M, Taleb S, Walker JA, Nus M, et al. Type-2 innate lymphoid cells control the development of atherosclerosis in mice. *Nat Commun.* (2017) 8:15781. doi: 10.1038/ncomms15781
53. Srikakulapu P, Upadhye A, Rosenfeld SM, Marshall MA, McSkimming C, Hickman AW, et al. Perivascular adipose tissue harbors atheroprotective IgM-producing B cells. *Front Physiol.* (2017) 8:719. doi: 10.3389/fphys.2017.00719

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

MINI REVIEW
published: 08 February 2019
doi: 10.3389/fimmu.2019.00170

Heterogeneity of NK Cells and Other Innate Lymphoid Cells in Human and Murine Decidua

Paola Vacca[1]*, *Laura Chiossone*[2], *Maria Cristina Mingari*[3,4] and *Lorenzo Moretta*[1]

[1] Department of Immunology, IRCCS Bambino Gesù Children's Hospital, Rome, Italy, [2] Innate Pharma Research Labs, Innate Pharma, Marseille, France, [3] Department of Experimental Medicine (DIMES) and Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy, [4] UOC Immunology, IRCCS Ospedale Policlinico, San Martino, Genoa, Italy

Innate lymphoid cells (ILCs) represent a heterogeneous group of cells lacking genetically rearranged antigen receptors that derive from common lymphoid progenitors. Five major groups of ILCs have been defined based on their cytokine production pattern and developmental transcription factor requirements: namely, natural killer (NK) cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue-inducer (LTi) cells. ILC1s, ILC2s, and ILC3s mirror the corresponding T helper subsets (Th1, Th2, and Th17, respectively) and produce cytokines involved in defense against pathogens, lymphoid organogenesis, and tissue remodeling. During the first trimester of pregnancy, decidual tissues contain high proportion of decidual NK (dNK) cells, representing up to 50% of decidual lymphocytes, and ILC3s. They release peculiar cytokines and chemokines that contribute to successful pregnancy. Recent studies revealed that ILCs display a high degree of plasticity allowing their prompt adaptation to environmental changes. Decidual NK cells may derive from peripheral blood NK cells migrated when pregnancy establishes or from *in situ* differentiation of hematopoietic precursors. Previous studies showed that human and murine decidua contain dNK cells, tissue resident NK cells, and ILC3s, all characterized by unique phenotypic and functional properties, most likely induced by decidual microenvironment to favor the establishment and the maintenance of pregnancy. Thus, during the early phase of pregnancy, the simultaneous presence of different ILC subsets further underscores the complexity of the cellular components of decidual tissues as well as the role of decidual microenvironment in shaping the plasticity and the function of ILCs.

Keywords: human and murine pregnancy, innate lymphoid cells (ILCs), natural killer (NK) cells, tolerance, decidua

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Alexander Rölle,
Nationales Centrum für Tumorerkrankungen (NCT), Germany
Alessandra Zingoni,
Sapienza University of Rome, Italy

***Correspondence:**
Paola Vacca
paola.vacca@opbg.net

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

Received: *30 November 2018*
Accepted: *21 January 2019*
Published: *08 February 2019*

Citation:
Vacca P, Chiossone L, Mingari MC and Moretta L (2019) Heterogeneity of NK Cells and Other Innate Lymphoid Cells in Human and Murine Decidua. Front. Immunol. 10:170. doi: 10.3389/fimmu.2019.00170

INTRODUCTION

ILCs represent an extended family of developmentally related hematopoietic cells that differ from T and B cells because they do not undergo somatic rearrangements of antigen-specific receptors. Notably, ILCs mirror the function of T cell subsets and contribute to host innate immune defenses, lymphoid organogenesis, and tissue remodeling. Based on their transcription factor (TF) profile ILCs have been recently classified in five groups including: (1) Natural Killer (NK cells); (2) ILC1s; (3) ILC2s; (4) ILC3s; and (5) Lymphoid Tissue-inducer cells (LTi) (1, 2). All ILCs derive from

a common lymphoid progenitor (CLP) expressing the inhibitor of DNA binding 2 (ID2) TF. The CLPs differentiate into common innate lymphoid precursors (CILPs) that, in turn, can differentiate into common helper innate lymphoid precursors (CHILPs) or into NK precursors (NKPs). Moreover, CHILPs can subsequently give rise to LTi precursors (LTiPs) that differentiate into LTi cells or to ILC precursors (ILCPs) that give rise to ILC1s, ILC2s, and ILC3s, while NKPs differentiate toward NK cells. Notably each differentiation step is driven by specific TF (**Figure 1**) (3–5).

NATURAL KILLER CELLS

NK cells display cytolytic activity against virus-infected and tumor cells and are characterized by the ability to rapidly release pro-inflammatory cytokines and chemokines involved in early inflammatory responses (6, 7). NK cell function is regulated by an array of inhibitory and activating receptors (8–13). Inhibitory NK receptors include Killer Ig-like Receptors (KIRs) in humans and Ly49 receptors in mice, that recognize classical MHC class-I molecules, and the heterodimer CD94/NKG2A able to interact with non-classical MHC class-I molecules (13, 14). Activating receptors include NKp46, NKG2D, and DNAM-1, in both humans and mice, and NKp30 and NKp44 that are expressed only by human NK cells. Other surface triggering molecules, such as 2B4 and NKp80, mainly function as co-receptors, enhancing natural cytotoxicity induced by triggering receptors. The most mature NK cells also express CD16, the low-affinity receptor for the Fc region of G-type immunoglobulins (IgG) responsible for antibody-dependent cell-mediated cytotoxicity (ADCC). In human, two NK cell subsets can be identified on the basis of CD16 and CD56 surface expression (15). $CD56^{dim}$ NK cells, co-expressing CD16 and KIRs, are predominant in peripheral blood (PB), display potent cytolytic activity and rapidly release IFN-γ, whereas the poorly cytolytic $CD56^{bright}CD16^{-}CD94/NKG2A^{+}KIR^{-}$ NK cells are mostly found in tissues and secondary lymphoid organs where they are responsible for long-lasting production of chemokines and cytokines (16). Several studies demonstrated that NK cells, as other immune cells including ILCs, derive from a $ID2^{+}$ hematopoietic precursor cell through the sequential acquisition of receptors and functions that allow the identification of distinct stages of development. Induction of NK cell commitment and further development require the expression of specific TFs as well as the exposure to a peculiar cytokine milieu. The TFs that drive NK cell differentiation are thymocyte selection-associated high mobility group box (TOX) and nuclear factor, interleukin 3 regulated (NFIL3, also known as E4BP4) (17). Achievement of later maturational stages also requires Eomesodermin (Eomes) and T-box transcription factor (Tbet) expression, which promotes the expression of cytolytic machinery and IFN-γ, respectively. Regarding the cytokine requirement, IL-15 is critical not only for the development of NK cells but also for their survival proliferation and function (2, 4, 5, 18). It is now clear that NK cell development not only occurs in the bone marrow (BM) but also in other peripheral lymphoid and non-lymphoid organs. Indeed, *ex vivo* maturational stages of NK cell differentiations have been identified in some tissues (e.g., thymus, tonsil, liver, and decidua) based on surface markers expression. In this context, NK cells have been extensively characterized in human and mouse decidual tissues.

During the first trimester of pregnancy, NK cells reach 40–70% of total lymphocytes present in the decidua, representing the main lymphoid population and display unique phenotypic and functional features (19–23). Human decidua NK (dNK) cells are characterized by $CD56^{bright}CD16^{-}KIR^{+}CD9^{+}CD49a^{+}$ phenotype, are poorly cytolytic and produce low amount of IFN-γ, as compared to PB-NK cells (24, 25) (**Figure 2**). Conversely, they secrete cytokines and chemokines e.g., VEGF, SDF-1, and IP-10 that promote neo-angiogenesis, tissue remodeling, immune modulation, and placentation (26–29). Moreover, dNK cells induce regulatory T cells (Tregs) that play a major role in the inhibition of maternal immune response and in tolerance induction (30, 31). In a recent paper, single-cell RNA sequencing of cells isolated from decidua and from the corresponding PB during the first trimester of pregnancy demonstrated the existence of three different NK cell subsets. These dNK subsets display a characteristic immunomodulatory profile and can specifically interact with other cells present in decidual microenvironment. The resulting cross-talk appears to play an important role in the control of successful pregnancy (32). It is of note that the microenvironment of different tumors displays an immunosuppressive milieu similar to that of decidua (33). Thus, a type of microenvironment playing a functional role in physiological condition, may instead favor tumor growth by suppressing the anti-tumor immune response. In particular, it has been shown that different types of cells present in the decidual microenvironment could exert a potent immunosuppressive activity inhibiting the function of NK cells (34–37). During murine gestation, metastatic spread is enhanced regardless of the tumor type and the decrease of NK cell activity is responsible of the observed increase in tumor metastases (33). It has been shown that human dNK cells express both inhibitory and activating KIRs specific for HLA-C molecules that are present at the trophoblast cell surface during the first trimester of pregnancy (30). Interactions occurring between KIRs and HLA-C molecules on trophoblast appear to play a relevant role in the induction of fetus-maternal tolerance (38, 39). In addition to KIRs, other receptors involved in the maintenance of pregnancy may be expressed by dNK cells. Of particular interest is NKG2C that upon binding to its corresponding ligand HLA-E, mediates the activation of NK cell function (23). In this context, the expression of NKG2C by dNK cells may play a key role in the control of cytomegalovirus (CMV) intrauterine infection during pregnancy (40). Notably, the frequency of $NKG2C^{+}$ dNK cells increases during repeated pregnancies as compared to the first pregnancy. $NKG2C^{+}$ dNK cell subset displays unique transcriptome and receptor profile and may sustain both vascularization and placentation during pregnancy (41). Recent studies provided evidence that $NKG2C^{+}$ NK cells can specifically discriminate among different peptides bound to HLA-E. In particular, HLA-E-bound peptides derived from the leader sequence of HLA-G

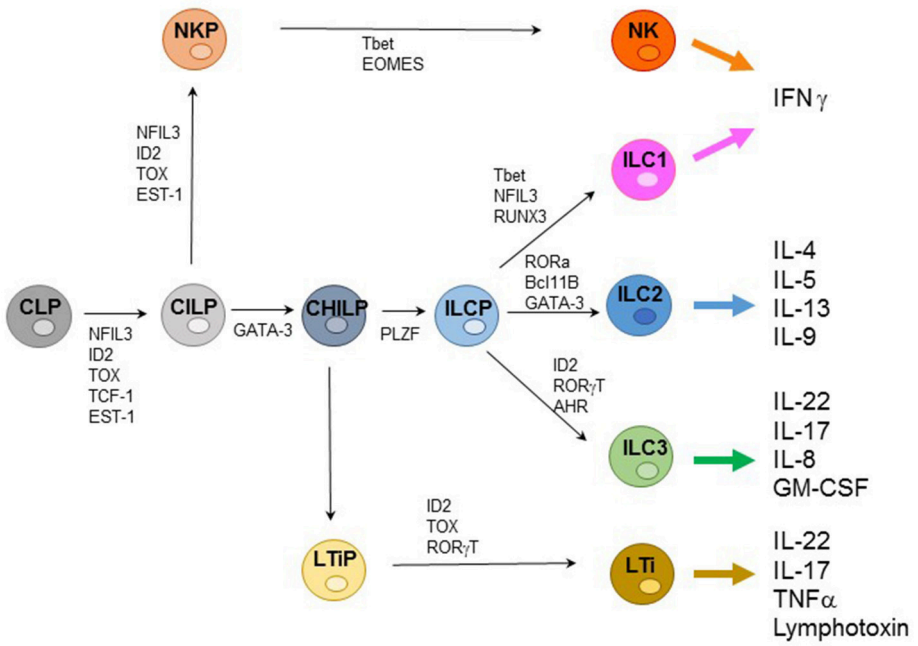

FIGURE 1 | Representative ILC development and cytokine production. Common lymphoid progenitors (CLPs) differentiate into common innate lymphoid precursors (CILPs) that can further give rise to common helper innate lymphoid precursors (CHILPs) or into NK precursors (NKPs). CHILPs give rise to LTi precursors (LTiPs) that differentiate into LTi cells or to ILC precursors (ILCPs) that differentiate into ILC1s, ILC2s, and ILC3s, while NKPs differentiate toward NK cells. Specific transcription factors (TFs) required step are indicated.

have been shown to induce an expansion of "adaptive" NK cells characterized by a high proliferative capacity and cytotoxicity (42, 43). Since HLA-G is mainly expressed by trophoblast cells it is possible to speculate that NKG2C and HLA-E binding to HLA-G peptides may play a relevant, still poorly explored, role in the maintenance of pregnancy.

The actual origin of dNK cells is not fully defined. Previous studies provided evidences that human decidual tissue contains $CD34^+$ hematopoietic cell precursors expressing IL-15/IL-2 receptor β-chain, IL-7 receptor α-chain and mRNA encoding for E4BP4 and ID2 TF. Upon culture they could undergo *in vitro* differentiation into mature NK cells that display a phenotypical and functional profile similar to that of dNK cells. These observations strongly suggest that $dCD34^+$ cells display a commitment to the NK cell lineage. Indeed, their differentiation occurs either in the presence of suitable growth factors or even upon co-culture with decidua-derived stromal cells strongly suggesting that dNK cells may derive from $CD34^+$ precursors already present in the decidua (44). It has also been proposed that, since decidual microenvironment produces large amounts of attractant chemokines, dNK cells can also be recruited from periphery into decidual tissues when pregnancy establishes (45, 46). In particular, PB-NK cells migrating into decidua acquire both phenotypic and functional features typical of dNK cells thanks to the factors present in the local microenvironment (45, 47, 48). Notably, hematopoietic precursors are also found in decidua and uterus of pregnant mice. These precursors are committed to the NK cell lineage and undergo differentiation to NK cells in decidua and uterus during early pregnancy. In addition to precursors a large proportions of immature NK cells are found in decidua and uterus. These cells undergo rapid *in situ* proliferation/maturation. Immature murine dNK cells display low cytolytic activity and IFN-γ production. Moreover, dNK cells express high levels of Ly49 receptors, usually expressed by PB-NK cells. This resemble the expression of KIRs by human dNK cells (49). Moreover, it has been shown in mice that PB-NK cells display limited homing capacity to decidua and uterus, thus indicating that the recruitment from PB can only marginally contribute to the accumulation of NK cells in decidua and uterus. Thus, it is conceivable that the decidual microenvironment plays a key role in stimulating and supporting such rapid and unique NK cell differentiation (49). These observations allowed to identify decidua and uterus as novel sites for PB-NK cell differentiation as previously described for other peripheral sites (50). On the other hand, a recent study provided evidence that in mice the primary source of NK cells during pregnancy are tissue resident (tr) NK cells displaying a high proliferative capacity (51). Phenotypic and functional analysis of decidua and uterus-NK cells provided evidence of a previously unexpected high plasticity of NK cells. Indeed, the local microenvironment was found to shape the NK cell features during development and contribute to the acquisition of regulatory, rather than pro-inflammatory, function. These important correlations between mouse and human dNK cells may offer suitable tools for understanding the

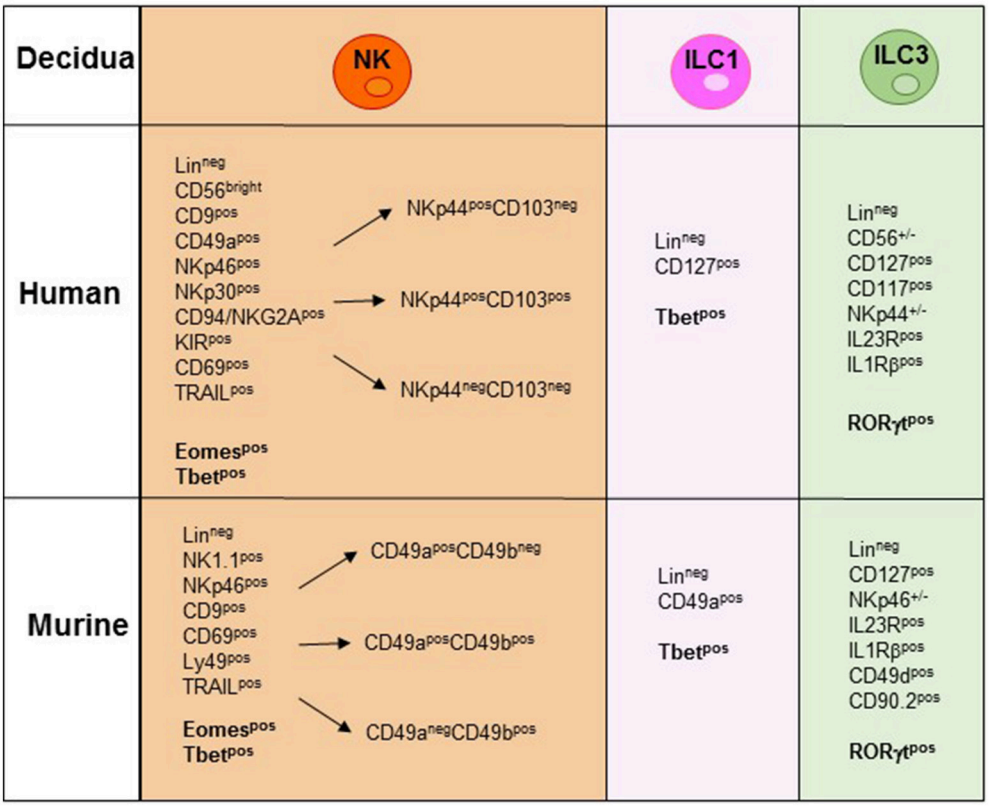

FIGURE 2 | NK/ILC subsets present in human and murine decidua during the early phase of pregnancy. In the figure are indicated the surface markers and the transcription factors (TFs) expressed by the different human and murine NK/ILCs subsets. Lineage^{neg} (CD3$^-$, CD19$^-$, CD14$^-$, CD123$^-$, CD34$^-$).

immune-regulation at the maternal-fetal interface and possibly, to clarify the pathogenesis of pregnancy-related diseases.

GROUP 1 ILC

In addition to NK cells, ILC1s are another important source of IFNγ in peripheral tissues. However, ILC1s are more proficient in the production of TNF-α and, different from NK cells, they mainly reside within peripheral organs (52, 53). Whether ILC1s have also cytotoxic capabilities is currently unclear. While expressing very low levels of granzymes and perforin, they can induce TRAIL-mediated target cell killing. In addition, thanks to their ability to produce IFN-γ, ILC1s provide innate defenses against intracellular bacteria and protozoa (54, 55). The development of ILC1 depends on Tbet but not on Eomes, necessary for the development of mature NK cells. Although ILC1s express markers in common with NK cells and ILC3s (NK1.1 in mice and NKp44 and NKp46 in humans and mice, respectively), they can be identified thanks to the expression of CD127, CD49a, and TRAIL both in humans and mice. In humans, two subsets of ILC1 are described in the intestine: (1) NCR$^-$Tbet$^+$IFN-γ$^+$ cells and (2) NKp44$^+$CD103$^+$ intraepithelial ILC1s (iILC1s) (56, 57). In particular, the first subset is characterized by high expression of CD127 and CD161 but lacks CD56, CD94, granzyme B and perforin (typical of mature NK cells). It express Tbet but not Eomes and reside in the lamina propria. The iILC1s share features in common with NK cells including the expression of CD56, the lack of CD127 and the localization in tonsils and in the intraepithelial space in the intestine. These cells are CD103$^+$ and NKp44$^+$ and express CD9 and CD49a, typical markers of dNK cells. Since CD103$^+$ cells are Eomes$^+$ and perforin$^+$, it is possible that they represent a subset of NK cells rather than ILC1s.

In mice, Tbet$^+$Eomes$^+$ NK cells and Tbet$^+$Eomes$^-$ ILC1s represent two distinct lineages of differentiation, with Eomes$^+$ NK cells originating from the BM and Tbet$^+$ Eomes$^-$ cells developing in peripheral organs (58). CD3$^-$NK1.1$^+$ cells characterized by Eomes$^{low/neg}$ expression have been described in murine peripheral organs. In the liver, Eomes^{low} cells are found to be trNK cells characterized by a CD3$^-$NK1.1$^+$CD49a$^+$DX5$^-$ phenotype (53). However, the absence of Eomes expression, together with the presence of Tbet, rather suggests their belonging to the ILC1s (54). Notably, while in the liver the expression of CD49a is confined to Eomes$^-$ cells, in decidua and uterus also a large proportion of Eomes$^+$ cells are CD49a$^+$, supporting the concept that CD49a expression alone does not allow discrimination between Eomes$^+$NK cells and Eomes$^-$ILC1 (58) (**Figure 2**). Previous studies in mice have shown that decidua and uterus NK cells express high levels of Eomes. Although NK1.1$^+$Eomes$^-$ ILC1s increased during

pregnancy and specifically expanded in second pregnancies, $Eomes^+$NK cells continued to represent the majority of uterine and decidual $CD3^-NK1.1^+$ cells. Importantly, both $Eomes^+$ and $Eomes^-$ subsets expressed Tbet. Moreover, based on CD49a, DX5 and Eomes expression, uterus and decidual cells could be further subdivided into different subsets, namely ILC1s ($Eomes^-CD49a^+DX5^-IFN\gamma^{low}TNF^{high}$), common NK (cNK) cells ($Eomes^+CD49a^-DX5^+IFN\text{-}\gamma^{high}TNF^{low}$), and two peculiar subsets of NK cells ($Eomes^+CD49a^+DX5^-IFN\gamma^+TNF^+$ and $Eomes^+CD49a^+DX5^+IFN\text{-}\gamma^+TNF^+$) that share phenotypic and functional features with cNK cells and the formerly described tissue resident NK (trNK) cells (53). A very recent study provided the first whole-genome transcriptome profile of the different ILC subsets present in decidua and uterus of mice during pregnancy. These results highlight the marked differences existing between the uterine resident $CD49a^+$ trNK cells and the ILC1s (59). The abundance of $Eomes^+$ cells in uterus and decidua suggests that they may derive from hematopoietic precursors of BM origin. However, as described above, dNK cells may also derive from accumulation of circulating immature cNK cells that upon exposure to tissue microenvironment acquire typical features of uterine NK cells including CD49a expression. Similarly, to mice, in human decidua, $Eomes^+$ cells can be divided in three different subsets on the basis of NKp44 and CD103 expression. The $CD103^+$ cells represent the major source of IFN-γ among dNK cells and may play a relevant role in the early inflammatory phase of pregnancy. Altogether, these studies indicate that the majority of ILCs present both in human and murine decidua are $Eomes^+$ NK cells. Moreover, only in mice it is possible to identify also $Eomes^-$ ILC1s (60–62). Notably, the decidual microenvironment may shape the conversion of a peculiar subset of ILC one into another. For example, in mice, TGF-β can induce the conversion of $CD49a^-CD49b^+Eomes^+$NK cells into $CD49a^+CD49b^-Eomes^{low}$ ILC1. Since this conversion may occur also in tumor microenvironment it may represent a further mechanism of tumor escape as ILC1 are characterized by reduced capacities to control tumor growth (63).

GROUP 2 ILC

ILC2s have been originally identified in mice, they depend on GATA binding protein-3 (GATA3) TF for their development (64), and release IL-5, IL-9, IL-13, and small amounts of IL-4 in response to IL-25 and IL-33 stimulation. This cell subset plays an important role in the immune response against helminthic infections and is involved in allergic immune responses. In mice ILC2s are detectable in several tissues, including lymph nodes, fat-associated lymphoid clusters, spleen, liver, intestine, and airways while in humans are mainly found in lung and intestine (2). Studies in mice, demonstrated that ILC2s derive from an $ID2^+$ precursor present in the BM and that their development is driven by RORα TF. ILC2s can be identified thanks to the expression of CRTH2, CD127, and CD25. They also express ICOS, which promotes ILC2 survival and cytokine production (65). They share with NK cells, ILC1s and ILC3s the expression of a number of activating and inhibitory receptors such as CD161, NKp30, KLRG1, and PD1 that can regulate their activation and function (66, 67).

The presence of ILC2s in decidual tissues is debated and may depend on the gestation phase. They are detectable in the uterine wall but not in decidua and endometrium both in humans and in mice. ILC2s have been detected in the uterus of both virgin and pregnant mice as well as in myometrium (Myo) and in mesometrial lymphoid aggregates (MLAp) (61, 62). Nfil3 TF is strictly required for the development and the expansion of uterine ILC2s in mice; indeed, in the uterus of virgin and pregnant $Nfil^{-/-}$ mice ILC2s are not present. Importantly, thanks to their ability to release IL-5 in response to IL-25 and IL-33, ILC2s are involved in the control of the eosinophil homeostasis that, in turn, may play a role in the remodeling of uterine mucosa (68, 69). A study in humans provided evidence that ILC2s are abundant during preterm and term gestation at the fetal-maternal interface (70).

GROUP 3 ILC

ILC3s represent a heterogeneous cell subset particularly abundant in mucosal tissues where they contribute to defenses against pathogens and to epithelial tissue homeostasis (1, 2, 71, 72). ILC3s are originally identified in the fetus and defined as LTi as they play a key role in driving lymphoid organogenesis. This capacity is partially related to the expression of lymphotoxin-alpha (LT-α) and LT-β that promote interactions with LTβ receptor (LTβ-R) expressing stromal cells. Upon engagement of LTβ-R, stromal cells upregulate adhesion molecules and secret chemokines that collectively promote the formation of lymph node anlagen (34, 73). Notably, cells with similar phenotypic characteristics have been identified also in adult secondary lymphoid organs and are defined as LTi-like cells. In the adult two subsets of ILC3s have recently been identified in mucosal tissues. They can be distinguished by the expression, or lack of NKp46 in mice and NKp44 in humans. ILC3s share common phenotypic features with both LTi-like cells and NK cells and express the RORγt TF, required for their differentiation and function. ILC3s, thanks to the ability to release IL-17 and IL-22, may contribute to host defenses by recruiting neutrophils and inducing the production of antimicrobial peptides (2, 4, 74, 75). Moreover, they are thought to induce tissue remodeling after acute inflammation (76). Although fetal LTi cells and adult ILC3s were previously considered to belong to the same ILC group, recent evidences revealed that they derive from two different precursors, namely, LTiP and ILCP, respectively, and follow separate developmental pathways.

Studies in mice demonstrated that, similarly to ILC2s, also ILC3s are present in virgin and pregnant uterus, in particular, in pregnant mice, they were enriched in Myo and MLAp but not in decidual tissues. During pregnancy, ILC3 numbers were higher than in virgin mice. Notably, the development of ILC3s was not dependent on the Nfil3 TF. However, in $Nfil3^{-/-}$ mice ILC3 numbers were lower than in wild-type mice (61, 62).

In humans, ILC3s have been identified in decidual and endometrial tissues and include both NCR^+ and NCR^- cell subsets (**Figure 2**). Decidual ILC3s express the hallmark of the ILC3 lineage, i.e., RORγt TF, CD127, CD117, IL-23R, and IL-1R. Human decidual $NKp44^+$ILC3s not only produce IL-22, but are also the main source of IL-8 and GM-CSF while NCR^-ILC3s mainly produce IL-17 and TNF-α (77, 78). These data are in line with those obtained in mice (49, 60). It is of note that a successful pregnancy requires an early inflammatory phase, necessary for implantation, while, at later stages a regulatory/immunosuppressive phase is needed to prevent fetal rejection (79). Since ILC3s release cytokines/chemokines involved in neutrophil recruitment/activation, neo-angiogenesis, tissue remodeling and placentation, they may actually play a key role not only in the early inflammatory phase but also in the induction of a tolerogenic status. Indeed, ILC3-derived IL-8 and GM-CSF are crucial for the recruitment of peripheral neutrophils into decidual tissues and for their activation and function. In turn, recruited neutrophils are necessary in the early inflammatory phase for a successful implantation. Thereafter, decidual neutrophils produce HB-EGF and IL1RA favoring the induction of tolerance (80). Moreover, decidual ILC3s interact with decidual stromal cells inducing the up-regulation of adhesion molecules on these cells. Notably, data on the role of ILC3s during pregnancy contributed to clarify the general involvement of these cells in tissues remodeling, inflammation and neo-angiogenesis. Regarding pregnancy, their effect on trophoblast invasion and placentation indicate that non-only dNK, but also ILC3s play a relevant role in the early phases of pregnancy (34, 35, 77).

CONCLUSIONS

In this review, we recapitulate current knowledge on the presence and the role of NK cells and different ILCs in decidual tissues. Altogether, data highlight the complexity of uterine and decidual NK and ILC subsets. Such complexity, particularly during the first trimester of pregnancy, may reflect the effect of peculiar decidual microenvironment in shaping the features of both NK and ILC subsets. Although further analysis is clearly required to define their involvement in the establishment and maintenance of pregnancy, it is possible to speculate that a deficit of a peculiar NK or ILC subset or their altered function may result in pregnancy failure consequent to uncontrolled infection or deficient tissue and vessels formation.

AUTHOR CONTRIBUTIONS

All authors discussed together the general outline of the article. PV, LC, and LM wrote the first draft that was subsequently reviewed by MCM. Thereafter, all authors contributed to the elaboration of the final version of the manuscript.

FUNDING

Supported by grants awarded by Associazione Italiana Ricerca per la Ricerca sul Cancro (AIRC)- Special Project 5 × 1,000 Molecular Clinical Oncology Extension Program no. 9962 (LM) and 5 × 1000 Immunity in Cancer Spreading and Metastasis (ISM) no. 21147 (LM); AIRC IG 2017 Id. 19920 (LM), AIRC 2014 Id. 15283 (LM), Ministero della Salute RF-2013, GR-2013-02356568 (PV).

REFERENCES

1. Diefenbach A, Colonna M, Romagnani C. The ILC world revisited. *Immunity* (2017) 46:327–32. doi: 10.1016/j.immuni.2017.03.008
2. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
3. Eberl G, Di Santo JP, Vivier E. The brave new world of innate lymphoid cells. *Nat Immunol.* (2014) 16:1–5. doi: 10.1038/ni.3059
4. Montaldo E, Vacca P, Moretta L, Mingari MC. Development of human natural killer cells and other innate lymphoid cells. *Semin Immunol.* (2014) 26:107–13. doi: 10.1016/j.smim.2014.01.006
5. Montaldo E, Vacca P, Vitale C, Moretta F, Locatelli F, Mingari MC, et al. Human innate lymphoid cells. *Immunol Lett.* (2016). 179:2–8. doi: 10.1016/j.imlet.2016.01.007
6. Chiossone L, Dumas PY, Vienne M, Vivier E. Natural killer cells and other innate lymphoid cells in cancer. *Nat Rev Immunol.* (2018) 18:671–88. doi: 10.1038/s41577-018-0061-z
7. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science* (2011) 33144–9. doi: 10.1126/science.1198687
8. Moretta L, Locatelli F, Pende D, Marcenaro E, Mingari MC, Moretta A. Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *Blood* (2011) 117:764–71. Epub 2010/10/05. doi: 10.1182/blood-2010-08-264085
9. Moretta L, Montaldo E, Vacca P, Del Zotto G, Moretta F, Merli P, et al. Human natural killer cells: origin, receptors, function, and clinical applications. *Int Arch Allergy Immunol.* (2014) 164:253–64. doi: 10.1159/000365632
10. Moretta L, Pietra G, Montaldo E, Vacca P, Pende D, Falco M, et al. Human NK cells: from surface receptors to the therapy of leukemias and solid tumors. *Front Immunol.* (2014) 5:87. doi: 10.3389/fimmu.2014.00087
11. Vacca P, Montaldo E, Croxatto D, Moretta F, Bertaina A, Vitale C, et al. NK cells and other innate lymphoid cells in hematopoietic stem cell transplantation. *Front Immunol.* (2016) 7:188. doi: 10.3389/fimmu.2016.00188
12. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol.* (2001) 19:197–223. doi: 10.1146/annurev.immunol.19.1.197
13. Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, et al. Receptors for HLA class-I molecules in human natural killer cells. *Annu Rev Immunol.* (1996) 14:619–48. doi: 10.1146/annurev.immunol.14.1.619
14. Ortaldo JR, Young HA. Mouse Ly49 NK receptors: balancing activation and inhibition. *Mol Immunol.* (2005) 42:445–50. doi: 10.1016/j.molimm.2004.07.024
15. Caligiuri MA. Human natural killer cells. *Blood* (2008) 112:461–9. doi: 10.1182/blood-2007-09-077438
16. Freud AG, Caligiuri MA. Human natural killer cell development. *Immunol Rev.* (2006) 214:56–72. doi: 10.1111/j.1600-065X.2006.00451.x

17. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature* (2014) 508:397–401. doi: 10.1038/nature13047
18. Cooper MA, Bush JE, Fehniger TA, VanDeusen JB, Waite RE, Liu Y, et al. *In vivo* evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood* (2002) 100:3633–8. doi: 10.1182/blood-2001-12-0293
19. Vacca P, Mingari MC, Moretta L. Natural killer cells in human pregnancy. *J Reprod Immunol.* (2013) 97:14–9. doi: 10.1016/j.jri.2012.10.008
20. Vacca P, Moretta L, Moretta A, Mingari MC. Origin, phenotype and function of human natural killer cells in pregnancy. *Trends Immunol.* (2011) 32:517–23. doi: 10.1016/j.it.2011.06.013
21. Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol.* (2013) 31:387–411. doi: 10.1146/annurev-immunol-032712-100003
22. Moffett-King A, Entrican G, Ellis S, Hutchinson J, Bainbridge D. Natural killer cells and reproduction. *Trends Immunol.* (2002) 23:332–3. doi: 10.1016/S1471-4906(02)02261-5
23. Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med.* (2003) 198:1201–12. doi: 10.1084/jem.20030305
24. Kopcow HD, Allan DS, Chen X, Rybalov B, Andzelm MM, Ge B, et al. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc Natl Acad Sci USA*. (2005) 102:15563–8. doi: 10.1073/pnas.05078 35102
25. Vacca P, Pietra G, Falco M, Romeo E, Bottino C, Bellora F, et al. Analysis of natural killer cells isolated from human decidua: evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. *Blood* (2006) 108:4078–85. doi: 10.1182/blood-2006-04-017343
26. Vacca P, Cantoni C, Prato C, Fulcheri E, Moretta A, Moretta L, et al. Regulatory role of NKp44, NKp46, DNAM-1 and NKG2D receptors in the interaction between NK cells and trophoblast cells. Evidence for divergent functional profiles of decidual versus peripheral NK cells. *Int Immunol.* (2008) 20:1395–405. doi: 10.1093/intimm/dxn105
27. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med.* (2006) 12:1065–74. doi: 10.1038/nm1452
28. Hanna J, Mandelboim O. When killers become helpers. *Trends Immunol.* (2007) 28:201–6. doi: 10.1016/j.it.2007.03.005
29. Le Bouteiller P, Tabiasco J. Killers become builders during pregnancy. *Nat Med.* (2006) 12:991–2. doi: 10.1038/nm0906-991
30. Vacca P, Cantoni C, Vitale M, Prato C, Canegallo F, Fenoglio D, et al. Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and immunosuppression. *Proc Natl Acad Sci USA*. (2010) 107:11918–23. doi: 10.1073/pnas.1001749107
31. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol.* (2010) 63:601–10. doi: 10.1111/j.1600-0897.2010.00852.x
32. Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, et al. Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature* (2018) 563:347–53. doi: 10.1038/s41586-018-0698-6
33. Mauti LA, Le Bitoux MA, Baumer K, Stehle JC, Golshayan D, Provero P, et al. Myeloid-derived suppressor cells are implicated in regulating permissiveness for tumor metastasis during mouse gestation. *J Clin Invest.* (2011) 121:2794–807. doi: 10.1172/JCI41936
34. Vacca P, Montaldo E, Vitale C, Croxatto D, Moretta L, Mingari MC. MSC and innate immune cell interactions: a lesson from human decidua. *Immunol Lett.* (2015) 168:170–4. doi: 10.1016/j.imlet.2015.05.006
35. Croxatto D, Vacca P, Canegallo F, Conte R, Venturini PL, Moretta L, et al. Stromal cells from human decidua exert a strong inhibitory effect on NK cell function and dendritic cell differentiation. *PLoS ONE* (2014) 9:e89006. doi: 10.1371/journal.pone.0089006
36. Abumaree MH, Chamley LW, Badri M, El-Muzaini MF. Trophoblast debris modulates the expression of immune proteins in macrophages: a key to maternal tolerance of the fetal allograft? *J Reprod Immunol.* (2012) 94:131–41. doi: 10.1016/j.jri.2012.03.488
37. Kammerer U, Eggert AO, Kapp M, McLellan AD, Geijtenbeek TB, Dietl J, et al. Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. *Am J Pathol.* (2003) 162:887–96. doi: 10.1016/S0002-9440(10)63884-9
38. Moffett A, Loke C. Immunology of placentation in eutherian mammals. *Nat Rev Immunol.* (2006) 6:584–94. doi: 10.1038/nri1897
39. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol.* (2002) 2:656–63. doi: 10.1038/nri886
40. Siewiera J, El Costa H, Tabiasco J, Berrebi A, Cartron G, Le Bouteiller P, et al. Human cytomegalovirus infection elicits new decidual natural killer cell effector functions. *PLoS Pathogens* (2013) 9:e1003257. doi: 10.1371/journal.ppat.1003257
41. Gamliel M, Goldman-Wohl D, Isaacson B, Gur C, Stein N, Yamin R, et al. Trained memory of human uterine NK cells enhances their function in subsequent pregnancies. *Immunity* (2018) 48:951–62 e5. doi: 10.1016/j.immuni.2018.03.030
42. Hammer Q, Ruckert T, Romagnani C. Natural killer cell specificity for viral infections. *Nat Immunol.* (2018) 19:800–8. doi: 10.1038/s41590-018-0163-6
43. Rolle A, Meyer M, Calderazzo S, Jager D, Momburg F. Distinct HLA-E peptide complexes modify antibody-driven effector functions of adaptive NK cells. *Cell Rep.* (2018) 24:1967–76 e4. doi: 10.1016/j.celrep.2018.07.069
44. Vacca P, Vitale C, Montaldo E, Conte R, Cantoni C, Fulcheri E, et al. CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proc Natl Acad Sci USA*. (2011) 108:2402–7. doi: 10.1073/pnas.1016257108
45. Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, et al. Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood* (2008) 111:3108–15. doi: 10.1182/blood-2007-08-105965
46. Santoni A, Carlino C, Stabile H, Gismondi A. Mechanisms underlying recruitment and accumulation of decidual NK cells in uterus during pregnancy. *Am J Reprod Immunol.* (2008) 59:417–24. doi: 10.1111/j.1600-0897.2008.00598.x
47. Keskin DB, Allan DS, Rybalov B, Andzelm MM, Stern JN, Kopcow HD, et al. TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci USA*. (2007) 104:3378–83. doi: 10.1073/pnas.0611098104
48. Drake PM, Gunn MD, Charo IF, Tsou CL, Zhou Y, Huang L, et al. Human placental cytotrophoblasts attract monocytes and CD56(bright) natural killer cells via the actions of monocyte inflammatory protein 1alpha. *J Exp Med.* (2001) 193:1199–212.
49. Chiossone L, Vacca P, Orecchia P, Croxatto D, Damonte P, Astigiano S, et al. *In vivo* generation of decidual natural killer cells from resident hematopoietic progenitors. *Haematologica* (2014) 99:448–57. doi: 10.3324/haematol.2013.091421
50. Shi FD, Ljunggren HG, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. *Nat Rev Immunol.* (2011) 11:658–71. doi: 10.1038/nri3065
51. Sojka DK, Yang L, Plougastel-Douglas B, Higuchi DA, Croy BA, Yokoyama WM. Cutting edge: local proliferation of uterine tissue-resident NK cells during decidualization in mice. *J Immunol.* (2018) 201:2551–6. doi: 10.4049/jimmunol.1800651
52. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* (2015) 517:293–301. doi: 10.1038/nature14189
53. Sojka DK, Plougastel-Douglas B, Yang L, Pak-Wittel MA, Artyomov MN, Ivanova Y, et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *eLife* (2014) 3:e01659. doi: 10.7554/eLife.01659
54. Klose CS, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
55. McKenzie AN, Spits H, Eberl G. Innate lymphoid cells in inflammation and immunity. *Immunity* (2014) 41:366–74. doi: 10.1016/j.immuni.2014.09.006
56. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol.* (2013) 14:221–9. doi: 10.1038/ni.2534
57. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and

IL-15-responsive IFN-gamma-producing cells. *Immunity* (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
58. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *J Exp Med.* (2014) 211:563–77. doi: 10.1084/jem.20131560
59. Filipovic I, Chiossone L, Vacca P, Hamilton RS, Ingegnere T, Doisne JM, et al. Molecular definition of group 1 innate lymphoid cells in the mouse uterus. *Nat Commun.* (2018) 9:4492. doi: 10.1038/s41467-018-06918-3
60. Montaldo E, Vacca P, Chiossone L, Croxatto D, Loiacono F, Martini S, et al. Unique Eomes(+) NK cell subsets are present in uterus and decidua during early pregnancy. *Front Immunol.* (2015) 6:646. doi: 10.3389/fimmu.2015.00646
61. Boulenouar S, Doisne JM, Sferruzzi-Perri A, Gaynor LM, Kieckbusch J, Balmas E, et al. The residual innate lymphoid cells in NFIL3-deficient mice support suboptimal maternal adaptations to pregnancy. *Front Immunol.* (2016) 7:43. doi: 10.3389/fimmu.2016.00043
62. Doisne JM, Balmas E, Boulenouar S, Gaynor LM, Kieckbusch J, Gardner L, et al. Composition, development, and function of uterine innate lymphoid cells. *J Immunol.* (2015) 195:3937–45. doi: 10.4049/jimmunol.1500689
63. Gao Y, Souza-Fonseca-Guimaraes F, Bald T, Ng SS, Young A, Ngiow SF, et al. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nat Immunol.* (2017) 18:1004–15. doi: 10.1038/ni.3800
64. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity* (2012) 37:634–48. doi: 10.1016/j.immuni.2012.06.020
65. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Richard-Le Goff O, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol.* (2006) 7:1217–24. doi: 10.1038/ni1395
66. Taylor S, Huang Y, Mallett G, Stathopoulou C, Felizardo TC, Sun MA, et al. PD-1 regulates KLRG1(+) group 2 innate lymphoid cells. *J Exp Med.* (2017) 214:1663–78. doi: 10.1084/jem.20161653
67. Salimi M, Xue L, Jolin H, Hardman C, Cousins DJ, McKenzie AN, et al. Group 2 innate lymphoid cells express functional NKp30 receptor inducing type 2 cytokine production. *J Immunol.* (2016) 196:45–54. doi: 10.4049/jimmunol.1501102
68. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* (2013) 502:245–8. doi: 10.1038/nature12526
69. Robertson SA, Mau VJ, Young IG, Matthaei KI. Uterine eosinophils and reproductive performance in interleukin 5-deficient mice. *J Reprod Fertility* (2000) 120:423–32.
70. Xu Y, Romero R, Miller D, Silva P, Panaitescu B, Theis KR, et al. Innate lymphoid cells at the human maternal-fetal interface in spontaneous preterm labor. *Am J Reprod Immunol.* (2018) 79:e12820. doi: 10.1111/aji.12820
71. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* (2009) 457:722–5. doi: 10.1038/nature07537
72. Cupedo T, Crellin NK, Papazian N, Rombouts EJ, Weijer K, Grogan JL, et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127(+) natural killer-like cells. *Nat Immunol.* (2009) 10:66–74. doi: 10.1038/Ni.1668
73. Scandella E, Bolinger B, Lattmann E, Miller S, Favre S, Littman DR, et al. Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nat Immunol.* (2008) 9:667–75. doi: 10.1038/ni.1605
74. Satoh-Takayama N, Serafini N, Verrier T, Rekiki A, Renauld JC, Frankel G, et al. The chemokine receptor CXCR6 controls the functional topography of interleukin-22 producing intestinal innate lymphoid cells. *Immunity* (2014) 41:776–88. doi: 10.1016/j.immuni.2014.10.007
75. Rankin LC, Girard-Madoux MJ, Seillet C, Mielke LA, Kerdiles Y, Fenis A, et al. Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nat Immunol.* (2016) 17:179–86. doi: 10.1038/ni.3332
76. Sonnenberg GF, Artis D. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. *Nat Med.* (2015) 21:698–708. doi: 10.1038/nm.3892
77. Vacca P, Montaldo E, Croxatto D, Loiacono F, Canegallo F, Venturini PL, et al. Identification of diverse innate lymphoid cells in human decidua. *Mucosal Immunol.* (2015) 8:254–64. doi: 10.1038/mi.2014.63
78. Vacca P, Vitale C, Munari E, Cassatella MA, Mingari MC, Moretta L. Human innate lymphoid cells: their functional and cellular interactions in decidua. *Front Immunol.* (2018) 9:1897. doi: 10.3389/fimmu.2018.01897
79. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann NY Acad Sci.* (2011) 1221:80–7. doi: 10.1111/j.1749-6632.2010.05938.x
80. Croxatto D, Micheletti A, Montaldo E, Orecchia P, Loiacono F, Canegallo F, et al. Group 3 innate lymphoid cells regulate neutrophil migration and function in human decidua. *Mucosal Immunol.* (2016) 9:1372–83. doi: 10.1038/mi.2016.10

Conflict of Interest Statement: LC is employee of Innate Pharma.

The remaining 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: 13 February 2019
doi: 10.3389/fimmu.2019.00217

Fingolimod Alters Tissue Distribution and Cytokine Production of Human and Murine Innate Lymphoid Cells

Ahmet Eken[1,2]*, *Mehmet Fatih Yetkin*[3]*, *Alperen Vural*[4], *Fatma Zehra Okus*[1,2], *Serife Erdem*[1,2], *Zehra Busra Azizoglu*[1,2], *Yesim Haliloglu*[1,2], *Mustafa Cakir*[1,2], *Enes Mehmet Turkoglu*[2], *Omer Kilic*[2], *Irfan Kara*[4], *Hamiyet Dönmez Altuntaş*[1], *Mohamed Oukka*[5]*, *Mehmet Serdar Kutuk*[6], *Meral Mirza*[3] and *Halit Canatan*[1,2]

[1] Erciyes University School of Medicine, Department of Medical Biology, Kayseri, Turkey, [2] Betül-Ziya Eren Genome and Stem Cell Center (GENKOK), Kayseri, Turkey, [3] Department of Neurology, Erciyes University School of Medicine, Kayseri, Turkey, [4] Department of Ear Nose and Throat, Erciyes University School of Medicine, Kayseri, Turkey, [5] Department of Immunology, University of Washington, Seattle, WA, United States, [6] Department of Obstetrics and Gynecology, Erciyes University School of Medicine, Kayseri, Turkey

Sphingosine-1 phosphate receptor 1 (S1PR1) is expressed by lymphocytes and regulates their egress from secondary lymphoid organs. Innate lymphoid cell (ILC) family has been expanded with the discovery of group 1, 2 and 3 ILCs, namely ILC1, ILC2 and ILC3. ILC3 and ILC1 have remarkable similarity to CD4+ helper T cell lineage members Th17 and Th1, respectively, which are important in the pathology of multiple sclerosis (MS). Whether human ILC subsets express S1PR1 or respond to its ligands have not been studied. In this study, we used peripheral blood/cord blood and tonsil lymphocytes as a source of human ILCs. We show that human ILCs express S1PR1 mRNA and protein and migrate toward S1P receptor ligands. Comparison of peripheral blood ILC numbers between fingolimod-receiving and treatment-free MS patients revealed that, *in vivo*, ILCs respond to fingolimod, an S1PR1 agonist, resulting in ILC-penia in circulation. Similarly, murine ILCs responded to fingolimod by exiting blood and accumulating in the secondary lymph nodes. Importantly, *ex vivo* exposure of ILC3 and ILC1 to fingolimod or SEW2871, another S1PR1 antagonist, reduced production of ILC3- and ILC1- associated cytokines GM-CSF, IL-22, IL-17, and IFN-γ, respectively. Surprisingly, despite reduced number of lamina propria-resident ILC3s in the long-term fingolimod-treated mice, ILC3-associated IL-22, IL-17A, GM-CSF and antimicrobial peptides were high in the gut compared to controls, suggesting that its long term use may not compromise mucosal barrier function. To our knowledge, this is the first study to investigate the impact of fingolimod on human ILC subsets *in vivo* and *ex vivo*, and provides insight into the impact of long term fingolimod use on ILC populations.

Keywords: S1PR1, ILC3, ILC1, Fingolimod, FTY720, SEW2871, multiple sclerosis

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Girdhari Lal,
National Centre for Cell Science (NCCS), India
Matthew Ross Hepworth,
University of Manchester, United Kingdom
Cyril Seillet,
Walter and Eliza Hall Institute of Medical Research, Australia

***Correspondence:**
Ahmet Eken
ahmet.eken@gmail.com
Mehmet Fatih Yetkin
drfatihmehmet@gmail.com
Mohamed Oukka
moukka@uw.edu

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

Received: *08 October 2018*
Accepted: *25 January 2019*
Published: *13 February 2019*

Citation:
Eken A, Yetkin MF, Vural A, Okus FZ, Erdem S, Azizoglu ZB, Haliloglu Y, Cakir M, Turkoglu EM, Kilic O, Kara I, Dönmez Altuntaş H, Oukka M, Kutuk MS, Mirza M and Canatan H (2019) Fingolimod Alters Tissue Distribution and Cytokine Production of Human and Murine Innate Lymphoid Cells. Front. Immunol. 10:217. doi: 10.3389/fimmu.2019.00217

INTRODUCTION

S1P receptor 1 (S1PR1) is a G-protein coupled receptor expressed by endothelial cells and lymphocytes. Upon binding to its natural ligand S1P, S1PR1 activates various signaling cascades, including Ras–Erk, PI3K–Akt, and the small G proteins Rac and Rho (1, 2). S1PR1 plays a critical role in the egress of T and B cells out of thymus and lymphoid organs (3–5). Egress regulation is achieved by a gradient of S1P across lymphoid tissues and blood or lymph, which is created by tight regulation of S1P production by kinases SPHK1 and SPHK2 and its reversible or irreversible degradation by S1P lyase or phosphatases, respectively (2, 6–8). Multiple S1PR1 agonists/antagonists have been discovered. Fingolimod (FTY720 or Gilenya^{TM}) is a structural analog of sphingosine-1 that blocks lymphocyte egress into blood or lymph by inducing S1PR1 internalization (9). Fingolimod was approved by FDA in 2010 as the first oral disease modifying pill for the treatment of MS. FTY720, however, is not very specific and can bind S1PR1, S1PR3, S1PR4, and S1PR5 (9). Thus, adverse effects including bradycardia have been reported (10). Moreover, although approved for the treatment of MS (11), in some patients, cessation or initiation of fingolimod therapy resulted in exacerbation of MS and formation of tumefactive lesions in the brain (12–16). Other S1PR1 agonists such as ponesimod, siponimod, and ozanimod are also in clinical trials recruiting MS patients (17–19). Ozanimod and siponimod are more specific than fingolimod and bind S1PR1 and S1PR5. S1PR1 antagonists/agonists are also tested for other conditions besides MS, including psoriasis, graft vs. host disease (GVHD) and inflammatory bowel diseases (IBD) (17–19). Therefore, the potential for the broader use of S1PR1 modulators for the treatment of a host of autoimmune conditions underlines the need for a better understanding of S1PR1 functions in various cell types in the body.

In this regard, the role of S1PR5 in murine natural killer (NK) cells has been studied (20). A report demonstrated that the tissue distribution of NK cells was regulated by S1PR5, thus S1PR5-deficient NK cells are enriched in the lymph nodes (LNs) and bone marrow (BM) and are reduced in circulation. Human NK cells also have recently been shown to use S1PR5 (21). Innate lymphoid cell family (ILC) has recently been expanded with the discovery of helper-T-cell-like innate lymphoid cell subsets ILC1, ILC2, and ILC3s (22–29). ILC3s emerged as the innate counterpart of Th17 cells. As such, ILC3s have been shown to play an essential role at the mucosal surfaces by establishing a de-colonized zone between the microbial community and the epithelial cells through IL-22 cytokine they produce (30, 31). In addition, ILC3s were shown to be crucial in fighting infections in murine models (32). More importantly, ILC3 involvement in the chronic inflammatory diseases such as Crohn's and colitis have been established in murine models as either pathogenic or protective agents in our and others' works (33–41). Increased ILC3 cellular presence or activity have been reported in the affected tissues or circulation in several other autoimmune disease patients from rheumatoid arthritis, psoriasis to MS warranting further study of these cells in human pathologies (42, 43). Although a population of ILC3s express NKp46 in mice and NKp44 in humans, it is completely unknown how S1P receptors regulate human or murine ILC3 biology or how drugs against S1P receptors would impact a critical mucosal innate cell population.

In the present study, we aimed to understand the role S1P receptors play in ILC biology, tissue distribution and function using tonsil or cord blood derived ILCs, fingolimod receiving MS patients and murine models. We show that S1PR1 may regulate human ILC1 and ILC3 chemotaxis and that S1PR1 ligands fingolimod and SEW2871 have immunomodulatory effects on human ILC3s. We also show that although long term fingolimod treatment of mice reduces intestinal ILC3 numbers, it does not reduce IL-17A, IL-22 or antimicrobial peptides, sparing the barrier immunity intact.

MATERIALS AND METHODS

Human Samples

Peripheral blood samples were taken from patients at Erciyes University School of Medicine, Department of Neurology. Informed consent was obtained from donors after the nature and possible consequences of the studies were explained. Revised McDonald Diagnostic Criteria for MS (Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria) was used for diagnosis of RRMS (44). All subjects in the MS patient/no treatment cohort were off immunomodulatory and immunosuppressive drugs at the time of study and for about 3 months before the blood samples were taken. Control subjects lack any known autoimmune condition or a family history of autoimmunity. Tonsils were taken after they were being discarded by Erciyes University Hospital Surgery Department. Cord Blood samples were taken from discarded cord following labor at Erciyes University Gevher Nesibe Hospital. The research protocols were approved by the Ethics Committee at Erciyes University. All methods for human studies involving human samples were performed in accordance with the relevant guidelines and regulations.

Mice

The research protocols involving mice were approved by the Animal Ethics Committee at Erciyes University. All methods for mice studies were performed in accordance with the relevant guidelines and regulations. C57BL/6 or IL-23RGFP reporter mice were housed under specific pathogen free conditions. 0.5 mg/kg fingolimod was administered daily intraperitoneally for 15-to 30 days. As control, PBS was given. SEW2871 (10006440 256414-75-2) and FTY720-P (10008639) were purchased from Cayman. For gavage feeding, Gilenya (Novartis) 0.5 mg tablets were reconstituted in Phosphate-Buffered Saline (PBS) and administered daily at 0.5 mg/kg dose. One hundred microgram (in 100 μl PBS) anti-CD40 (FGK4.5, Bioxcell) was injected intraperitoneally 2-days prior to sacrificing mice.

Isolation, Staining, and Culture of Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood via Ficoll-Paque Plus (GE17-1440-03) based on the manufacturer's instructions. Mice lymphocytes from lymph

nodes, spleen or intestines were isolated as previously described (45). For intestinal lamina propria preps, 2–3 cm piece from ileum was processed for lamina propria lymphocyte isolation. Single cell suspension was stained for the relevant antibodies after blocking 5 min with Human TruStainFcX (BioLegend, San Diego, CA, USA) in Staining Buffer (2% FBS in PBS) with proper dilution. The cells were. gated and sorted via FACSAria III (BD Biosciences) according to the protocol by Mjösberg and Spits (46).

Human ILC3s were cultured in the following cocktail of cytokines, IL-2, IL-7, IL-23, IL-1β, 20 ng/ml each (47). ILC1 cells were cultured in the cocktail containing cytokines, IL-2, IL-7, IL-12, 20 ng/ml each.

Antibodies and Reagents

Alexa Fluor® 700 anti-human CD3, (clone: HIT3a), FITC-anti human TCRαβ (clone: IP26), FITC-anti-human TCRγδ (clone: B1), APC/Cy7 Anti-Human CD127 (I-7Ra), (clone: A019D5), PE anti-human CD161, (clone: HP-3G10), Brilliant Violet 421TM anti-human CD117 (c-kit), (clone 104D2), PE/Cy7 anti-human CD294 (CRTH2), (clone: BM16), APC anti-human CD336 (NKp44), (clone: 325110), Alexa Fluor® 488 anti-human CD19, (clone: HIB19), FITC anti-human CD94, (clone:DX22), FITC anti-human CD1a, (clone: HI149), FITC anti-human CD11c, (clone: 3.9), FITC anti-human CD123, (clone: 6H6), anti-human CD303 (BDCA-2), (clone:201A), FITC anti-human CD14, (clone: 63D3), FITC anti-human FcεRIα, (clone: NP4D6), FITC anti-human CD34,(clone: 561), APC/Cy7 anti-human IFN-γ, (clone: 4S.B3) all from BioLegend. Anti-Human CD363 (S1PR1) eFluor® 660, (clone: SW4GYPP, ThermoFisher), Mouse IgG1 K Isotype Control eFluor® 660, (clone: P3.6.2.8.1, ThermoFisher). Anti-mouse CD3) APC, (clone: 17A2), anti-mouse NK1.1 Alexa Fluor® 647, (clone: PK136), anti-mouse B220 APC/Cy7 or FITC, (clone:RA3-6B2), anti-mouse CD45-Percp Cy5.5, (clone:30-F11), anti-mouse CD90.2 PE Cy7, (clone:30-H12), anti-mouse CD11b APC, (clone:M1/70), anti-mouse GATA3 PE (Clone: 16E10A23), anti-mouse Rorγt (Clone: 2F7-2).

Real-Time qPCR

Sorted or cultured ILC subsets were spun and lysed with lysis buffer from RNeasy kit (Qiagen, Hilden, Germany), in most studies 3–5 samples were pooled. RNA was extracted. cDNA was synthesized using iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA). Light Cycler® 480 Instrument and SYBR Green method was used to detect PCR products. Relative gene expression levels were calculated by ΔΔCT method. Expression was normalized over 18S ribosomal RNA message. Primer sequences are given in **Figure S6**.

Chemotaxis Assay

Briefly, sorted ILC subsets were rested in serum free media for 4 h at 37°C (5% CO_2). Then 1×10^4 to 2×10^4 cells per insert (depending on the availability of sorted cell number) loaded into the polycarbonate inserts (Corning) with 5 μm pores in 300 μl of serum free medium. In the bottom wells, 500 nM ligand containing medium was added. As control, serum free media, used. After 4 h of culture at 37°C, number of cells migrating to the wells was quantified by Flow cytometry and charted as percent migrated cell vs. treatment. For pretreatment experiments, ILCs were cultured for 2 h in the presence of serum free media (RPMI 1640, Gibco), or media with 1 μm FTY720, or SEW2871, after they are washed, the cells were seeded in the inserts. At the bottom, 1 μm FTY720 was added. Cells migrating to bottom wells were counted by Flow cytometry, SPHERO™ AccuCount Particles.

Enzyme-Linked Immunosorbent Assay (ELISA)

Sorted human ILCs were cultured in 96-well plates at 37°C, 5% CO2 incubator for 2–3 days in medium supplemented with charcoal stripped FBS and ILC polarizing cytokines as described above. The supernatants were collected for ELISA. For whole colon/small intestine cultures, 1 cm piece of tissue from the ileum, or proximal colon was cut from fingolimod or vehicle-treated mice. The tissues were cleaned under sterile conditions as previously described (37) and cultured in 500 μl complete media supplemented with anti-anti at 37°C, 5% CO_2 incubator for 2 days in 24-well plates. Supernatants were collected for ELISA. Collected supernatants from human cells or mouse intestinal tissues were used to run ELISA for human or murine IL-22 (BioLegend #434505 and #436305), IL-17A (BioLegend #433915 and #432505), and GM-CSF (BioLegend #432005 and #432205), respectively. Manufacturer's protocol was followed.

Statistical Analyses

Two tailed, Unpaired Student's *t*-test and GraphPad Prism 6 was used for significance analyses. $P < 0.05$ is accepted as statistically significant.

RESULTS

Human ILC1 and ILC3 Express S1PR1 and Respond to Its Ligands

To assess the expression and functionality of S1P receptors in human ILC subsets, we first analyzed the RNA-Seq and microarray datasets available at GEO database for murine intestinal ILC3s and Th17 cells (48) and human tonsil ILC3 (49) and NK cells. Previously S1PR5 was shown to be expressed more abundantly than other S1P receptors and to regulate NK cell lymphoid tissue egress (20, 21). We observed very low S1PR5 message by both murine and human ILC3s compared with other S1P receptors (**Figure S1A**). We then, sort purified ILCs from human tonsils and cord blood since these two organs have been documented to contain relatively enriched number of ILCs. ILC gating was done based on Mjösberg and Spits (46). Lineage negative (TCRαβ-, TCRγδ-, CD34-, CD123-, CD94- , CD14-, BDCA2-, FcεRIα-, CD1a-, CD11c-, CD19-, B220-) CD3-CD161+CD127+cKit+ cells were sorted as ILC3, Lineage-CD3-CD161+CD127+cKit- cells were sorted as ILC1 and Lineage-CD3-CD161+CD127+CRTH2+ cells sorted as ILC2 (**Figure 1A**). As documented, human tonsils contained around 40 % NKp44+ ILC3s, whereas cord blood contained mostly NKP44- ILC3 fraction (50). Cord blood or peripheral blood ILC3 gate has been recently shown to contain precursors

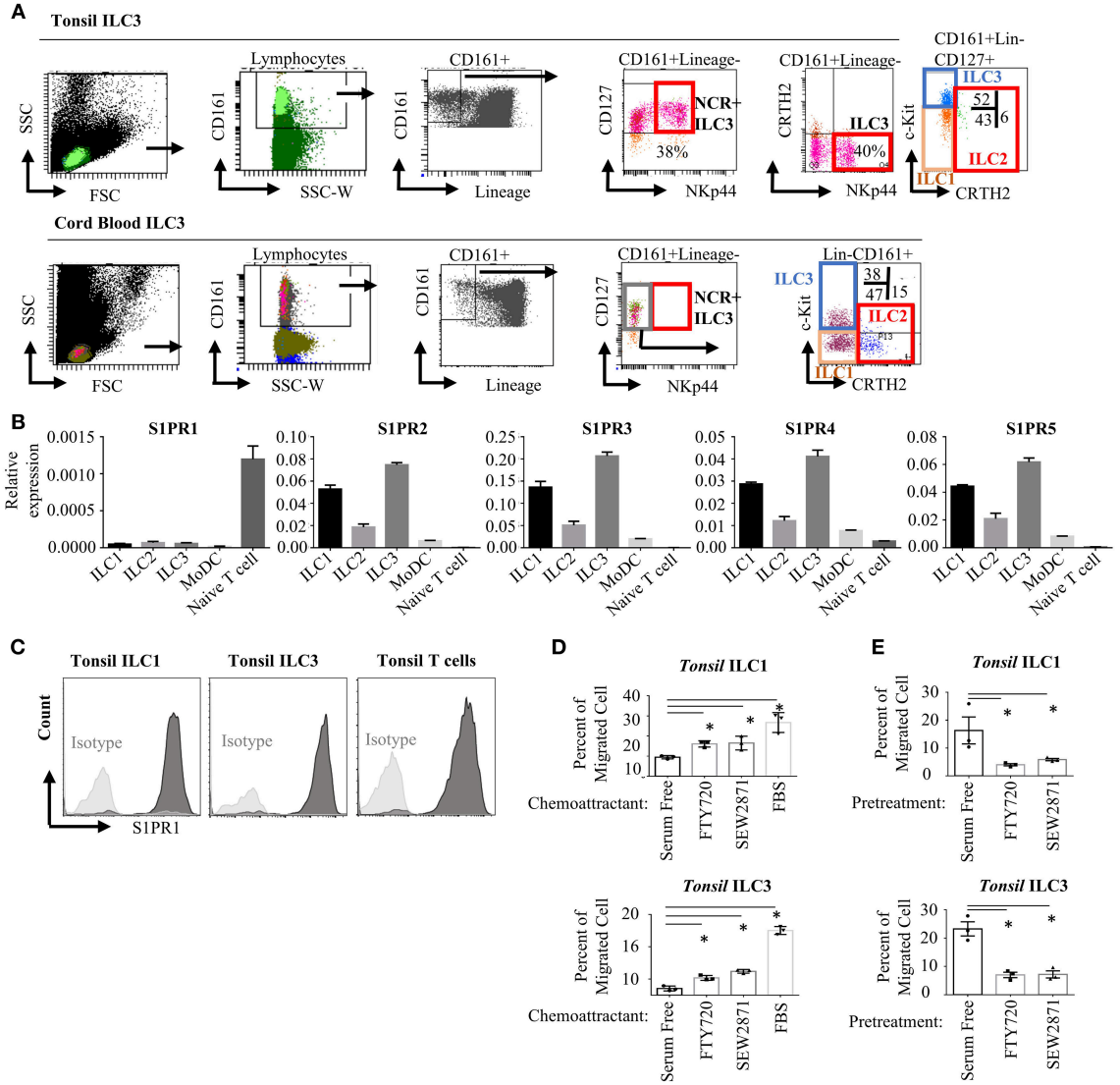

FIGURE 1 | Human ILC subsets express S1P receptors and migrate toward S1P analogs *in vitro*. **(A)** Gating strategy for human ILC subset isolation from tonsil and cord blood. **(B)** Sorted ILC subsets were used to determine relative gene expression of S1P receptors (S1PR1, S1PR2, S1PR3, S1PR4, S1PR5) via real time qPCR. **(C)** Human tonsil ILC1 and ILC3 were stained for S1PR1 to determine protein expression, a representative histogram flow plot with isotype control. **(D)** Migration of ILC1 and ILC3 cultured in serum free media toward FTY720 and SEW2871 gradient. Percentage of cells migrating into lower chamber of a trans-well plate quantified. **(E)** Migration of ILC1 and ILC3 pretreated with serum free media, FTY720 or SEW2871 for 2 h toward FTY720. Percentage of cells migrating was charted. *$p < 0.05$. Experiments in **(B–E)** were performed three separate times.

for all ILCs and named as precursor ILCs (pILCs). This should be kept in mind when blood ILC3s were discussed in the remainder of the paper. Sorted ILC1, ILC2, and ILC3 from tonsils expressed S1PR2-S1PR5 receptors mRNA at higher levels than T and monocyte derived dendritic cells (moDC). In contrast, S1PR1 message levels were significantly higher in T cells compared with ILCs and moDC. S1PR1 gene expression in human ILCs were comparable to that of moDCs (**Figure 1B**). Compared to other S1P receptors S1PR1 expression by human ILCs were low. We also examined the expression of S1P receptors by real time qPCR in the murine intestinal ILC3 and ILC2/ILC1s. Similar to humans, S1PR1 was expressed at lower levels than other S1P receptors, however murine ILC3 and ILC2/ILC1s expressed comparable S1PR1 to intestinal T cells (**Figure S1B**). Flow cytometric assessment of S1PR1 expression revealed high level S1PR1 protein expression by human ILC1 and ILC3s on the cell surface (**Figure 1C** and **Figure S1E**). We next tested the functionality of the S1P receptors in human ILC1 and ILC3 by a chemotaxis assay. To this end, we used two analogs of S1P, FTY720-P (fingolimod) and SEW2871. FTY720 binds four of five S1P receptors (but not S1PR2), SEW2871 however is more specific and binds exclusively S1PR1. Both ILC1s and ILC3s were able to migrate toward high gradients of both FTY720 and SEW2871, or fetal bovine serum (which

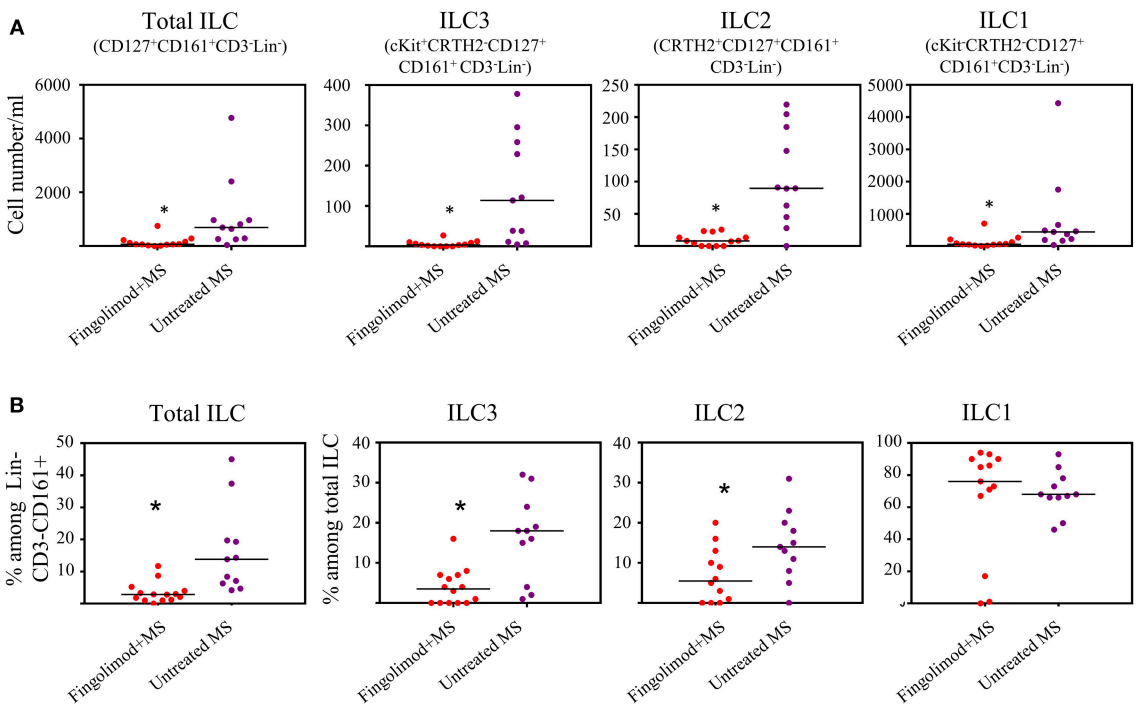

FIGURE 2 | Fingolimod causes ILC-penia in human peripheral blood. **(A)** absolute number of total or subsets of ILCs in the peripheral blood of MS patients treated with fingolimod ($n = 14$) or untreated control MS patients ($n = 11$) per 10 ml peripheral blood. Total ILCs were gated as $CD127^+CD161^+CD3^-Lin^-$ cells, ILC3s as $cKit^+CRTH2^-CD127^+$ $CD161^+$ $CD3^-Lin^-$, ILC2s as $CRTH2^+CD127^+CD161^+$ $CD3^-Lin^-$, and ILC1s as $cKit^-CRTH2^-CD127^+$ $CD161^+CD3^-Lin^-$. **(B)** percentages of ILCs in "A". *Indicates $p < 0.05$.

contains high levels of S1P) compared with serum free media suggesting that S1PR1 may regulate human ILC1 and ILC3 chemotaxis (**Figure 1D**). Furthermore, to assess which S1P receptors used by ILCs to migrate, we pre-treated ILCs with serum free media, FTY720 (1 μM) or SEW2871 (1 μM) for 2 h. The latter two treatments are expected to cause internalization of four S1P receptors (S1PR1, S1PR3, S1PR4, and S1PR5) or S1PR1 alone, respectively, rendering ILCs unresponsive to S1P. Such pre-treatment of ILC1 and ILC3 with SEW2871 blocked migration almost as efficiently as FTY720 pretreatment, suggesting that S1PR1 plays a major role in the migration of human ILC1s and ILC3s (**Figure 1E**). Additionally, treatment of ILC1 with pertussis toxin, a G-protein coupled receptor inhibitor, blocked cell migration toward fingolimod suggesting that S1PR1 may regulate human ILC migration (**Figure S1C**). Although pretreatment of ILCs with FTY720 or SEW2871 blocked their migration toward FTY720 *ex vivo*, the cells were able to migrate toward serum even after pretreatment FTY720 or SEW2871 suggesting other ligands and receptors may compensate their migration *ex vivo* (**Figure S1D**) (51). These data collectively show that human ILC1s and ILC3s express S1P receptors and thus respond to ligands *ex vivo*, and that S1PR1 plays an important role in human ILC1 and ILC3 migration. Unfortunately, we could not include ILC2 in our chemotaxis assays due low yield of cell number in the sorts.

Fingolimod Receiving MS Patients Have ILC-Penia

Next, we explored whether human ILCs respond to S1P agonists *in vivo*. To this end we recruited 14 patients with relapsing remitting MS (RRMS) who are under fingolimod therapy and compared blood ILC subset numbers to that of RRMS patients who were not receiving fingolimod (n=11). Age and sex information for the patients were provided in **Table S1**. Gating strategy for human peripheral blood ILCs is shown in **Figure S2A**. We observed dramatic reductions in the absolute number of total ILCs, and all subsets of ILCs in the peripheral blood of MS patients who are under fingolimod therapy compared to control MS patients not on fingolimod (**Figures 2A,B**). The percentages of total ILCs among $CD161^+CD3^-Lin^-$ cells, and of ILC1s and ILC2s among $CD127^+CD161^+CD3^-Lin^-$ total ILCs were also reduced. These data collectively indicate that human ILCs respond to S1P receptor agonists and suggests that fingolimod sequesters peripheral blood human ILCs in LNs.

Fingolimod Alters ILC Distribution Across Lymphoid Organs and Gut in Mice

Recently Huang et al. reported that murine ILC2 tissue trafficking is regulated by S1P, especially the egress from intestinal tissue to lung during inflammation (52). Yet it is unclear if, in

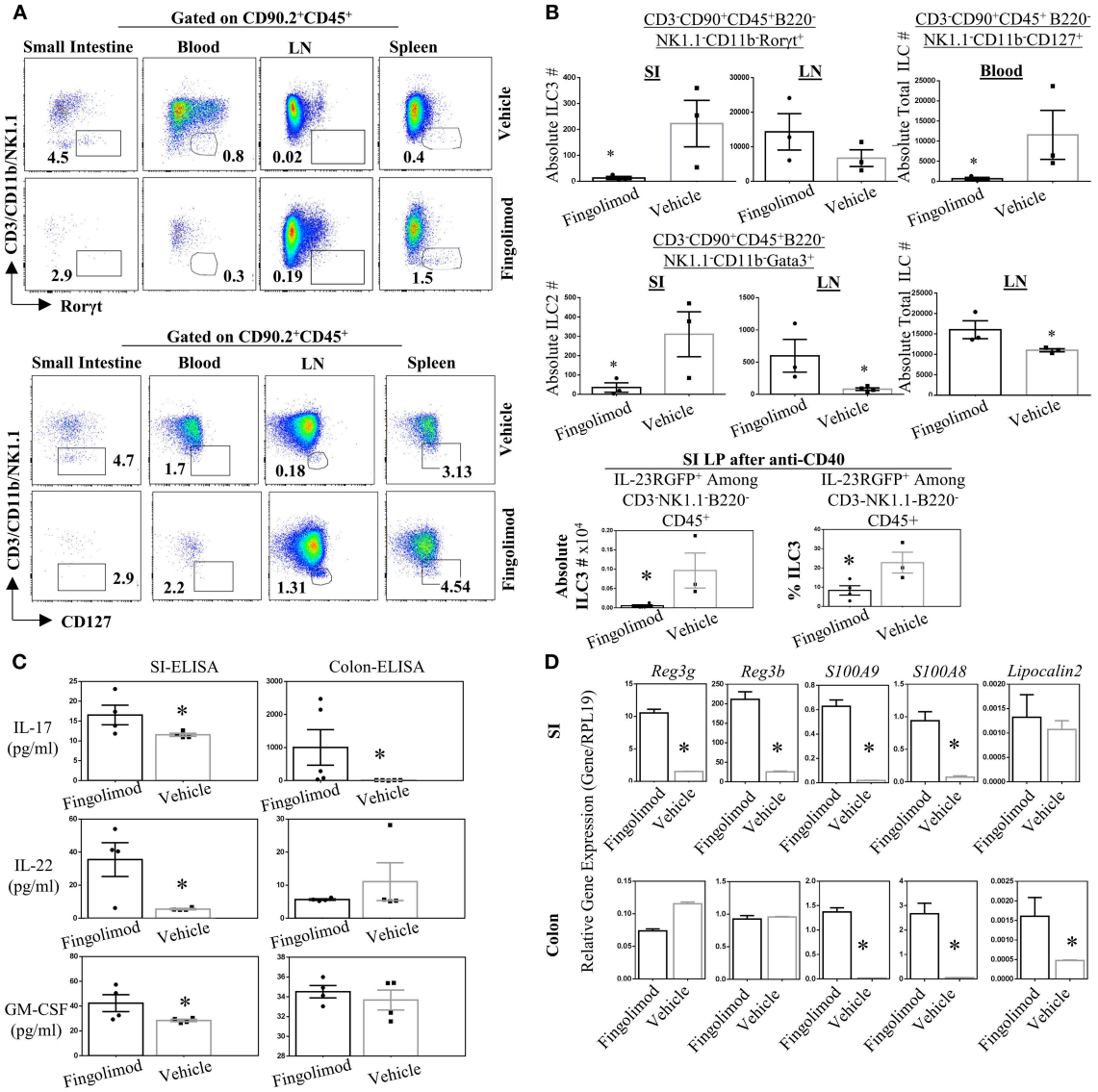

FIGURE 3 | Fingolimod causes ILC-penia, augments lymph node ILC numbers, and decreases small intestine lamina propria ILC3 numbers in mice but does not reduce antimicrobial peptide production. **(A)** A representative flow plot of the percentages of total ILCs in the small intestine lamina propria blood, inguinal lymph node (LN) and spleen of mice gavage-fed with fingolimod or vehicle for 30 days. The plots show live cells gated on CD90.2+CD45+B220- which then plotted as CD3/NK1.1/CD11b vs. Rorγt (top panel) or Gata3 (bottom panel). **(B)** Absolute number of Rorγt+ ILC3s (top panel), of Gata3+ ILC2s (middle panel) in the steady state mice after fingolimod of vehicle-gavage feeding for 30 days. Top panel, right, shows total ILCs (CD90.2-CD45+CD3-B220-CD11b-NK1.1-CD127+) in the blood of steady state mice following 30 days of fingolimod or vehicle treatment. The bottom most panel shows absolute number or percentages of IL-23RGFP+ ILC3s in the small intestine lamina propria 2-days after anti-CD40 injection following 30 days fingolimod or vehicle treatment. Three-to-four mice per group were used. Experiment was repeated 3 times. **(C)** 1 cm piece of ileum from fingolimod or vehicle-injected mice (for 30 days) were cultured 48 h and the supernatants were assessed with ELISA for the production of indicated ILC3-associated cytokines. Four to five of mice per group. **(D)** 1 cm piece of ileum or colon from fingolimod or vehicle-injected mice for 30 days was examined for gene expression of indicated antimicrobial peptides via real-time qPCR. *Indicates $p < 0.05$.

humans or murine models, long term fingolimod use alters ILC3 distribution across tissues, and consequently the associated cytokine production. Given the critical role of ILC3s in the establishment of barrier immunity, we quantified the number of ILC subsets in the lymphoid organs and gut of healthy B6 mice treated daily with fingolimod intraperitoneally for 30 days (**Figure 3**). Then the mice were sacrificed to harvest organs in a non-inflammatory setting. In some experiments, 2 days prior to terminating the experiment, we injected mice intraperitoneally with 100 μg of anti-CD40 to expand and boost the detection of gut ILC3s (37). anti-CD40 injection have been shown to promote IL-23 production by DCs and expand ILC3s. in the gut (37). Murine ILC detection was done based on our previous publication (45) and gate strategy for every organ is given in

Figure S3. After B220-CD45+CD90.2+ cells were gated, CD3-CD11b-NK1.1- Rorγt+ cells were defined as ILC3s, and CD3-CD11b-NK1.1-Gata3+ cells defined as ILC2s. CD3-CD11b-NK1.1-CD127+ cells were accepted as total ILCs. We also used IL-23RGFP reporter mice for fingolimod injections/gavage feeding which marks ILC3s without the need for intracellular Rorγt+ staining (**Figures S3B,C**). In the blood, using either IL-23RGFP mice or Rorγt+ staining, detection of a specific classical ILC3 was not possible (**Figure 3A, Figures S2B, S3A**). Similarly, using Gata3 staining, detection of a specific ILC2 subset was also difficult (**Figure S4A**). For these reasons, we gated CD90.2+CD45+CD3-CD11b-NK1.1-CD127+ fraction to mark total blood ILCs or ILC precursors. This fraction of blood ILCs were significantly diminished following fingolimod injections (**Figure 3B**). Conversely, in the inguinal LN of mice injected with fingolimod we detected significantly higher absolute numbers of total ILCs (**Figure 3B**), suggesting that, similar to T and B cells, they rely on S1P receptors for egress from secondary lymphoid organs (**Figures 3A,B**). Moreover, in the lamina propria of small intestine and LN we could show significantly lower number of Rorγt+ILC3s and Gata3+ILC2s after 30 days of Fingolimod injections (**Figures 3A,B** and **Figure S4A**). This reduction in ILC3s in the small intestine could also be recapitulated after anti-CD40 injections (**Figure 3C**, bottom panel; **Figure S4C**). The changes in the frequency of ILCs in the blood, lymph nodes or small intestine lamina propria upon fingolimod treatment were similar to those of CD45+ lymphocytes, T and B cells, suggesting that fingolimod affects distribution of most lymphocytes in a similar way, **Figures S4B,F**. Importantly, fingolimod treated mice did not show any observable health issues or weakness during the 20–30 days of treatment.

Genetic deletion or antibody-mediated depletion of ILC3s from mice was previously associated with bacterial translocation across intestine, and into blood (53). Therefore, to further assess whether fingolimod-induced intestinal ILC3 depletion translates to any changes in the barrier immunity, we quantified the expression of various ILC3-associated cytokines (IL-17A and F, IL-22, GMCSF, IL-10) and antimicrobial proteins (Reg3γ, Reg3B, S100A9, S100A8) in the intestines of mice following fingolimod injections (**Figures 3C,D**). To our surprise, we detected high levels of anti-microbial Reg3γ, Reg3B, S100A9, S100A8 in the ileum of mice treated with fingolimod intraperitoneally compared with controls. In the colon, only S100A9, S100A8, and lipocalin 2 messages were highly upregulated. Intestinal *ex vivo* culture supernatants also revealed significantly higher IL-17A, IL-22, and GM-CSF protein production by the fingolimod treated group suggesting that despite the drop in the cell number, the intestinal tissue of mice which received intraperitoneal fingolimod is able produce antimicrobial peptides and IL-22 or IL-17A.

We next wondered whether route of fingolimod administration had an effect in our observations. Therefore, we gavage-fed the mice with fingolimod for 2-weeks and then following anti-CD40 treatment, quantified the number of ILCs in the gut, and examined gut and skin tissue for antimicrobial peptide and ILC3-associated cytokine production (**Figures S4C–E**). The results were almost identical to that of mice treated intraperitoneally with fingolimod. ILCs were greatly reduced in number in the colon and small intestine lamina propria in orally fingolimod-treated mice compared with controls (**Figure S4C**). More importantly, both in the gut and skin, antimicrobial peptides Reg3γ, Reg3B, and IL-22 cytokine gene expression were upregulated (**Figures S4C,E**). Collectively these results argue that fingolimod use, though alters tissue distribution of ILC3s, does not reduce antimicrobial peptide or Th17-associated cytokine production in the gut or skin.

S1PR1 Ligands Have Immunomodulatory Effects on ILC1 and ILC3

Next, we explored the egress-independent impact of S1P analogs on human ILC3 and ILC1. We first cultured ILC3s and ILC1s sorted from healthy human tonsils or cord blood in charcoal-treated-FBS supplemented media (which is stripped of endogenous S1P) with different concentrations of fingolimod or SEW2871 for 3 days. ILC3 cultures are activated with a cytokine cocktail of IL-2/IL-7/IL-1B/IL-23 (50 ng/ml each). We did not observe a toxic effect of the drugs on ILC3 (**Figure S5**) or ILC1s (not shown) at doses below 10 μM when assessed by staining of apoptosis markers ANNEXINV and 7-AAD. Tonsil ILC3s produced GM-CSF, IL-22 and IL-17, when stimulated with IL-2/IL-7/IL-1B/IL-23 cytokine cocktail, and S1P analogs inhibited secretion and gene expression of these cytokines in a dose dependent manner (**Figures 4A,B**). As previously reported cord blood ILC3 subsets did not produce conventional ILC3 cytokines IL-22 or IL-17A (47). They produced GM-CSF in large quantities and S1P analogs also inhibited GMCSF production in a dose dependent manner (**Figure 4B**). We also checked the surface NKP44 expression by ILC3s cultured in the presence of S1P FTY720 (**Figure 4C**). IL-2/IL-7/IL-1B/IL-23 cytokine cocktail stimulation upregulated NKP44 surface expression by human tonsil ILC3s. Fingolimod exposure downregulated its surface expression in a dose-dependent manner (**Figure 4C**). Unlike ILC3s, fingolimod and SEW2871 inhibited IFN-γ production by ILC1 only at very high concentrations (10 μM) (**Figures 5A–C**). These *ex-vivo* treatment data contrast with *in vivo* fingolimod exposure data and suggest that, the observed increase in Th17-associated cytokines in the fingolimod injected mice gut *in vivo*, may come from sources other than ILC3s.

DISCUSSION

This study provides novel information regarding the regulation of ILCs by S1P analogs in humans primarily, and in mice. We show that all ILC subsets in humans express S1PR1 at the mRNA message level and specifically ILC1 and ILC3 at the protein level and migrate toward S1PR1 analogs *ex vivo*. Our data show that fingolimod, a currently used S1P analog, can sequester ILCs in secondary lymphoid organs in humans and mice, thus number of circulating blood ILCs as well as gut resident ILC3s drop drastically. This drop in the intestinal ILC3s, however, does not result in impaired production of mucosal cytokines, or anti-microbial peptides. Lastly, we provide evidence

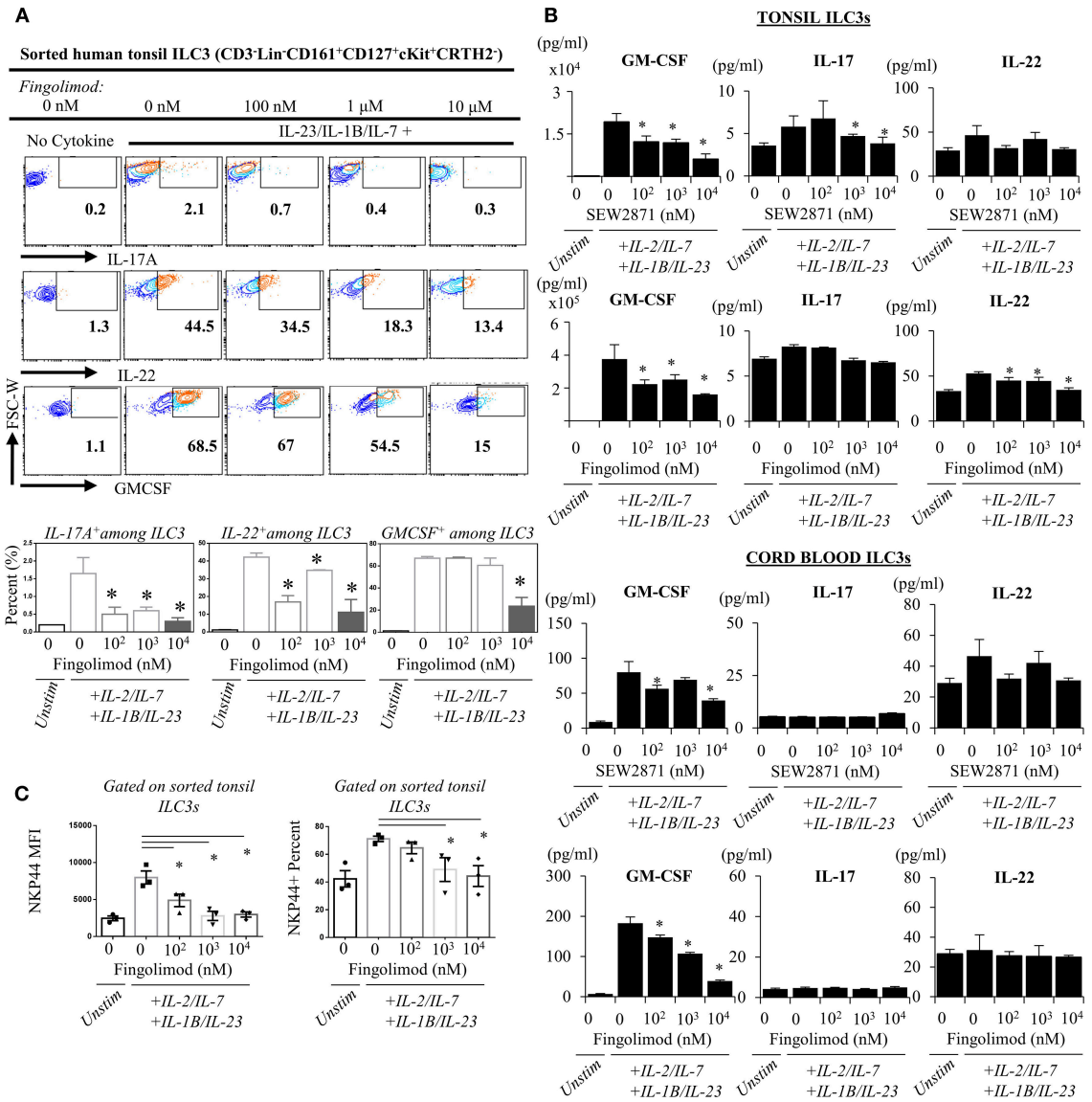

FIGURE 4 | *Ex vivo* exposure of human ILC3s to S1P analogs inhibits production of ILC3-associated cytokines. **(A)** Sorted ILC3s from tonsils (gated as Lin-CD3-CD161+CD127+ckit+ CRTH2-) were cultured in complete medium at increasing doses of FTY720 and activated with IL-2, IL-23, IL-7, and IL-1B (20 ng/ml each) for 2–3 days, and PMA (50 ng/ml) /Inonomycin (1 μg/ml) stimulated for 4 h at 37°C for intracellular staining of indicated cytokines, representative flow plots (left) and quantified bar graphs for percentages of cells producing the indicated cytokines (right). **(B)** ELISA was performed from supernatants of "A" and SEW2871 exposed tonsil-derived (top panel) cord blood-derived (bottom panel) ILC3 cultures for ILC3-asssociated cytokines cord-derived ILC3s. **(C)** NKP44 surface expression by ILC3s were examined via flow cytometry, percent and mean fluorescence intensity (MFI) was quantified after culture with FTY720 for 3 days. *Indicates $p < 0.05$. The experiments were performed with triplicates and repeated for 3 times.

as to immunomodulatory effects of fingolimod on human ILC3s and ILC1s.

To the best of our knowledge, there is only a single recent report in which a murine ILC subset, ILC2s, was studied in relation to S1P/fingolimod (52). Interestingly, in that study, fingolimod injections were reported to block ILC2 migration from intestines to lung or bone marrow during an inflammatory state, thus increasing their abundance in the intestinal tissue. ILC3s have not been studied in that report (52). Moreover, there is no available research as to how and if S1P or its synthetic analogs regulate human non-NK ILC subsets. ILC egress from secondary lymphoid organs have not been investigated to this day. First studies by Gasteiger et al. in mice suggested that both lymphoid and non-lymphoid tissue residents ILCs are sedentary and are mostly maintained by local self-renewal during steady state (54). Using parabiotic mice, they also showed that even during two distinct forms of inflammatory state, tissue resident ILCs are of mostly host derived (54). More recently, Lim et al.

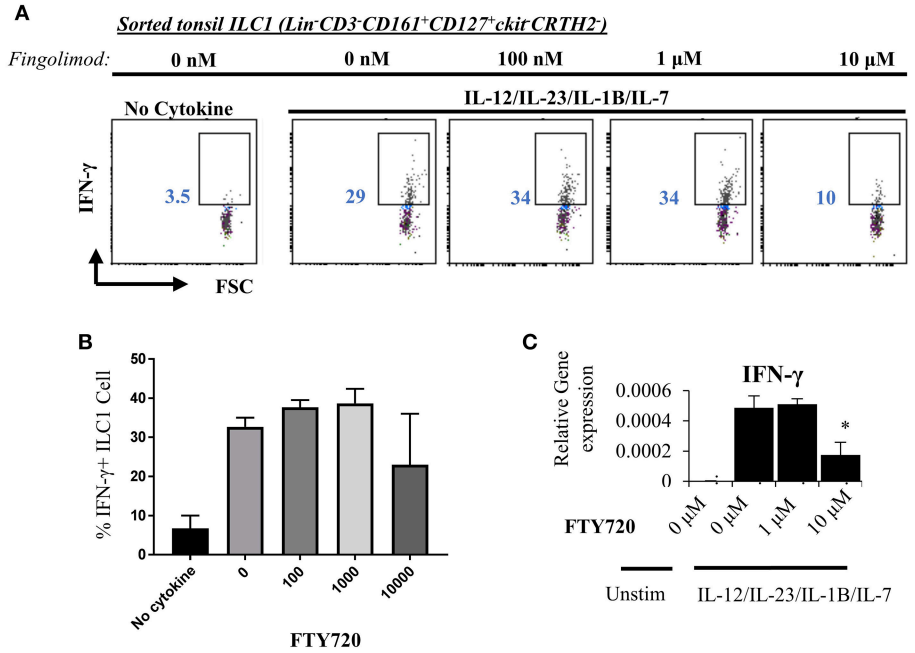

FIGURE 5 | Fingolimod does not alter IFN-γ production by ILC1 at low doses. **(A)** ILC1s sorted from tonsils (gated as Lin-CD3-CD161+CD127+ckit-CRTH2-) were cultured in charcoal stripped FBS-supplemented media containing IL-12/IL-23/IL-1B/IL-7, 20 ng each, with increasing concentrations of FTY70 for 3 days IFN-γ production was measured with intracellular staining (**A**, a representative plot) and quantified **(B)** and mRNA levels were quantified via real time qPCR **(C)**.

showed that, the circulating blood ILC3 population in humans contained a number of ILC precursors which can migrate into tissues, and *in situ*, differentiate into all three subsets of ILCs (47). This study employed a combination of *in vivo* adoptive transfer as well as serial dilution and cloning studies *ex vivo* (47). Our data on human ILCs in fingolimod-treated MS patients, combined with fingolimod-injected mice, show that ILC circulation in both species could be interrupted by fingolimod. The increase in the LNs and the decrease in a non-lymphoid tissue, small intestine, show that egress from secondary lymphoid tissue to lymph is regulated by S1P receptors. Given that FTY720 binds four of the S1P receptors, and ILCs can migrate toward SEW2871, a selective S1PR1 agonist, and SEW2871 pre-treatment blocks ILC1/ILC3 migration as efficiently as FTY720 pre-treatment, we propose that S1PR1 may be the major receptor for migration of human ILC1 and ILC3 toward S1P.

Second important piece of information our present study reveals is that long term fingolimod use does not compromise barrier immunity-associated cytokines, or antimicrobial peptides. Several studies showed that ILC3s and Th17 cells are crucial for homeostasis in the gut (28, 31). ILC3s are critical in various processes in the gut including tolerance induction (55), production of antimicrobial proteins (56), epithelial regeneration (53, 57), in addition to their role in protective immunity against fungal or extracellular pathogens. Whether the number of ILC3s in the small intestine changes over extended period of fingolimod use, and consequently impact those processes is a legitimate concern. Our results are interesting in that, although intestinal lamina propria resident ILC3s drop in numbers, probably due to entrapment of circulating ILCs in the LNs, ILC3-signature cytokines and antimicrobial peptides remained significantly high after prolonged fingolimod injections.

Lastly our data provide evidence for immunomodulatory effects of S1P analogs on human ILCs. S1P was previously shown to inhibit IFN-γ and IL-4 production by CD4+ T cells (58). Others also reported that FTY720 was able to inhibit IL-33/IL12-induced IFN-γ production by CD8+ T cells (59). Our data reveal that SEW2871, a selective S1PR1 agonist, and FTY720, a non-selective S1PR1 agonist can both block GM-CSF, IL-17A, and IL-22 production by ILC3s in a dose-dependent manner *ex vivo*. However, intraperitoneal injection or oral administration of fingolimod did not result in the reduction of these cytokines *in vivo* in the intestinal tissue, despite a drop in ILC3 numbers in the small intestine lamina propria. This suggests that sources other than ILC3s such as Th17, γδ T, mucosal-associated invariant T (MAIT) cells or neutrophils may contribute to the production of these cytokines. It appears that long term fingolimod injection may have activated remaining sources of such cytokines in the gut, as a result, more IL-22, GM-CSF and IL-17A is produced compared with vehicle treated mice intestinal tissue cultures.

In summary, this work reveals that human and murine ILCs utilize S1P receptors for egress from secondary lymphoid organs, and that ILCs could be targeted by S1P analogs in both humans and mice.

ETHICS STATEMENT

All protocols were approved by the local ethics committee for animal research (#2017/458). All the experiments were performed in accordance with the relevant guidelines and

regulations established by Erciyes University Institutional Review Board in accordance with Helsinki Guidelines.

AUTHOR'S NOTE

Part of the results in this study were presented as poster and oral presentation in the AAI 2018 meeting in Texas, USA, and WIRM Meeting in 2017 in Davos, Switzerland, respectively.

AUTHOR CONTRIBUTIONS

AE, AV, MY, HD, HC, MM, MO, and MK designed the study, reviewed the manuscript interpreted the results. FO, SE, and ZA, performed cell sorting, and most experiments. YH, OK, ET, and IK performed chemotaxis experiments and reviewed the manuscript. MC helped with oral fingolimod treatment experiments in mice. MK provided cord blood samples. AV and IK organized tonsil samples. AE wrote manuscript and supervised the study.

FUNDING

This work was supported partly by the Erciyes University BAP grant, TOA-2016-6130; TUBITAK grants, 215S725 and 315S315 to AE; partly by RG-1802-30486 to MO.

ACKNOWLEDGMENTS

We are grateful to Erciyes University Gevher Nesibe Hospital ENT Department Surgeons, Genkok administrative personnel and Erciyes University Animal Vivarium personnel at DEKAM.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00217/full#supplementary-material

Figure S1 | Expression levels of S1P receptors by murine and human ILC subsets. **(A)** S1P receptor expression by murine intestinal ILC3 and Th17 RNASeq data from GSE70596 (left). S1P receptor expression by human ILC3 and NK cells, microarray data obtained from GSE63197 (right). **(B)** Relative gene expression of S1P receptors by murine intestinal ILC2, ILC3, and T cells measured by real time qPCR. **(C)** Sorted human ILC1s are pretreated with either serum free media alone or with pertussis toxin for 2 h, then cell migration toward 1 μM FTY20 was quantified using trans-well migration assay. Percentage of cells migrating to bottom chamber was shown. **(D)** Sorted human total ILCs cells were pretreated with either serum free media, or FTY720, or SEW2871 for 2 h, then cell migration toward FBS was quantified using trans-well migration assay. No-FBS condition measures spontaneous migration toward serum free media. **(E)** Sorted human Tonsil ILC1 (CD3-Lin-CD161+CD127+cKit-CRTH2-), ILC3 (CD3-Lin-CD161+CD127+cKit+CRTH2-) and T cells (CD3+Lin-CD161-) were stained with S1PR1 or isotype antibody. * indicates p value < 0.05.

Figure S2 | Gating strategy for PBMCs obtained from humans and mice. **(A)** A representative sequential gating for human peripheral blood ILC subsets. Top panel shows untreated MS patient blood PBMCs, bottom panel shows fingolimod receiving-patient PBMCs. **(B)** A representative sequential gating of mouse peripheral blood ILC3s for a blood sample obtained from IL-23RGFP reporter mouse.

Figure S3 | Murine ILC gating Strategy. **(A)** Gating of mouse ILCs using Gata3 and Rorγt staining in blood, spleen, small intestine (SI) inguinal lymph node (LN). **(B,C)** Gating of ILC3s in the small intestine (SI) using IL-23RGFP reporter mice. A representative flow plot for one mouse.

Figure S4 | Oral fingolimod administration decreases murine small intestine lamina propria ILC3 numbers in mice but does not reduce antimicrobial peptide production. **(A)** Representative flow plots for Gata3+ ILC2 distribution in the organs of fingolimod- or vehicle fed mice for 30 days. **(B)** Absolute number of CD3+ T and B220+ B or CD45+ total lymphocytes in the blood, mesenteric lymph node (LN) and small intestine of fingolimod- or vehicle fed mice for 30 days. **(C)** Absolute number of total lymphocytes, $CD45^{medium}CD90.2^{high}$ ILC3s in the small intestine or colon lamina propria of anti-CD40 injected mice, day 2 of injection. Five mice per group were used. Experiment was repeated 2 times. **(D)** 1 cm piece of ileum or colon from mice treated orally with fingolimod or vehicle for 15 days was examined for gene expression of indicated antimicrobial peptides and cytokines via real-time qPCR. **(E)** 1 cm^2 piece of skin from mice treated orally with fingolimod or vehicle for 15 days was examined for gene expression of indicated antimicrobial peptides and cytokines via real-time qPCR. Five mice per group were used. Skins were pooled and run as technical triplicates. **(F)** Small intestine lamina propria lymphocytes were isolated from 30-day fingolimod treated mice, B220 vs. CD45 or FSC vs. CD45 flow plots were shown for one mouse per group. *Indicates $p < 0.05$.

Figure S5 | Fingolimod does not have toxic effects on human ILC3 below 10 μM doses. A representative flow plot for 7AAD and ANNEXIN V staining of sorted ILC3 ($CD3^-Lin^-CD161^+CD127^+cKit^+CRTH2^-$) cultured in the presence of absence of activating cytokines for 3 days at varying fingolimod doses (Top panel). The percentages of early apoptotic ($ANNEXINV^+7AAD^-$), late apoptotic ($ANNEXINV^+7AAD^+$) and live ($ANNEXINV^-7AAD^-$) cells quantified.

Figure S6 | Primer list.

Table S1 | MS Patient age and sex information.

REFERENCES

1. Chi H. Sphingosine-1-phosphate and immune regulation: Trafficking and beyond. *Trends Pharmacol Sci.* (2011) 32:16–24. doi: 10.1016/j.tips.2010.11.002
2. Rosen H, Stevens RC, Hanson M, Roberts E, Oldstone MBA. Sphingosine-1-Phosphate and Its Receptors: Structure, Signaling, and Influence. *Annu Rev Biochem.* (2013) 82:637–62. doi: 10.1146/annurev-biochem-062411-130916
3. Cinamon G, Matloubian M, Lesneski MJ, Xu Y, Low C, Lu T, et al. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol.* (2004) 5:713–20. doi: 10.1038/ni1083
4. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* (2004) 427:355–60. doi: 10.1038/nature02284
5. Allende ML, Dreier JL, Mandala S, Proia RL. Expression of the Sphingosine 1-Phosphate Receptor, S1P1, on T-cells Controls Thymic Emigration. *J Biol Chem.* (2004) 279:15396–401. doi: 10.1074/jbc.M314291200
6. Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* (2007) 316:295–8. doi: 10.1126/science.1139221
7. Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P Gradients. *Science* (2005) 309:1735–9. doi: 10.1126/science.1113640
8. Cyster JG, Schwab SR. Sphingosine-1-Phosphate and Lymphocyte Egress from Lymphoid Organs. *Annu Rev Immunol.* (2012) 30:69–94. doi: 10.1146/annurev-immunol-020711-075011
9. Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate

receptor agonists. *Science* (2002) 296:346–9. doi: 10.1126/science.1070238
10. Koyrakh L, Roman MI, Brinkmann V, Wickman K. The Heart Rate Decrease Caused by Acute FTY720 Administration Is Mediated by the G Protein-Gated Potassium Channel IKACh. *Am J Transplant* (2005) 5:529–36. doi: 10.1111/j.1600-6143.2005.00754.x
11. Cohen JA, Barkhof F, Comi G, Hartung H-P, Khatri BO, Montalban X, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N Engl J Med*. (2010) 362:402–15. doi: 10.1056/NEJMoa0907839
12. Bourdette D, Gilden D. Fingolimod and multiple sclerosis: four cautionary tales. *Neurology* (2012) 79:1942–3. doi: 10.1212/WNL.0b013e3182735edf
13. Jander S, Turowski B, Kieseier BC, Hartung H-P. Emerging tumefactive multiple sclerosis after switching therapy from natalizumab to fingolimod. *Mult Scler J*. (2012) 18:1650–2. doi: 10.1177/1352458512463768
14. Gross CM, Baumgartner A, Rauer S, Stich O. Multiple sclerosis rebound following herpes zoster infection and suspension of fingolimod. *Neurology* (2012) 79:2006–7. doi: 10.1212/WNL.0b013e3182735d24
15. Centonze D, Rossi S, Rinaldi F, Gallo P. Severe relapses under fingolimod treatment prescribed after natalizumab. *Neurology* (2012) 79:2004–5. doi: 10.1212/WNL.0b013e3182735c7a
16. Visser F, Wattjes MP, Pouwels PJW, Linssen WHJP, van Oosten BW. Tumefactive multiple sclerosis lesions under fingolimod treatment. *Neurology* (2012) 79:2000–3. doi: 10.1212/WNL.0b013e3182735cb3
17. *Search of: siponimod - List Results - ClinicalTrials.gov*. Available onlline at: https://clinicaltrials.gov/ct2/results?cond=&term=siponimod&cntry1=&state1=&Search=Search (Accessed October 7, 2017).
18. *Search of: ponesimod - List Results - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/results?cond=&term=ponesimod&cntry1=&state1=&Search=Search (Accessed October 7, 2017).
19. *Search of: ozanimod - List Results - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/results?cond=&term=ozanimod&cntry1=&state1=&recrs= (Accessed October 7, 2017).
20. Walzer T, Chiossone L, Chaix J, Calver A, Carozzo C, Garrigue-Antar L, et al. Natural killer cell trafficking *in vivo* requires a dedicated sphingosine 1-phosphate receptor. *Nat Immunol.* (2007) 8:1337–44. doi: 10.1038/ni1523
21. Drouillard A, Mathieu A-L, Marçais A, Belot A, Viel PharmD S, Mingueneau M, et al. S1PR5 is essential for human natural killer cell migration toward sphingosine-1 phosphate. *J Allergy Clin Immunol*. (2018) 141:2265–8.e1. doi: 10.1016/j.jaci.2017.11.022
22. Melo-Gonzalez F, Hepworth MR. Functional and phenotypic heterogeneity of group 3 innate lymphoid cells. *Immunology* (2017) 150:265–75. doi: 10.1111/imm.12697
23. Klose CSN, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol.* (2016) 17:765–74. doi: 10.1038/ni.3489
24. Eberl G, Di Santo JP, Vivier E. The brave new world of innate lymphoid cells. *Nat Immunol.* (2014) 16:1–5. doi: 10.1038/ni.3059
25. Eberl G, Colonna M, Di Santo JP, McKenzie ANJ. Innate lymphoid cells. Innate lymphoid cells: a new paradigm in immunology. *Science* (2015) 348:aaa6566. doi: 10.1126/science.aaa6566
26. Montaldo E, Vacca P, Vitale C, Moretta F, Locatelli F, Mingari MC, et al. Human innate lymphoid cells. *Immunol Lett*. (2016) 179:2–8. doi: 10.1016/j.imlet.2016.01.007
27. Montaldo E, Juelke K, Romagnani C. Group 3 innate lymphoid cells (ILC3s): Origin, differentiation, and plasticity in humans and mice. *Eur J Immunol.* (2015) 45:2171–82. doi: 10.1002/eji.201545598
28. McKenzie ANJ, Spits H, Eberl G. Innate lymphoid cells in inflammation and immunity. *Immunity* (2014) 41:366–74. doi: 10.1016/j.immuni.2014.09.006
29. Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity* (2014) 41:354–65. doi: 10.1016/j.immuni.2014.09.005
30. Satoh-Takayama N, Vosshenrich CAJ, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* (2008) 29:958–70. doi: 10.1016/j.immuni.2008.11.001
31. Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Bérard M, Kleinschek M, et al. RORγt+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol.* (2011) 12:320–6. doi: 10.1038/ni.2002
32. Sonnenberg GF, Monticelli LA, Elloso MM, Fouser LA, Artis D. CD4+ Lymphoid Tissue-inducer cells promote innate immunity in the gut. *Immunity* (2011) 34:122–34. doi: 10.1016/j.immuni.2010.12.009
33. Geremia A, Arancibia-Cárcamo CV, Fleming MPP, Rust N, Singh B, Mortensen NJ, et al. IL-23–responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med*. (2011) 208:1127–33. doi: 10.1084/jem.20101712
34. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and−23 Control Plasticity of CD127+ Group 1 and Group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity* (2015) 43:146–60. doi: 10.1016/j.immuni.2015.06.019
35. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, et al. Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγt(+) innate lymphocytes. *Immunity* (2010) 33:736–51. doi: 10.1016/j.immuni.2010.10.017
36. Klose CSN, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. *Nature* (2013) 494:261–5. doi: 10.1038/nature11813
37. Eken A, Singh AK, Treuting PM, Oukka M. IL-23R+ innate lymphoid cells induce colitis via interleukin-22-dependent mechanism. *Mucosal Immunol.* (2014) 7:143–54. doi: 10.1038/mi.2013.33
38. Qiu J, Guo X, Chen Z-ME, He L, Sonnenberg GF, Artis D, et al. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity* (2013) 39:386–99. doi: 10.1016/j.immuni.2013.08.002
39. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* (2010) 464:1371–5. doi: 10.1038/nature08949
40. Griseri T, Arnold IC, Pearson C, Krausgruber T, Schiering C, Franchini F, et al. Granulocyte macrophage colony-stimulating factor-activated eosinophils promote interleukin-23 driven chronic colitis. *Immunity* (2015) 43:187–99. doi: 10.1016/j.immuni.2015.07.008
41. Cox JH, Kljavin NM, Ota N, Leonard J, Roose-Girma M, Diehl L, et al. Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. *Mucosal Immunol.* (2012) 5:99–109. doi: 10.1038/mi.2011.54
42. Shikhagaie MM, Germar K, Bal SM, Ros XR, Spits H. Innate lymphoid cells in autoimmunity: emerging regulators in rheumatic diseases. *Nat Rev Rheumatol.* (2017) 13:164–73. doi: 10.1038/nrrheum.2016.218
43. Eken A, Donmez-Altuntas H. Innate Lymphoid Cells (Non-NK ILCs). In: Isvoranu G. Editor. *Lymphocyte Updates - Cancer, Autoimmunity and Infection*. London: InTech (2017). p. 129–48.
44. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol.* (2018) 17:162–73. doi: 10.1016/S1474-4422(17)30470-2
45. Singh AK, Eken A, Fry M, Bettelli E, Oukka M. DOCK8 regulates protective immunity by controlling the function and survival of RORγt+ ILCs. *Nat Commun.* (2014) 5:4603. doi: 10.1038/ncomms5603
46. Mjösberg J, Spits H. Human innate lymphoid cells. *J Allergy Clin Immunol.* (2016) 138:1265–76. doi: 10.1016/j.jaci.2016.09.009
47. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell* (2017) 168:1086–1100.e10. doi: 10.1016/j.cell.2017.02.021
48. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, et al. An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. *Cell* (2015) 163:381–93. doi: 10.1016/j.cell.2015.08.061
49. Montaldo E, Teixeira-Alves LG, Glatzer T, Durek P, Stervbo U, Hamann W, et al. Human RORγt+CD34+ cells are lineage-specified progenitors of group 3 RORγt+ Innate Lymphoid Cells. *Immunity* (2014) 41:988–1000. doi: 10.1016/j.immuni.2014.11.010
50. Hazenberg MD, Spits H. Human innate lymphoid cells. *Blood* (2014) 124:700–9. doi: 10.1182/blood-2013-11-427781
51. Ledgerwood LG, Lal G, Zhang N, Garin A, Esses SJ, Ginhoux F, et al. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat Immunol.* (2008) 9:42–53. doi: 10.1038/ni1534

52. Huang Y, Mao K, Chen X, Sun M-A, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science* (2018) 359:114–9. doi: 10.1126/science.aam5809
53. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science* (2012) 336:1321–5. doi: 10.1126/science.1222551
54. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science* (2015) 350:981–5. doi: 10.1126/science.aac9593
55. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4+ T cells. *Science* (2015) 348:1031–5. doi: 10.1126/science.aaa4812
56. Cash HL, Whitham C V, Behrendt CL, Hooper L V. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* (2006) 313:1126–30. doi: 10.1126/science.1127119
57. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol.* (2011) 12:383–90. doi: 10.1038/ni.2025
58. Dorsam G, Graeler MH, Seroogy C, Kong Y, Voice JK, Goetzl EJ. Transduction of multiple effects of sphingosine 1-phosphate (S1P) on T cell functions by the S1P1 G protein-coupled receptor. *J Immunol.* (2003) 171:3500–7. doi: 10.4049/jimmunol.171.7.3500
59. Ottenlinger F, Schwiebs A, Pfarr K, Wagner A, Grüner S, Mayer CA, et al. Fingolimod targeting protein phosphatase 2A differently affects IL-33 induced IL-2 and IFN-γ production in CD8 $^{+}$ lymphocytes. *Eur J Immunol.* (2016) 46:941–51. doi: 10.1002/eji.201545805

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

ORIGINAL RESEARCH
published: 05 March 2019
doi: 10.3389/fimmu.2019.00366

Differential Effects of the Absence of Nkx2-3 and MAdCAM-1 on the Distribution of Intestinal Type 3 Innate Lymphoid Cells and Postnatal SILT Formation in Mice

Dóra Vojkovics[1,2], *Zoltán Kellermayer*[1,2], *Fanni Gábris*[1,2], *Angela Schippers*[3], *Norbert Wagner*[3], *Gergely Berta*[4], *Kornélia Farkas*[5] *and Péter Balogh*[1,2]*

[1] Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Pécs, Hungary, [2] Lymphoid Organogenesis Research Group, Szentágothai Research Center, University of Pécs, Pécs, Hungary, [3] Department of Pediatrics, University Hospital RWTH, Aachen, Germany, [4] Central Electron Microscope Laboratory, Department of Medical Biology, Medical School, University of Pécs, Pécs, Hungary, [5] Department of Bioanalytics, Medical School, University of Pécs, Pécs, Hungary

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Wenkai Ren,
South China Agricultural University, China
Kamalakannan Rajasekaran,
Genentech, Inc., United States

***Correspondence:**
Péter Balogh
balogh.peter@pte.hu

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

Received: 30 November 2018
Accepted: 13 February 2019
Published: 05 March 2019

Citation:
Vojkovics D, Kellermayer Z, Gábris F, Schippers A, Wagner N, Berta G, Farkas K and Balogh P (2019) Differential Effects of the Absence of Nkx2-3 and MAdCAM-1 on the Distribution of Intestinal Type 3 Innate Lymphoid Cells and Postnatal SILT Formation in Mice. Front. Immunol. 10:366. doi: 10.3389/fimmu.2019.00366

Seeding of leukocytes to developing lymphoid tissues in embryonic and early postnatal age and to the mucosa throughout adulthood depends on the interaction between endothelial MAdCAM-1 addressin and its cognate ligand α4β7 integrin. Nkx2-3 as a transcriptional regulator of MAdCAM-1 controls vascular patterning in visceral lymphoid tissues in mice, and has been identified as a susceptibility factor for inflammatory bowel diseases in humans, associated with lymphoid neogenesis in the inflamed intestines. The role of Nkx2-3 in the organogenesis of the solitary intestinal lymphoid tissues (SILTs) involving type 3 innate lymphoid cells (ILC3) is still unknown. Here we investigated the effect of Nkx2-3 on the postnatal distribution of intestinal ILC3s and the development of SILTs, comparing these to mice lacking MAdCAM-1, but preserving Nkx2-3. At 1 week of age small intestines (SI) contained significantly higher number of ILC3s relative to the colon, with a substantial reduction in MAdCAM-1$^{-/-}$ mice compared to C57BL/6 controls. One week later SI ILC3 number decreased in all genotypes, the number of colonic ILC3 of both Nkx2-3-deficient and Nkx2-3-heterozygous mice significantly increased. On the fourth postnatal week a further reduction of SI ILC3s was observed in both Nkx2-3-deficient and Nkx2-3-heterozygous mice, while in the colon the number of ILC3s showed a significant reduction in all genotypes. At 1 week of age only sporadic SILT components were present in all genotypes. By the second week mice deficient for either Nkx2-3 or MAdCAM-1 showed absence of SILT maturation compared to their relevant controls, lacking mature isolated lymphoid follicles (ILF). By the fourth week both Nkx2-3-deficient and Nkx2-3-heterozygous mice showed a similar distribution of ILFs relative to cryptopatches (CP), whereas in MAdCAM-1$^{-/-}$ mice CPs and immature ILFs were present, mature ILFs were scarce. Our data demonstrate that the complete absence of MAdCAM-1 partially impairs intestinal seeding of ILC3s and causes partial

blockade of SILT maturation, without affecting peripheral lymph node development. In contrast, the inactivation of Nkx2-3 permits postnatal seeding, and its blocking effect on SILT maturation prevails at later stage, thus other adhesion molecules may compensate for the intestinal homing of ILC3s in the absence of MAdCAM-1.

Keywords: NKX2-3, MAdCAM-1, ILC3, isolated lymphoid follicle, cryptopatch

INTRODUCTION

The intestinal lymphoid tissues comprise a large and complex network with diverse developmental and structural features of its components. The development of programmed lymphoid tissues of the gut, such as the ileal Peyer's patches (PP) and colonic patches, is initiated during the embryonic period, forming separate T- and B-cell compartments, thus representing secondary lymphoid tissues. In contrast, cryptopatches (CP) and isolated lymphoid follicles (ILF) form postnatally and lack defined T-cell territories, representing an "instructed" form of secondary lymphoid tissues, influenced by environmental and dietary factors. Upon inflammatory conditions ILFs may further evolve into tertiary lymphoid tissues (1).

Despite their developmental and structural differences, PPs and CP/ILFs have common developmental requirements and cellular interactions. Critically, lymphoid tissue inducer (LTi) cells identified by c-kit, IL-7Rα, CD45, and α4β7 integrin and lack of mature T- and B-cell associated markers participate in the initiation of PP formation prenatally and colonic CP/ILF development in the postnatal period, similarly to their involvement in initiating embryonic lymph node formation (2). These cells are related to type 3 innate lymphoid cells (ILC3) expressing retinoic acid receptor-related orphan receptor (RORγt) (3, 4). Subsequent tissue-specific colonization of PP anlagen by circulating mature lymphocytes requires the recognition of MAdCAM-1 addressin displayed by mucosal high endothelial venules (HEVs) via α4β7 integrin (5, 6). Similarly, ILF formation is initiated from pre-existing cryptopatches, where adult LTi-equivalent ILC3 cells are hypothesized to support the eventual transformation into follicles (7).

An important tissue-specific factor involved in the regulation of MAdCAM-1 addressin is Nkx2-3 homeodomain transcription factor (8). Nkx2-3, a member of Nkx family, is expressed in the spleen, midgut, hindgut, and pharyngeal endoderm (9) and is necessary for the development of visceral mesoderm and later for the formation of several stromal cells of secondary lymphoid organs, including endothelial cells as well as intestinal and vascular smooth muscle cells (10). In mice the absence of Nkx2-3 causes a lymph node-like switch of vascular patterning in spleen (11), characterized by the appearance of ectopic high endothelial venules (HEVs) expressing peripheral lymph node addressin (PNAd), and replacement of MAdCAM-1 with PNAd in PP HEVs in the early postnatal period (12). Consequently in mice lacking Nkx2-3 PPs are smaller and less numerous (13). Interestingly, in the absence of Nkx2-3 MAdCAM-1expression is retained during embryogenesis, and disappears gradually from endothelial cells, but it still persists on follicular stromal cells (14). In humans Nkx2-3 may contribute to the regulation of colorectal stem cell niche through the maintenance of local myofibroblast identity (15). In genome-wide association studies (GWAS) single nucleotide polymorphisms of Nkx2-3 have been linked to inflammatory bowel diseases, often associated with ectopic intestinal lymphoid tissue formation (16, 17).

Although Nkx2-3 is crucial for the normal development of spleen and PP, its role in CP/ILF organogenesis is still unknown, including its effect on the intestinal distribution of ILC3s. As the postnatal SILT formation may recapitulate several events of secondary lymphoid organogenesis, we hypothesized that the lack of Nkx2-3 as transcriptional regulator of MAdCAM-1, or the genomic absence of MAdCAM-1 itself as two different models for MAdCAM-1 deficiency, may influence the distribution of ILC3s and the postnatal formation of SILT. Our findings reveal considerably different kinetics for both CP/ILF formation and ILC3 distribution between small intestine and colon, indicating that different forms of MAdCAM-1 deficiency variably influence the development of distinct mucosal lymphoid tissues.

MATERIALS AND METHODS

Mice

MAdCAM-$1^{-/-}$ ($Madcam1^{tm1.2Nwag}$) mice on C57BL/6J background(18) and Nkx$2.3^{-/-}$ mice backcrossed onto BALB/cJ background (13) were maintained at the Department of Immunology and Biotechnology, University of Pécs. BALB/cJ and C57BL/6J mice were obtained from The Jacksons Laboratory, Bar Harbor, USA. In order to ensure comparable environmental conditions from birth, for Nkx2-3 deficiency cage-matched Nkx2-3 heterozygotes were used as control. Mice were kept in standard housing conditions prior to the experiments for at least 1 week. All procedures involving live animals were conducted in accordance with the guidelines set out by the Ethics Committee on Animal Experimentation of the University of Pécs, Hungary, under license number BA02/2000-16/2015, with approval for the use of genetically modified organisms under license number SF/27-1/2014 issued by the Ministry of Rural Development, Hungary.

Antibodies and Reagents

For flow cytometry anti-Thy-1.2 (CD90.2)-PerCPCy5.5 (clone 53-2.1) was purchased from BioLegend, anti-CD3-FITC (clone KT3), anti-CD19-FITC (clone 1D3), and biotinylated anti-CD45 (clone IBL-3/16) were produced and labeled in our lab, the latter visualized with Streptavidin PE-Cy7 (Biolegend). Anti-PNAd mAb (clone MECA-79) was purchased from BD Biosciences, FITC-conjugated goat anti-rat IgG was purchased from Vector Laboratories. Anti-RORγt mAb (clone Q31-378) conjugated

with AlexaFluor647 and membrane permeabilization buffer were purchased from BD Biosciences. For tissue immunofluorescence the following rat mAbs were produced in our lab: anti-CD45 mAb (clone IBL-3/16) conjugated with FITC, anti-B220 mAb (clone RA3-6B2) labeled with CF647, anti-Thy-1 mAb (clone IBL-1) conjugated with TAMRA, anti-MAdCAM-1 (clone MECA-367) IgG purified from hybridoma supernatant using Protein G chromatography. The architecture of peripheral lymph nodes was determined using a cocktail of anti-CD21/35-FITC (clone 7G6, produced in our lab), anti-Thy-1-TAMRA, and anti-B220-CF647. For ELISA horseradish peroxidase conjugated rabbit anti-mouse polyclonal antibody (Dako) was used.

Flow Cytometry

Lamina propria lymphocytes were isolated by a modification of a previously described protocol (19). Briefly, intestinal parts were cut open longitudinally and the PPs were cut out from the small intestine. In case of 1 and 2 week old mice at least 3 intestines were pooled. Colons and small intestines were washed in PBS and then shaken for 10–20 min in DMEM containing 10 mM EDTA (Sigma Aldrich) to remove the epithelial layer. Intestinal parts were washed thoroughly in ice cold PBS, cut into 3–5 mm pieces, and digested at 37°C in DMEM containing 0.6 mg/ml Collagenase D (Sigma Aldrich) and 5 U/ml DNAse I (Sigma Aldrich). After 20 min the supernatant was collected by passing through 70 μm cell strainer (Greiner Bio-One), to the remaining tissue fragments freshly digesting medium was added and further incubated until completely dissociated. Mononuclear cells were separated with density gradient centrifugation using discontinuous 40%/80% (w/v) Percoll (Sigma Aldrich). The separated cells were washed in PBS, and labeled for flow cytometry first with a cocktail of mAbs against surface markers, followed by permeabilization and staining for RORγt. Measurements were carried out on a BD FACS Canto II flow cytometer. Within the lymphoid gate, at least five thousand CD45+CD3-CD19- cells were collected and analyzed using the FCS Express Software. ILC3 cells were determined as CD45+CD3-CD19-CD90/Thy1+RORγt+ cells.

Histology

For analyzing SILT composition 8μm cryostat sections were cut at 4–6 different planes 100–150 μm apart from Swiss rolls prepared from small intestines and colons of 1, 2, and 4 week old mice. SILT structures were defined according to size, morphology, and cellular composition as follows: CP were Thy-1++/B220-, immature ILFs were Thy-1++/B220+, and mature ILF were defined as Thy-1±/B220++. For immunofluorescence labeling frozen sections were fixed in cold acetone for 10 min and then blocked in 5% BSA in PBS for 20 min. Multiple fluorescence labeling was performed by incubating the sections with a cocktail of fluorochrome-labeled mAbs against CD45, Thy-1, and B220 in PBS for 45 min followed by washing. For intestinal addressin expression frozen sections were incubated first with 5 μg/ml anti-MAdCAM-1 IgG in PBS, followed by washing and detection using FITC-conjugated anti-rat IgG (BD Biosciences). After saturation with 50x diluted normal rat serum sections were incubated with DyLight594-conjugated MECA-79 anti-mouse PNAd antibody (kindly provided by Eugene C. Butcher, Stanford University) and anti-mouse CD45-CF647 conjugate. For peripheral lymph node analysis sections prepared at median plane inguinal lymph nodes were incubated with a cocktail of FITC anti-CD21/35 to detect follicular dendritic cells, TAMRA anti-Thy-1 against T cells and CF-647-conjugated anti-B220 mAb against B cells. After mounting with 1:1 PBS:glycerol, sections were viewed with an Olympus BX61 fluorescence microscope, and representative images were created with an Olympus Fluo-View FV-1000 laser scanning confocal imaging system. For single color labeling of MAdCAM-1 or PNAd acetone-fixed cryostat sections of small intestine or colon were incubated with anti-MAdCAM-1 or anti-PNAd mAb at 1 μg/ml antibody followed by FITC-conjugated anti-rat IgG (Vector Laboratories), using Hoechst 33,342 nuclear counterstaining in the mounting. The labeling intensities were quantified using Image J software.

Immunization and ELISA

To induce an anti-ovalbumin immune response, mice were immunized with 50 μl of 50 mg/ml OVA with complete Freund's adjuvant in the left footpads on day 0 and day 7. Mice were sacrificed on day 21 and serum was collected. To determine the anti-OVA IgG response, ELISA plates (Nunc Maxisorp, Thermo Scientific) were coated with 5 μg/ml ovalbumin (Sigma-Aldrich, Budapest) overnight in PBS, followed by saturation with 0.1% gelatine in PBA-0.1% Tween-20 (Sigma–Aldrich, Budapest, Hungary) for 1 h at 37°C. After saturation and washing in PBS-Tween diluted sera of mice were added to blocked plates, followed by washing. Next, horseradish peroxidase conjugated rabbit anti-mouse polyclonal antibody was added, and after incubation peroxidase activity was detected using ortho-phenylenediamine and H_2O_2 (Sigma-Aldrich, Budapest, Hungary) in citrate-phosphate buffer, pH: 5.0. The reaction was stopped with 4 M H_2SO_4. Samples were measured at 492 nm in duplicates.

Quantitative RT-PCR

Total RNA from inguinal lymph node, mesenteric lymph node and Peyer's patch homogenates was isolated using NucleoSpin RNA (Macherey-Nagel GmbH). Purity and concentration of RNA was analyzed by NanoDrop. cDNA was synthetized using High Capacity cDNA RT Kit (Life Technologies). RT-PCR was run on an ABI-PRISM 7,500 machine in duplicates using previously described SYBR green primers (11). Results are shown as percentage of β-actin housekeeping gene.

Statistical Analysis

Data was analyzed using IBM® SPSS® Statistics software (Version 22). A Mann-Whitney test was applied to compare groups with non-normally distributed data. Data are represented as mean ± SEM. Statistical significance was $p < 0.05$.

RESULTS

Postnatal Distribution of Intestinal ILC3 Is Perturbed in the Absence of Nkx2-3 and MAdCAM-1

Although the absence of Nkx2-3 causes transcriptional blockade of MAdCAM-1 expression (8, 14), our previous studies indicated

that the distribution of colonic lamina propria ILC3s in young adult mice are differentially affected in the lack of Nkx2-3 compared to the absence of MAdCAM-1 itself, so in Nkx2-$3^{-/-}$ mice the colonic ILC3 number was higher, whereas in MAdCAM-$1^{-/-}$ mice ILC3 numbers were significantly less (20). In this work first we sought to determine how these two different forms of MAdCAM-1 deficiency affect the postnatal population kinetics of ILC3s along the entire intestinal tract. ILC3s isolated from the lamina propria at various postnatal ages were identified using flow cytometry as CD45+CD3-CD19-CD90+RORγt+ cells (**Figure 1A**).

Using this strategy a systemic analysis of postnatal colonization of ILC3 cells in the small intestine and colon was performed. The number of ILC3s in the small intestine (siILC3) was higher than colonic ILC3s (cILC3s) in all mouse strains at each time points. On the first postnatal week siILC3s were present at the highest number, and in the absence of Nkx2-3 there were no significant alterations compared to control Nkx2-3 heterozygous mice. In MAdCAM-$1^{-/-}$ small intestines a considerably lower number of siILC3s was found compared to C57BL/6 controls; however, this decrease did not reach statistical significance.

On the second postnatal week siILC3 numbers substantially decreased in all mouse strains. We found that siILC3 numbers were the highest in mice lacking Nkx2-3, while lowest in MAdCAM-$1^{-/-}$ mice.

By the fourth postnatal week siILC3s decreased significantly in each mouse strain except C57BL/6 mice, where a slight increase in ILC3 number was observed compared to the second week. In Nkx2-$3^{-/-}$ mice the siILC3 number was significantly lower than in heterozygous controls. In MAdCAM-$1^{-/-}$ mice siILC3 numbers were the lowest at each investigated time points, and both cILC3 and siILC3s decreased continuously until the fourth postnatal week.

At the first postnatal week both Nkx2-$3^{+/-}$ and C57BL/6 wild-type mouse strains and Nkx2-3 mutants showed a similar range of absolute numbers of cILC3s, while in MAdCAM-$1^{-/-}$ mice the cILC3 numbers showed an approximately three-fold reduction at this time point.

On the second postnatal week, cILC3 numbers showed an around four-fold increase in both Nkx2-$3^{+/-}$ and Nkx2-$3^{-/-}$ mice. In contrast, cILC3 numbers in the absence of MAdCAM-1 further decreased, similarly to that in C57BL/6 control group.

By the fourth postnatal week in both Nkx2-$3^{+/-}$ and Nkx2-$3^{-/-}$ mice the number of cILC3 dropped considerably, although the absolute cILC3 numbers were still higher than on the first postnatal week. In contrast to the small intestine, where siILC3 number was significantly lower in the absence of Nkx2-3, in the colon of Nkx2-3-deficient mice the ILC3 cell number was higher. In contrast, in MAdCAM-$1^{-/-}$ mice cILC3 absolute numbers decreased to an almost undetectable level, while in the control C57BL/6 group cILC3 numbers were similar to that on the first week (**Figure 1C**).

These data indicate that the different forms of MAdCAM-1-deficiency variably affect the postnatal distribution pattern of ILC3s along the intestinal tract, with a generalized absence of MAdCAM-1 in MAdCAM-$1^{-/-}$ mice causing the most severe alterations.

Altered SILT Maturation in the Absence of Nkx2-3 and MAdCAM-1

Our earlier studies showed that in the absence of Nkx2-3 the perturbed lymphocyte distribution is coupled with an altered vascular pattern in PPs of young adult mice, including the gradual replacement of MAdCAM-1 by PNAd (12). To test how this altered addressin pattern affects the development of SILT, next we compared the various differentiation stages of the SILT spectrum between mice lacking either Nkx2-3 or MAdCAM-1 and wild-type controls during the first 4 weeks of postnatal period. As controls for Nkx2-$3^{-/-}$ mice we used heterozygous littermates in order to maintain identical environmental conditions. SILT structures were identified with multiple immunofluorescence staining of colonic sections as described in the Materials and Methods. Immature and mature ILFs were distinguished also according to their morphology, as mature ILFs (matILF) are larger and often contain B220+ germinal centers while immature ILFs (imILFs) are smaller with less B220+ cells, and also with a less compacted organization (**Figure 2A**). The statistical analyses of the SILT maturation in different genotypes at various ages and in different gut segments are summarized on **Figures 2B,C**.

On the first postnatal week only CPs and imILFs were present in the colon of each mouse strain and no matILFs were found. The distribution of these immature SILT structures in Nkx2-$3^{-/-}$ and MAdCAM-$1^{-/-}$ mice were similar to their relevant Nkx2-$3^{+/-}$ and C57BL/6 control groups. Interestingly the ratio of CP was higher in C57BL/6 and MAdCAM-$1^{-/-}$ mice and these strains had less imILFs than either Nkx2-$3^{+/-}$ or Nkx2-$3^{-/-}$ mice.

On the second postnatal week matILFs also started to appear in Nkx2-3 heterozygous mice, while in the Nkx2-$3^{-/-}$ group matILFs were present only at a very low ratio. In MAdCAM-$1^{-/-}$ mice we observed no matILFs at all. There were no significant differences in CP and imILF ratios between the Nkx2-3-deficient and heterozygous mice, and MAdCAM-1-deficient and wild-type C57BL/6 mice, respectively. Similarly to the first week, CPs were present at a lower ratio in Nkx2-$3^{+/-}$ and Nkx2-$3^{-/-}$ than in C57BL/6 and MAdCAM-$1^{-/-}$ mice. Furthermore, imILF ratios were almost the same in each genotype at this time point.

On the fourth week matILFs were present in the colonic lamina propria of each mouse strain, but in MAdCAM-$1^{-/-}$ colons their ratio was significantly lower compared to the wild-type C57BL/6 control group. In the absence of Nkx2-3, the matILF ratio was also lower than in Nkx2-$3^{+/-}$ littermate controls. ImILFs were observed at a higher ratio in MAdCAM-1 knock-out (KO) mice than in the control C57BL/6 group, while CP ratio was lower in MAdCAM-$1^{-/-}$ compared to C57BL/6 mice. Nkx2-$3^{-/-}$ mice had a similar imILF percentage as in Nkx2-$3^{+/-}$ mice but a higher ratio of CPs.

Our data demonstrate that the different forms of MAdCAM-1-deficiency variably affect colonic SILT development, as in the absence of Nkx2-3 ILF maturation is only slightly altered in comparison to the relevant wild-type group, while MAdCAM-$1^{-/-}$ mice had a more profound delay in colonic SILT maturation. These findings also demonstrate that although the absence of MAdCAM-1 delays colonic SILT maturation in the

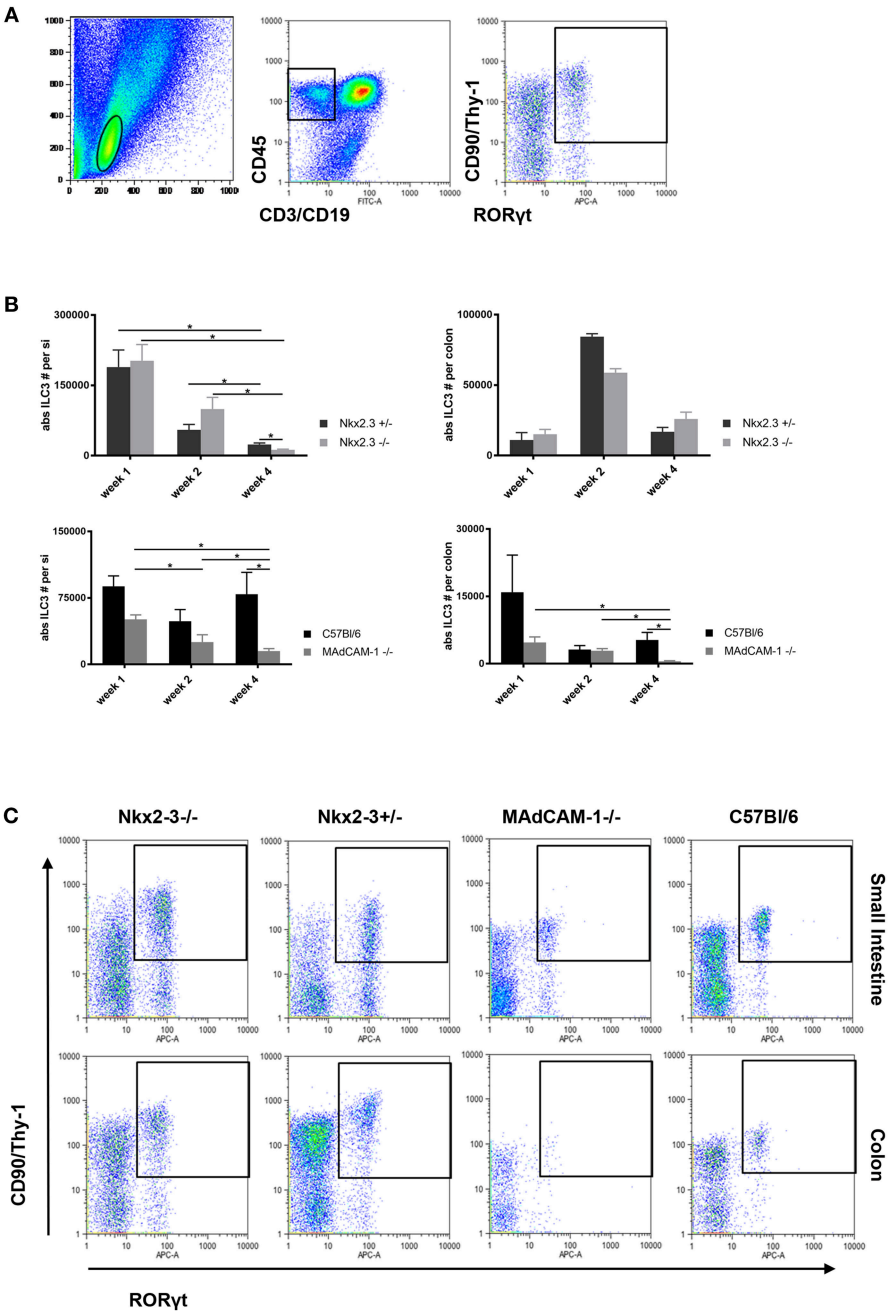

FIGURE 1 | Different distribution of ILC3 in the postnatal gut in Nkx2-3$^{-/-}$ and MAdCAM-1$^{-/-}$ mice. **(A)** Gating strategy for the identification of ILC3s defined on the basis the lymphoid gate (left: FSC/SSC ellipse) and CD45+/non-T/B lineage (middle: rectangle) combined with CD90/RORγt (right, rectangle; representative example from a small intestine of a 4 weeks old C57BL/6 mouse). **(B)** Kinetics of the absolute number of ILC3s in Nkx2-3$^{-/-}$ and Nkx2-3$^{+/-}$ mice (top) and MAdCAM-1$^{-/-}$ and wild-type C57BL/6 (bottom) intestine (small intestine: left, colon: right) at various ages as indicated ($n = 3$–7, mean ± SEM, $^*p < 0.05$). **(C)** Representative example of the appearance of ILC3s in the small intestine and colon of Nkx2-3$^{-/-}$, Nkx2-3$^{+/-}$, MAdCAM-1$^{-/-}$, and wild-type C57BL/6 mice at 4 weeks of age.

early postnatal period, it does not prevent it. This delay is most pronounced on the second postnatal week, when Nkx2-3$^{-/-}$ mice developed a few matILFs, while in MAdCAM-1$^{-/-}$ mice matILFs appeared only on the fourth postnatal week at a greatly decreased ratio.

Altered mRNA for PNAd Core Proteins and Modifying Enzymes in MAdCAM-1$^{-/-}$ Peyer's Patches

MAdCAM-1 is the main addressin recruiting lymphocytes to Peyer's patches (PP) and the intestine. As the lack of Nkx2-3

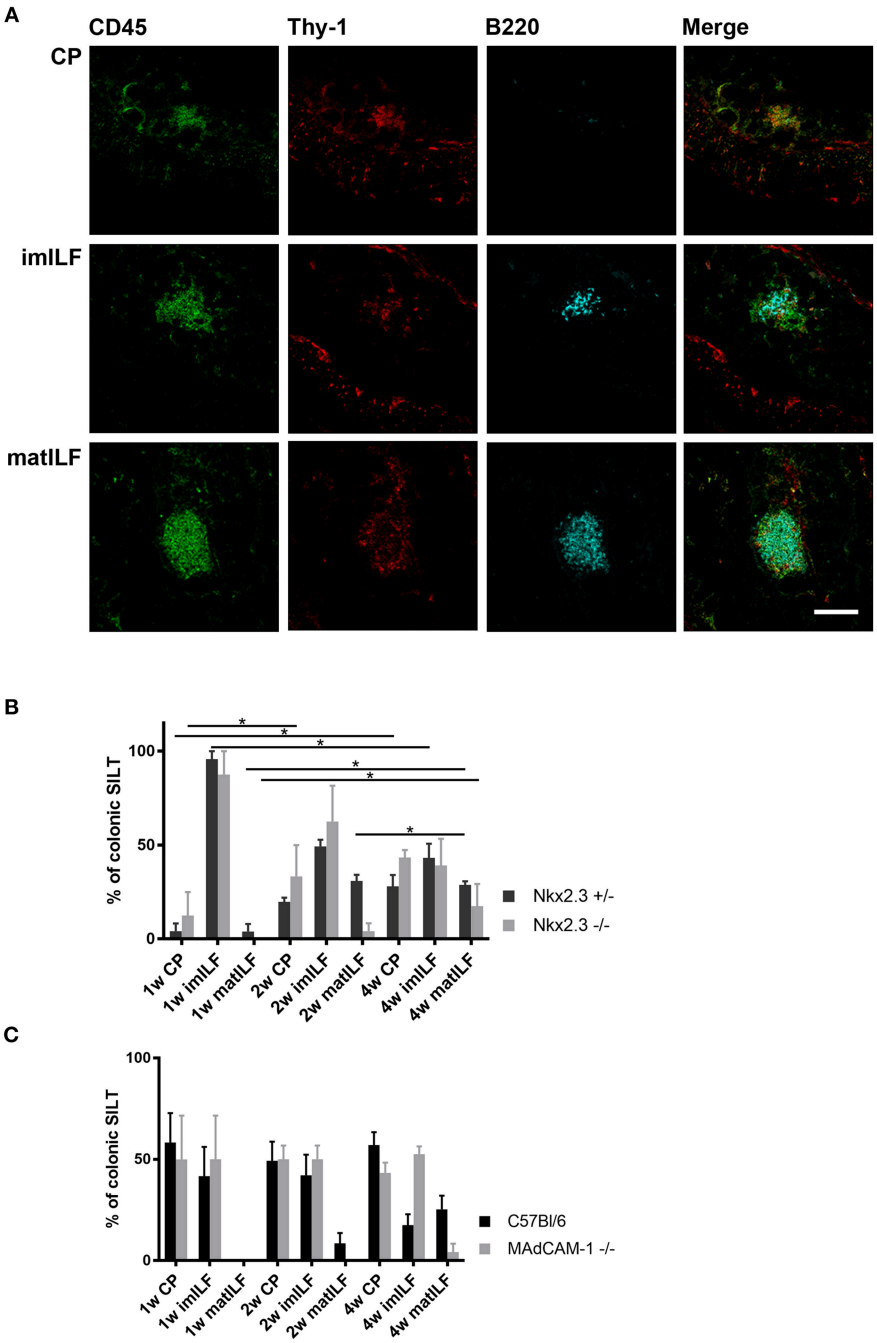

FIGURE 2 | Composition of postnatal colonic SILT spectrum. **(A)** Representative samples for cryptopatches (CP), immature (imILF), and mature (matILF) isolated lymphoid follicles of a 4 weeks old Nkx2-3$^{+/-}$ mouse using CD45/Thy-1/B220 combined immunofluorescence staining with the markers as indicated (scale bar = 100 μm). **(B)** Composition of colonic SILT spectrum in Nkx2-3$^{+/-}$ (black filled) and Nkx2-3$^{-/-}$ (gray filled) mice at 1, 2, and 4 weeks of age as indicated; $n = 4$; mean ± SEM, *$p < 0.05$). **(C)** Composition of SILT spectrum in the colon of wild-type C57BL/6 (black filled) and MAdCAM-1$^{-/-}$ (gray filled) mice at 1, 2, and 4 weeks of age as indicated ($n = 4$; mean ± SEM *$p < 0.05$).

causes increased mRNA levels for various PNAd backbone proteins and modifying enzymes in PPs lacking endothelial MAdCAM-1 (12), next we studied whether similar alterations occur in MAdCAM-1$^{-/-}$ mice. Using qPCR analysis of cDNA from PPs of 8–10 weeks old MAdCAM-1$^{-/-}$ mice we observed a significant increase of two PNAd backbone proteins Endomucin and Podocalyxin-like protein in MAdCAM-1$^{-/-}$ PPs compared to wild-type controls, while the increase in CD34 did not reach statistical significance. The mRNA for Glycam1 and Nepmucin did not show any difference compared to control

PPs (**Figure 3A**). Among the modifying enzymes important in creating the PNAd epitope, mRNA for the betaGal beta-1,3-N-acetylglucosaminyltransferase 3 sulfotransferase (B3gnt3) and alpha-(1, 3)-fucosyltransferase (Fut7) did not show statistically significant differences, whereas N-acetylglucosamine 6-O sulfotransferase (Chst4) was significantly elevated in MAdCAM-$1^{-/-}$ PPs, resulting in a prominent expression of MECA-79 PNAd epitope (**Figure 3B**). These alterations are different from those reported earlier in Nkx2-$3^{-/-}$ mice, where the most robust alteration of mRNA expression for PNAd core proteins involved Glycam1, but the significant increase of Chst4 was also observed (12).

Correlation of ILC3 Distribution and Regional Expression of Vascular Addressin in the Mucosa

To investigate whether the ILC3 distribution is correlated with the altered expression of MAdCAM-1 in Nkx2-$3^{-/-}$ mice or PNAd in MAdCAM-$1^{-/-}$ mice, next we compared the expression level using quantitative immunofluorescence and Image J analysis.

In Nkx2-$3^{-/-}$ mice we found a higher level of MAdCAM-1 expression at week 1 and even a minor increase by the second postnatal week, while by the 4th postnatal week the MAdCAM-1 labeling intensity was statistically below that of the Nkx2-3 heterozygotes in the small intestine. In contrast, the colonic MAdCAM-1 expression in Nkx2-$3^{-/-}$ samples was continuously below that of heterozygotes, suggesting in different gut segments the loss of MAdCAM-1 expression follows different kinetics (**Figure 4A**). Furthermore, the MAdCAM-1 expression does not correlate with the local ILC3 numbers detailed above (**Figure 1B**).

In the small intestine of MAdCAM-$1^{-/-}$ mice we observed a slight increase of PNAd expression by the second week below that of C57BL/6 mice, which continued to increase significantly by the fourth week, exceeding that of wild-type controls. Correlating the number of ILC3s with the alterations of small intestinal PNAd in C57BL/6 mice the ILC3 number remained relatively stable, while the PNAd expression steadily increased, whereas in MAdCAM-$1^{-/-}$ mice was accompanied by significantly decreased ILC3 numbers.

The colonic PNAd expression in MAdCAM-$1^{-/-}$ mice showed a significant increase already by the second week compared to the controls, followed by a further increase by the fourth week (**Figure 4B**). However, ILC3 cell numbers decreased in both MAdCAM$^{-/-}$ and wild-type mice, where at fourth week of age the colonic ILC3 cells were barely detectable in MAdCAM-$1^{-/-}$ mice (**Figure 1B**).

Preserved Lymph Node Architecture and Normal T Cell-Dependent Antibody Response in the Absence of MAdCAM-1

During the embryonic development and early postnatal maturation of peripheral lymph nodes (pLN) HEVs display MAdCAM-1 recognized by α4β7 integrin (2, 4). Therefore, we next investigated whether the formation and structure of pLNs is affected by the absence of MAdCAM-1 similarly to the SILT. Using multicolor immunofluorescence for T and B cells and follicular dendritic cells we found no noticeable differences in the lymphoid compartmentalization and follicular stromal organization in young adult (8 week old) mice (**Figure 5**).

To examine how the absence of MAdCAM-1 affects the induction of a local T-dependent immune response, mice received two subcutaneous injections of ovalbumin (OVA) in their footpads 7 days apart. Twenty-one days after the first injection mice were sacrificed and serum was collected. We determined the anti-OVA IgG response using a custom-made indirect ELISA. Interestingly, we found that lack of MAdCAM-1 did not inhibit the production of IgG against ovalbumin as neither MAdCAM-$1^{-/-}$ nor Nkx2-$3^{-/-}$ mice had significantly lower antibody levels compared to their appropriate controls (data not shown).

These results indicate that, in addition to allowing the establishment of normal architecture, in the absence of MAdCAM-1 the development of a T-dependent antibody response in peripheral lymph nodes is preserved.

DISCUSSION

Type 3 innate lymphoid cells (ILC3s) have recently been demonstrated to play a role in several immunological processes associated with normal mucosal lymphoid tissue formation and also inflammation and epithelial regeneration in inflammatory bowel diseases (IBD). IBD manifests as a consequence of genetic susceptibility, aberrant immunological responsiveness against commensal bacteria, and bacterial dysbiosis of the intestine (21, 22). Amongst other genetic factors, Nkx2-3 has been identified as a susceptibility factor for both ulcerative colitis and Crohn's disease in humans, characterized by ectopic lymphoid neogenesis (23). In these events ILC3 subsets in a close relationship with their intestinal stromal microenvironment produce a range of cytokines, and as putative antigen-presenting cells, they can also influence T-cell responses against commensal bacteria (24, 25). To exert local activities, various lymphoid cells need to recognize MAdCAM-1 addressin displayed by mucosal high endothelial venules and lamina propria vessels, which also offers a potential target mechanism for IBD amelioration, using either anti-α4β7/α4β1 integrin antibodies or anti-MAdCAM-1 therapy, respectively (26). Although the efficiency of ILC3s critically depends on their intestinal colonization, it is not yet known how the regulatory or genomic absence of MAdCAM-1 affects the intestinal distribution of ILC3s and the maturation of colonic lymphoid follicles as immunological effector sites.

As a critical postnatal regulator promoting MAdCAM-1 expression (8, 27), we studied mice deficient for Nkx2-3 transcription factor. Although in Nkx2-3 KO mice endothelial MAdCAM-1 gradually disappears and is replaced by PNAd during the first month (4), the lack of Nkx2-3 does not abrogate the expression of non-endothelial MAdCAM-1. Therefore its effect appears to be lineage-restricted (14), in contrast to the MAdCAM-$1^{-/-}$ mice (18). We also noted increased

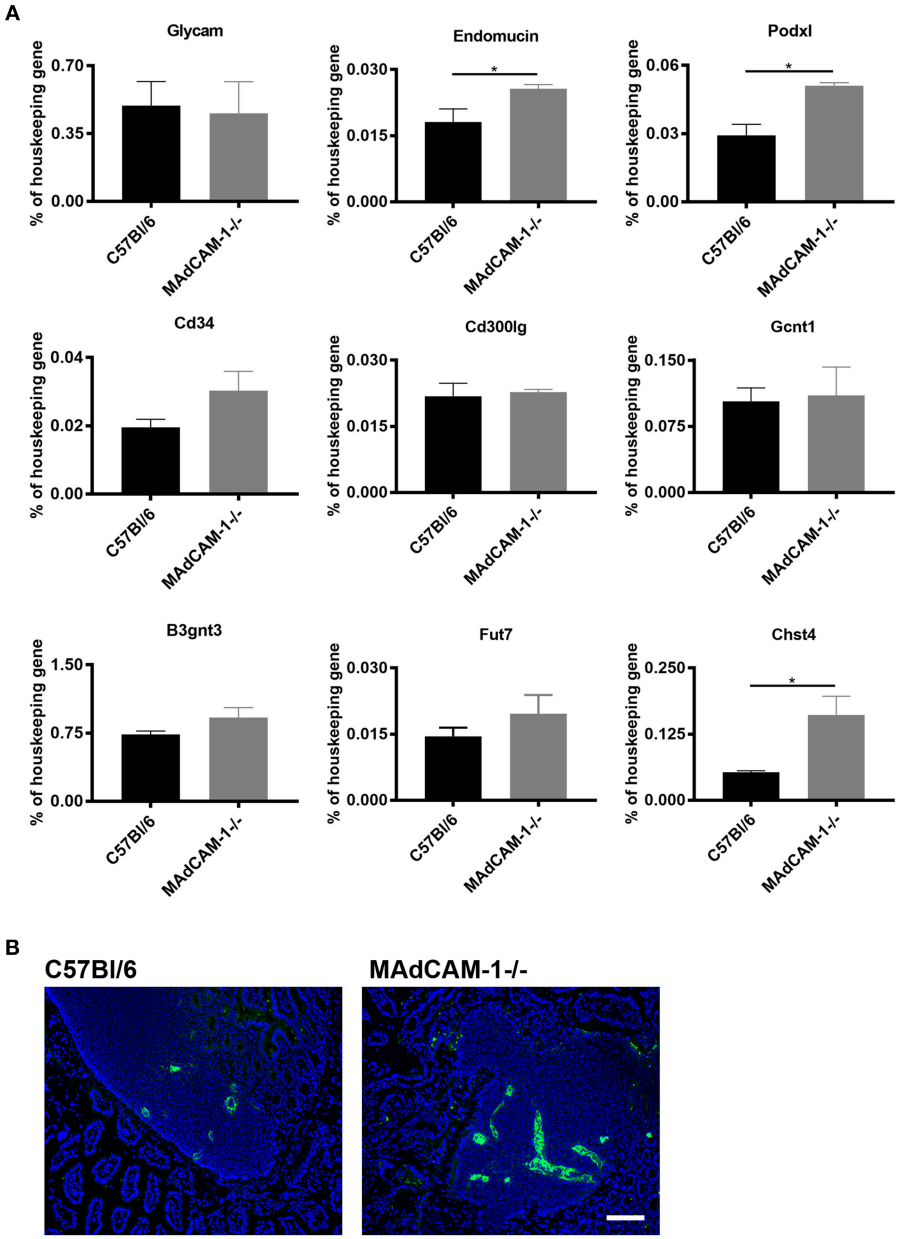

FIGURE 3 | Comparison of expression of mRNA expression for peripheral lymph node addressin core proteins and glycosylation enzymes in Peyer's patches between MAdCAM-$1^{-/-}$ and wild-type mice and the expression of MECA-79 PNAd epitope. **(A)** qPCR analyses were performed for the various core proteins (GlyCAM, endomucin, podocalyxin-like protein, CD34, and nepmucin) and glycosylation enzymes, expressed as a mRNA level relative to β-actin ($n = 6$; mean ± SEM, in a duplicate measurement; *$p < 0.05$). **(B)** Immunofluorescence detection of MECA-79 epitope (green) in wild-type (left) and MAdCAM-$1^{-/-}$ Peyer's patches with Hoechst nuclear counterstaining (blue) (representative example of a cohort of 3 mice; scale bar = 100 μm).

expression of PNAd epitope MECA-79; however, comparison of PNAd core protein and glycosylation enzyme mRNA expression alterations to those in Nkx2-3-deficient mice reported earlier (12) show notable differences. In Nkx2-$3^{-/-}$ mice the most dramatic alteration of mRNA expression was the robust increase of Glycam1 mRNA; in contrast, in MAdCAM-$1^{-/-}$ mice the mRNA for Glycam1 was unaltered, while mRNA for podocalyxin-like protein (Pdxn), endomucin and, to a lesser degree, CD34 increased. Moreover, in both cases sulfotransferase enzyme Chst4 increased. These findings indicate that in the two different models of MAdCAM-1 deficiency the compensatory upregulation of PNAd epitopes follows different mRNA expression patterns.

Irrespectively of their genotype and background, small intestines host more ILC3 cells than the colon, with the peak occurring on the first postnatal week. At this period the

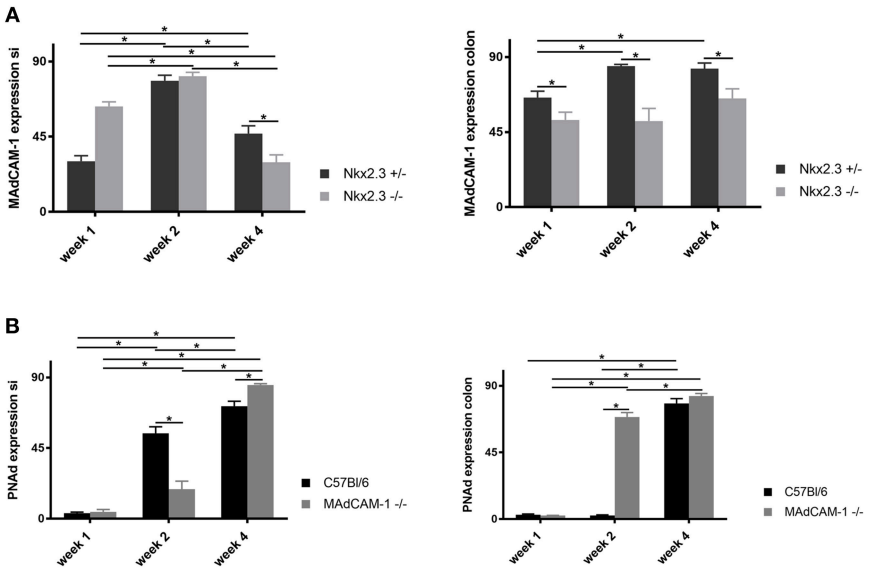

FIGURE 4 | Quantification of the expression of endothelial addressins in the postnatal period. **(A)** Relative pixel intensities (mean gray values, *y*-axis) of MAdCAM-1 labeling of small intestine (left) and colon (right) in Nkx2-3$^{+/-}$ (black filled) and Nkx2-3$^{-/-}$ (gray filled) at the periods indicated at the *x*-axis ($n = 5$, mean ± SEM, in a duplicate measurement; *$p < 0.05$). **(B)** Relative pixel intensities (mean gray values, *y*-axis) of PNAd labeling of small intestine (left) and colon (right) in C57BL/6 (black filled) and MAdCAM-1$^{-/-}$ (gray filled) at the periods indicated at the *x*-axis ($n = 5$, mean ± SEM, in a duplicate measurement; *$p < 0.05$).

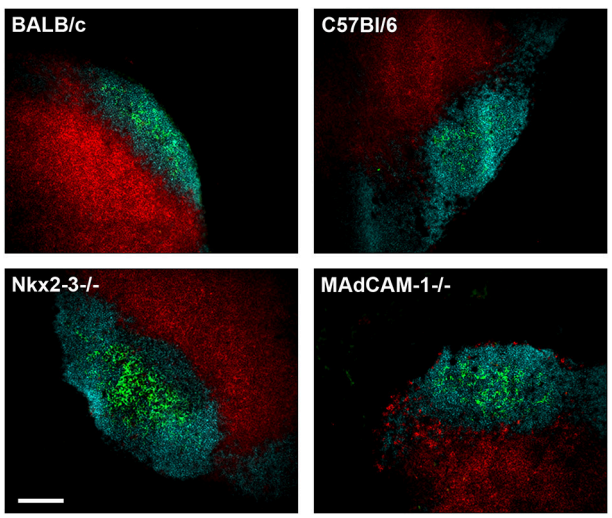

FIGURE 5 | Preserved structure of pLN in the absence of MAdCAM-1. Cryostat sections from inguinal lymph nodes of 6 weeks old Nkx2-3$^{-/-}$, Nkx2-3$^{+/-}$, MAdCAM-1$^{-/-}$, and C57BL/6 mice were stained for B cells (B220/turquoise), T cells (Thy-1/red), and follicular dendritic cells (CD21/35/green) as indicated (scale bar: 100 μm. Representative image, the staining was repeated twice).

dominant addressin in the peripheral lymph nodes of wild-type and Nkx2-3-deficient mice is MAdCAM-1 (4, 12), and we noted no significant difference between Nkx2-3 deficient and heterozygous samples, indicating efficient seeding of ILC3s to the small intestine mucosa. However, the number of cells showed substantial differences between Nkx2-3-deficient and heterozygous mice on a BALB/c background, and C57BL/6-background MAdCAM-1$^{-/-}$ and wild-type mice, which may be related to the delayed maturation of siILF in C57BL/6 mice, as reported earlier (28). On the other hand, at later periods Nkx2-3-deficient mice showed a reduced number of small intestinal ILC3s, coupled with a markedly lower frequency of mature ILFs, presumably reflecting the effect of the progressive reduction of endothelial MAdCAM-1 expression.

Interestingly, the pattern of PNAd core protein and glycosylation enzyme expression in PPs of MAdCAM-1$^{-/-}$ mice is different compared to both Nkx2-3$^{-/-}$ and wild-type C57BL/6 mice, and it also allows the appearance of MECA-79 epitope. It remains to be investigated, whether in a fashion similar to Nkx2-3$^{-/-}$ PPs, the MAdCAM-1/α4β7-dependent mucosal mechanism is also replaced—at least partially—by an L-selectin/PNAd-dependent homing (12). Nevertheless, the appearance of cryptopatches and their initial maturation into imILFs is in agreement with previous data on the formation of cryptopatches being independent from α4β7 integrin-MAdCAM-1 interaction (27).

In contrast, colonic ILC3 dispersion showed strikingly different kinetics. Compared to the small intestines, on the first week we found relatively fewer ILC3 cells in all genotypes. However, by the second week the colonic ILC3 number increased in Nkx2-3-deficient and heterozygous mice, whereas it was further reduced in both MAdCAM-1$^{-/-}$ and C57BL/6 mice, similar to the difference between the small intestines of BALB/c and C57BL/6 mice (28, 29). This reduction continued in MAdCAM-1$^{-/-}$ mice to a virtually undetectable level, whereas it remained unchanged in C57BL/6 samples. Furthermore, the lack of MAdCAM-1 caused a more dramatic reduction in the

colonic ILC3 number compared to that in the small intestine, indicating that in the small intestine potential alternative endothelial addressins may partially compensate for the absence of MAdCAM-1, in addition to other differences of developmental requirements for small intestinal and colonic ILF formation (30). With regard to the effect of the absence of Nkx2-3 in young adult mice on other innate lymphoid subsets, we found an increased level of Th17 and Treg cells in the colon (20); however, it remains to be investigated how other ILC subsets are affected either in Nkx2-3$^{-/-}$ or MAdCAM-deficient mice. Our recent findings also indicate the expression of Nkx2-3 in VAP-1-positive myofibroblasts cells, thus the modulatory effect on ILC3 distribution is likely to be mediated through stromal components (20).

Differences in the maturation of colonic ILFs can be attributed to several factors. Importantly, mice on a C57BL/6 background showed a delayed maturation, evidenced through the cryptopatch:ILF ratio, but by the fourth postnatal week C57BL/6 mice had a similar ratio of mature ILFs as in Nkx2-3-deficient mice. Interestingly, the relatively stable number of CPs in C57BL/6 mice at this age was associated with fewer immature ILFs, suggesting that in these mice the course of CP-imILF maturation may be delayed compared to CP appearance and/or imILF-matILF transformation, in a fashion similar to the difference between C57BL/6 and BALB/c mice observed in the small intestine (28, 29). Furthermore, in MAdCAM-1$^{-/-}$ mice the appearance of CPs and their initial maturation into immature ILFs largely correspond to those of wild-type C57BL/6, suggesting MAdCAM-1 independence (27), but its further maturation into mature ILFs is drastically blocked (although not completely abolished) in MAdCAM-1$^{-/-}$ mice. In this process the increased expression of PNAd addressin appears to be unable to compensate for the loss of MAdCAM-1 in sustaining immature-to-mature ILF transition, also reflected to a lesser degree in Nkx2-3$^{-/-}$ mice with partially preserved MAdCAM-1 expression.

Lastly, we also investigated the systemic effect of MAdCAM-1 deficiency on the formation and immune responsiveness, as α4β7 integrin-MAdCAM-1 also participates in peripheral lymph nodes formation during the embryonic period, similarly to the development of PPs (4). We found that, although both PP development and ILF maturation are blocked in MAdCAM-1$^{-/-}$ mice, the peripheral lymph nodes showed no detectable structural and functional differences compared to wild-type mice, suggesting the involvement of other endothelial ligands in the local accumulation of ILC3/lymphoid tissue inducer (LTi) cells in the lymph node anlage (31). The partial reservation of ILC3s seeding (with the potential of perpetuating the intestinal lymphoid neogenesis) to mucosal locations or for LTi subset of ILC3s in embryonic lymph node anlage even in the complete absence of MAdCAM-1 may reflect some degree of endothelial plasticity in addressin display, which may question the efficacy of anti-adhesion therapeutic interventions in IBD. It remains to be determined how the vasculature in developing peripheral lymph nodes and mucosal territories can collect leukocytes, including ILC subsets, in the absence of MAdCAM-1, thus expanding the involvement if other adhesion molecules to be considered as potential targets in limiting intestinal inflammatory diseases.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

DV and PB devised experiments. DV, ZK, FG, and PB performed experiments. GB operated the confocal microscope. ZK, DV, and PB analyzed and interpreted the results. KF performed statistical analysis. AS and NW evaluated data, edited and commented on manuscript, which was written by DV, ZK, and PB.

FUNDING

ZK is supported by the ÚNKP-17-4-I-PTE-83 New National Excellence Program of the Ministry of Human Capacities of Hungary and the postdoctoral research grant of the Faculty of Medicine, University of Pécs. AS and NW are supported by the Deutsche Forschungsgemeinschaft (SCHI 1170/2-1 and WA 1127/4-1). PB is supported by the Hungarian Scientific Research Fund (OTKA K108429). This project is supported by PEPSYS-GINOP-2.3.2-15-2016-00050 and EFOP-3.6.1-16-2016-00004.

REFERENCES

1. Buettner M, Lochner M. Development and function of secondary and tertiary lymphoid organs in the small intestine and the colon. *Front Immunol*. (2016) 7:342. doi: 10.3389/fimmu.2016.00342
2. Mebius RE. Organogenesis of lymphoid tissues. *Nat Rev Immunol*. (2003) 3:292–303. doi: 10.1038/nri1054
3. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol*. (2004) 5:64–73. doi: 10.1038/ni1022
4. Mebius RE, Streeter PR, Michie S, Butcher EC, Weissman IL. A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4+ CD3- cells to colonize lymph nodes. *Proc Nat Acad Sci USA*. (1996) 93:11019–24. doi: 10.1073/pnas.93.20.11019
5. Adachi S, Yoshida H, Kataoka H, Nishikawa S. Three distinctive steps in Peyer's patch formation of Mmurine embryo. *Int Immunol*. (1997) 9:507–14. doi: 10.1093/intimm/9.4.507
6. Hashi H, Yoshida H, Honda K, Fraser S, Kubo H, Awane M, et al. Compartmentalization of Peyer's patch anlagen before lymphocyte eEntry. *J Immunol*. (2001) 166:3702–9. doi: 10.4049/jimmunol.166.6.3702
7. Ivanov II, Diehl GE, Littman DR. Lymphoid tissue inducer cells in intestinal immunity. *Curr Top Microbiol Immunol*. (2006) 308:59–82. doi: 10.1007/3-540-30657-9_3
8. Pabst O, Forster R, Lipp M, Engel H, Arnold HH. NKX2.3 is required for MAdCAM-1 expression and homing of lymphocytes in spleen

and mucosa-associated lymphoid tissue. *EMBO J*. (2000) 19:2015–23. doi: 10.1093/emboj/19.9.2015
9. Pabst O, Schneider A, Brand T, Arnold HH. The mouse Nkx2-3 homeodomain gene is expressed in gut mesenchyme during pre- and postnatal mouse development. *Dev Dynam*. (1997) 209:29–35. doi: 10.1002/(SICI)1097-0177(199705)209:1<29::AID-AJA3>3.0.CO;2-Z
10. Brendolan A, Rosado MM, Carsetti R, Selleri L, Dear TN. Development and function of the mammalian spleen. *Bioessays*. (2007) 29:166–77. doi: 10.1002/bies.20528
11. Czömpöly T, Lábadi Á, Kellermayer Z, Olasz K, Arnold HH, Balogh P. Transcription Factor Nkx2-3 controls the vascular identity and lymphocyte homing in the spleen. *J Immunol*. (2011) 186:6981–9. doi: 10.4049/jimmunol.1003770
12. Kellermayer Z, Mihalj M, Labadi A, Czompoly T, Lee M, O'Hara E, et al. Absence of Nkx2-3 homeodomain transcription factor reprograms the endothelial addressin preference for lymphocyte homing in Peyer's patches. *J Immunol*. (2014) 193:5284–93. doi: 10.4049/jimmunol.1402016
13. Pabst O, Zweigerdt R, Arnold HH. Targeted disruption of the homeobox transcription factor Nkx2-3 in mice results in postnatal lethality and abnormal development of small intestine and spleen. *Development*. (1999) 126:2215–25.
14. Wang C, Biben C, Robb L, Nassir F, Barnett L, Davidson N, et al. Homeodomain factor Nkx2-3 controls regional expression of leukocyte homing coreceptor MAdCAM-1 in specialized endothelial cells of the viscera. *Dev Biol*. (2000) 224:152–67. doi: 10.1006/dbio.2000.9749
15. Hsia LT, Ashley N, Ouaret D, Wang LM, Wilding J, Bodmer WF. Myofibroblasts are distinguished from activated skin fibroblasts by the expression of AOC3 and other associated markers. *Proc Natl Acad Sci USA*. (2016) 12:113. doi: 10.1073/pnas.1603534113
16. Fisher S, Tremelling M, Anderson C, Gwilliam R, Bumpstead S, Prescott N, et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. *Nat Gen*. (2018) 40:710–2. doi: 10.1038/ng.145
17. Parkes M, Barrett J, Prescott N, Tremelling M, Anderson C, Fisher S, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Gen*. (2007) 39:830–2. doi: 10.1038/ng2061
18. Schippers A, Leuker C, Pabst O, Kochut A, Prochnow B, Gruber AD, et al. Mucosal addressin cell adhesion molecule-1 controls plasma-cell migration and function in the small intestine of mice. *Gastroenterol*. (2009) 137:924–33. doi: 10.1053/j.gastro.2009.05.039
19. Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Berard M, Kleinschek M et al. RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol*. (2011) 12:320–6. doi: 10.1038/ni.2002
20. Kellermayer Z, Vojkovics D, Dakah TA, Bodó K, Botz B, Helyes Z, et al. IL-22-independent protection from colitis in the absence of Nkx2.3 transcription factor in mice. *J Immunol*. (2019) 202:ji1801117. doi: 10.4049/jimmunol.1801117
21. Geremia A, Arancibia-Cárcamo CV. Innate lymphoid cells in intestinal inflammation. *Front Immunol*. (2017) 8:1296. doi: 10.3389/fimmu.2017.01296
22. Tamboli CP, Neut C, Desreumaux P, Colombel JF. Dysbiosis in inflammatory bowel disease. *Gut*. (2004) 53:1–4. doi: 10.1136/gut.53.1.1
23. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol*. (2008) 8:458–66. doi: 10.1038/nri2340
24. Kellermayer Z, Vojkovics D, Balogh P. Innate lymphoid cells and their stromal microenvironments. *Immunol Lett*. (2017) 189:3–9. doi: 10.1016/j.imlet.2017.04.008
25. Hepworth M, Monticelli L, Fung T, Ziegler C, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature*. (2013) 498:113–7. doi: 10.1038/nature12240
26. Zundler S, Becker E, Weidinger C, Siegmund B. Anti-adhesiontherapies in inflammatory bowel disease-molecular and clinical aspects. *Front Immunol*. (2017) 8:891. doi: 10.3389/fimmu.2017.00891
27. Wang C, McDonough JS, McDonald KG, Huang C, Newberry RD. Alpha4beta7/MAdCAM-1 interactions play an essential role in transitioning cryptopatches into isolated lymphoid follicles and a nonessential role in cryptopatch formation. *J Immunol*. (2008) 181:4052–61. doi: 10.4049/jimmunol.181.6.4052
28. Hamada H, Hiroi T, Nishiyama Y, Takahashi H, Masunaga Y, Hachimura S, et al. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol*. (2002) 168:57–64. doi: 10.4049/jimmunol.168.1.57
29. Pabst O, Herbrand H, Worbs T, Friedrichsen M, Yan S, Hoffmann MW, et al. Cryptopatches and isolated lymphoid follicles: dynamic lymphoid tissues dispensable for the generation of intraepithelial lymphocytes. *Eur J Immunol*. (2005) 35:98–107. doi: 10.1002/eji.200425432
30. Knoop KA, Butler BR, Kumar N, Newberry RD, Williams IR. Distinct developmental requirements for isolated lymphoid follicle formation in the small and large intestine: RANKL is essential only in the small intestine. *Am J Pathol*. (2011)179:1861–71. doi: 10.1016/j.ajpath.2011.06.004
31. Berlin-Rufenach C, Otto F, Mathies M, Westermann J, Owen MJ, Hamann A, et al. Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. *J Exp Med*. (1999) 189:1467–78. doi: 10.1084/jem.189.9.1467

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

MINI REVIEW
published: 12 March 2019
doi: 10.3389/fimmu.2019.00405

Physiological Regulation of Innate Lymphoid Cells

Nicolas Jacquelot[1,2], *Kylie Luong*[1] *and Cyril Seillet*[1,2]*

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

Discovery of innate lymphoid cells (ILCs) have provoked a paradigm shift in our understanding of the immune protection. Their constitutive presence and activity at the body's barrier surfaces ensure the maintenance of the tissue homeostasis and immune protection. This complex family has distinct and non-redundant functions that can have both beneficial and detrimental effects on disease outcome. The capacity of ILCs to perform their function effectively relies on their ability to sense and integrate intrinsic and extrinsic signals. Recent studies have shown that ILCs are not only sensitive to pathogen-derived stimuli but are also very well equipped to sense host-derived signals such as neuropeptides, hormones, and metabolites. The integration of these signals represents a complex and constant cross-talk between the immune system and the physiological systems of the body, including the nervous, endocrine, digestive, and reproductive systems. The physiological regulation of ILCs constitutes an important step in our understanding of the events leading to the protective and pathological properties of these cells. This review summarizes the recent advances in the understanding of the regulation of ILCs by physiological signals and their consequences on the maintenance of tissue homeostasis.

Keywords: innate lymphoid cells, immunity, homeostasis, neuroendocrine regulation, metabolites, hormones, neuropeptides

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Linda Quatrini,
Bambino Gesù Ospedale Pediatrico, Italy
Tanel Mahlakõiv,
Cornell University, United States
Fotios Karagiannis,
Universitätsklinikum Bonn, Germany

***Correspondence:**
Cyril Seillet
seillet@wehi.edu.au

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

Received: *21 December 2018*
Accepted: *15 February 2019*
Published: *12 March 2019*

Citation:
Jacquelot N, Luong K and Seillet C (2019) Physiological Regulation of Innate Lymphoid Cells. Front. Immunol. 10:405. doi: 10.3389/fimmu.2019.00405

INTRODUCTION

With the discovery of an innate counterpart of the T lymphocytes mirroring key aspect of their phenotype and function, the innate lymphoid cells (ILCs) have forced immunologists to rethink the immunological architecture that confers immune protection. Despite recent evidence that ILCs can be mobilized from blood (1, 2), ILCs are considered to mainly reside within tissues (3). Their activity is not modulated by antigen-specific receptors but rather through a complex integration of cytokines, alarmins, and physiological signals derived from their micro-environment.

Divided in 3 main groups, group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s) are associated with T helper (Th) 1, Th2, and Th17 functions, respectively, while the natural killer (NK) cells are analogous to the $CD8^+$ cytotoxic T cells. ILCs express particular sets of receptors encoded by specific transcriptomic signatures that are imprinted in a tissue-specific manner and therefore ILCs are well equipped to sense host-derived signals (**Figure 1**) (5). Constitutive sensing and integration of these endogenous signals are essential to ILC activity and maintenance of tissue homeostasis. Dysregulation of ILC responses lead to the development of inflammation. ILC1s are mainly involved in the early protection against virus (6) and bacteria (7, 8) through the secretion of interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), however their dysregulation in adipose tissues leads to the development of metabolic disorders and

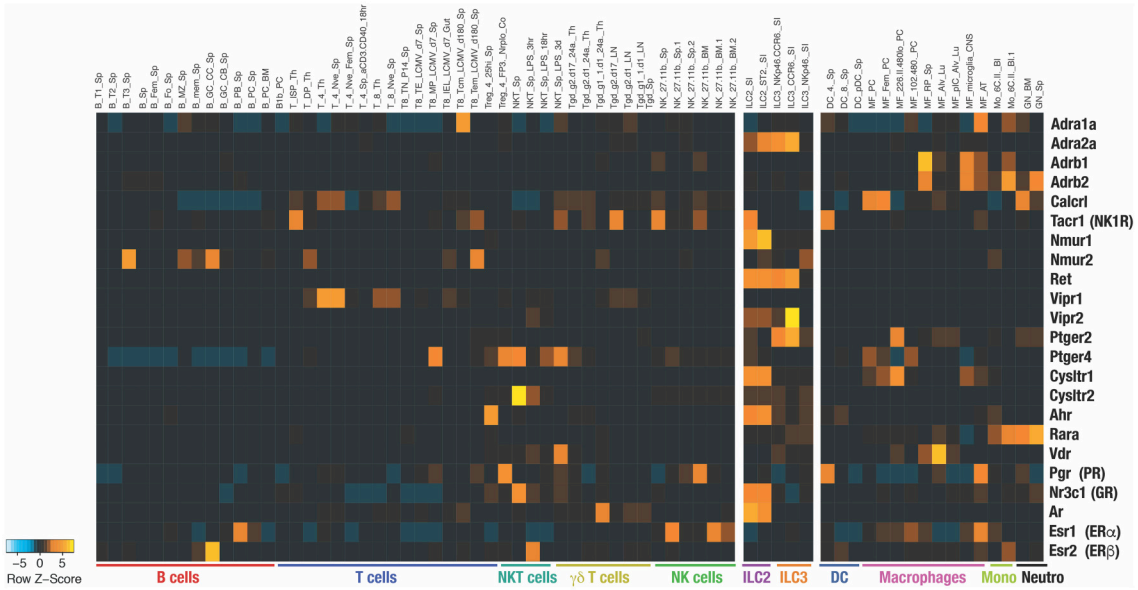

FIGURE 1 | Heatmap shows the RNA expression of the indicated receptors among different immune populations. The data have been extracted from Immunological Genome Project (https://www.immgen.org) (4). Name of each population extracted from ImmGen database are indicated on top of the heatmap.

obesity (9). ILC2s are an early source of interleukin (IL)-5 and IL-13 (10–12). ILC2 activity allows the emergence to type 2 immune responses characterized by goblet cell differentiation, recruitment of eosinophils, basophils, and mast cells which is critical for protection against infection with helminths and viruses but, when uncontrolled, also drive allergic responses and metabolic disorder (13–15). ILC3s produce IL-22 in the gut to protect against intestinal inflammation (16–18). In this review we propose that physiological signals are integrated by ILCs and modulate their constitutive activity in a tissue- and time-specific manner.

NEURO-ENDOCRINE REGULATION OF ILC RESPONSES

ILCs predominately reside at mucosal barrier surfaces and have been shown to be in close anatomical proximity to neurons and glial cells of the autonomic nervous system (ANS). The ANS is divided into (i) the sympathetic nervous system (SNS), which predominates during "fight-or-flight" situations and prepares the body for physical activity and (ii) the parasympathetic nervous system (PNS), which regulates basic body functions during resting conditions, such as digestion, energy conservation, and storage. The expression of specific neuroregulators by the ANS allows tissue-specific regulation of innate cells.

Neuromedin U (NMU)

Neuromedin U (NMU) is a neuropeptide found throughout the body and is highly expressed in the gastrointestinal tract by lamina propria enteric cholinergic neurons (19). NMU plays a plethora of physiological roles, including regulating food intake (20). NMU signals through two receptors, (i) *Nmur1*, mainly expressed on peripheral tissues, and (ii) *Nmur2*, mainly expressed within the central nervous system (CNS). Amongst immune cells, *Nmur1* is specifically expressed on ILC2 (19, 21, 22). Infection with parasitic helminth *N. brasiliensis* is sensed by mucosal neurons, inducing the secretion of NMU in response, which in turn promotes the secretion of IL-5, IL-13, and amphiregulin by ILC2s (19, 21). Deficiency in NMUR signaling leads to impaired type 2 responses and poor control of worm infection. Parasite expulsion critically relies on ILC2 activity through the specific recruitment of eosinophils, basophils, and mast cells and the induction of goblet cell hyperplasia (10) (**Figure 2**).

In the lung, synergistic effects of IL-25 and NMU promote ILC2 proliferation and the secretion of IL-5 and IL-13, resulting in exacerbated allergic inflammation (19, 22). Mice lacking *Nmur1* show reduced ILC2 numbers after house dust mite (HDM) challenge and decreased type 2 allergic airway inflammation (19, 22). Altogether, these studies show how neuronal cues can shape ILC responses to generate a rapid and optimal immune response.

Norepinephrine and β2-Adrenergic Receptor Signaling

Norepinephrine is released by the SNS and signals through β_1- and β_2-adrenergic receptors (β_2AR). NK cell cytotoxicity and expression of IFNγ, granzyme B and perforin are reduced when β_2AR-signaling is engaged (23, 24).

Norepinephrine signaling exerts its inhibitory effect on ILC2s proliferation through binding to β_2AR (25) (**Figure 2**). Administration of β_2AR agonist Clenbuterol during *N. brasiliensis* infection inhibits ILC2 effector functions, leading to reduced eosinophil recruitment, goblet cell hyperplasia and

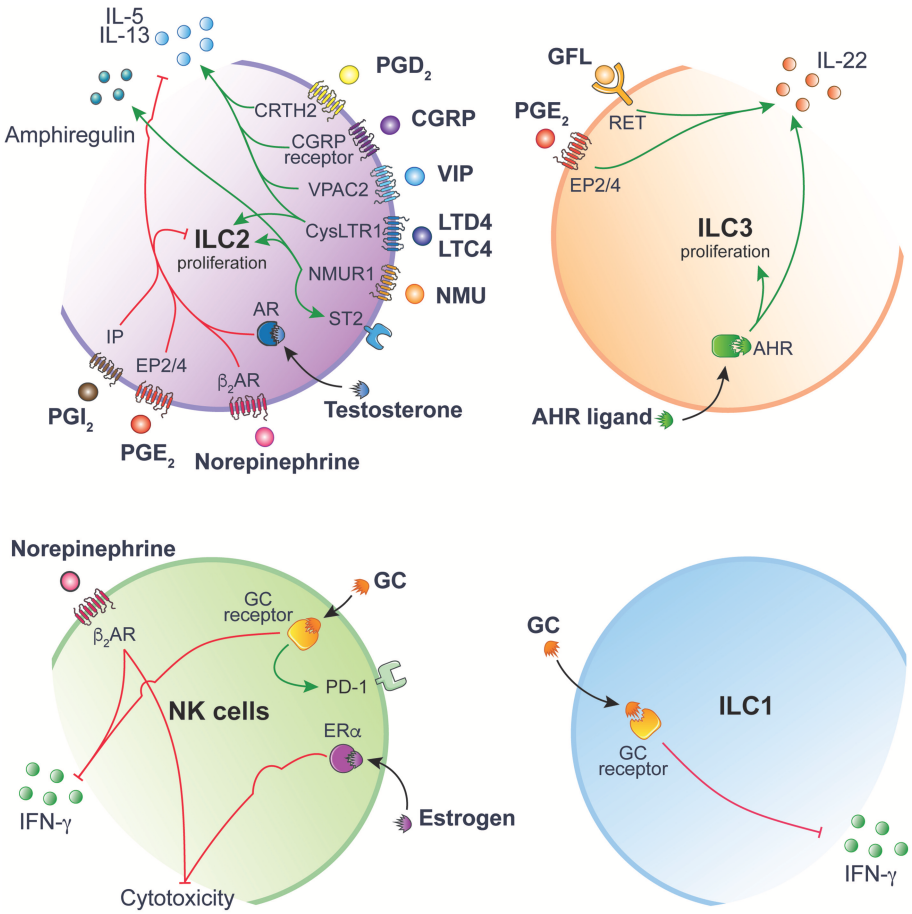

FIGURE 2 | ILC function is tightly regulated by ligand-receptor interactions. Inhibitory and activating intracellular pathways are colored in red and green, respectively. This figure has been drawn using Servier Medical Art (https://smart.servier.com) and modified by the authors under the following terms: Creative Commons Attribution 3.0 Unported License.

consequently increased worm burden. Conversely, mice lacking the β_2AR show increased ILC2 infiltration and exaggerated type 2 response to *N. brasiliensis* infection. Interestingly, β_2AR-signaling specifically inhibits lung and enteric ILC2s, but not Th2 cells, by regulating cell intrinsic proliferation during type 2 inflammatory response (25).

Vasoactive Intestinal Peptide (VIP)

VIP is a neuropeptide expressed throughout the nervous system and has been found in neurons that innervate the lung and gut mucosa (26). VIP is involved in number of physiological processes, including coordinating gastrointestinal motility, mucus, and enzymatic secretions in response to feeding, synchronizing the central circadian rhythm (27) and also skews the differentiation of T cells toward Th2 and T regulatory cells (28, 29). Enteric and lung ILC2s stimulated with VIP through VIP receptor type 2 (VPAC2) promotes a type 2 response. The circadian release of VIP in response to feeding induces a rhythmic expression of IL-5 by ILC2s (**Figure 2**), resulting in increased systemic eosinophil numbers in a circadian manner (30).

IL-5 stimulates the production of VIP by acting directly on nociceptors, creating an inflammatory signal loop that promotes allergic inflammation (31). Noxious environmental respiratory stimuli, such as capsaicin or OVA peptide challenge, induces bronchial hyperresponsiveness and airway inflammation through the activation of lung NaV1.8$^+$ nociceptor. Ablation of NaV1.8$^+$ nociceptor reduces the activation of lung resident ILC2 and Th2 cells, thus reducing bronchial hyperresponsiveness. Administering VPAC2 antagonist leads to decreased ILC2 activation, decreased expression of inflammatory marker ST2 and decreased production of IL-5 and IL-13 (31) (**Figure 2**). This positive feedback loop between sensory nociceptors and ILC2s may be a mechanism to prime and enhance the sensitivity of sensory nociceptors to environmental stimuli.

Calcitonin Gene-Related Protein (CGRP) and Neurotransmitter Gamma-Aminobutyric Acid (GABA)

CGRP is a neuropeptide involved in nociception but also a potent vasodilator found throughout the body in perivascular

innervation (32). During an immune response, CGRP is secreted by specialized epithelial cells called pulmonary neuroendocrine cells (PNECs). PNECs are closely associated with lung ILC2s and amplify ILC2-mediated type 2 airway inflammation in response to environmental allergens (33). In CGRP receptor-deficient mice (CalcrlKO), immune cell infiltration is reduced after HDM allergen challenge. Similarly, mice lacking PNECs (AsclCKO) show blunted type 2 immune response to ovalbumin (OVA) peptide allergen. However, when compared to control lungs, AsclCKO lungs show pronounced reduction in CGRP and inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Whilst disrupting neurotransmitter GABA synthesis in PNECs does not directly affect immune response, blocking GABA signaling prevents overproduction of mucus and IL-13 and reduce goblet cell hyperplasia during allergic airway inflammation (34). As asthmatic patients have a higher number of PNECs, disrupting the communication between PNECs and ILC2s may be a potential therapeutic avenue to alleviate allergic asthma symptoms (**Figure 2**).

Neurotrophic Factors and RET Receptor

Enteric ILC3s respond to neurotrophic factors released by closely associated mucosal glial cells (35). Glial cells are support cells to neurons and enteric glial cells which sense the commensal products and alarmins in a Myd88-dependent manner (35). Glial-derived neurotrophic factor family of ligands (GFL) act though ILC3 tyrosine kinase receptor RET to maintain gut defense. RET signaling in ILC3s directly controls innate IL-22 expression (**Figure 2**). When neurotrophic receptor RET is deleted in enteric ILC3s, ILC3-dervied IL-22 is reduced at steady state and during *C. rodentium* infection. Mice without RET showed greater propensity toward gut inflammation, leading altered microbial communities (35).

Glucocorticoids (GC)

The hypothalamic-pituitary-adrenal (HPA) axis is a crucial neuroendocrine regulatory axis for coordinating and responding to environmental cues. GC have potent immunosuppressive and anti-inflammatory effects. Endogenous GC production after stimulation of the HPA axis inhibits ILC1 function during both bacterial (36) and viral infections (37), thus, preventing immunopathology without impairing immune responses against infections. Whilst NK cells and ILC1 are sensitive to GC during the priming phase, only NK cells from the spleen and liver show higher levels of IFN-γ production in the absence of GC receptor (GR) signaling (36). The disparity in GC influence on ILC1 suggests that the mechanisms controlling IFN-γ production in ILC1 differ from NK cells during endotoxin tolerance. When GR is deleted in ILC1 and NK cells, mice show exacerbated endotoxin LPS-induced septic shock. Similarly, GC produced following MCMV infection is shown to induce *de novo* expression of checkpoint inhibitor programmed death-1 (PD-1/CD279) specifically on splenic NK cells but not in the liver NK cells or ILC1 (37). Expression of PD-1 limits IFN-γ production and is required for the protection of the host against necrotic and granulomatous inflammation. The tissue-specificity of GC-signaling on ILC1 and NK cells results from the integration of different cytokine signals within the tissue microenvironment, allowing the fine tuning and tailoring of the immune response during infection (**Figure 2**).

Estrogens

Estrogens are synthesized from cholesterol mainly in the ovaries and can diffuse through the membrane of cells and bind to their receptors in the cytosol. They can bind to the estrogen receptor (ER) α and β or act through "non-genomic" action, via modulation of intracellular signaling pathways (38). Some discrepancies exist regarding the described impact of estrogens on NK cells. While estrogen is shown to enhance the cytotoxicity of human NK-like cell line *in vitro* (39), others have reported that estrogen decreased the NK cell proliferation, cytotoxicity, and IFNγ production (40, 41). These conflicting effects of estrogens need to be carefully interpreted as the concentration used in these studies are greater than the physiological concentrations and therefore, may not reflect the actual effects of estrogens *in vivo*. As estrogens have multiple targets *in vivo*, it will be important to develop new models in which ER will be specifically deleted in NK cells to investigate the role of estrogens on this particular cell type.

Uterine ILC2 uniquely express ERα while ILC2 in other tissues do not (42). This specific pattern may explain why only uterine ILC2 have been shown to be sensitive to estrogen regulation (43). Deficiency in estrogen signaling using either $ER\alpha^{-/-}$ and $\beta^{-/-}$ mice, or in ovariectomized mice, revealed a decreased ILC2 numbers in the uterus. The role of uterine ILC2s in regulation and maintenance of the uterus homeostasis is not known and the consequences of the regulation of these cells by estrogens will require more investigation.

Androgens

The androgens such as testosterone and dihydrotestosterone mediate their effect via the androgen receptor (AR) and act as ER through genomic and non-genomic pathways. Regulation of ILCs by androgen signaling has been highlighted in ILC2. In mice, proportions and absolute numbers of ILC2 are greater in females compared to males (43). A similar trend is observed in humans, where female asthmatic patients have higher frequencies of ILC2s in the blood than asthmatic men (44). ILC2 progenitors express specifically the AR but not ER. Mechanistically, AR signaling directly inhibits the differentiation and proliferation of mature ILC2s in a cell intrinsic manner (43). IL-5 and IL-13 production is reduced in males compared to females and in testosterone stimulated ILC2s. This regulatory effect of testosterone on ILC2s protects males against the effects of allergen-induced lung inflammation when mice are challenged with house dust mite (HDM), IL-33 or *Alternaria alternata* (43, 44) (**Figure 2**).

METABOLITES DERIVED REGULATION

There is constant communication at the mucosal barriers between the environment and the host and considerable efforts are currently made to better understand the interplay between

the physiological systems and how key molecules, such as aryl hydrocarbon receptor (AHR) ligands and lipids influence local immunity and gut homeostasis.

Aryl Hydrocarbon Receptor Ligands

AHR acts as a toxin sensor and binds to diverse endogenous and exogenous chemicals. The food and gut ecosystem are natural sources of AHR inducers. These include indoles directly found in cruciferous vegetables or obtained following tryptophan degradation by the gut microbiota (45, 46). Gut resident ILCs highly express AHR (**Figure 1**). The lack of AHR is associated with reduced ILC3 numbers and decreased ILC3-derived IL-22 (47, 48) (**Figure 2**). AHR directly binds to the *Il22* promoter and act in concert with RORγt to induce *Il22* expression in ILC3 (49).

The regulation of AHR signaling is tightly controlled as prolonged activation can have detrimental effects (50). The metabolic clearance of AHR ligands is mediated by the cytochrome P4501 (CYP1) family enzymes. Interestingly, constitutive expression of CYP1 enzymes drastically reduces the availability of AHR ligands and lead to the loss of gut ILC3 and Th17 cells (50). Consequently, constitutive *Cyp1a* expression or complete loss of *Ahr* in mice increases their susceptibility to *C. rodentium* associated with impeded IL-22 production (48–50). These studies highlight how homeostatic regulation of the availability of AHR ligands by intestinal epithelial cells provide critical feedback to immune cells, thus shaping mucosal protection.

Leukotrienes

Leukotrienes (LT) are derived from the catabolism of the arachidonic acid. The cysteinyl leukotriene receptor 1 (CysLTR1), one of the LT receptors, is highly expressed on lung ILC2s (51, 52). LTD_4 induces ILC2 secretion of IL-4, IL-5, and IL-13 through CysLT1R engagement (**Figure 2**). Administration of LTC_4 and LTD_4 *in vivo* synergizes with IL-33 to activate lung ILC2 and promote lung ILC2 proliferation, IL-5 expression and lung eosinophilia (52). Deficiency in LT receptors does not affect the maintenance of ILC2 but impairs the functional response of ILC2 during *N. Brasiliensis* or *Alternaria* species infection (51, 52). Human ILC2 function is also enhanced upon LT stimulation resulting in higher IL-13 production and increased expression of IL-33/IL-25 receptors, thus promoting their responsiveness to these cytokines (53). Collectively, these results demonstrate the role of LTs in promoting lung inflammation and type 2 responses through the direct activation of lung ILC2. Given the role of ILC2s in allergy and asthma, targeting this LT-CysLTR pathway is of great interest and may provide an effective therapeutic strategy to constrain ILC2-mediated inflammation (52, 54, 55).

Prostaglandins

Prostaglandins (PG) are also derived from the arachidonic acid and exhibit different roles on ILCs. Whilst PGE2 and PGI2 signaling inhibit ILC functions (56, 57), PGD_2 promotes the activation, migration, and accumulation of ILC2 in inflamed lung (58–60). PGE_2 and PGI_2 signal through the PGE_2 receptors and the prostacyclin receptor (IP), respectively. IP is almost exclusively expressed by ILC2, PGE_2 receptor 2 (EP2) is mainly expressed by ILC3 and PGE_2 receptor 4 (EP4) is found on ILC1, NK cells, and ILC2 (**Figure 1**).

The PGD_2 receptor, CRTH2, was first identified on Th2 cells (61) and is now widely used to distinguish human ILC2 from other ILC subsets (62). PGD_2 in lungs amplifies type 2 immunity synergistically with IL-33/IL-25 stimulation, subsequently enhancing ILC2-derived IL-13 and chemotaxis (59, 63). This effect can be prevented by lipotoxin A_4 in human ILC2 (63) highlighting the complex interactions between these molecules. In pathological conditions, CRTH2-deficient ILC2s do not accumulate in inflamed lungs and IL-4 and IL-13 production is impaired (58) highlighting the importance of the PGD_2-CRTH2 axis in ILC2 regulation and control of lung inflammation and allergic disease exacerbations (**Figure 2**).

IP mainly act as a negative regulator of ILC function. Zhou et al. have demonstrated the negative impact of PGI_2 on ILC2 functions (57). ILC2 stimulated with IL-33 and a PGI_2 analog, cicaprost, show reduced cell proliferation and IL-5 and IL-13 productions (57). When IP-deficient mice are challenged intranasally with *Alternaria alternata* extracts, ILC2s accumulate in the lung and cells show enhanced IL-5 and IL-13 expression, resulting in increased eosinophils infiltration and lung inflammation (**Figure 2**). Similarly, PGE_2 abrogates IL-33-induced ILC2 proliferation and cytokine production in mice (56) and humans (64). Deletion of EP4 exacerbates lung inflammation associated with ILC2-mediated eosinophil recruitments and increased ILC2-derived IL-5 and IL-13 in response to *Alternaria alternata*. Taken together, these studies reveal an evolutionarily conserved role of PGE_2-EP2/4 pathway in negatively controlling ILC2 activity (**Figure 2**).

Recently, a physiological role of PGE_2-EP4 signaling in activating the ILC3/IL-22 axis has been described. Suppression of PGE_2 synthesis with indomethacin leads to the development of LPS-induced systemic inflammation and septic shock, resulting in elevated serum TNFα and IL-6 levels, increased spleen weight, translocation of gut bacteria and accumulation of neutrophils in the peritoneal cavity (65). LPS-induced systemic inflammation is prevented by using EP4 agonists. PGE_2 contributes to systemic inflammation through acting on the homeostatic production of IL-22 by ILC3 (**Figure 2**). Suppressing PGE_2 by indomethacin administration inhibits IL-22–IL-22R signaling pathway in intestinal epithelial cells, which lead to the downregulation of critical proteins involved in mucosal integrity such as RegIIIβ, RegIIIγ, Fut2, mucins, and molecules forming tight junctions (65). This study highlights the critical role of physiological mediators that contributes to the crosstalk between the innate immune system, gut epithelium and microflora, which confers optimal protection against systemic inflammation.

CONCLUSION

The studies summarized in this review reveal a direct influence of the neuroendocrine system and metabolites on the different

ILC subsets at barrier surfaces to maintain tissue homeostasis and appropriate responses during infection. They collectively demonstrate the complex interactions between neuropeptides, hormones and metabolites with ILCs and offer new opportunities to manipulate ILC responses in disease and allergy. Modulating these physiological pathways may present less side effects than synthetic drug-based strategies. Because of the synergistic or antagonistic effects between these mediators, it will be important to explore these regulatory pathways in models where the mediator or its receptor(s) can be deleted in a spatio-temporal manner. The recent ILC transcriptional analyses and database made available by Immgen Consortium have highlighted the capacity of these cells to sense a myriad of physiological signals and it will be now crucial to discover how ILCs integrate these signals to fine tune the immune response to prevent immunopathology without impairing infection control.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by grants and fellowships from the National Health and Medical Research Council (NHMRC) of Australia (CS; APP1098832 and APP1165443), Australian Research Career development Fellowship (CS; APP1123000), Cure Cancer Australia and Cancer Australia through the Cancer Australia Priority-driven Cancer Research Scheme (NJ; APP1163990), and the Foundation ARC pour la Recherche sur le cancer (NJ). This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIIS.

REFERENCES

1. Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science.* (2018) 359:114–9. doi: 10.1126/science.aam5809
2. Stier MT, Zhang J, Goleniewska K, Cephus JY, Rusznak M, Wu L, et al. IL-33 promotes the egress of group 2 innate lymphoid cells from the bone marrow. *J Exp Med.* (2018) 215:263–81. doi: 10.1084/jem.20170449
3. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science.* (2015) 350:981–5. doi: 10.1126/science.aac9593
4. Heng TS, Painter MW, Immunological Genome Project Consortium, Elpek K, Lukacs-Kornek V, Mauermann N, et al. Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol.* (2008) 10:1091-4. doi: 10.1038/ni1008-1091
5. Ricardo-Gonzalez RR, Van Dyken SJ, Schneider C, Lee J, Nussbaum JC, Liang HE, et al. Tissue signals imprint ILC2 identity with anticipatory function. *Nat Immunol.* (2018) 10:1093–9. doi: 10.1038/s41590-018-0201-4
6. Weizman OE, Adams NM, Schuster IS, Krishna C, Pritykin Y, Lau C, et al. ILC1 confer early host protection at initial sites of viral infection. *Cell.* (2017) 171:795–808.e712. doi: 10.1016/j.cell.2017.09.052
7. Klose CSN, Flach M, Möhle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell.* (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
8. Abt MC, Lewis BB, Caballero S, Xiong H, Carter RA, Sušac B, et al. Innate immune defenses mediated by two ILC subsets are critical for protection against acute *Clostridium difficile.* infection. *Cell Host Microbe.* (2015) 18:27–37. doi: 10.1016/j.chom.2015.06.011
9. Boulenouar S, Michelet X, Duquette D, Alvarez D, Hogan AE, Dold C, et al. Adipose type one innate lymphoid cells regulate macrophage homeostasis through targeted cytotoxicity. *Immunity.* (2017) 46:273–86. doi: 10.1016/j.immuni.2017.01.008
10. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature.* (2010) 463:540–4. doi: 10.1038/nature08636
11. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* (2010) 464:1367–70. doi: 10.1038/nature08900
12. Saenz SA, Siracusa MC, Monticelli LA, Ziegler CG, Kim BS, Brestoff JR, et al. IL-25 simultaneously elicits distinct populations of innate lymphoid cells and multipotent progenitor type 2 (MPPtype2) cells. *J Exp Med.* (2013) 210:1823–37. doi: 10.1084/jem.20122332
13. Scanlon ST, McKenzie AN. Type 2 innate lymphoid cells: new players in asthma and allergy. *Curr Opin Immunol.* (2012) 24:707–12. doi: 10.1016/j.coi.2012.08.009
14. Molofsky AB, Nussbaum JC, Liang HE, Van Dyken SJ, Cheng LE, Mohapatra A, et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J Exp Med.* (2013) 210:535–49. doi: 10.1084/jem.20121964
15. Monticelli LA, Buck MD, Flamar AL, Saenz SA, Tait Wojno ED, Yudanin NA, et al. Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation. *Nat Immunol.* (2016) 17:656–65. doi: 10.1038/ni.3421
16. Rankin LC, Girard-Madoux MJ, Seillet C, Mielke LA, Kerdiles Y, Fenis A, et al. Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nat Immunol.* (2016) 17:179–86. doi: 10.1038/ni.3332
17. Behnsen J, Jellbauer S, Wong CP, Edwards RA, George MD, Ouyang W, et al. The cytokine IL-22 promotes pathogen colonization by suppressing related commensal bacteria. *Immunity.* (2014) 40:262–73. doi: 10.1016/j.immuni.2014.01.003
18. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature.* (2009) 457:722–5. doi: 10.1038/nature07537
19. Klose CSN, Mahlakõiv T, Moeller JB, Rankin LC, Flamar AL, Kabata H, et al. The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation. *Nature.* (2017) 549:282–6. doi: 10.1038/nature23676
20. Howard AD, Wang R, Pong SS, Mellin TN, Strack A, Guan XM, et al. Identification of receptors for neuromedin U and its role in feeding. *Nature.* (2000) 406:70–4. doi: 10.1038/35017610
21. Cardoso V, Chesné J, Ribeiro H, García-Cassani B, Carvalho T, Bouchery T, et al. Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature.* (2017) 549:277–81. doi: 10.1038/nature23469
22. Wallrapp A, Riesenfeld SJ, Burkett PR, Abdulnour RE, Nyman J, Dionne D, et al. The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation. *Nature.* (2017) 549:351–6. doi: 10.1038/nature24029
23. Sun Z, Hou D, Liu S, Fu W, Wang J, Liang Z. Norepinephrine inhibits the cytotoxicity of NK92MI cells via the beta2adrenoceptor/cAMP/PKA/pCREB signaling pathway. *Mol Med Rep.* (2018) 17:8530–5. doi: 10.1016/j.expneurol.2012.05.008
24. De Lorenzo BH, de Oliveira Marchioro L, Greco CR, Suchecki D. Sleep-deprivation reduces NK cell number and function mediated by beta-adrenergic signalling. *Psychoneuroendocrinology.* (2015) 57:134–43. doi: 10.1016/j.psyneuen.2015.04.006
25. Moriyama S, Brestoff JR, Flamar AL, Moeller JB, Klose CSN, Rankin LC, et al. beta2-adrenergic receptor-mediated negative regulation of group 2 innate lymphoid cell responses. *Science.* (2018) 359:1056–61. doi: 10.1126/science.aan4829
26. Said SI, Rosenberg RN. Vasoactive intestinal polypeptide: abundant immunoreactivity in neural cell lines and normal nervous tissue. *Science.* (1976) 192:907–8. doi: 10.1126/science.1273576
27. Aton SJ, Colwell CS, Harmar AJ, Waschek J, Herzog ED. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nat Neurosci.* (2005) 8:476–83. doi: 10.1038/nn1419

28. Ibrahim H, Barrow P, Foster N. Transcriptional modulation by VIP: a rational target against inflammatory disease. *Clin Epigenetics.* (2011) 2:213–22. doi: 10.1007/s13148-011-0036-4
29. Delgado M, Ganea D. Vasoactive intestinal peptide: a neuropeptide with pleiotropic immune functions. *Amino Acids.* (2013) 45:25–39. doi: 10.1007/s00726-011-1184-8
30. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature.* (2013) 502:245–8. doi: 10.1038/nature12526
31. Talbot S, Abdulnour RE, Burkett PR, Lee S, Cronin SJ, Pascal MA, et al. Silencing nociceptor neurons reduces allergic airway inflammation. *Neuron.* (2015) 87:341–54. doi: 10.1016/j.neuron.2015.06.007
32. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol Rev.* (2014) 94:1099–142. doi: 10.1152/physrev.00034.2013
33. Sui P, Wiesner DL, Xu J, Zhang Y, Lee J, Van Dyken S, et al. Pulmonary neuroendocrine cells amplify allergic asthma responses. *Science.* (2018) 360:eaan8546. doi: 10.1126/science.aan8546
34. Xiang YY, Wang S, Liu M, Hirota JA, Li J, Ju W, et al. A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. *Nat Med.* (2007) 13:862–7. doi: 10.1038/nm1604
35. Ibiza S, García-Cassani B, Ribeiro H, Carvalho T, Almeida L, Marques R, et al. Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. *Nature.* (2016) 535:440–3. doi: 10.1038/nature18644
36. Quatrini L, Wieduwild E, Guia S, Bernat C, Glaichenhaus N, Vivier E, et al. Host resistance to endotoxic shock requires the neuroendocrine regulation of group 1 innate lymphoid cells. *J Exp Med.* (2017) 214:3531–41. doi: 10.1084/jem.20171048
37. Quatrini L, Wieduwild E, Escaliere B, Filtjens J, Chasson L, Laprie C, et al. Endogenous glucocorticoids control host resistance to viral infection through the tissue-specific regulation of PD-1 expression on NK cells. *Nat Immunol.* (2018) 19:954–62. doi: 10.1038/s41590-018-0185-0
38. Gruber CJ, Tschugguel W, Schneeberger C, Huber JC. Production and actions of estrogens. *N Eng J Med.* (2002) 346:340–52. doi: 10.1056/NEJMra000471
39. Sorachi K, Kumagai S, Sugita M, Yodoi J, Imura H. Enhancing effect of 17 beta-estradiol on human NK cell activity. *Immunol Lett.* (1993) 36:31–5. doi: 10.1016/0165-2478(93)90065-A
40. Souza SS, Castro FA, Mendonça HC, Palma PV, Morais FR, Ferriani RA, et al. Influence of menstrual cycle on NK activity. *J Reprod Immunol.* (2001) 50:151–9. doi: 10.1016/S0165-0378(00)00091-7
41. Hao S, Li P, Zhao J, Hu Y, Hou Y. 17beta-estradiol suppresses cytotoxicity and proliferative capacity of murine splenic NK1.1+ cells. *Cell Mol Immunol.* (2008) 5:357–64. doi: 10.1038/cmi.2008.44
42. Bartemes K, Chen CC, Iijima K, Drake L, Kita H. IL-33-responsive group 2 innate lymphoid cells are regulated by female sex hormones in the uterus. *J Immunol.* (2018) 200:229–36. doi: 10.4049/jimmunol.1602085
43. Laffont S, Blanquart E, Savignac M, Cénac C, Laverny G, Metzger D, et al. Androgen signaling negatively controls group 2 innate lymphoid cells. *J Exp Med.* (2017) 214:1581–92. doi: 10.1084/jem.20161807
44. Cephus JY, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, et al. Testosterone Attenuates Group 2 Innate Lymphoid Cell-Mediated Airway Inflammation. *Cell Rep.* (2017) 21:2487–99. doi: 10.1016/j.celrep.2017.10.110
45. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol.* (2003) 43:309–34. doi: 10.1146/annurev.pharmtox.43.100901.135828
46. Stockinger B, Di Meglio P, Gialitakis M, Duarte JH. The aryl hydrocarbon receptor: multitasking in the immune system. *Annu Rev Immunol.* (2014) 32:403–32. doi: 10.1146/annurev-immunol-032713-120245
47. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol.* (2011) 13:144–51. doi: 10.1038/ni.2187
48. Kiss EA, Vonarbourg C, Kopfmann S, Hobeika E, Finke D, Esser C, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science.* (2011) 334:1561–5. doi: 10.1126/science.1214914
49. Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity.* (2012) 36:92–104. doi: 10.1016/j.immuni.2011.11.011
50. Schiering C, Wincent E, Metidji A, Iseppon A, Li Y, Potocnik AJ, et al. Feedback control of AHR signalling regulates intestinal immunity. *Nature.* (2017) 542:242–5. doi: 10.1038/nature21080
51. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol.* (2013) 132:205–13. doi: 10.1016/j.jaci.2013.03.048
52. von Moltke J, O'Leary CE, Barrett NA, Kanaoka Y, Austen KF, Locksley RM. Leukotrienes provide an NFAT-dependent signal that synergizes with IL-33 to activate ILC2s. *J Exp Med.* (2017) 214:27–37. doi: 10.1084/jem.20161274
53. Salimi M, Stöger L, Liu W, Go S, Pavord I, Klenerman P, et al. Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. *J Allergy Clin Immunol.* (2017) 140:1090–100.e1011. doi: 10.1016/j.jaci.2016.12.958
54. Yokomizo T, Nakamura M, Shimizu T. Leukotriene receptors as potential therapeutic targets. *J Clin Invest.* (2018) 128:2691–701. doi: 10.1172/JCI97946
55. Kim AS, Doherty TA. New and emerging therapies for asthma. *Ann Allergy Asthma Immunol.* (2016) 116:14–7. doi: 10.1016/j.anai.2015.08.001
56. Zhou Y, Wang W, Zhao C, Wang Y, Wu H, Sun X, et al. Prostaglandin E2 inhibits group 2 innate lymphoid cell activation and allergic airway inflammation through E-prostanoid 4-cyclic adenosine monophosphate signaling. *Front Immunol.* (2018) 9:501. doi: 10.3389/fimmu.2018.00501
57. Zhou W, Toki S, Zhang J, Goleniewksa K, Newcomb DC, Cephus JY, et al. Prostaglandin I2 signaling and inhibition of group 2 innate lymphoid cell responses. *Am J Respir Crit Care Med.* (2016) 193:31–42. doi: 10.1164/rccm.201410-1793OC
58. Wojno ED, Monticelli LA, Tran SV, Alenghat T, Osborne LC, Thome JJ, et al. The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol.* (2015) 8:1313–23. doi: 10.1038/mi.2015.21
59. Chang JE, Doherty TA, Baum R, Broide D. Prostaglandin D2 regulates human type 2 innate lymphoid cell chemotaxis. *J Allergy Clin Immunol.* (2014) 133:899–901.e893. doi: 10.1016/j.jaci.2013.09.020
60. Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie AN, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol.* (2014) 133:1184–94. doi: 10.1016/j.jaci.2013.10.056
61. Nagata K, Tanaka K, Ogawa K, Kemmotsu K, Imai T, Yoshie O, et al. Selective expression of a novel surface molecule by human Th2 cells *in vivo*. *J Immunol.* (1999) 162:1278–86.
62. Mjösberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol.* (2011) 12:1055–62. doi: 10.1038/ni.2104
63. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci Transl Med.* (2013) 5:174ra126. doi: 10.1126/scitranslmed.3004812
64. Maric J, Ravindran A, Mazzurana L, Björklund ÅK, Van Acker A, Rao A, et al. Prostaglandin E2 suppresses human group 2 innate lymphoid cell function. *J Allergy Clin Immunol.* (2018) 141:1761–73.e6. doi: 10.1016/j.jaci.2017.09.050
65. Duffin R, O'Connor RA, Crittenden S, Forster T, Yu C, Zheng X, et al. Prostaglandin E2 constrains systemic inflammation through an innate lymphoid cell-IL-22 axis. *Science.* (2016) 351:1333–8. doi: 10.1126/science.aad9903

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

ORIGINAL RESEARCH
published: 29 March 2019
doi: 10.3389/fimmu.2019.00649

Commensal and Pathogenic Bacteria Indirectly Induce IL-22 but Not IFNγ Production From Human Colonic ILC3s via Multiple Mechanisms

*Moriah J. Castleman[1], Stephanie M. Dillon[1], Christine M. Purba[1], Andrew C. Cogswell[2], Jon J. Kibbie[1], Martin D. McCarter[3], Mario L. Santiago[1], Edward Barker[2] and Cara C. Wilson[1]**

[1] Division of Infectious Disease, Department of Medicine, University of Colorado Anschutz Medical, Aurora, CO, United States, [2] Department of Microbial Pathogens and Immunity, Rush University Medical Center, Chicago, IL, United States, [3] Department of Surgery, University of Colorado Anschutz Medical, Aurora, CO, United States

OPEN ACCESS

Edited by:
Joanna Kirman,
University of Otago, New Zealand

Reviewed by:
Carolina V. Arancibia,
University of Oxford, United Kingdom
Jorge Caamano,
University of Birmingham,
United Kingdom

***Correspondence:**
Cara C. Wilson
cara.wilson@ucdenver.edu

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

Received: 05 December 2018
Accepted: 11 March 2019
Published: 29 March 2019

Citation:
Castleman MJ, Dillon SM, Purba CM, Cogswell AC, Kibbie JJ, McCarter MD, Santiago ML, Barker E and Wilson CC (2019) Commensal and Pathogenic Bacteria Indirectly Induce IL-22 but Not IFNγ Production From Human Colonic ILC3s via Multiple Mechanisms. Front. Immunol. 10:649. doi: 10.3389/fimmu.2019.00649

Innate lymphoid cells (ILCs) are a diverse family of cells that play critical roles in mucosal immunity. One subset of the ILC family, Group 3 ILCs (ILC3s), has been shown to aid in gut homeostasis through the production of IL-22. IL-22 promotes gut homeostasis through its functional effect on the epithelial barrier. When gut epithelial barrier integrity is compromised, such as in Human Immunodeficiency Virus (HIV) infection and inflammatory bowel disease (IBD), microbes from the gut lumen translocate into the lamina propria, inducing a multitude of potentially pathogenic immune responses. In murine models of bacterial infection, there is evidence that bacteria can induce pro-inflammatory IFNγ production in ILC3s. However, the impact of diverse translocating bacteria, particularly commensal bacteria, in dictating IFNγ versus IL-22 production by human gut ILC3s remains unclear. Here, we utilized an *in vitro* human lamina propria mononuclear cell (LPMC) model to evaluate ILC3 cytokine production in response to a panel of enteric Gram-positive and Gram-negative commensal and pathogenic bacteria and determined potential mechanisms by which these cytokine responses were induced. The percentages of IL-22-producing ILC3s, but not IFNγ-producing ILC3s, were significantly increased after LPMC exposure to both Gram-positive and Gram-negative commensal or pathogenic bacterial stimuli. Stimulation of IL-22 production from ILC3s was not through direct recognition of bacterial antigen by ILC3s, but rather required the help of accessory cells within the LPMC population. CD11c+ myeloid dendritic cells generated IL-23 and IL-1β in response to enteric bacteria and contributed to ILC3 production of IL-22. Furthermore, ligation of the natural cytotoxicity receptor NKp44 on ILC3s in response to bacteria stimulation also significantly increased the percentage of IL-22-producing ILC3s. Overall, these data demonstrate that human gut microbiota, including commensal bacteria, indirectly modulate colonic ILC3 function to induce IL-22, but additional signals are likely required to induce IFNγ production by colonic ILC3s in the setting of inflammation and microbial translocation.

Keywords: human, innate lymphoid cells, commensal bacteria, colonic mucosa, NKp44, myeloid dendritic cells, IL-23, IL-1β

INTRODUCTION

Innate Lymphoid Cells (ILCs) descend from the lymphoid lineage, but unlike T cells and B cells lack rearranged antigen receptors (1). Rather than responding to antigen priming, ILCs respond in an antigen-independent manner through cytokine stimulation to produce effector cytokines (2) or through engagement of germline encoded receptor stimulation (3–5). ILCs are categorized into three groups based on the expression of specific master transcription factors and effector function (6). Group 1 ILCs (ILC1, NK cells) express T-bet and produce the pro-inflammatory cytokine IFNγ in response to IL-12 and IL-18; Group 2 ILCs (ILC2s) express GATA3 and produce IL-4, IL-13, and IL-5 in response to IL-25 and IL-33; and Group 3 ILCs (ILC3s) express RORγt and AHR and typically produce IL-22, GM-CSF, IL-17, and LT-α1β2 in response to IL-23 and IL-1β (6). ILCs are constitutively found in the gastrointestinal tract (7). ILC3s are of particular importance to gut mucosal immunity (7–9) via the secretion of effector cytokines that act directly on epithelial cells (10). Epithelial cells express the IL-22 receptor (IL-22R) (11) and IL-22 production by ILC3s promotes epithelium proliferation, survival, mucus production, upregulation of fucosylation, and in some studies increased gene expression of antimicrobial peptides (12–16). To date, the majority of studies characterizing gut ILC3 function have utilized murine models. Examination of human ILC3 function have primarily focused on tonsil tissue and demonstrated that the mechanisms contributing to IL-22 production include stimulation by the cytokines IL-23 and IL-1β (3, 17, 18) with synergistic enhancement of IL-22 production observed in the presence of the natural cytotoxicity receptor NKp44 engagement (3). Few studies have directly investigated factors driving IL-22 production by human gut ILC3s, although one study observed a requirement for IL-23, IL-1β, and IL-7, with synergy again being induced in the presence of NKp44 signaling (3).

Epithelial barrier damage and loss of function in gut-associated diseased states have correlated with alterations in ILC frequency and function. In Inflammatory Bowel Disease (IBD), patients with Crohn's disease have a loss of colonic or ileum IL-22-producing ILCs (including ILC3s) (18, 19) and an increase in IFNγ/IL-17A-producing ILCs (20–22). In Human Immunodeficiency Virus (HIV) infection, loss of colonic IL-22-producing ILCs has been reported (23). Similarly, reduced frequencies of IL-22/IL-17-producing ILCs during Simian Immunodeficiency Virus (SIV) infection (the non-human primate model of HIV) were noted (24–28). Furthermore, we and others have reported increased frequencies of IFNγ-producing ILCs both in people living with HIV (PLWH) who were not receiving anti-retroviral therapy (ART) (29) and during SIV infection (27). Since IFNγ alters epithelial tight junctions and upregulates epithelial cell expression of TNFα receptor which results in further epithelial cell damage (30, 31), increased frequencies of IFNγ-producing ILC3s may be an additional contributor to epithelial barrier breakdown. When the epithelial barrier is compromised, translocation of gut-associated bacteria into the lamina propria (LP) exposes immune cells to bacteria of different species or magnitude than what these cells typically encounter in the healthy human gut (32, 33). We previously demonstrated that increased frequencies of colonic IFNγ-producing ILCs in PLWH correlated with alterations in mucosa-associated bacterial communities (dysbiosis), specifically with increased relative abundance of Gram-negative commensal *Prevotella* species (29). Understanding the bacteria-specific cytokine responses of ILC3s and the mechanisms by which protective or deleterious cytokines are produced are critical to determining the effect of ILC3s on gut homeostasis, not only for their role in enteric bacterial immunity, but also for their role in influencing epithelial cell function in disease states.

Murine studies highlighted a complex role for gut microbiota in ILC subset development and functional production of IL-22 (13, 34, 35). IL-22 production by ILC3s protected against an enteric pathogen *Citrobacter rodentium* (34, 36, 37), and prevented systemic dissemination of the commensal *Alcaligenes* species in mice (15). Fucosylation of epithelial cells induced by ILC3 production of IL-22 contributed to host defense against murine *S. typhimurium* infection (38). Furthermore, murine ILC3s negatively regulated microbe-specific T cells in the gut to limit pathological responses to commensal bacteria (39, 40). While these studies support a homeostatic role for ILC3s in microbiota-associated gut responses in mice, gut inflammatory ILC3s in response to bacteria have been reported. ILC3s produced IFNγ in response to infection with *Salmonella typhimurium* (41) and IFNγ/IL-17 in response to infection with the *Helicobacter hepaticus* (42). Furthermore, ILC3-associated IFNγ/IL-17 production in response to *H. hepaticus* was linked to the development of colitis (42) highlighting a potentially deleterious role of ILC3 cytokine production. *In vitro* exposure of human ILC3s have also suggested a plasticity in cytokine production with the capacity to produce IFNγ or IL-22 dependent on the cytokine milieu (18, 21). These observations raise the possibility that human gut ILC3s may also have the capacity to produce IL-22 or IFNγ in response to exposure to different types of bacteria.

In this study, we hypothesized that pathogenic enteric bacteria would induce pro-inflammatory cytokine production (IFNγ) from human lamina propria ILC3s, whereas commensal bacteria would primarily elicit protective (IL-22) cytokine production. To address this, we utilized an *in vitro* human colonic mononuclear cell model (43, 44) to investigate ILC3 cytokine profiles induced in response to a panel of whole Gram-negative and Gram-positive, commensal and pathogenic bacteria and the mechanisms driving these responses. Overall, our observations provide insight into the ILC3 role in enteric bacteria immunity and their contribution to the inflammatory environment in disease states where microbes translocate through a compromised epithelial barrier.

MATERIALS AND METHODS

Human Tissue Samples

Human colonic tissue samples were acquired from patients undergoing elective abdominal surgery at the University of Colorado Hospital and are categorized as discarded tissue from macroscopically normal sites. Samples from patients

that underwent chemotherapy or radiation within 8 weeks of tissue collection were not included in the study. Other criteria for tissue exclusion include those with Inflammatory Bowel Disease, HIV infection or treatment with immunosuppressive drugs. Intraepithelial mononuclear cells (IEMC) or lamina propria mononuclear cells (LPMC) were isolated from tissue samples as previously described (43, 44) and stored in liquid nitrogen until use. Human tonsillar tissue samples were acquired from pediatric patients from Colorado Children's Hospital. Tonsillar mononuclear cells (TMCs) were isolated as previously described (45). All patients undergoing surgery signed a release to allow unrestricted use of discarded tissue and protected patient information was de-identified to the laboratory investigators. This research was reviewed by the Colorado Multiple Institutional Review Board (COMIRB) at the University of Colorado Anschutz Medical Campus and was granted exempt research status.

Preparation of Bacterial Stocks

Growth of anaerobic bacteria was performed using a BD GasPak EZ Anaerobe Pouch System according to manufacturer's instructions (BD Diagnostics, Franklin Lakes, NJ). *Prevotella stercorea* (DSM No. 18206, Braunschweig, Germany) was grown in liquid chopped meat broth (Hardy Diagnostics, Santa Maria, CA) supplemented with 1% Trace Minerals (ATCC), 1% Vitamin Supplements (ATCC), 0.05% Tween80, 29.7 mM acetic acid, 8.1 mM propionic acid and 4.4 mM butyric acid (Sigma-Aldrich) under anaerobic conditions at 37°C for 5–7 days. *Ruminococcus bromii* (ATCC# 27255) was grown in liquid chopped meat broth (Hardy Diagnostics) under anaerobic conditions at 37°C for 1–2 days. The long term stock of *Bifidobacterium longum* subsp *infantis* (ATCC 15697) was grown in liquid chopped meat broth (Hardy Diagnostics) under anaerobic conditions at 37°C for 2–3 days and the working stock was grown on Brucella plates (Teknova, Hollister, CA) under anaerobic conditions at 37°C for 2–3 days. *Acinetobacter junii* (ATCC 17908) was grown using Nutrient Agar plates (Edge Biologicals, Memphis, TN) under aerobic conditions at 26°C for 1–2 days. *Salmonella typhimurium* (ATCC 35986) was grown on LB agar plates (Sigma-Aldrich) under aerobic conditions at 37°C for 1–2 days. Long term stocks of all bacteria were prepared using 10% glycerol and single-use working stocks were prepared using DPBS. All stocks were stored at −80°C and bacterial cell counts were determined using the BD Cell Viability Kit (BD Bioscience).

In vitro Stimulation of LPMCs With Whole Bacteria

For the *in vitro* stimulations, human colonic LPMCs were thawed as previously described (43, 44) and cultured in RPMI with 10% human AB serum (Gemini Bioproducts, West Sacramento, CA), 1% Penicillin/Streptomycin/Glutamine (Life Technologies, Grand Island, NY), and 500 μg/ml Zosyn (Piperacillin and Tazobactam, Wyeth, Madison, NY) at a concentration of 1.0×10^6 million cells per mL in a 48 well plate. LPMCs were exposed to a panel of Gram-positive and Gram- negative bacteria detailed in **Supplemental Table 1** including mucosa-associated colonic bacteria previously shown to be increased or decreased in relative abundance during HIV-1 infection (46, 47): Gram-negative *Prevotella stercorea* and *Acinetobacter junii* (increased) and Gram-positive *Ruminococcus bromii* (decreased), as well as the Gram-positive probiotic *Bifidobacterium infantis* and the Gram-negative pathogen *Salmonella typhimurium*. Broad spectrum antibiotics including Penicillin, Streptomycin, Piperacillin, and Tazobactam were present throughout the time in culture to prevent bacterial overgrowth.

For assays examining ILC3 responses, whole bacteria were added to cell cultures at a ratio of 2.5 bacteria to 1 LPMC and incubated for 16 h at 37°C + 5% CO_2, followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 h. Cells were then collected for flow cytometry as described below.

For blocking experiments: human colonic LPMCs were first exposed to blocking antibodies targeting IL-23 p19, IL-1β (R & D Systems, Minneapolis, MN) or IL-7 (Biolegend) at 5 ug/mL or NKp44 (Biolegend) at 10 ug/mL for 30 min followed by incubation with *A. junii* as described above. Cells were then collected for flow cytometry as described below. The concentration of blocking antibodies was optimized for use by measurement of IL-22 + ILC3s or IL-22 + Lineage negative cells (for IL-7) in response to recombinant cytokine stimulation (50 ng/mL of IL-23, IL-1β, or IL-7) or bead ligation (for NKp44) with a dose curve of blocking antibody treatment (**Supplemental Figure 1**). The addition of recombinant IL-7 prevented the identification of ILC3s (defined as CD127+ which is IL-7Rα) and instead gating for IL-22 was determined on lineage- cells. Antibodies and controls used to block are listed in **Supplemental Table 2**.

For depletion experiments: CD11c+ cells (mDC) or CD3+ (T cells) were depleted from LPMCs using the EasySep PE Positive Selection Kit according to manufacturer's instructions (StemCell Technologies, Vancouver, Canada) and the antibody PE-CD11c (Biolegend, San Diego, CA) or PE-CD3 (Tonbo, San Diego, CA) followed by incubation with *A. junii* as described above. Greater than 90.25% of CD11c+ mDCs were depleted from total LPMCs. Greater than 91.92% of CD3+ T cells were depleted from total LPMCs. Cells were then collected for flow cytometry as described below.

For the measurement of secreted cytokines, LPMCS were plated at a concentration of 2.0×10^6 million cells per mL in a 96 well plate and exposed to whole bacteria *R. bromii* or *A. junii* at a ratio of 2.5 bacteria to 1 LPMC and incubated for 24 h at 37°C + 5% CO_2. Supernatant was collected and saved at −20°C until use. IL-23, IL-1β, and IL-7 were measured in the supernatant using the U-PLEX Assay according to manufacturer's instructions and quantified on the QuickPlex SQ 120 Instrument (Mesoscale Discovery, Rockville, MD).

For assays examining antigen presenting cell responses (mDC, B cell, and Macrophages), whole bacteria were added to cell cultures at 2.5 bacteria to 1 LPMC and incubated for 4 h at 37°C + 5% CO_2, followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 16 h. Cells were then collected for flow cytometry as described below.

In vitro Stimulation of LPMCs With Bacterial Cell Surface Components

Human colonic LPMCs were exposed to either 1 μg/mL of the TLR2 ligand, lipoteichoic acid (LTA) from *B. subtilis* (InvivoGen,

San Diego, CA), or the TLR4 ligand, lipopolysaccharide (LPS) from *E. coli* (InvivoGen) for 16 h at 37°C + 5% CO_2, followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 h. Cells were then collected for flow cytometry as described below.

In vitro Stimulation of LPMCs With Recombinant Cytokines or NKp44 Activation

Human colonic LPMCs were exposed to 50 ng/mL IL-23 or IL-1β (R & D Systems), or the combination of both, or IL-2 or IL-7 (Tonbo) for 16 h at 37°C + 5% CO_2 followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 h. For NKp44 activation experiments; 20 ug/mL of anti-NKp44-biotin (clone P44-8, Biolegend) was combined with Anti-Biotin MACSiBead Particles (Miltenyi Biotec) according to manufacturer's instructions. The beads were then added to LPMCs at a ratio of 5 beads to 1 LPMC for 16 h at 37°C + 5% CO_2 followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 h. Cells were then collected for flow cytometry as described below.

In vitro Stimulation of Tonsil Mononuclear Cells

Human tonsil mononuclear cells (TMCs) were cultured in RPMI with 10% human AB serum (Gemini Bioproducts), 1% penicillin/streptomycin/glutamine (Life Technologies), and 500 μg/ml Zosyn (Wyeth) at a concentration of 1.0×10^6 million cells per mL in a 48 well plate. TMCs were exposed to whole bacteria added to cell cultures at a ratio of 2.5 bacteria to 1 LPMC, or to the combination of 50 ng/mL IL-23 and IL-1β, or NKp44 activation beads at a ratio of 5 beads to 1 TMC and incubated for 16 h at 37°C + 5% CO_2, followed by the addition of Golgi Plug Transport Inhibitor (BD Bioscience) for 4 h. Cells were then collected for flow cytometry as described below.

Flow Cytometry Protocol for Surface and Intracellular Staining

Using flow cytometry, viable CD45+ single cell lymphocytes were identified followed by identification of ILC3s as follows: Lineage-CD127+CD117+. The lineage negative cocktail comprised antibodies targeting CD3, CD20, CD13, CD123, CD303, CD34, FCεR1α, CD11c, and CRTH2. All data were acquired on an LSRII flow cytometer (BD Biosciences). Routine quality control using the Cytometer Setup and Tracking feature within the BD FACSDiva software version 6.1.2 (BD Biosciences) was performed daily. All antibodies and clones used for staining are listed in **Supplemental Table 3**.

For *ex vivo* phenotyping of ILC3s: IEMCs or LPMCs were thawed and surface stained to identify ILC subsets for expression of NKp44, CD56, CCR6, TLR2, TLR4, and TLR5 and intranuclear stained for the transcription factors RORγt, AHR, T-bet and EOMES using the Foxp3/Transcription Factor buffer set according to manufacturer's instructions (Thermo Fisher Scientific, Frederick, MD).

For *in vitro* culture examination of ILC3s; LPMCs were collected after stimulations described above and surface stained to identify ILC3s and subsets (NKp44) followed by intracellular staining for cytokines IL-22, IFNγ, and IL-17 using Fix and Perm Cell fixation and permeabilization buffer set according to manufacturer's instructions (Thermo Fisher Scientific, Frederick, MD).

For *in vitro* culture examination of antigen presenting cells; cells were collected after stimulation and surface stained to identify mDCs (CD45+ Viable Myeloid CD3- CD19- HLA-DR+ CD11c+), B cells (CD45+ Viable Lymphocyte CD3-CD19+) or Macrophages (CD45+ Viable Myeloid CD3- CD19-HLA-DR+ CD11c-) [as defined by Smith et al. and Bain and Mowat (48–50)] followed by intracellular staining for cytokines IL-12/IL-23 p40, IL-23p19, and IL-1β using Fix and Perm Cell fixation and permeabilization reagents according to manufacturer's instructions (Thermo Fisher Scientific). Only cells that expressed both subunits of the cytokine IL-23 (IL-12/IL-23p40 and IL-23p19) were considered to be IL-23+ cells.

In vitro Stimulation of Purified ILC3s

ILC3s were isolated from colonic LPMCs and on average purified to 91.79% purity (**Supplemental Figure 2**) by sorting using the MoFlo Astrios EQ (Beckman Coulter, Indianapolis, IN). ILC3s were sorted from Viable CD45+ single cell lymphocytes that were Lineage-CD127+CD117+ as described above. Purified ILC3s were then exposed to either 50 ng/mL IL-23 and IL-1β (R & D Systems) or whole bacteria at a ratio of 1 bacteria to 1 ILC3 and incubated for 24 h at 37°C + 5% CO_2. Supernatant was collected and saved at −20°C until use. Secreted IL-22 was measured in the supernatant using the IL-22 U-PLEX Assay according to manufacturer's instructions and quantified on the QuickPlex SQ 120 Instrument (Mesoscale Discovery, Rockville, MD).

Data Analysis

Each patient who provided a tissue specimen for research is considered a single sample for data analysis, and figure legends indicate how many samples were examined for each assay using the following terminology: N = number of patients samples. All flow cytometer data analysis was done using FlowJo v10.0. All statistical analysis and graphing were performed using GraphPad Prism v6.00 for Windows (GraphPad Software, La Jolla California). Paired *t*-test was used to determine statistical differences between conditions as indicated in figure legend. Data sets without a minimum number of 25 ILC3 events captured using flow cytometry were excluded from analysis.

RESULTS

ILC3s Are Phenotypically Similar in the Intraepithelial and Lamina Propria Layers of the Human Colon

Human ILC1 subsets were previously shown to be phenotypically different between the colonic layers (22). We therefore sought to determine if ILC3s that reside in the intraepithelial layer (IE) of the human colon (closer to the intestinal lumen where gut bacteria reside) are phenotypically different to ILC3s that

reside within the lamina propria layer (LP). The frequency and phenotype of ILC3 in these locations were determined with ILC3s in colonic human tissue identified as CD45+ viable Lineage- CD127+ CD117+ (**Figure 1A**). ILC3s were more frequent as a fraction of CD45+ cells in the LP (1.16% ± 0.27) compared to the IE layer (0.40% ± 0.09) (**Figure 1B**).

NKp44, CD56, and CCR6, have previously been utilized to identify subsets of ILC3s (17, 24, 29, 41) therefore expression of these markers was next evaluated on colonic ILC3s. The majority of ILC3s expressed NKp44 or CCR6 in both layers (NKp44: IE: 79.13% ± 5.25, LP: 74.21% ± 5.04; CCR6: IE: 66.68% ± 8.40, LP: 68.61% ± 3.27) (**Figures 1C,D**). On average, less than half of LP and IE ILC3s expressed CD56 (LP: 45.08% ± 6.68; IE: 33.58% ± 5.04) (**Figures 1C,D**).

As expected (51), the majority of ILC3s expressed the master transcription factor RORγt (IE: 60.60% ± 12.07, LP: 61.42% ± 10.10) and frequencies of RORγt + ILC3s as a percent of viable CD45 + lymphocytes were not significantly different between the IE and LP layers (**Figure 1E** and **Supplemental Figure 3**). Expression of AHR in ILC3s was lower than that of RORγt (**Figure 1E** and **Supplemental Figure 3**) but similar between tissue layers (LP: 18.23% ± 4.24, IE: 9.35% ± 1.77). Of the AHR-expressing ILC3s, the majority also co-expressed RORγt (LP: 76.77% ± 8.84, IE: 82.44% ± 9.74). Less than 1% of ILC3s expressed T-bet in both layers of the colon (LP: 0.15% ± 0.06, IE: 0.67% ± 0.49) (**Figure 1E** and **Supplemental Figure 3**). Low frequencies of EOMES expressing ILC3s were also quantified in both layers (LP: 1.56% ± 0.62, IE: 3.16% ± 0.91). Overall, of the markers examined, ILC3s were phenotypically similar between the intraepithelial and lamina propria layer in the normal human colon.

Enteric Bacteria Stimulate Production of IL-22 but Not IFNγ From ILC3s When Exposed to Total LPMCs

Mimicking the state where the epithelial barrier is damaged and LP immune cells are exposed to bacteria from the colonic lumen, we utilized an *in vitro* model of lamina propria mononuclear cells (LPMCs) to investigate the ILC3 response to whole enteric bacteria. LPMCs were exposed to a panel of whole bacteria representing Gram-positive and Gram-negative commensal bacteria reported to be altered in various diseases associated with epithelial barrier damage (**Supplemental Table 1**). This panel included commensal Gram-positive *Ruminococcus bromii* (Rb) which is decreased in relative abundance in colonic mucosa of people living with HIV (PLWH) and Gram-negative *Acinetobacter junii* (Aj) and *Prevotella stercorea* (Ps), which were increased in relative abundance (46, 47). The enteric gram-negative pathogen *Salmonella typhimurium* (St) which may contribute to the onset of IBD symptoms (52) and to which PLWH are at an increased risk of acquiring *S. typhiumurium*-bacteremia (53, 54) as well as the probiotic

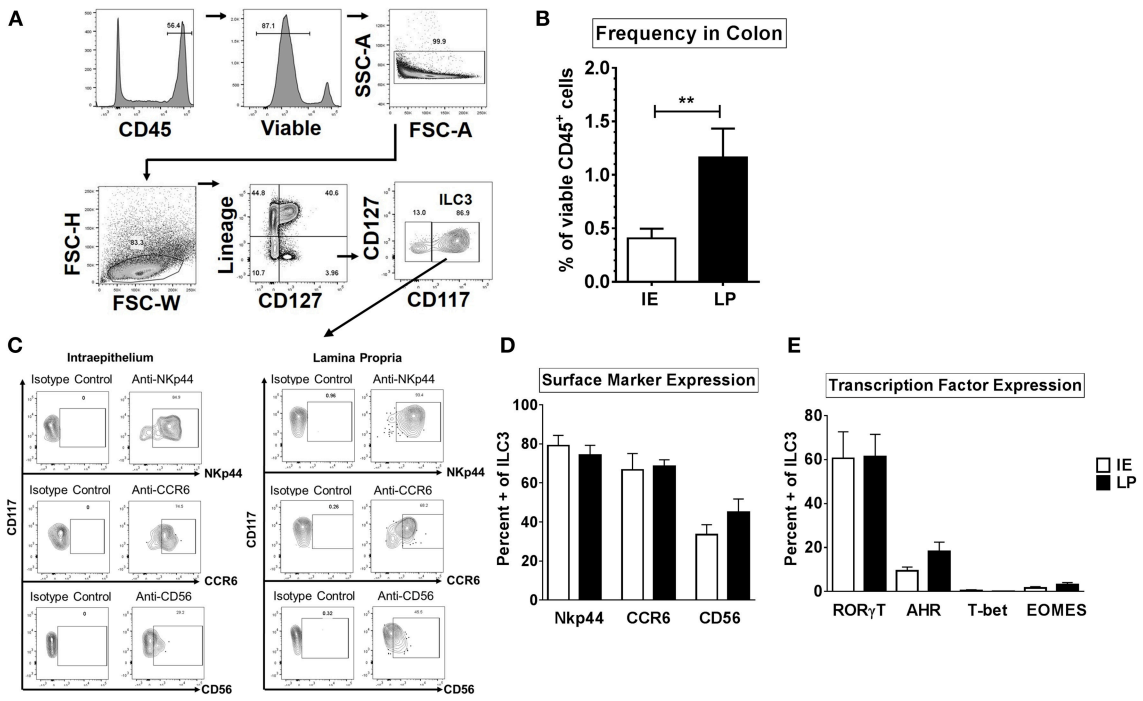

FIGURE 1 | ILC3s are phenotypically similar between the intraepithelial and lamina propria layers of the human colon. **(A)** Representative flow cytometry gating strategy to identify ILC3s in human colonic tissue. **(B)** Frequencies of ILC3s in the colon in the intraepithelial (IE) and lamina propria (LP) layer as a percent of viable CD45+ cells. IE: $N = 6$, LP: $N = 6$. **(C)** Representative flow plots gated on ILC3s for the expression of surface markers NKp44, CD56, or CCR6. **(D)** Percentages of ILC3s expressing the surface markers NKp44, CD56, or CCR6 *ex vivo*. IE: $N = 6$, LP: $N = 6$. **(E)** Percentages of ILC3s expressing the transcription factors RORγt, AHR, T-bet, or EOMES *ex vivo*. IE: $N = 7$, LP: $N = 7$. Bars are mean + S.E.M. Statistical analysis performed was paired t test. $^{**}p < 0.01$.

Gram-positive *Bifidobacterium longum* sp *infantis* (Bi), which is used therapeutically to reestablish a protective microbiome (55, 56) were also included. Bacterial experiments were performed in the presence of broad spectrum antibiotics in order to inhibit bacterial overgrowth. All bacteria tested, irrespective of gram-staining and commensal or pathogenic nature, induced production of IL-22 in a fraction of ILC3s relative to the no stimulation condition (Bi: 30.0% ± 7.13, Rb: 18.76% ± 6.43, Aj: 21.61% ± 3.02, Ps: 28.58% ± 3.54, St: 32.21% ± 4.65) (**Figures 2A–C**). Of the bacteria tested, none induced a significant increase in IL-17A+ ILC3s above the no stimuli control (data not shown: less than 0.80% IL-17+ ILC3).

To determine if bacterial surface components were important in driving the ILC3 IL-22 response to whole bacteria, LPMCs were exposed to Gram-positive and Gram-negative bacterial cell surface components (LTA and LPS, respectively). The percentage of IL-22+ ILC3s was similarly increased in response to both bacterial surface antigens (LTA: 17.98% ± 3.09, LPS: 15.18% ± 2.44) (**Figure 2D**).

ILC3s that produced IFNγ were associated with inflammatory responses in murine infection with bacteria (41) or bacteria-driven murine colitis (42). We next measured the frequencies of IFNγ-producing LP ILC3s in response to whole bacteria. Low frequencies of IFNγ expressing ILC3s were observed in response to exposure of LPMC to each bacteria (Bi: 0.63% ± 0.50, Rb: 0.06% ± 0.04, Aj: 1.51% ± 0.81, Ps: 0.50% ± 0.35, St: 1.77% ± 0.68), however no significant increase above the no stimuli control was detected (**Supplemental Figure 4**). Furthermore, IFNγ+ ILC3s were not induced by exposure to LTA and LPS (data not shown). Overall, these data indicate that the bacteria tested did not drive the production of IFNγ by ILC3s in our *in vitro* culture system.

Purified ILC3s Do Not Produce IL-22 in Response to Enteric Bacteria

To assess if ILC3s produced IL-22 in direct response to bacteria, ILC3s were purified from the LP (**Supplemental Figure 2**) and exposed *in vitro* to either Gram-positive (*R. bromii)*, or Gram-negative (*A. junii)* bacteria or to recombinant IL-23 and IL-1β. After 24 h, the levels of secreted IL-22 were measured. Exposure of isolated ILC3s to IL-23 and IL-1β induced IL-22 production (86.74 pg/mL ± 29.60), whereas purified ILC3s did not produce IL-22 in response to either bacterial species compared to no stimulation control (C: 0.92 pg/mL ± 0.19, *Rb*: 1.00 pg/mL ±

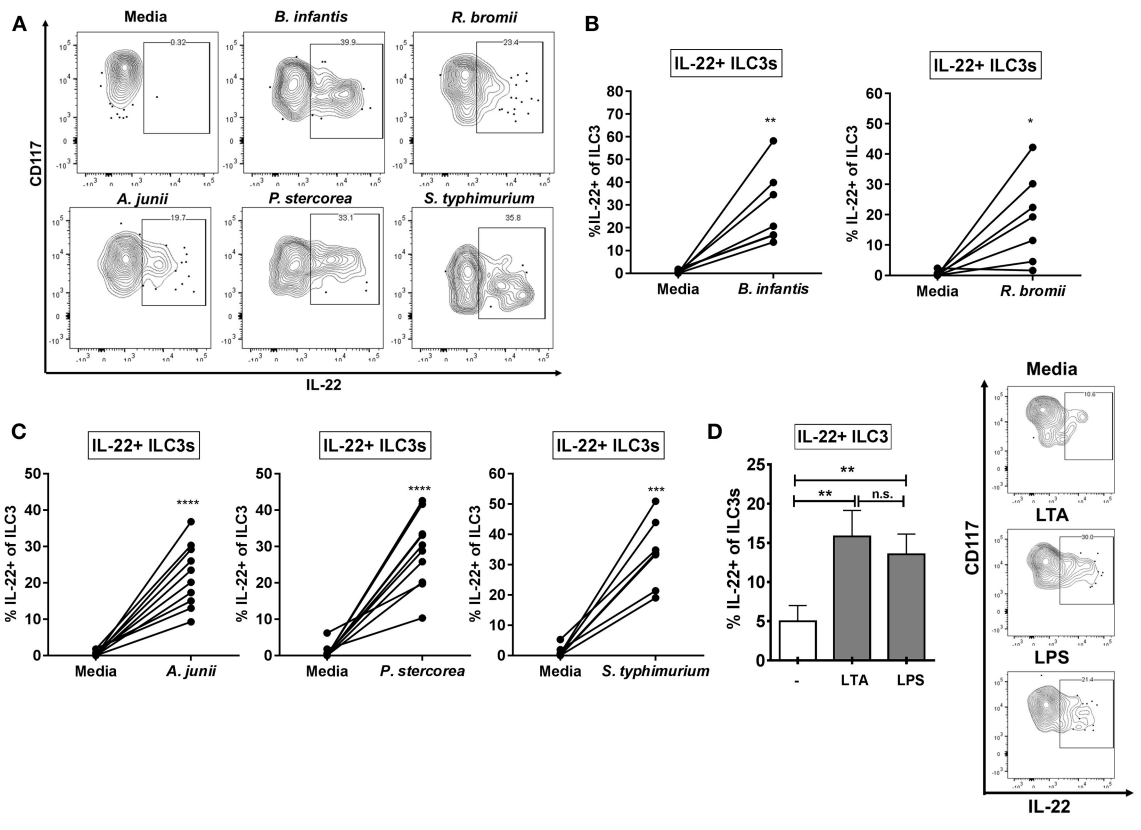

FIGURE 2 | ILC3s produce IL-22 in response to Gram-positive and Gram-negative enteric bacteria. **(A)** Representative flow cytometry demonstrating cytokine staining for IL-22 gated on ILC3s after LPMC exposure to enteric bacteria *in vitro*. **(B,C)** Percentages of IL-22+ ILC3s after LPMC exposure to enteric bacteria or no bacterial control. $N = 6–10$. **(D)** Percentages of IL-22+ ILC3s after LPMC exposure to lipoteichoic acid (LTA) or lipopolysaccharide (LPS) or no stimulation control. Representative flow cytometry demonstrating cytokine staining for IL-22 gated on ILC3s after LPMC exposure to bacterial cell surface components. $N = 6$. Bars are mean + S.E.M. Statistical analysis performed was paired t test as indicated. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. n.s., not significant.

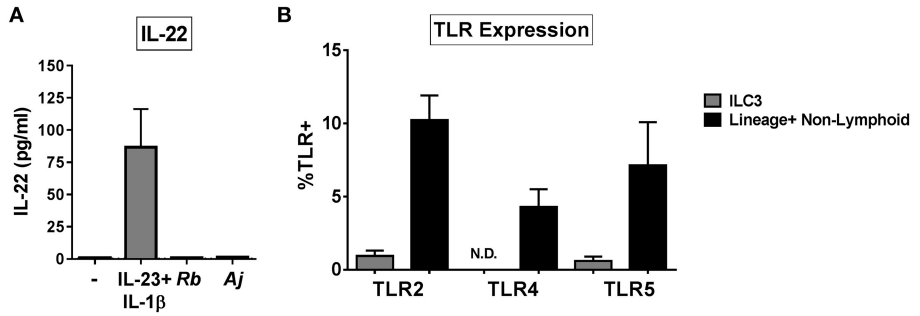

FIGURE 3 | ILC3s do not respond directly to bacteria by producing IL-22. **(A)** Quantification of IL-22 (pg/mL) in the supernatant of purified ILC3s exposed to recombinant IL-23+ IL-1β (50 ng/mL) or *R. bromii* (Rb) or *A. junii* (Aj) *in vitro* at a ratio of 1 ILC3 to 1 bacterium or no stimulation control. $N = 3$. **(B)** Percentages of LPMCs stained *ex vivo* for TLR2, TLR4, or TLR5 expression gated on ILC3s or Lineage positive non-lymphoid cells. $N = 6$. N.D., not detected. Bars are mean + S.E.M.

0.24, *Aj*: 1.42 pg/mL ± 0.38) (**Figure 3A**). In keeping with the lack of response to direct bacterial stimulation, cell surface expression of bacterial Pattern Recognition Receptors (PRRs) TLR2, TLR4, and TLR5 on colonic ILC3s *ex vivo* were low or not detected (TLR2: 0.94% ± 0.36, TLR4: 0%, TLR5: 0.60% ± 0.29) compared to Lineage+ non-lymphoid cells (TLR2: 10.23% ± 1.68, TLR4: 4.28% ± 1.22, TLR5: 7.12% ± 2.96) (**Figure 3B**). These data suggest that human colonic ILC3s do not produce IL-22 in direct response to the enteric bacteria tested, but require additional stimulation from accessory cells in LPMCs to induce IL-22.

IL-23 and IL-1β Contribute to the ILC3 IL-22 Response to Gut Bacteria

Given that recombinant IL-23 and IL-1β stimulated production of IL-22 from purified ILC3s, the levels of secreted IL-23 and IL-1β following exposure of LPMCs to commensal Gram-positive (*R. bromii*) or to Gram-negative (*A. junii*) bacteria were next evaluated. *R. bromii* and *A. junii* significantly induced the secretion of IL-23 (*Rb*: 21.57 pg/mL ± 3.54, *Aj*: 234.4 pg/mL ± 57.23) from LPMCs (**Figure 4A**) above background. Both bacteria also induced significant production of IL-1β (*Rb*: 34.86 pg/mL ± 7.62, *Aj*: 247.6 pg/mL ± 74.49) from LPMCs (**Figure 4B**). Although both commensal bacteria stimulated significant production of these cytokines, *A. junii* induced 10.8 fold more IL-23 and 7.1 fold more IL-1β than *R. bromii* (**Figures 4A,B**).

To evaluate the relative contribution of IL-23 and IL-1β independently and in combination to ILC3 induction of IL-22, LPMC were exposed to recombinant IL-23 and/or IL-1β. Both recombinant cytokines individually significantly increased the percentage of IL-22+ ILC3s (C: 1.28% ± 1.22, IL-23: 17.59% ± 4.20, IL-1β: 17.41% ± 7.06) compared to the unstimulated control (**Figure 4C**). Although the combination of recombinant IL-23 and IL-1β significantly increased the percentage of IL-22+ ILC3s (11.07% ± 3.66) compared to unstimulated control, the combination response was not synergistic (**Figure 4C**).

To determine if production of IL-23 and/or IL-1β from LPMCs drives IL-22 induction in ILC3s in response to bacteria, blocking antibodies directed against IL-23, IL-1β, or both cytokines were added to LPMC cultures before exposure to the commensal *A. junii*. The frequency of IL-22 producing ILC3s in response to *A. junii* was significantly reduced by an average of 38.0% when blocking IL-23 and 38.5% when blocking IL-1β compared to stimulation with *A. junii* and the control IgG antibody (**Figure 4D**). The combination of blocking both IL-23 and IL-1β also significantly reduced the frequencies of IL-22 + ILC3s in response to *A. junii* by 40.1% (anti-IL-23: 14.46% ± 3.30, anti-IL-1β: 16.13% ± 4.71, anti-IL-23+ anti-IL1β: 15.28% ± 4.04), but did not lead to a synergistic reduction (**Figure 4D**).

Given the lack of complete abrogation of the ILC3 IL-22 response to bacteria when blocking IL-23 and IL-1β, the role of other cytokines (IL-7, IL-2) reported to promote ILC3 phenotype (57) were next investigated. Although secreted IL-7 was detected in unstimulated LPMC cultures, levels of IL-7 did not increase after LPMCs were exposed to commensal Gram-positive bacteria (*R. bromii*) or to Gram-negative bacteria (*A. junii)* (**Supplemental Figure 5A**). In keeping with this lack of production in presence of bacteria, blocking IL-7 alone did not reduce the frequency of IL-22+ ILC3s (**Supplemental Figure 5B**). Blocking IL-7 in combination with blocking IL-23 and IL-1β did not further reduce the frequency of IL-22+ ILC3s compared to blocking IL-23 and IL-1β (**Supplemental Figure 5B**). T cells are a major producer of IL-2 among other cytokines, however, depletion of CD3+ T cells from LPMCs did not alter the percentage of IL-22+ ILC3s generated in response to *A. junii* (**Supplemental Figure 5C**) and the addition of recombinant IL-2 to LPMCs did not significantly increase the percentage of IL-22+ ILC3s (data not shown). Altogether these data indicate that IL-2 and IL-7 do not have a major role in LPMCs in promoting IL-22 production by ILC3s in response to enteric bacteria in this culture system.

Myeloid Dendritic Cells Contribute to IL-22 Production of ILC3s in Response to Bacteria by Production of IL-23 and IL-1β

To identify potential cellular sources of IL-23 and IL-1β in response to *A. junii* and *R. bromii*, production of IL-23 and IL-1β by mDCs (CD3- CD19- HLA-DR+ CD11c+), macrophages (defined as CD3- CD19- HLA-DR+ CD11c-) (48–50) and

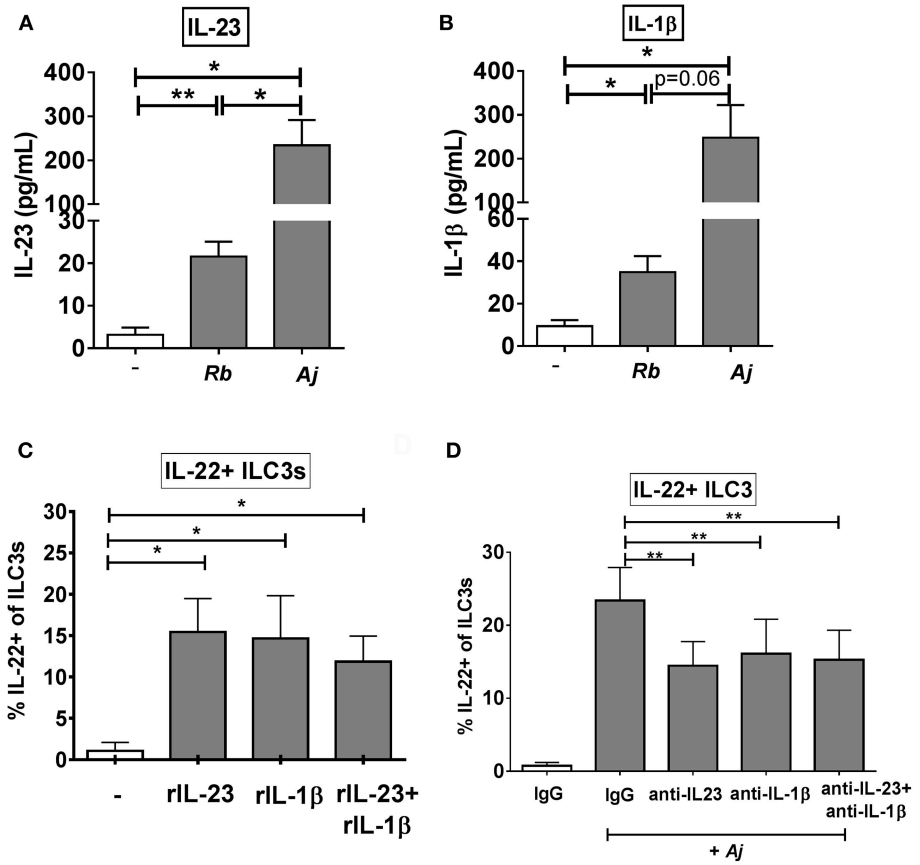

FIGURE 4 | IL-23 and IL-1β modulate the ILC3 IL-22 response to enteric bacteria. **(A)** Quantification of IL-23 (pg/mL) or **(B)** IL-1β (pg/mL) in the supernatant of LPMCs exposed to *R. bromii* (Rb) or *A. junii* (Aj) or no bacteria control. $N = 5$. **(C)** Percentages of IL-22+ ILC3s after LPMC exposure to recombinant IL-23, IL-1β, or IL-23+ IL-1β (50 ng/mL) or no stimulation control. $N = 5$. **(D)** Percentages of IL-22+ ILC3s after LPMC exposure to no bacteria control or *A. junii* (Aj) in the presence of 5 ug/mL blocking antibodies targeting IL-23 and/or IL-1β or the antibody isotype control IgG. $N = 8$. Bars are mean + S.E.M. Statistical analysis performed was paired *t*-test as indicated. $^{*}p < 0.05$, $^{**}p < 0.01$.

CD19+ B cells in LPMCs were determined by intracellular cytokine staining and flow cytometry following exposure to bacteria (**Figure 5A**). *A. junii*, but not *R. bromii* significantly increased the percentage of IL-23+ mDCs (**Figure 5B** and **Supplemental Figure 6A**). Although low frequencies of IL-23+ macrophages following *A. junii* stimulation were detected, this was not statistically different compared to no bacteria stimulation (**Supplemental Figures 7A,B**). Both *R. bromii* and *A. junii* significantly increased the percentages of IL-1β+ mDCs compared to no bacteria stimulation (**Figure 5C** and **Supplemental Figure 6B**). Exposure of LPMC to *R. bromii* or *A. junii* did not significantly induce IL-1β+ macrophages above no stimuli although IL-1β+ macrophages were detected (**Supplemental Figures 7D,E**). No significant increases in IL-23+ or IL-1+ B cells were observed in response to either bacteria stimuli (**Supplemental Figure 7**). Taken together, these observations suggested that mDCs in LPMCs were a major producer of the canonical cytokines known to drive IL-22 production by ILC3s. To verify the contribution of these cells to bacteria-induced IL-22 production by ILC3s, CD11c+ mDCs were depleted from LPMCs followed by exposure to *A. junii* and frequencies of IL-22+ ILC3s were determined. Depletion of mDCs significantly reduced the frequency of IL-22+ ILC3s in response to *A. junii* by 40.9% compared to total LPMCs (**Figure 5D**).

NKp44 Ligation Contributes to the Gut ILC3 IL-22 Response to Enteric Bacteria

Recent work suggests a functional role for the natural cytotoxicity receptor NKp44 in driving cytokine responses of ILC3s (3), thus we sought to determine if NKp44 is critical to the IL-22 response of human colonic ILC3s to enteric bacteria. As noted previously, the majority of colon ILC3s expressed Nkp44 directly *ex vivo* (**Figure 1C**). Following exposure to enteric bacteria *in vitro*, a small increase in the percentage of ILC3s expressing NKp44 was noted (**Figure 6A**). Examination of the IL-22+ ILC3s after stimulation with bacteria revealed that the majority of IL-22-producing cells were also NKp44+ (**Figure 6B**). Direct ligation of NKp44 by crosslinking beads led to a significant increase in the percentage of IL-22+ ILC3s compared to the no bead control (**Figure 6C**). To evaluate the contribution of NKp44 to the ILC3 IL-22 response to

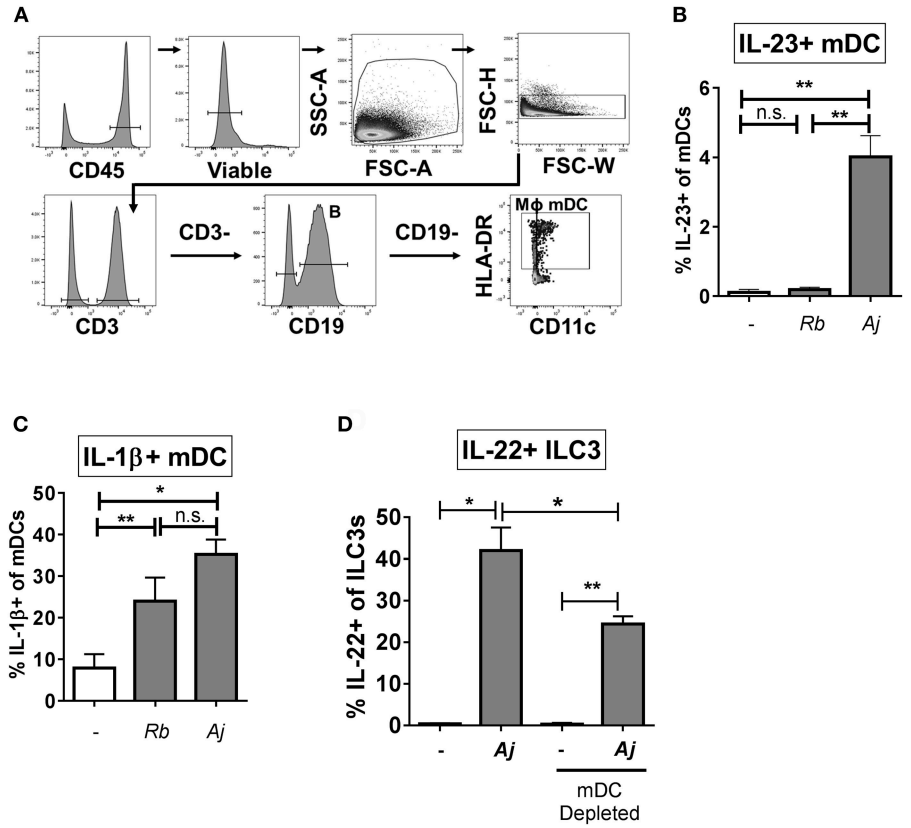

FIGURE 5 | Myeloid dendritic cells producing IL-23 and IL-1β contribute to the ILC3 IL-22 response to bacteria. **(A)** Representative flow cytometry demonstrating gating strategy to identify antigen presenting cells (mDC; myeloid dendritic cells, B: B cells, MΦ; macrophages) after LPMC exposure to bacteria *in vitro*. **(B)** Percentages of IL-23+ or **(C)** IL-1β+ mDCs after LPMC exposure to *R. bromii* (Rb) or *A. junii* (Aj) or no bacteria control. $N = 4$. **(D)** Percentages of IL-22+ ILC3s after LPMC exposure to *A. junii* (Aj) or no bacteria control with CD11c mDC depletion and no depletion control. $N = 3$. Bars are mean + S.E.M. Statistical analysis performed was paired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$. n.s., not significant.

bacteria, NKp44 was blocked in the presence of commensal *A. junii.* Compared to the IgG control, blocking NKp44 during *A. junii* stimulation resulted in a significant (IgG control: 31.08% ± 4.69, anti-NKp44: 25.52% ± 3.63) but incomplete reduction of IL-22+ ILC3s (**Figure 6D**). To determine if NKp44 promotion of IL-22 in ILC3s in response to bacteria was complementary or redundant with IL-23 and IL-1β, NKp44 was blocked in combination with blocking of IL-23 and IL-1β. Blocking Nkp44, IL-23, and IL-1β in combination did not further reduce the percentage of IL-22+ ILC3s generated in response to *A. junii* compared to only blocking IL-23 and IL-1β (**Figure 6D**).

Bacteria-Induced IL-22 Production by ILC3s Is Enhanced in LPMCs Relative to TMCs

To evaluate if the ILC3 IL-22 response to bacteria is unique to the colonic environment, tonsil mononuclear cells (TMCs) were stimulated with commensal bacteria *R.bromii* and *A.junii* as well as the combination of recombinant IL-23 + IL-1β or NKp44 activating beads and induction of IL-22 by ILC3s determined by flow cytometry. The combination of IL-23+IL-1β significantly induced IL-22 production by tonsillar ILC3s as expected (**Figure 7A**). A small but significant increase in the percentage of IL-22+ ILC3s was observed following exposure to both commensal bacteria tested, whereas a similarly small increase in IL-22+ ILC3s following ligation of NKp44 was not statistically significant (**Figure 7A**). NKp44 expression on tonsillar ILC3s was characterized *ex vivo* and was not found to differ significantly from NKp44 expression on colon ILC3s (T: 60.17% ± 4.19, L: 74.21% ± 5.05) (**Figure 7B**). Despite the majority of the tonsillar ILC3s expressing the receptor NKp44, stimulation with NKp44 activation beads resulted in significantly lower percentages of IL-22+ ILC3s (T: 1.11% ± 1.11, L: 16.89% ± 0.12) compared to the colon (**Figure 7C**). Furthermore, when stimulated with bacteria, the percentage of tonsillar IL-22+ ILC3s was significantly lower in response to *R. bromii* (T: 1.11% ± 0.37, L: 18.67% ± 5.41) and *A. junii* (T: 0.93% ± 0.30, L: 21.38% ± 2.81) compared to the colon (**Figures 7D,E**). Although there was minimal induction of IL-22+ ILC3s in response to NKp44 ligation in tonsil cultures, the canonical cytokines, IL-23+IL-1β similarly induced ILC3 IL-22 responses (T: 8.15% ± 1.61, L: 10.79% ± 3.11) between the colon and the tonsil (**Figure 7F**).

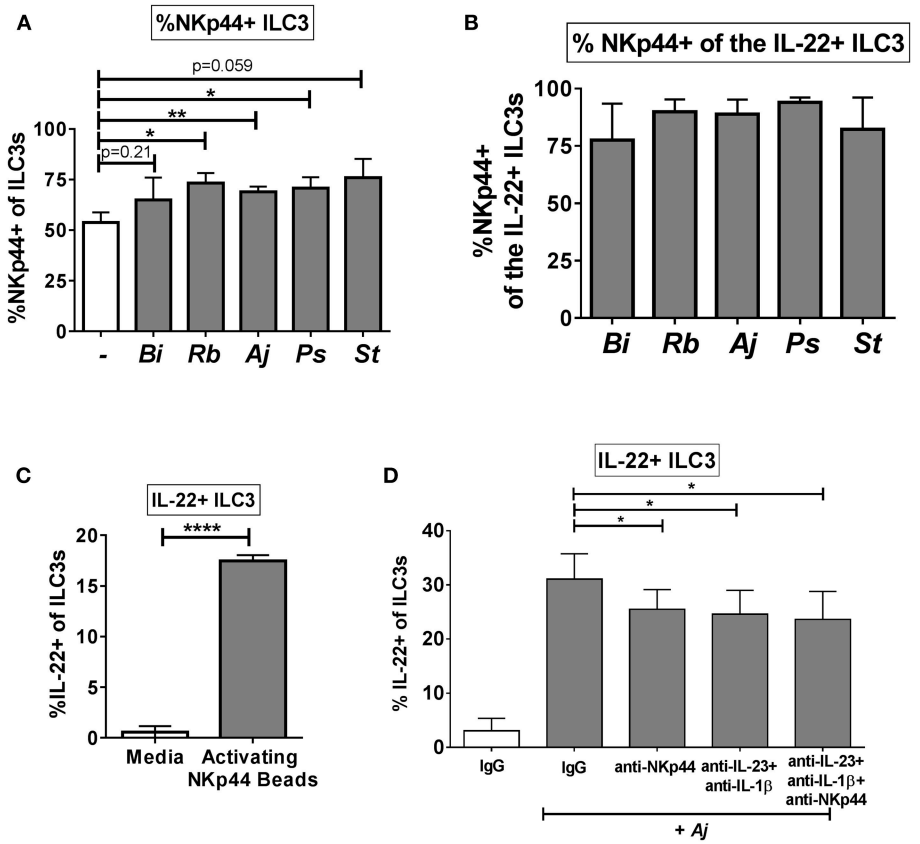

FIGURE 6 | NKp44 contributes to the gut ILC3 IL-22 response to bacteria. **(A)** Percentages of NKp44 + ILC3s after LPMC exposure to enteric bacteria or no bacteria control. $N = 5$. **(B)** Percentages of NKp44+ when gating on IL-22 + ILC3s after LPMC exposure to enteric bacteria. $N = 5$. **(C)** Percentages of IL-22+ ILC3s after LPMC stimulation with NKp44 cross-linking beads. $N = 3$. **(D)** Percentages of IL-22+ ILC3s after LPMC exposure to no bacteria control or *A. junii* (Aj) in the presence of blocking antibodies targeting IL-23 and IL-1β (5 ug/mL) or NKp44 (10 ug/mL) or the antibody isotype controls IgG. $N = 5$. Bars are mean + S.E.M. Statistical analysis performed was paired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

DISCUSSION

Numerous murine studies have highlighted the importance of gut ILC3s in immunity to bacteria, but few studies have directly investigated how human lamina propria ILC3s respond to enteric bacteria, particularly commensal bacteria, and the mechanisms driving these responses. The major findings of the present study demonstrate that: (1) both Gram-positive and Gram-negative commensal and pathogenic bacteria induced similar frequencies of IL-22-producing ILC3s, (2) ILC3 production of IL-22 was not mediated through direct ILC3 recognition of bacteria, but rather mediated indirectly by mDCs, (3) IL-22 production was partly dependent on IL-23 and IL-1β, (4) and ligation of the NKp44 receptor stimulated IL-22 production. Overall, this work expands on the basic biology of human gut ILC3s and provides insight into their contribution to the innate immune response to enteric bacteria.

Similar frequencies of IL-22-producing ILC3s were induced irrespective of bacterial cell surface structure by Gram stain (i.e., Gram-positive or Gram-negative) or characterization as a human commensal or pathogenic bacteria suggesting a commonality between bacteria which drive ILC3 cytokine responses. In the context of gut ILC3 biology, this suggests that different enteric bacteria induce immune responses by LPMCs that are then "sensed" by ILC3s as the same. Using purified ILC3s, we demonstrated that induction of IL-22 was not due to direct recognition of bacteria or bacterial antigens. Presumably, direct recognition of bacteria by purified ILC3s would occur through external expression of TLRs specific to bacterial ligands. In keeping with this concept and a lack of direct induction of IL-22 by bacteria, we detected minimal expression of bacteria-associated TLRs on colonic ILC3s. This contrasts to a previous report demonstrating TLR2 expression by ILC3s in human duodenum (58) suggesting possible tissue site differences in PRR expression. Importantly, production of IL-22 by tonsillar ILC3s in response to TLR2 ligand required co-stimulation with IL-2, IL-15, or IL-23 (59).

We determined that IL-23 and IL-1β were important for bacteria-driven IL-22 production by human gut ILC3s similar to multiple murine studies highlighting IL-23-mediated regulation of IL-22 production in the context of pathogenic *C. rodentium* infection (12, 36) and responses to bacterial flagella (60, 61).

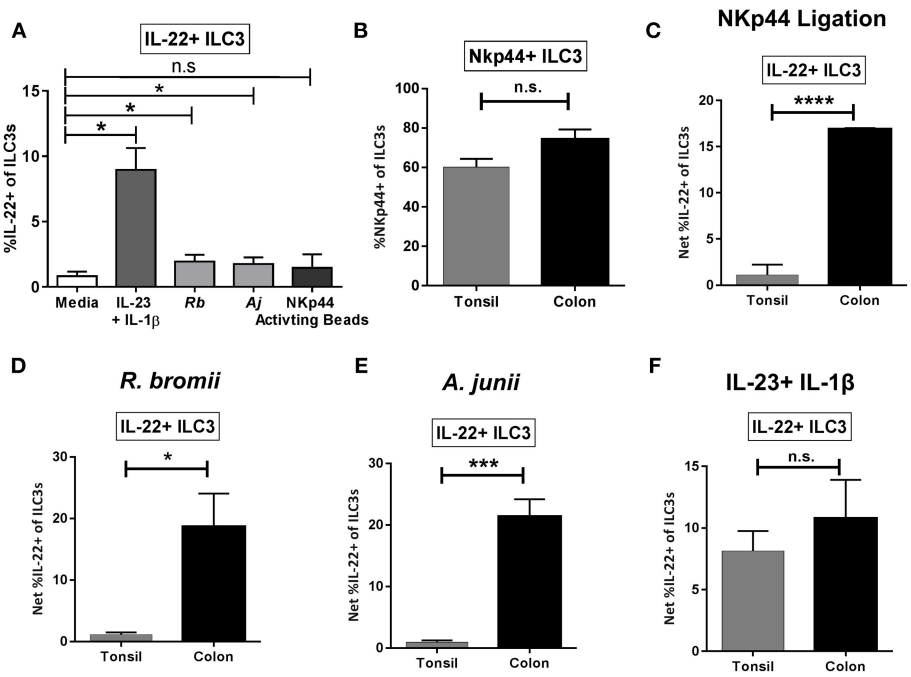

FIGURE 7 | Tonsillar and gut ILC3 IL-22 responses to stimulation. **(A)** Percentages of IL-22+ ILC3s after TMC exposure *in vitro* to *R. bromii*, *A. junii*, NKp44 cross-linking beads, recombinant IL-23+ IL-1β (50 ng/mL) or no stimulation control. $N = 5$. Bars are mean + S.E.M. Statistical analysis performed was paired *t*-test. $^{*}p < 0.05$, n.s., not significant. **(B)** Percentages of ILC3s expressing NKp44 *ex vivo.* TMC: $N = 3$, LPMC: $N = 9$. **(C)** Percentages of IL-22+ ILC3s after TMC or LPMC stimulation *in vitro* with NKp44 cross-linking beads. TMC: $N = 5$, LPMC: $N = 3$. **(D)** Percentages of IL-22+ ILC3s after TMC or LPMC stimulation with *R. bromii.* TMC: $N = 5$, LPMC: $N = 7$. **(E)** Percentages of IL-22+ ILC3s after TMC or LPMC stimulation with *A. junii.* TMC: $N = 5$, LPMC: $N = 11$. **(F)** Percentages of IL-22+ ILC3s after TMC or LPMC exposure to recombinant IL-23 + IL-1β (50 ng/mL). TMC: $N = 5$, LPMC: $N = 6$. Bars are mean + S.E.M. Statistical analysis performed was unpaired *t*-test. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s., not significant.

IL-1β signaling through the IL-1R1 and MyD88 pathway was also shown to be critical for murine ILC3 production of IL-22 (62) and in human secondary lymphoid tissue, continuous IL-1β signaling was required to preserve the ILC capacity to produce IL-22 (63). Interestingly, in our *in vitro* model, frequencies of IL-22-producing ILC3s were not synergistically increased by the combined addition of IL-23 and IL-1β or decreased with antibody-mediated blocking of both cytokines in the context of bacteria exposure, suggesting that IL-23 and IL-1β may stimulate colonic ILC3 production of IL-22 in a redundant manner. Alternatively, ILC3s that express the IL-23R could also be the same subset that express the IL-1R indicating that only a fraction of ILC3s have to capacity to be stimulated in this manner. Taken together, these observations highlight a role for accessory cell mediated production of IL-23 and IL1β in regulating IL-22 from human gut ILC3s in response to enteric bacteria.

The importance of crosstalk between accessory cells such as mDCs and ILCs for induction of IL-22 has been implicated in a number of studies including the observation that a loss of a subset of mDCs correlated with a loss of IL-22-producing ILCs during SIV infection (25). Additionally, mouse intestinal mDC production of IL-23 in response to flagella derived from *Salmonella* was important in the stimulation of IL-22 from ILC3s (60, 61) and a need for physical contact between DCs and ILCs was necessary for the protective IL-22 response to infection with *C. rodentium* (64). We provide evidence that depletion of mDC resulted in a decrease in IL-22 production by ILC3s; however, purified colonic ILC3s responded directly to IL-23 and IL-1β, suggesting that contact dependence between human colonic ILC3s and mDCs may not be a requirement for induction of IL-22. Importantly, despite removing mDCs, complete abrogation of the bacteria-induced IL-22 response in ILC3s was not achieved, highlighting that other accessory cell types may contribute to the regulation of bacteria-specific ILC3 responses in the colon. Indeed, we detected low percentages of IL-23 and IL-1β-expressing macrophages in response to representative commensal bacteria in a subset of donors, suggesting that macrophages have the potential to contribute to the ILC3 IL-22 response to bacteria. A number of studies have implicated monocytes in driving IL-22 production. For example, soluble factors from LPS-activated human monocytes stimulated IL-22 from tonsillar ILC3s (17). In murine studies CX_3CR1^+ phagocytes (including mDCs and macrophages) stimulated a protective IL-22 ILC3 response during *C. rodentium* infection (65, 66) and mice deficient in CX_3CR1^+ phagocytes had impaired IL-22 production by ILC3s leading to increased microbial translocation and bacterial dissemination (65). Thus, exposure of multiple types of human gut antigen presenting cells to bacteria and the subsequent induction of IL-23 and IL-1β likely contributes to the production of IL-22 by ILC3s.

In Crohn's disease patients, loss of NKp44+ ILC3s from the ileum correlated with an increase in pro-inflammatory T cell subsets implying a role for NKp44+ ILC3s in mucosal regulation (67). In our study, we show that the majority of IL-22-producing ILC3s expressed the NKp44 receptor and direct ligation of the NKp44 receptor induced IL-22 from colonic ILC3s. Interestingly, the role of NKp44 in directly driving IL-22 production was unique to the gut and not observed with tonsillar ILC3s in keeping with a previous report on NKp44 ligation of tonsillar ILC3s (3). This is intriguing since we show that there are similar percentages of NKp44-expressing ILC3s *ex vivo,* as well as, similar percentages of IL-22-expressing ILC3s after *in vitro* exposure to exogenous IL-23 and IL-1β in both the tonsil and colon. In the context of bacterial stimulation, blocking NKp44 signaling partially reduced the percentage of IL-22-producing ILC3s demonstrating that NKp44 also plays a contributory role in cytokine stimulation of ILC3s. Ligands for NKp44 have been identified during pathological conditions such as tumor development or immortalized cell lines of cancerous origin, and include an isoform of the protein MLL5, proliferating cell nuclear antigen (PCNA), and platelet derived growth factor (PDGF)-DD (5, 68, 69). Further studies will be needed to determine if these, or yet to be identified ligands, can stimulate IL-22 production by ILC3s in our *in vitro* model. Interestingly, NKp44 has been shown to directly bind to bacteria (70) as well as viral hemagglutinin of influenza (71), thus, investigations will need to be undertaken to determine if enteric bacteria are an additional source of NKp44L. It is possible that other factors not identified here may contribute to the IL-22 response by ILC3s by bacteria. A recent study determined that IL-18 production by mDCs, in conjunction with IL-15, was able to induce IL-22 in tonsillar ILC3s after longer term exposure (14 days) to these cytokines (72). It is therefore possible, that similar combinations of signals could induce IL-22 from colonic ILC3s, although if there is a role for enteric bacteria in initiating this signaling cascade would need to be determined.

Reports have indicated that IFNγ production by ILC3s is possible in the context of murine models of GI bacterial infection (41, 42). Furthermore, *in vitro* human tonsillar ILC3s can be functionally plastic in terms of cytokine production including a switch from IL-22 to IFNγ production dependent on the cytokine milieu (18, 21). In this current study, exposure of LPMC to enteric bacteria did not induce a significant increase in IFNγ+ ILC3s suggesting that while our *in vitro* model has the appropriate microenvironment to drive IL-22 production from ILC3s, additional signals would be required for robust IFNγ production. It is possible that cellular movement as is seen *in vivo* could promote interactions that may be needed for IFNγ induction in response to bacteria, and the *in vitro* model used in this study may not recapitulate those interactions. It is also important to note that in our model system, broad spectrum antibiotics were present to prevent bacterial overgrowth. Thus, functional responses are likely driven by static whole bacteria and/or processed bacterial antigens. Perhaps when bacteria are able to be metabolically active and/or replicate, additional components (such as virulence factors in regards to *S. typhimurium*) would be produced leading to the subsequent production of IFNγ from ILC3s.

To the best of our knowledge, this study is the first to undertake an extensive evaluation of the mechanisms by which *in vitro* exposure to whole enteric commensal and pathogenic bacteria drive human colonic ILC3 cytokine production. Here we demonstrate that IL-22 production was driven indirectly in LPMCs, mediated in part by mDCs, and driven by multiple mechanisms including IL-23, IL-1β, and NKp44 signaling. The complexity of the gut environment was emphasized by the observation that the combination of these processes did not fully account for all of the bacteria-driven IL-22 responses. Remarkably, IL-22 production from ILC3s were induced in response to all bacteria tested, potentially highlighting an evolutionarily conserved response by ILC3s to both commensal and pathogenic bacterial antigens. IL-22 has important gut homeostatic functions and thus the production of IL-22 by ILC3s would be a critical component of the innate response to enteric pathogenic challenge. This function of ILC3s may serve as a means to repair the epithelial barrier during disease states when there is a breach in epithelium integrity to prevent the induction of further inflammation and damage by translocating microbes. Our *in vitro* observations highlight that production of IL-22 by ILC3s in response to commensal bacteria is also likely a significant component of GI tract bacterial immunity.

AUTHOR CONTRIBUTIONS

MC, SD, AC, EB, and CW designed the study and interpreted the work. MC, SD, and CW wrote the manuscript. MC and CP performed experiments. MM and MS provided tissue specimens. JK provided technical assistance and interpretation of the work. All authors contributed to manuscript revision, and read and approved the submitted version.

FUNDING

This work was supported by National Institute of Health Grants R01 AI118983 (EB, CW) and T32 AI007405 (MC).

ACKNOWLEDGMENTS

We are grateful to the Flow Cytometry Shared Resource at the University of Colorado Cancer Center (P30CA046934) for technical assistance with sorting ILC3s. We also acknowledge the help of Sabrina Nesladek, Allison Christians, and other members of the Wilson lab for technical assistance with processing of the tissue specimens.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00649/full#supplementary-material

REFERENCES

1. Sonnenberg GF, Artis D. Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. *Immunity.* (2012) 37:601–10. doi: 10.1016/j.immuni.2012.10.003
2. Eberl G, Colonna M, Di Santo JP, McKenzie AN. Innate lymphoid cells: a new paradigm in immunology. *Science.* (2015) 348:aaa6566. doi: 10.1126/science.aaa6566
3. Glatzer T, Killig M, Meisig J, Ommert I, Luetke-Eversloh M, Babic M, et al. RORgammat(+) innate lymphoid cells acquire a proinflammatory program upon engagement of the activating receptor NKp44. *Immunity.* (2013) 38:1223–35. doi: 10.1016/j.immuni.2013.05.013
4. Killig M, Glatzer T, Romagnani C. Recognition strategies of group 3 innate lymphoid cells. *Front Immunol.* (2014) 5:142. doi: 10.3389/fimmu.2014.00142
5. Barrow AD, Edeling MA, Trifonov V, Luo J, Goyal P, Bohl B, et al. Natural killer cells control tumor growth by sensing a growth factor. *Cell.* (2018) 172:534–48 e519. doi: 10.1016/j.cell.2017.11.037
6. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
7. Sonnenberg GF. Regulation of intestinal health and disease by innate lymphoid cells. *Int Immunol.* (2014) 26:501–7. doi: 10.1093/intimm/dxu052
8. Bostick JW, Zhou L. Innate lymphoid cells in intestinal immunity and inflammation. *Cell Mol Life Sci.* (2016) 73:237–52. doi: 10.1007/s00018-015-2055-3
9. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol.* (2016) 17:765–74. doi: 10.1038/ni.3489
10. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol.* (2011a) 12:383–90. doi: 10.1038/ni.2025
11. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity.* (2004) 21:241–54. doi: 10.1016/j.immuni.2004.07.007
12. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med.* (2008) 14:282–9. doi: 10.1038/nm1720
13. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol.* (2009) 10:83–91. doi: 10.1038/ni.1684
14. Sanos SL, Vonarbourg C, Mortha A, Diefenbach A. Control of epithelial cell function by interleukin-22-producing RORgammat+ innate lymphoid cells. *Immunology.* (2011) 132:453–65. doi: 10.1111/j.1365-2567.2011.03410.x
15. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science.* (2012) 336:1321–5. doi: 10.1126/science.1222551
16. Lindemans CA, Calafiore M, Mertelsmann AM, O'Connor MH, Dudakov JA, Jenq RR, et al. Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. *Nature.* (2015) 528:560–4. doi: 10.1038/nature16460
17. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature.* (2009) 457:722–5. doi: 10.1038/nature07537
18. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and−23 control plasticity of CD127(+) group 1 and group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity.* (2015) 43:146–60. doi: 10.1016/j.immuni.2015.06.019
19. Takayama T, Kamada N, Chinen H, Okamoto S, Kitazume MT, Chang J, et al. Imbalance of NKp44(+)NKp46(-) and NKp44(-)NKp46(+) natural killer cells in the intestinal mucosa of patients with Crohn's disease. *Gastroenterology.* (2010) 139:882–92, 892 e881–3. doi: 10.1053/j.gastro.2010.05.040
20. Geremia A, Arancibia-Carcamo CV, Fleming MP, Rust N, Singh B, Mortensen NJ, et al. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med.* (2011) 208:1127–33. doi: 10.1084/jem.20101712
21. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat. Immunol.* (2013) 14:221–9. doi: 10.1038/ni.2534
22. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity.* (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
23. Zhang Z, Cheng L, Zhao J, Li G, Zhang L, Chen W, et al. Plasmacytoid dendritic cells promote HIV-1-induced group 3 innate lymphoid cell depletion. *J Clin Invest.* (2015) 125:3692–703. doi: 10.1172/JCI82124
24. Reeves RK, Rajakumar PA, Evans TI, Connole M, Gillis J, Wong FE, et al. Gut inflammation and indoleamine deoxygenase inhibit IL-17 production and promote cytotoxic potential in NKp44+ mucosal NK cells during SIV infection. *Blood.* (2011) 118:3321–30. doi: 10.1182/blood-2011-04-347260
25. Klatt NR, Estes JD, Sun X, Ortiz AM, Barber JS, Harris LD, et al. Loss of mucosal CD103+ DCs and IL-17+ and IL-22+ lymphocytes is associated with mucosal damage in SIV infection. *Mucosal Immunol.* (2012) 5:646–57. doi: 10.1038/mi.2012.38
26. Xu H, Wang X, Liu DX, Moroney-Rasmussen T, Lackner AA, Veazey RS. IL-17-producing innate lymphoid cells are restricted to mucosal tissues and are depleted in SIV-infected macaques. *Mucosal Immunol.* (2012) 5:658–69. doi: 10.1038/mi.2012.39
27. Li H, Richert-Spuhler LE, Evans TI, Gillis J, Connole M, Estes JD, et al. Hypercytotoxicity and rapid loss of NKp44+ innate lymphoid cells during acute SIV infection. *PLoS Pathog.* (2014) 10:e1004551. doi: 10.1371/journal.ppat.1004551
28. Xu H, Wang X, Lackner AA, Veazey RS. Type 3 innate lymphoid cell depletion is mediated by TLRs in lymphoid tissues of simian immunodeficiency virus-infected macaques. *FASEB J.* (2015) 29:5072–80. doi: 10.1096/fj.15-276477
29. Dillon SM, Castleman MJ, Frank DN, Austin GL, Gianella S, Cogswell AC, et al. Inflammatory colonic innate lymphoid cells are increased during untreated HIV-1 infection and associated with markers of gut dysbiosis and mucosal immune activation. *J Acquir Immune Defic Syndr.* (2017) 76:431–7. doi: 10.1097/QAI.0000000000001523
30. Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR. Interferon-γ and tumor necrosis factor-α synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. *Am J Pathol.* (2005) 166:409–19. doi: 10.1016/S0002-9440(10)62264-X
31. Beaurepaire C, Smyth D, McKay DM. Interferon-gamma regulation of intestinal epithelial permeability. *J Interferon Cytokine Res.* (2009) 29:133–44. doi: 10.1089/jir.2008.0057
32. Marchetti G, Tincati C, Silvestri G. Microbial translocation in the pathogenesis of HIV infection and AIDS. *Clin Microbiol Rev.* (2013) 26:2–18. doi: 10.1128/CMR.00050-12
33. Pastorelli L, De Salvo C, Mercado JR, Vecchi M, Pizarro TT. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Front Immunol.* (2013) 4:280. doi: 10.3389/fimmu.2013.00280
34. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity.* (2008) 29:958–70. doi: 10.1016/j.immuni.2008.11.001
35. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, et al. An IL-23R/IL-22 circuit regulates epithelial serum amyloid A to promote local effector Th17 responses. *Cell.* (2015) 163:381–93. doi: 10.1016/j.cell.2015.08.061
36. Sonnenberg GF, Monticelli LA, Elloso MM, Fouser LA, Artis D. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity.* (2011b) 34:122–34. doi: 10.1016/j.immuni.2010.12.009
37. Guo X, Qiu J, Tu T, Yang X, Deng L, Anders RA, et al. Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection. *Immunity.* (2014) 40:25–39. doi: 10.1016/j.immuni.2013.10.021
38. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. *Science.* (2014) 345:1254009. doi: 10.1126/science.1254009

39. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature.* (2013) 498:113–7. doi: 10.1038/nature12240
40. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells. *Science.* (2015) 348:1031–5. doi: 10.1126/science.aaa4812
41. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature.* (2013) 494:261–5. doi: 10.1038/nature11813
42. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature.* (2010) 464:1371–5. doi: 10.1038/nature08949
43. Howe R, Dillon S, Rogers L, McCarter M, Kelly C, Gonzalez R, et al. Evidence for dendritic cell-dependent CD4(+) T helper-1 type responses to commensal bacteria in normal human intestinal lamina propria. *Clin Immunol.* (2009) 131:317–32. doi: 10.1016/j.clim.2008.12.003
44. Dillon SM, Rogers LM, Howe R, Hostetler LA, Buhrman J, McCarter MD, et al. Human intestinal lamina propria CD1c+ dendritic cells display an activated phenotype at steady state and produce IL-23 in response to TLR7/8 stimulation. *J Immunol.* (2010) 184:6612–21. doi: 10.4049/jimmunol.1000041
45. Miller SM, Miles B, Guo K, Folkvord J, Meditz AL, McCarter MD, et al. Follicular regulatory T cells are highly permissive to R5-tropic HIV-1. *J Virol.* (2017) 91:e00430–17. doi: 10.1128/JVI.00430-17
46. Dillon SM, Lee EJ, Kotter CV, Austin GL, Dong Z, Hecht DK, et al. An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal Immunol.* (2014) 7:983–94. doi: 10.1038/mi.2013.116
47. Dillon SM, Lee EJ, Donovan AM, Guo K, Harper MS, Frank DN, et al. Enhancement of HIV-1 infection and intestinal CD4+ T cell depletion *ex vivo* by gut microbes altered during chronic HIV-1 infection. *Retrovirology.* (2016) 13:5. doi: 10.1186/s12977-016-0237-1
48. Smith PD, Smythies LE, Shen R, Greenwell-Wild T, Gliozzi M, Wahl SM. Intestinal macrophages and response to microbial encroachment. *Mucosal Immunol.* (2011) 4:31–42. doi: 10.1038/mi.2010.66
49. Bain CC, Mowat AM. The monocyte-macrophage axis in the intestine. *Cell Immunol.* (2014b) 291:41–8. doi: 10.1016/j.cellimm.2014.03.012
50. Bain CC, Mowat AM. Macrophages in intestinal homeostasis and inflammation. *Immunol Rev.* (2014a) 260:102–17. doi: 10.1111/imr.12192
51. Bjorklund AK, Forkel M, Picelli S, Konya V, Theorell J, Friberg D, et al. The heterogeneity of human CD127(+) innate lymphoid cells revealed by single-cell RNA sequencing. *Nat Immunol.* (2016) 17:451–60. doi: 10.1038/ni.3368
52. Schultz BM, Paduro CA, Salazar GA, Salazar-Echegarai FJ, Sebastian VP, Riedel CA, et al. A potential role of salmonella infection in the onset of inflammatory bowel diseases. *Front Immunol.* (2017) 8:191. doi: 10.3389/fimmu.2017.00191
53. Hung CC, Hung MN, Hsueh PR, Chang SY, Chen MY, Hsieh SM, et al. Risk of recurrent nontyphoid Salmonella bacteremia in HIV-infected patients in the era of highly active antiretroviral therapy and an increasing trend of fluoroquinolone resistance. *Clin Infect Dis.* (2007) 45:e60–67. doi: 10.1086/520681
54. Taramasso L, Tatarelli P, Di Biagio A. Bloodstream infections in HIV-infected patients. *Virulence.* (2016) 7:320–8. doi: 10.1080/21505594.2016.1158359
55. d'Ettorre G, Ceccarelli G, Giustini N, Serafino S, Calantone N, De Girolamo G, et al. Probiotics reduce inflammation in antiretroviral treated, HIV-infected individuals: results of the "Probio-HIV" clinical trial. *PLoS ONE.* (2015) 10:e0137200. doi: 10.1371/journal.pone.0137200
56. Eom T, Kim YS, Choi CH, Sadowsky MJ, Unno T. Current understanding of microbiota- and dietary-therapies for treating inflammatory bowel disease. *J Microbiol.* (2018) 56:189–98. doi: 10.1007/s12275-018-8049-8
57. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proc Natl Acad Sci USA.* (2010) 107:10961–6. doi: 10.1073/pnas.1005641107
58. Marafini I, Monteleone I, Di Fusco D, Cupi ML, Paoluzi OA, Colantoni A, et al. TNF-alpha producing innate lymphoid cells (ILCs) are increased in active celiac disease and contribute to promote intestinal atrophy in mice. *PLoS ONE.* (2015) 10:e0126291. doi: 10.1371/journal.pone.0126291
59. Crellin NK, Trifari S, Kaplan CD, Satoh-Takayama N, Di Santo JP, Spits H. Regulation of cytokine secretion in human CD127(+) LTi-like innate lymphoid cells by Toll-like receptor 2. *Immunity.* (2010) 33:752–64. doi: 10.1016/j.immuni.2010.10.012
60. Van Maele L, Carnoy C, Cayet D, Songhet P, Dumoutier L, Ferrero I, et al. TLR5 signaling stimulates the innate production of IL-17 and IL-22 by CD3(neg)CD127+ immune cells in spleen and mucosa. *J Immunol.* (2010) 185:1177–85. doi: 10.4049/jimmunol.1000115
61. Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, Hohl TM, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity.* (2012) 36:276–87. doi: 10.1016/j.immuni.2011.12.011
62. Reynders A, Yessaad N, Vu Manh TP, Dalod M, Fenis A, Aubry C, et al. Identity, regulation and *in vivo* function of gut NKp46+RORgammat+ and NKp46+RORgammat- lymphoid cells. *EMBO J.* (2011) 30:2934–47. doi: 10.1038/emboj.2011.201
63. Hughes T, Becknell B, Freud AG, McClory S, Briercheck E, Yu J, et al. Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. *Immunity.* (2010) 32:803–14. doi: 10.1016/j.immuni.2010.06.007
64. Tumanov AV, Koroleva EP, Guo X, Wang Y, Kruglov A, Nedospasov S, et al. Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. *Cell Host Microbe.* (2011) 10:44–53. doi: 10.1016/j.chom.2011.06.002
65. Manta C, Heupel E, Radulovic K, Rossini V, Garbi N, Riedel CU, et al. CX(3)CR1(+) macrophages support IL-22 production by innate lymphoid cells during infection with *Citrobacter rodentium*. *Mucosal Immunol.* (2013) 6:177–88. doi: 10.1038/mi.2012.61
66. Longman RS, Diehl GE, Victorio DA, Huh JR, Galan C, Miraldi ER, et al. CX(3)CR1(+) mononuclear phagocytes support colitis-associated innate lymphoid cell production of IL-22. *J Exp Med.* (2014) 211:1571–83. doi: 10.1084/jem.20140678
67. Li J, Doty AL, Tang Y, Berrie D, Iqbal A, Tan SA, et al. Enrichment of IL-17A(+) IFN-gamma(+) and IL-22(+) IFN-gamma(+) T cell subsets is associated with reduction of NKp44(+) ILC3s in the terminal ileum of Crohn's disease patients. *Clin Exp Immunol.* (2017) 190:143–53. doi: 10.1111/cei.12996
68. Rosental B, Brusilovsky M, Hadad U, Oz D, Appel MY, Afergan F, et al. Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. *J Immunol.* (2011) 187:5693–702. doi: 10.4049/jimmunol.1102267
69. Baychelier F, Sennepin A, Ermonval M, Dorgham K, Debre P, Vieillard V. Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. *Blood.* (2013) 122:2935–42. doi: 10.1182/blood-2013-03-489054
70. Esin S, Batoni G, Counoupas C, Stringaro A, Brancatisano FL, Colone M, et al. Direct binding of human NK cell natural cytotoxicity receptor NKp44 to the surfaces of mycobacteria and other bacteria. *Infect Immun.* (2008) 76:1719–27. doi: 10.1128/IAI.00870-07
71. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* (2001) 31:2680–9. doi: 10.1002/1521-4141(200109)31:9<2680::AID-IMMU2680gt;3.0.CO;2-A
72. Victor AR, Nalin AP, Dong W, McClory S, Wei M, Mao C, et al. IL-18 Drives ILC3 proliferation and promotes IL-22 production via NF-kappaB. *J Immunol.* (2017) 199:2333–42. doi: 10.4049/jimmunol.1601554

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: 04 April 2019
doi: 10.3389/fimmu.2019.00623

ILC2s—Trailblazers in the Host Response Against Intestinal Helminths

Tiffany Bouchery[1]*, *Graham Le Gros*[2] *and Nicola Harris*[1]*

[1] Department of Immunology and Pathology, Monash University, AMREP, Melbourne, VIC, Australia, [2] Allergic & Parasitic Diseases Programme, Malaghan Institute of Medical Research, Wellington, New Zealand

Group 2 innate lymphoid cells (ILC2s) were first discovered in experimental studies of intestinal helminth infection—and much of our current knowledge of ILC2 activation and function is based on the use of these models. It is perhaps not surprising therefore that these cells have also been found to play a key role in mediating protection against these large multicellular parasites. ILC2s have been intensively studied over the last decade, and are known to respond quickly and robustly to the presence of helminths—both by increasing in number and producing type 2 cytokines. These mediators function to activate and repair epithelial barriers, to recruit other innate cells such as eosinophils, and to help activate T helper 2 cells. More recent investigations have focused on the mechanisms by which the host senses helminth parasites to activate ILC2s. Such studies have identified novel stromal cell types as being involved in this process—including intestinal tuft cells and enteric neurons, which respond to the presence of helminths and activate ILC2s by producing IL-25 and Neuromedin, respectively. In the current review, we will outline the latest insights into ILC2 activation and discuss the requirement for—or redundancy of—ILC2s in providing protective immunity against intestinal helminth parasites.

Keywords: ILC2, helminth, type 2 immunity, activation, regulation, tuft cell, neuron

OPEN ACCESS

Edited by:
Andrew McKenzie, University of Cambridge, United Kingdom

Reviewed by:
Rick M. Maizels, University of Edinburgh, United Kingdom
Christoph Wilhelm, University of Bonn, Germany

***Correspondence:**
Tiffany Bouchery
tiffany.bouchery@monash.edu
Nicola Harris
nicola.harris@monash.edu

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

Received: *19 December 2018*
Accepted: *08 March 2019*
Published: *04 April 2019*

Citation:
Bouchery T, Le Gros G and Harris N (2019) ILC2s—Trailblazers in the Host Response Against Intestinal Helminths. Front. Immunol. 10:623. doi: 10.3389/fimmu.2019.00623

INTRODUCTION

Intestinal helminths have co-evolved with mammals and constitute a diverse but extremely successful group of pathogens infecting over one billion people worldwide, mostly in impoverished countries (1). Three main laboratory models of intestinal helminth infection have been used to study Group 2 Innate Lymphoid Cells (ILC2s). Two of these are rodent parasites (namely *Nippostrongylus brasiliensis* (Nb) and *Heligmosomoides polygyrus* (Hp)), that model human hookworm infection (2). Nb has a short life-cycle, that unlike its human counterpart causes only an acute infection, but nicely mimics the infectious lifecycle of the human hookworms by migrating from the skin to the lungs by the bloodstream, before being coughed up to finish its life cycle in the small intestine (2). Hp in the contrary, is a strictly enteric parasite, however like most human helminths this parasite establishes a chronic infection in its host and is strongly immuno-modulatory (2). The third model species, *Trichuris muris* (Tm) is used as a model of human whipworm infection, and is a non-migrating parasite that resides in the lumen of the large intestine (3).

Despite the diversity both in terms of biology of helminths, and their lifecycle within their host, the mammalian immune response against these parasites is remarkably conserved and is dominated by a type 2 cell mediated response, which is characterized by IgE antibody production, eosinophilia, mastocytosis, and the differentiation of type 2 macrophages (M2, activated either by IL-4 or IL-13) in response to the production of the canonical type 2 cytokines interleukin-4 (IL-4), IL-5, and IL-13 (4). More recently, the discovery of ILC2s, has forced us to re-evaluate the paradigm of what constitutes a protective immune response against these parasites.

ILC2s were first identified in 2010 as non T cell receptor (TCR) non B cell receptor (BCR) bearing cells that are enriched at mucosal sites of Nb infected mice (5). These innate cells were initially described as IL-25 responsive, and subsequently called ILC2 in a series of later publications that showed the importance of ILC2s for immune protection against helminths in primary infection (6–8). ILC2s are now understood to form part of a greater population of innate lymphoid cells, which also encompasses ILC1s and ILC3s, and are defined as lacking lineage markers (markers that define T cells, B cells, NK cells, myeloid cells, granulocytes, dendritic cells, and hematopoietic stem cells) in addition to expressing the transcription factors Gata-3 and (Retinoic Acid Receptor- Related Orphan Receptor Alpha) ROR-α (9). ILCs all originate from a common helper-like innate lymphoid precursor (CHILP) (10), whose development is regulated by Notch signaling and IL-7 (8, 11, 12). Id2, an inhibitor of E protein transcription factors, was been shown to be indispensable for ILC differentiation (6, 13). The factors driving the specific differentiation of ILC2s is still unclear, however it involves passage through an intermediate stage termed an ILC2-specific progenitor (ILC2P) (9). ILC2s express a variety of surface markers—most notably Chemoattractant Receptor-homologous molecule expressed on TH2 cells (CRTH2) (14), suppression of tumorigenicity 2 (ST-2), IL-17RB, CD127, CD80, MHCII and CD25, and produce the type 2 cytokines IL-13 and IL-5, as well as amphiregulin (8, 15).

Although early studies referred to ILC2s as a single population, these cells have more recently been described to exist as several subsets, termed natural (or tissue resident) ILC2s (nILC2s) and inflammatory ILC2s (iILC2s) (16). nILC2s are IL-33 responsive, express high levels of ST-2 and are not found in the circulation. By contrast iILC2s express high levels of Killer cell lectin-like receptor G1 (KLRG1) and IL-17RB, but low levels of ST2, and arise in response to IL-25 (16). Functional differences between iILC2s and nILC2s also exist, with iILC2s being described to produce more IL-13, whilst nILC2s exhibit a pro-repair phenotype and release IL-9 (16, 17). Plasticity between these subsets have been described with Notch ligands shown to promote the switch from nILC2 to iILC2 (12). Interestingly, the local microenvironment of the tissue or organ may influence the subset of ILC2s present. This hypothesis was recently confirmed by Huang and colleagues who reported that iILC2s expanded in the small intestine in response to helminth infection or exogenous IL-25 delivered intraperitoneally, but that intranasal administration of exogenous IL-25 did not induce iILC2s in the lungs (18). The authors hypothesized that iILC2s precursors are present in the small intestine, but not the lungs (18). Nevertheless, those iILC2s generated in the intestine were able to migrate to other organs, including the lungs, as demonstrated by elegant parabiosis experiments by the same authors (18). ILC2s from various tissue have a unique signature at steady state, as shown by single cells transcriptomic of ILC2s from gut, lung, skin and bone marrow (19). Furthermore, that different ILC2 subsets, or precursor subsets, may reside in different organs is supported by a recent study which intranasal administration of a unadjuvanted Fowlpox virus (FPV)-HIV vaccine caused nILC2s expansion locally within the lungs, whilst intramuscular administration of the same vaccine caused an expansion of iILC2s (20). Given that the existence of distinct ILC2 subsets is a relatively new finding we still have much to learn about its functional relevance and the majority of the literature discussed in this review reports work investigating ILC2s as a whole population rather than as distinct subpopulations.

Whilst the expansion and activation of ILC2s is likely to be beneficial for those living in regions endemic for helminths, their activity is more commonly associated with immune pathologies for those living in developed countries. These pathologies include airway hyperreactivity (21), allergen-induced lung inflammation (22, 23), and atopic dermatitis (24). Thus, an improved understanding of ILC2 activation and regulation is of great importance for human health. As a consequence, progress in this field demands that we understand the mechanisms involved in the activation and regulation of ILC2s as well as elucidating their full function. This review will focus on outlining the known role of helminth infection in promoting ILC2 activation, in addition to discussing their known functions during helminth infection. Specific attention will be given to the array of recent advances that have been reported using helminth models to study ILC2 function. Lastly, we outline what we believe are the most pressing questions for future research in this area.

THE ACTIVATION AND REGULATION OF ILC2S

Early and recent studies described the activation of ILC2s by a surprising array of stimuli including helminths, allergens, certain bacteria and even endogenous host molecules (8, 25–27). This is perhaps not surprising as it is now clear that ILC2s become activated in response to factors released by both stromal and immune cell populations in response to stress or tissue damage (as outlined below and in **Figure 1**). In the following paragraphs, we will discuss those pathways associated with the activation or regulation of ILC2 function to date.

Epithelial Cell Production of Alarmins

Alarmins, namely Interleukin-33 (IL-33), IL-25, and Thymic stromal lymphopoietin (TSLP), can be released from a variety of cells, but are especially rich within epithelial cells present in the skin and mucosal tissues (28–32). These cytokines cause both the proliferation and activation of ILC2s and are particularly good at eliciting the production of IL-13 and IL-5 (33), with some reports also detailing release of IL-4 or IL-9 in mice (17, 27, 33, 34). The

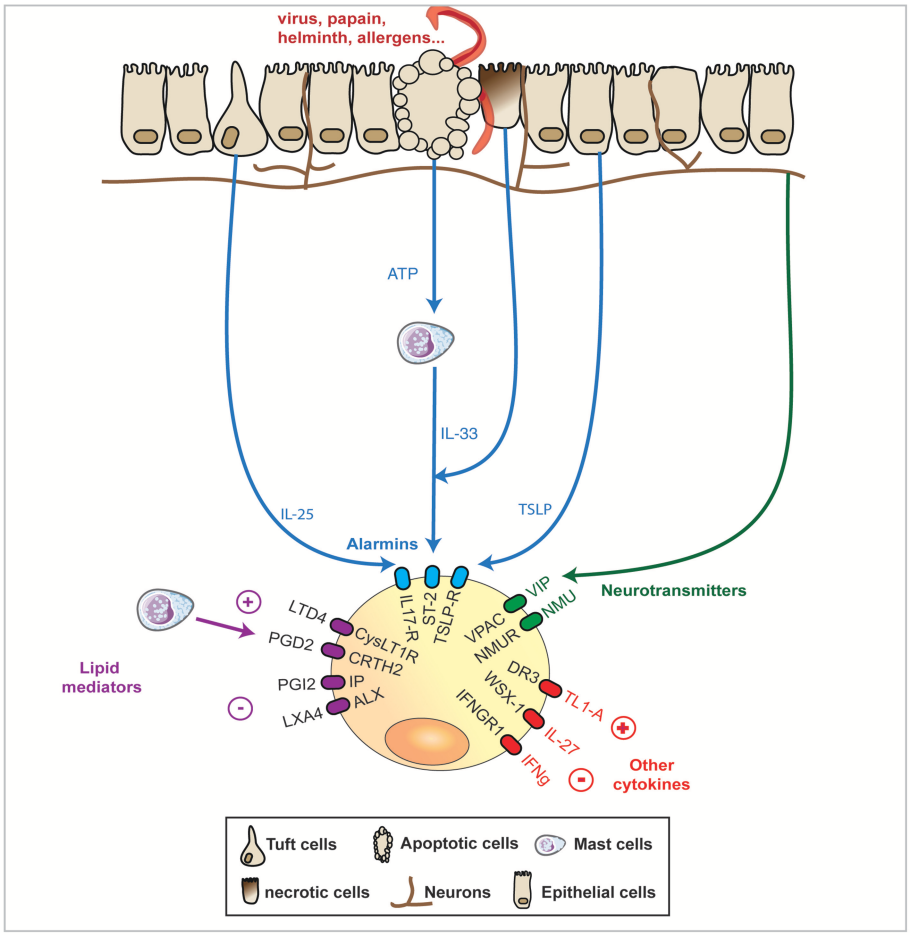

FIGURE 1 | In response to various stimuli such as infection or injury, mucosal epithelial barriers express a range of signals that ILC2s can integrate. The most studied and thought to be of utmost importance pathway of activation of ILC2 is formed by the "alarmins," IL-33, TSLP, and IL-25 that cause release of the canonical IL-13, IL-5, and amphiregulin cytokines expression. Upon damage, necrotic epithelial cells can release IL-33 while apoptotic epithelial cells, can release ATP, that further activates mast cells to release IL-33. Alternatively, epithelial cells can release TSLP or the newly identified and specialized chemosensory epithelial cells, namely tuft cells, can release IL-25. Recently mucosal sensory nervous system has been shown to detect helminths and protists and release in response Neuromedin (Nmu) or vasoactive intestinal polypeptide (VIP) that can both activates by themselves ILC2s or synergise with alarmins to potentiate ILC2 response. Finally, some cytokines and lipid mediators have emerged as potential controllers of ILC2s, with the prostaglandin PGI2 and the lipoxin LXA4 limiting ILC2 cytokines release, while the prostaglandin PGD2 and the leukotriene LTD4 can potentiate the same cytokine expression. The regulatory IL-27 cytokines as well as Interferon (IFN)g can dampen ILC2 activation.

relative importance of each alarmin in ILC2 activation has been extensively studied, but still forms an incomplete picture. Studies suggest that IL-33 is more potent that TSLP or IL-25 at inducing ILC2s in the context of allergic airway inflammation (35). However, in the context of atopic dermatitis both TSLP and IL-33 have been shown to play a crucial role in ILC2 activation and pathology development, with TSLP specifically controlling the itch response (29) and IL-33 being more important for causing the "atopic march" (typical progression of allergic disease going from atopic dermatitis, to food allergy, rhinitis, and asthma) (36). Lastly, redundancy between all three cytokines has been observed in the context of fibrosis and chronic intestinal inflammation (37). Interestingly, TSLP and IL-33 can act in a synergistic manner during the host response to chitin stimulation, with the combination of alarmins acting to potentiate type 2 cytokine production by ILC2 (35). Such synergy was also observed for human ILC2s cultured *in vitro*, where the presence of two or three alarmins together promoted ILC2 proliferation and survival, and cytokine production (38). The presence of TSLP as a member of the "alarmin cocktail" was deemed to be of particular importance (38). Although the ability of all three alarmins to participate in ILC2 activation may at first be confusing, it has been proposed that the relative importance of each alarmin is related to the specific tissue, infection or pathology in which the ILC2 response is involved (39). This view suggests that the three alarmins do not act in a purely redundant, or even synergistic manner, but that the relative levels of each alarmin found within a given tissue may act to match the ILC2 response to environment in which it is present. In line with this view, IL-33 has been reported to mediate the activation nILC2s, whilst IL-25 preferentially elicits iILC2

activation (16). However, to date the full implications of these findings in terms of protective immunity or immune-pathology remain unclear.

All three alarmins are released by epithelial cells, although to varying amounts within distinct mucosal tissues. TSLP and IL-33 can be released by alveolar epithelial cells type II in the respiratory tract, by keratinocytes in the skin or by epithelial cells in the intestine. IL-33 is expressed by fibroblast reticular cells (28, 40) in lymphoid organs and myofibroblasts in the intestine (33). Endothelial cells in the spleen and lymph-nodes, as well as in the intestine under inflammatory conditions have been shown to be another source of IL-33 (28, 40). In human, it has recently been described that endothelial cells, rather than epithelial cells in the lungs release IL-33 (41, 42).

By contrast IL-25 production appears to be restricted to a specialized chemosensory epithelial cells, called tuft cells (30, 32, 43). These cells, which are also commonly referred to as "brush" cells, are found in the epithelium of various organs including the intestine and respiratory tract (44). In the intestine, tuft-cell-derived IL-25 elicits IL-13 release by ILC2s, which in turn promotes the further expansion of IL-25 producing tuft cells, in a feed-forward amplification of the type 2 immune response (32). Of note, expansion of intestinal tuft cells in response to Tm and Nb infection has been shown to be dependent on chemosensory taste receptors, most notably on the transient receptor potential cation channel, subfamily M, member 5 (Trpm5) (30, 45) which causes release of acetylcholine and activation of nearby vagal nerve fibers (46). Employing a single cell RNA sequencing approach, Haber and colleagues recently showed that intestinal tuft cells constitute a heterogeneous population, with two main subtypes identified (47). Both subsets expressed IL-25, but only one also expressed TSLP (and was also CD45+). Interestingly, the TSLP+ tuft cell population was specifically expanded following Hp infection (47). These data raise the question as to whether tuft cells respond differently to distinct stimuli and whether they can also promote ILC2 activation via TSLP. In the respiratory tract brush cells were shown to be activated by leukotrienes in response to *Alternaria* to release IL-25, which in turn activated ILC2s (48, 49). Unlike what has been described for intestinal tuft cells (30), respiratory brush cell hyperplasia was found to be STAT6 independent (48). Whether this difference is due to the tissue location of the cells, or to the source of the stimuli remains unclear.

The tuft cell/ILC2 expansion loop in helminth infection is now well established, but what triggers helminth recognition by tuft cells remains unclear. Recent work elegantly demonstrated that protists can be detected by tuft cells by virtue of their secretion of the metabolic product succinate (50). Surprisingly, however, even though Nb was shown to produce succinate, the tuft response to Nb infection was succinate independent, indicating the presence of an alternative stimulatory signal from this parasite (32, 50, 51). Another open question is whether negative regulators of the tuft cell-ILC2 feed-forward loop exist. In this regard, it was recently described that A20 (*Tnfaip3*) expression by ILC2s is a negative regulator of their expansion in response to IL-25 release by tuft cells in the intestine (50). Of interest, mice deficient for A20 within ILC2s exhibited intestinal crypt hypertrophy, thickening of the surrounding muscularis and an increased frequency of secretory cells, which are all features observed following helminth infection of wildtype mice (50).

Nervous System

Sensory neurons have recently been shown by several groups (10, 52, 53) to contribute to the activation of an ILC2 response. The possibility that neurons may play a role in ILC2 activation was first raised by the finding that ILC2s expressed high levels of the Neuromedin U receptor 1 (Nmur1) (52). Nmur1 can be stimulated by its ligand, Neuromedin U (Nmu), which is typically expressed by cholinergic enteric neurons. Moreover, ILC2s have been reported to form close associations with Nmu+ neurons in both the lungs and intestine (52). Stimulation of ILC2s with Neuromedin U caused their prompt proliferation as well as eliciting expression of the type 2 cytokines IL-5, IL-13, the growth factors amphiregulin and colony stimulating factor 2 (Csf2) (10, 52). Interestingly, the response of ILC2s to Nmu was found to be more rapid than that observed for IL-33 or IL-25, suggesting that the neuronal/immune pathway could be a precocious threat sensor, activated even before the onset of tissue damage. In keeping with this hypothesis enteric neurons were shown to directly release Nmu in response to helminth products (52). That neuronal-ILC2 interactions play a functional role in helminth immunity was demonstrated by the more rapid expulsion of adult worms in Nb infected animals administered exogenous recombinant Nmu. NmU administration was also found to potentiate the response of ILC2s to IL-25, and to a lesser extent IL-33, as determined by IL-13 and IL-5 production *in vitro* (54). Synergy between Nmu and IL-25 was confirmed *in vivo* using an allergic airway inflammation model, with co-treatment increasing IL-13 and IL-5 level and eosinophils in the bronchoalveolar lavage (54). Furthermore, an *in vivo* synergistic role for IL-33 is also likely as Nmu deficient mice failed to exhibit increased numbers of ST2+ ILC2s (or nILC2s) in the lung of allergen challenged animals (54). Nmu release is not the only means by which neurons interact with ILC2s as a study investigating experimental allergic airway inflammation demonstrated a role for vasoactive intestinal peptide (VIP) in stimulating IL-5 release from ILC2s. VIP is produced by pulmonary neurons and can bind to the vasoactive intestinal peptide receptor 2 (VPAC2) present on ILC2s (53). Interestingly, IL-5 can in turn stimulate the release of VIP by sensory neurons, in yet another example of a feed-forward loop acting to amplify the type 2 immune response (53).

Cytokines Other Than Alarmins

In both mouse and human, ILC2s express high levels of the tumor necrosis factor (TNF)-receptor superfamily member DR3 (TNFRSF25). Administration of recombinant TL1A, a DR3 ligand, induced ILC2 expansion and DR3 deficient mice are unable to expel Nb -highlighting this cytokine as a possible positive regulator of ILC2 function (55, 56). ILC2s can also respond to Transforming growth factor (TGF)-β, however the outcome of the response remains unclear. Epidermal-derived TGF-β has been shown to enhance ILC2 recruitment and IL-13 expression in the lungs after house dust mite exposure (57).

By contrast TGF-β and IL-10 release by Tregs can suppress the production of type 2 cytokines by ILC2s *in vitro* and *in vivo* in an ovalbumin model of pulmonary allergy—although it did not impact on the proliferation and survival of these cells (58, 59). Given the contradictory nature of these reports more studies will be required to fully understand the impact of TGF-β on ILC2 function. Recently, skin ILC2s have been shown to be IL-18 responsive *in vitro*, causing IL-5 and IL-13 expression in these cells. This finding was further confirmed *in vivo* in an MC903 atopic like skin inflammation model, in which IL-18 deficient mice exhibit decreased ILC2s and eosinophils recruitment in the skin (19).

In terms of negative regulation, it is interesting to note that ILC2s can express the receptors for the anti-inflammatory cytokine IL-10, as well as for classical type 1 cytokines, such as Interferon (IFN)- γ and IL-27 (6, 60, 61). These cytokines are all able to suppress Th2 responses (62–64) and *in vitro* studies in which ILC2s were stimulated with IFN-γ, IFN-β, or IL-27 demonstrated that each of these cytokines could individually suppress the secretion of IL-13 and IL-5 by ILC2s (60, 65–68). A role for type 1 cytokines in restraining ILC2 function *in vivo* was demonstrated by adoptively transferring ILC2s isolated from the lung of wild type or IFNgr1$^{-/-}$ Rag2$^{-/-}$ mice into IL2rg$^{-/-}$Rag2$^{-/-}$ recipient mice (which lack endogenous ILC2 populations). Recipient mice were then given an intra-tracheal inoculation of recombinant IL-33 to activate the transferred ILC2s and the authors reported that ILC2s derived from IFNgr1$^{-/-}$ Rag2$^{-/-}$ mice produced higher quantities of type 2 cytokines compared to their WT counterparts (60). Mchedlidze and colleagues showed similar results using animals deficient in the IL-27 subunit Epstein-Barr virus induced gene 3 (Ebi3). *Ebi3*$^{-/-}$ mice infected with Nb exhibited higher numbers of lung ILC2s and higher levels of circulating type 2 cytokines. The authors further confirmed that IL-27 regulation of ILC2 activation was direct by *in vitro* stimulation (68).

Interaction With Innate Cells

Various innate cells have been shown to participate in the activation of ILC2s. IL-25 is known to be produced by eosinophils and mast cells (69), whilst IL-33 can be produced by mast cells (70, 71), and macrophages (72) indicating that these cells may be able to directly activate ILC2s. The activation of ILC2s can also result from interactions between innate cells and epithelial cells. IL-33—which is typically released as a procytokine—can be cleaved into its bioactive form by proteases released from mast cells and neutrophils (73, 74). This cleavage increases the potency of IL-33 by up to 10-fold, thus enhancing its ability to activate ILC2s (73, 74). In another example of epithelial cell-innate cell collaboration, intestinal epithelial cells release ATP that can activate mast cells, to secrete IL-33 and activate ILC2s (70, 75). More specifically, ATP activates mast cells by interacting with the adenosine receptor, P2X purinoceptor 7 (P2X7R) (70). Of note, ILC2s also express adenosine receptor and stimulation of bone marrow-derived ILC2s with the nonselective adenosine receptors agonist, 1-(6-samino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-d-ribofuranuronamide (NECA), resulted in decreased type 2 cytokine production by these cells (70, 76). In response to Nb or Hp infection, blockade of the A2B adenosine receptor (A2BAR) inhibited ILC2 expansion and treated mice failed to expel the adult worms. However, whether the cells targeted by A2BAR blockade were ILC2s or epithelial cells was unclear (77). Altogether these studies highlight an interesting role of adenosine receptors in regulating ILC2 responses—but the exact molecular mechanisms by this occurs will require further study.

Lipid Mediators

Arachidonic acid derivatives, including the cysteine leukotrienes LTC4 and LTE4, the prostaglandin PGE2, are well characterized for their role in the induction and control of type 2 inflammation (78). Not surprisingly, ILC2s have been shown to respond to various lipid mediators, which act either to activate or suppress the activity of these cells. Prostaglandin D2, a product of Prostaglandin D2 synthase, has been shown to promote ILC2 migration and IL-13 production both in humans and in mice (14, 79, 80). Indeed, the receptor for PGD2, CRTH2, has been highlighted as a useful marker of human ILC2s (81). Murine ILC2s express both CysLTR-1 and-2 and their ligands, LTC4, LTD4, and LTE4 have been shown to induce IL-13 and IL-5 expression by these cells (82, 83). In contrast, other eicosanoids, including lipoxin A4 (LXA4) and Prostaglandin I2 (PGI2) limited the activation of ILC2 and inhibited the production of type 2 cytokines (84, 85). Similar to the synergy noted between Nmu and alarmins, some leukotrienes (namely LTB4 and LT C4), have been shown to enhance the ability of IL-33 activate ILC2s in the context of lung inflammation (86) or helminth infection (83).

In summary, ILC2s are able to be activated by a large array of stimuli—a finding that, at least in part, explains the diversity of environmental triggers that can elicit a type 2 immune response. Further research as to how different stimuli co-operate to activate ILC2 and their possible relevance to different tissues and/or pathological settings will no doubt lead to the development of better therapeutics for type 2 mediated diseases. Last but not least it is likely that we have only exposed the "tip of the iceberg" in terms of identifying possible positive and negative regulators of ILC2 activation, and it is certain that this area will continue to yield exciting and novel insights into type 2 immune responses.

ILC2 FUNCTION DURING INTESTINAL HELMINTH INFECTION

The extensive research dedicated to the activation and regulation of ILC2s (outlined above) has provided many answers related to the possible function of these cells and has also raised many questions. One key question is what is the relative role of these cells in mediating protective immunity and tissue repair in response to helminth infection. The following section will discuss the current state of the art in terms of our understanding of ILC2 function during intestinal helminth infection.

A Role for ILC2s in Driving the Expulsion of Adult Worms

The expulsion of adult worms from the intestinal lumen has long been known to be associated with strong type 2 cytokine production, with IL-13 acting as a potent activator of epithelial

cell turnover, goblet cell hyperplasia and mucus secretion, and increased muscle contractility—culminating in what is commonly referred to as a "weep and sweep" response (87). Although much of the early work on this response centered on the contribution of Th2 cells, we now understand that ILC2s are an important contributor of IL-13 produced early on during infection (88). Indeed, one of the founding papers reporting the existence of ILC2s, demonstrated that adoptive transfer of ILC2s into the normally susceptible IL-13 deficient mice was sufficient to promote the expulsion of Nb (8). This proved that although ILC2s are a rare cell type, they can actively contribute to anti-helminth immunity. ILC2s also produce IL-5 (and are considered as the main source for this cytokines in allergies) and can mediate an early Th cell-independent tissue eosinophilia (8). However, although eosinophilia is a hallmark of helminth infection, they have been described to exhibit diverse, even contrasting, roles in terms of protective immunity and thus their full function remains unclear (89).

To date, ILC2s have been shown to contribute to the timely expulsion of a variety of helminths including Nb and Tm (8, 90, 91). Amongst these parasites, Nb is the most potent elicitor of the ILC2 response, and both the recruitment and activation of ILC2s by IL-33 has been shown to be required for the expulsion of this parasite. Of note, whilst IL-25 is not required for the eventual expulsion of Nb (8), it is important for the expulsion of Hp (92). Intriguingly however, in Hp primary infection, enhanced ILC2 numbers caused by IL-2 treatment (up to 5 times their basal level) were not sufficient to cause adult worm expulsion (34). However, this treatment resulted in reduced adult worm burden with increased numbers of L4 larvae trapped in the submucosa (34). Similarly, treatment of Tm infected mice with recombinant IL-25 treatment promotes parasite expulsion, however this study was completed before the discovery of ILC2s (93). All in all, IL-25-induced ILC2 expansion and activation appears to play an important role in promoting the expulsion of adult helminth parasites, but the relative contribution, of these cells to host immune responses against distinct parasites remains unclear. Nevertheless, alarmin release and nervous recognition of helminths resulting in increased Nmu expression all seem to be a general feature in the host response to intestinal helminth infection (10, 94).

Although ILC2s have created much excitement, it is important to note that in natural settings the activation of ILC2s alone is not sufficient to mediate protection against helminths. Indeed, Neill and colleagues demonstrated that transfer of ILC2s into Rag2-deficient mice (which lack B and T cells) was not sufficient to mediate worm expulsion (8). In this setting, ILC2 numbers were not sustained for long enough (more than 2 days) to allow expulsion of the worms and the authors suggested that Th2 cells might support ILC2 maintenance (8). Indeed, a series of later reports identified an interplay between ILC2s and the adaptive immune response, and this will be discussed later in the review.

The Contribution of ILC2s to Tissue Repair

Intestinal helminths are large multicellular pathogens that cause extensive tissue damage as they migrate through host tissues as larvae stages, and whilst they dwell within the intestine as adult worms. In recent years, is has become evident that type 2 immune responses evolved not only to limit parasite burdens, but also suppress excessive inflammation and to mediate the rapid repair of damaged tissues (95). To date, many of the investigations addressing the contribution for type 2 immunity to tissue repair have focused on IL-4 activated macrophages (95). However, studies addressing the possible contribution of ILC2s to repair are beginning to emerge. Amphiregulin (an epidermal growth factor) has long been known to be required for protective immunity following Tm infection (96), and ILC2s were later reported to represent a potent source of this cytokine (97). Studies investigating the role of IL-9 in helminth immunity noted that IL-9 deficient mice infected with Nb exhibited enhanced lung damage and delayed worm expulsion (17, 33, 98). The same authors demonstrated that IL-9 functioned as a survival factor for ILC2, which in turn provided the amphiregulin required for efficient lung repair following parasitic migration through this organ (17, 33). Similarly, nILC2s have been shown to secrete amphiregulin leading to the differentiation and proliferation of epidermal growth factor receptor (EGFR) expressing epithelial cells following respiratory virus infection (97). ILC2s may also promote intestinal protection against damage as the transfer of ILC2s in an experimental model of colitis was shown to attenuate disease severity, through enhanced mucin production (99). IL-9 production by ILC2s has also directly been shown to limit type 1 inflammation in a sepsis induced model of acute lung inflammation (27). In this study, it was further shown that IL-33 activated ILC2 present in the lung produced IL-9 which acted to prevent lung endothelial cells from undergoing pyroptosis (a form of cell death), by virtue of its ability to limit caspase-1 activation (27).

Together these studies indicate that ILC2s can contribute to the modulation of inflammation and the promotion of tissue repair following a variety of environmental insults. However, it is possible that these cells also contribute to the pathology that can result from exaggerated or prolonged type 2 inflammatory responses. On this note nILC2s have recently been shown to constitutively express arginase-1 (Arg-1), and the selective absence of this gene within ILC2s resulted in an exacerbated emphysema in response to Nb infection (100). Similarly, IL-13 secretion by ILC2s present in the lungs has been demonstrated to in the disrupt tight junctions in asthmatic patients (101).

Adaptive Immune Response

Priming of Type 2 Immune Response

The cellular and molecular mechanisms that lead to the differentiation of naïve CD4+ T cells into type 2 cytokine producing T helper 2 (Th2) cells are still not fully understood. To date most of the work in this area has focused on the importance of dendritic cell (DC)—T cell interactions, however the discovery of ILC2s has widened our view of a DC centric world to appreciate the possible importance of ILC2s in initiating or modulating the Th2 response.

Studies using experimental mouse models in which ILC2s were preferentially depleted have revealed that ILC2s are required to promote Th2 cell responses in response to infection with Nb, or following the intranasal administration of the allergen papain

(102–104). More recently, tissue-specific ILC2s were shown to represent a critical source of the co-stimulatory molecule OX40 ligand (OX40L) in response to IL-33 stimulation (105). Binding of OX40L to OX40 on CD4 T cells was required for the development of both Th2 and GATA3+/– Treg responses in the lungs after Nb infection (105). In keeping with these findings, ICOS-ICOSL interactions between ILC2s and CD4 T cells have been shown to be required for optimal Treg expansion in response to IL-33 stimulation or Nb infection (67). ILC2s can also contribute to the development of Th2 cells in response to the murine helminth Hp infection by releasing IL-4 (34, 103). Although most studies indicate that murine ILC2s make little IL-4, this is in contrast to human ILC2s which can produce large quantities of this cytokine in response to combined stimulation with IL-33 and TSLP (106).

In addition to direct ILC2-T cell interactions, ILC2s can impact on DC function and have been shown to promote the migration of DCs from the tissues to the lymph node by virtue of producing IL-13 (102). Last but not least, ILC2s can present antigen directly to CD4 T cells (103) and CD4 T cells have been shown to support the continued survival of ILC2s by providing IL-2 (107, 108). Altogether, these studies highlight a complex interplay between ILC2s, DC and CD4 T cells that promotes the development of optimal adaptive type two immune responses.

Memory Immune Responses

ILC2s have now been reported to contribute to the amplitude of memory type 2 immune responses in a variety of models. In the first report, Halim and colleagues investigated the contribution of ILC2s to recall responses against papain (109). Here, DCs play a critical role by producing CCL-17 and CCL-22 to attract CCR4+ memory CD4 T cells. Interestingly the expression of CCL-17 and CCL-22 was triggered by type 2 cytokines released by ILC2s - and ILC2 ablation prior to papain re-challenge attenuated the number of Th2 cells present (109). In another example of ILC2 potentiation of memory type 2 responses, these cells were reported to critically contribute to the production of IL-13 in response to challenge infections with Nb allowing the rapid activation of M2 macrophages which were able to mediate both parasite killing and tissue repair (108). In this model, Th2 cells also contributed to the activation of M2 macrophages by producing IL-4, and were additionally found to promote the maintenance of ILC2s following challenge infection with Nb in an elegant example of ILC2-Th2 cell co-operation (108). Of note, short term treatment with recombinant IL-33 has been reported to induce the sustained activation (for over 1 year) of ILC2s both in helminth and glomerulosclerosis models indicating that—like memory cells—some ILC2s could be long lived cells (110, 111). Lastly, previously activated ILC2s were able to produced increased amounts of IL-13 when re-exposed to the same antigen, or even to an unrelated allergen or to IL-33 (110).

Humoral Immune Responses

The role of ILC2s in humoral immunity has just begun to be addressed. Recently, ILC2s isolated from the lungs of naive wild type mice were shown to promote the proliferation of B1-, as well as B2-, type B cells *in vitro*. ILC2-activated B cells produced IgM, IgG1, IgA, and IgE, with the production of IgM being IL-5 dependent (112). Given that antibodies, and in particular IgE and IgG have been implicated in protective immune responses against challenge infections with a variety of helminths (113, 114), the impact of ILC2 on B cell responses could be of great importance to helminth protection. Moreover, one of the functions of IgE is to arm basophils and mast cells, that in turn function to potentiate Th2 responses (115), or release inflammatory mediators (116) potentiating type 2 inflammation.

Helminth-Mediated Regulation of the ILC2 Response

Helminths have co-evolved with their host and typically form chronic infections in their host. These parasites are often described as masters of immunomodulation and a multitude of parasite-derived products have been identified that interfere with host immune responses (117). It is noteworthy that Nb, which is a potent elicitor of ILC2 responses, is expelled rapidly from its murine host, whilst Hp and Tm, which elicit only modest ILC2 responses, form chronic infections. Interestingly, resistant SJL mice have higher ILC2 responses to Hp than susceptible B6 mice (118). This raises the possibility that some helminths may attempt to evade host rejection by modulating ILC2 responses, and in line with this idea McSorley and colleagues recently reported that secretory products from Hp can attenuate allergic airway inflammation by blocking the release of IL-33 from epithelial cells (119). The authors went on to identify one of the proteins responsible, and termed this protein Hp-derived Alarmin Release Inhibitor (HpARI) (120). HpARI selectively binds to IL-33 and traps it in the nucleus, preventing its release during cell apoptosis. This was associated with a reduction in IL-13+ and IL-5+ ILC2s in lungs of mice exposed to *Alternaria*. Hp has also been shown to alter the composition of the intestinal microbiome increasing the availability of the bacterial metabolites, short chain fatty acids (SCFA). The authors went on to demonstrate that Hp-induced production of bacterial SCFAs was able to attenuate house dust mite-induced allergic asthma in mice (121). However, it would also be interesting the determine the impact of SCFAs on the host response to the parasite as butyrate, a SCFA, has recently been shown to directly block IL-13 and IL-5 expression by ILC2s (122), which have been associated with the weep and sweep response.

ILC2 RESPONSES AND HUMAN HELMINTH INFECTION

To date, the markers used to identify the various ILC populations in humans remain poorly defined, and very few studies have characterized ILC responses in the context of any human infection (123), let alone helminth infection. Instead most of our knowledge about human ILC2s are derived from studies of inflammatory diseases where ILC2s play a pathological role (*i.e.,* chronic rhinosinusitis, COPD, dermatitis, asthma) (124). Importantly however, these studies have revealed that ILC2s are present in various mucosal tissues of humans including the lungs, the gut and the skin—the same sites where helminths are typically found. In addition, many of the activation pathways described for

murine ILC2s, have now been confirmed in humans including the three alarmins (IL-33, IL-25, and TSLP), leukotrienes and tuft cells (38, 69, 125–129).

In terms of helminth infection, Boyd et al. (130) reported an increase of c-kit+ ILCs (similar to ILC2s and ILC3s in mice) in the circulation of people infected with helminth filariae (*Loa loa* or *Wuchereria bancrofti* or *Onchocerca volvulus*). These cells expressed IL-13 and were identified as Lineage (Lin)-/CD45+/cKit+/CD127+, but additionally expressed IL-10 and IL-17 (130). Both *Wuchereria* and *Onchocerca* helminths harbor the bacterial endosymbiont *Wolbachia*, which may bias the host immune response away from a type 2 response and toward a type 17 response and would explain the observed increase in both IL-13 and IL-17 expression. Yet, most of the patients studied were infected only with *Loa loa* (130), which unlike its relatives does not harbor *Wolbachia*. An alternative explanation could therefore be that the increased c-kit ILCs reported in infected patients may be largely LTis rather than ILC2s.

A second study assessed the ILC response in children infected with *Schistosoma haematobium* (131). Younger children exhibited lower numbers of circulating ILC2s, identified as Lin-CD45+CD127+CD294+CD161+, whilst ILC2 numbers in older children were similar between infected and control individuals. Following anthelmintic treatment, the number of ILC2s present in young children was restored to levels apparent in uninfected patients, suggesting that *Schistosoma* may suppress the ILC2 response (131). In *Schistosoma* infection, protective immunity is known to build up over time, and the "older" children had antibody titers indicative of the acquisition of immune protection (131). Determining whether the positive correlation between ILC2 numbers and increasing age simply reflects the slow acquisition of protective type 2 immunity against endemic helminths, or whether these cells actually play a causal role in promoting such protection will be an important question for the future.

Other unanswered questions include: Does the ILC2 response differ following infection with different families or species of helminths? Are there fundamental differences in the ability of young children or adults to generate ILC2s? Do ILC2 numbers correlate with disease phenotypes including resistant (non-infected but exposed), susceptible (clinically symptomatic, infected), or controller (clinically asymptomatic, infected) individuals? These questions may have fundamental importance for the design of successful vaccines against these widespread, and often debilitating, parasites.

CONCLUSIONS AND PERSPECTIVE

Our understanding and knowledge of ILC2s has expanded tremendously over the recent decade, yet much remains to be determined. Whilst the majority of existing research related to human ILC2s have focused on the "first-world" diseases related to allergic inflammation, we would argue that attention should also be given to the role of these cells during human helminth infection given the clear need for improved control of these parasites amongst developing societies. In addition to this, ongoing studies of ILC2s in the context of host-helminth interactions—either in mice or humans—are highly likely to continue to shed light on the activation, regulation, and function of these cells. In terms of allergic disease, mining helminths for molecules that suppress ILC2 responses could represent a promising avenue for the identification of novel therapeutics. Lastly, at a time when we are just beginning to understand the full importance of ILC2s in anti-helminth immune responses, Maizels and colleagues recently described the existence of another, as yet undefined but very rare innate immune cell, that is important for the expulsion of Hp (132). This report highlights that our understanding of type 2 immune responses and of host-helminth interactions is continually evolving. Although studies performed over the last decade led to the discovery of ILC2s, it is likely that continued efforts in this area will reveal many interesting, and perhaps even surprising, facets of the type 2 immune response.

AUTHOR CONTRIBUTIONS

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

REFERENCES

1. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. *J Clin Invest*. (2008) 118:1311–21. doi: 10.1172/JCI34261
2. Bouchery T, Volpe B, Shah K, Lebon L, Filbey K, LeGros G, et al. The study of host immune responses elicited by the model murine hookworms *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Curr Protoc Mouse Biol*. (2017) 7:236–86. doi: 10.1002/cpmo.34
3. Hurst RJM, Else KJ. *Trichuris muris* research revisited: a journey through time. *Parasitology*. (2013) 140:1325–39. doi: 10.1017/S0031182013001054
4. Harris NL, Loke P. Recent advances in Type-2-cell-mediated immunity: insights from helminth infection. *Immunity*. (2017) 47:1024–36. doi: 10.1016/j.immuni.2017.11.015
5. Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, et al. Identification of an interleukin (IL)-25–dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med*. (2006) 203:1105–16. doi: 10.1084/jem.20051615
6. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T_H2 cytokines by adipose tissue-associated c-$Kit^+Sca\text{-}1^+$ lymphoid cells. *Nature*. (2010) 463:540–4. doi: 10.1038/nature08636
7. Price AE, Liang H-E, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13–expressing cells in type 2 immunity. *Proc Natl Acad Sci USA*. (2010) 107:11489–94. doi: 10.1073/pnas.1003988107
8. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TKA, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. (2010) 464:1367–70. doi: 10.1038/nature08900
9. Hoyler T, Klose CSN, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity*. (2012) 37:634–48. doi: 10.1016/j.immuni.2012.06.020
10. Klose CSN, Flach M, Möhle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell*. (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030

11. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *J Exp Med*. (2012) 209:729–40. doi: 10.1084/jem.20111594
12. Zhang K, Xu X, Pasha MA, Siebel CW, Costello A, Haczku AF, et al. Cutting edge: notch signaling promotes the plasticity of group-2 innate lymphoid cells. *J Immunol*. (2017) 198:1798–803. doi: 10.4049/jimmunol.1601421
13. Colonna M. Innate lymphoid cells: diversity, plasticity, and unique functions in immunity. *Immunity*. (2018) 48:1104–17. doi: 10.1016/j.immuni.2018.05.013
14. Tait Wojno E, Monticelli L, Tran S, Alenghat T, Osborne L, Thome J, et al. The prostaglandin D2 receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol*. (2015) 8:1313–23. doi: 10.1038/mi.2015.21
15. Wojno EDT, Artis D. Emerging concepts and future challenges in innate lymphoid cell biology. *J Exp Med*. (2016) 213:2229–48. doi: 10.1084/jem.20160525
16. Huang Y, Guo L, Qiu J, Chen X, Hu-Li J, Siebenlist U, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential "inflammatory" type 2 innate lymphoid cells. *Nat Immunol*. (2015) 16:161–9. doi: 10.1038/ni.3078
17. Turner J-E, Morrison PJ, Wilhelm C, Wilson M, Ahlfors H, Renauld J-C, et al. IL-9–mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J Exp Med*. (2013) 210:2951–65. doi: 10.1084/jem.20130071
18. Huang Y, Mao K, Chen X, Sun M, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science*. (2018) 359:114–9. doi: 10.1126/science.aam5809
19. Ricardo-Gonzalez RR, Van Dyken SJ, Schneider C, Lee J, Nussbaum JC, Liang H-E, et al. Tissue signals imprint ILC2 identity with anticipatory function. *Nat Immunol*. (2018) 19:1093–9. doi: 10.1038/s41590-018-0201-4
20. Li Z, Jackson RJ, Ranasinghe C. Vaccination route can significantly alter the innate lymphoid cell subsets: a feedback between IL-13 and IFN-γ. *NPJ Vaccines*. (2018) 3:10. doi: 10.1038/s41541-018-0048-6
21. Kim HY, Chang Y-J, Subramanian S, Lee H-H, Albacker LA, Matangkasombut P, et al. Innate lymphoid cells responding to IL-33 mediate airway-hyperreactivity independent of adaptive immunity. *J Allergy Clin Immunol*. (2012) 129:216–227.e6. doi: 10.1016/j.jaci.2011.10.036
22. Chang Y-J, DeKruyff RH, Umetsu DT. The role of type 2 innate lymphoid cells in asthma. *J Leukoc Biol*. (2013) 94:933–40. doi: 10.1189/jlb.0313127
23. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol*. (2012) 129:191–198.e1–4. doi: 10.1016/j.jaci.2011.09.041
24. Imai Y, Yasuda K, Sakaguchi Y, Haneda T, Mizutani H, Yoshimoto T, et al. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci USA*. (2013) 110:13921–6. doi: 10.1073/pnas.1307321110
25. Hardman CS, Chen Y-L, Salimi M, Jarrett R, Johnson D, Järvinen VJ, et al. CD1a presentation of endogenous antigens by group 2 innate lymphoid cells. *Sci Immunol*. (2017) 2:eaan5918. doi: 10.1126/sciimmunol.aan5918
26. Pulendran B, Artis D. New paradigms in type 2 immunity. *Science*. (2012) 337:431–5. doi: 10.1126/science.1221064
27. Lai D, Tang J, Chen L, Fan EK, Scott MJ, Li Y, et al. Group 2 innate lymphoid cells protect lung endothelial cells from pyroptosis in sepsis. *Cell Death Dis*. (2018) 9:369. doi: 10.1038/s41419-018-0412-5
28. Pichery M, Mirey E, Mercier P, Lefrancais E, Dujardin A, Ortega N, et al. Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: *in situ* analysis using a novel Il-33-LacZ gene trap reporter strain. *J Immunol*. (2012) 188:3488–95. doi: 10.4049/jimmunol.1101977
29. Wilson SR, Thé L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. *Cell*. (2013) 155:285–95. doi: 10.1016/j.cell.2013.08.05
30. Howitt MR, Lavoie S, Michaud M, Blum AM, Tran SV, Weinstock JV, et al. Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. *Science*. (2016) 351:1329–33. doi: 10.1126/science.aaf1648
31. Gerbe F, Sidot E, Smyth DJ, Ohmoto M, Matsumoto I, Dardalhon V, et al. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature*. (2016) 529:226–30. doi: 10.1038/nature16527
32. von Moltke J, Ji M, Liang H-E, Locksley RM. Tuft-cell-derived IL-25 regulates an intestinal ILC2–epithelial response circuit. *Nature*. (2016) 529:221–5. doi: 10.1038/nature16161
33. Mohapatra A, Van Dyken SJ, Schneider C, Nussbaum JC, Liang H-E, Locksley RM. Group 2 innate lymphoid cells utilize the IRF4-IL-9 module to coordinate epithelial cell maintenance of lung homeostasis. *Mucosal Immunol*. (2016) 9:275–86. doi: 10.1038/mi.2015.59
34. Pelly VS, Kannan Y, Coomes SM, Entwistle LJ, Rückerl D, Seddon B, et al. IL-4-producing ILC2s are required for the differentiation of TH2 cells following *Heligmosomoides polygyrus* infection. *Mucosal Immunol*. (2016) 9:1407–17. doi: 10.1038/mi.2016.4
35. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J Allergy Clin Immunol*. (2013) 132:933–41. doi: 10.1016/j.jaci.2013.05.012
36. Han H, Roan F, Johnston L, Bryce PJ, Ziegler SF. Distinct tissue-specific functions for TSLP and IL-33 in the atopic march. *J Immunol*. (2016) 196:68.6.
37. Vannella KM, Ramalingam TR, Borthwick LA, Barron L, Hart KM, Thompson RW, et al. Combinatorial targeting of TSLP, IL-25, and IL-33 in type 2 cytokine-driven inflammation and fibrosis. *Sci Transl Med*. (2016) 8:337ra65. doi: 10.1126/scitranslmed.aaf1938
38. Camelo A, Rosignoli G, Ohne Y, Stewart RA, Overed-Sayer C, Sleeman MA, et al. IL-33, IL-25, and TSLP induce a distinct phenotypic and activation profile in human type 2 innate lymphoid cells. *Blood Adv*. (2017) 1:577–89. doi: 10.1182/bloodadvances.2016002352
39. Van Dyken SJ, Mohapatra A, Nussbaum JC, Molofsky AB, Thornton EE, Ziegler SF, et al. Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and γδ T cells. *Immunity*. (2014) 40:414–24. doi: 10.1016/j.immuni.2014.02.003
40. Moussion C, Ortega N, Girard J-P. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*: a novel 'Alarmin'? *PLoS ONE*. (2008) 3:e3331. doi: 10.1371/journal.pone.0003331
41. Howard CL, Decker DC, Swanson IA, Blaine KM, Nobrega MA, Sperling AI. A novel role of human lung endothelial cells in allergic airway disease by producing and responding to IL-33. *J Immunol*. (2018) 200:44.17.
42. Pivniouk VI, Rosenbaum D, Herrell A, Pivniouk O, Rafaels N, Mathias R, et al. Differential regulation of human and mouse IL33 expression in the lungs of human IL33 BAC transgenic mice. *J Immunol*. (2016) 196:120.2.
43. Gerbe F, Legraverend C, Jay P. The intestinal epithelium tuft cells: specification and function. *Cell Mol Life Sci*. (2012) 69:2907–17. doi: 10.1007/s00018-012-0984-7
44. Grencis RK, Worthington JJ. Tuft cells: a new flavor in innate epithelial immunity. *Trends Parasitol*. (2016) 32:583–5. doi: 10.1016/j.pt.2016.04.016
45. Nadjsombati MS, McGinty JW, Lyons-Cohen MR, Jaffe JB, DiPeso L, Schneider C, et al. Detection of succinate by intestinal tuft cells triggers a type 2 innate immune circuit. *Immunity*. (2018) 49:33–41.e7. doi: 10.1016/j.immuni.2018.06.016
46. Saunders CJ, Christensen M, Finger TE, Tizzano M. Cholinergic neurotransmission links solitary chemosensory cells to nasal inflammation. *Proc Natl Acad Sci USA*. (2014) 111:6075–80. doi: 10.1073/pnas.1402251111
47. Haber AL, Biton M, Rogel N, Herbst RH, Shekhar K, Smillie C, et al. A single-cell survey of the small intestinal epithelium. *Nature*. (2017) 551:333–9. doi: 10.1038/nature24489
48. Bankova LG, Yoshimoto E, Giannetti MP, Kanaoka Y, Austen KF, Barrett NA. The leukotriene E4 receptor, CysLT3r regulates airway chemosensory brush cell expansion. *J Allergy Clin Immunol*. (2018) 141:AB400. doi: 10.1016/j.jaci.2017.12.940
49. Bouchery T, Marsland BJ. Airway brush cells: not as "tuft" as you might think. *Sci Immunol*. (2018) 3:eaau8719. doi: 10.1126/sciimmunol.aau8719
50. Schneider C, O'Leary CE, von Moltke J, Liang H-E, Ang QY, Turnbaugh PJ, et al. A metabolite-triggered tuft cell-ILC2 circuit drives small intestinal remodeling. *Cell*. (2018) 174:271–284.e14. doi: 10.1016/j.cell.2018.05.014
51. Lei W, Ren W, Ohmoto M, Urban JF, Matsumoto I, Margolskee RF, et al. Activation of intestinal tuft cell-expressed Sucnr1 triggers type 2 immunity in the mouse small intestine. *Proc Natl Acad Sci USA*. (2018) 115:5552–7. doi: 10.1073/pnas.1720758115

52. Cardoso V, Chesné J, Ribeiro H, García-Cassani B, Carvalho T, Bouchery T, et al. Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature*. (2017) 549:277–81. doi: 10.1038/nature23469
53. Talbot S, Abdulnour R-EE, Burkett PR, Lee S, Cronin SJF, Pascal MA, et al. Silencing nociceptor neurons reduces allergic airway inflammation. *Neuron*. (2015) 87:341–54. doi: 10.1016/j.neuron.2015.06.007
54. Wallrapp A, Riesenfeld SJ, Burkett PR, Abdulnour R-EE, Nyman J, Dionne D, et al. The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation. *Nature*. (2017) 549:351–6. doi: 10.1038/nature24029
55. Yu X, Pappu R, Ramirez-Carrozzi V, Ota N, Caplazi P, Zhang J, et al. TNF superfamily member TL1A elicits type 2 innate lymphoid cells at mucosal barriers. *Mucosal Immunol*. (2014) 7:730–40. doi: 10.1038/mi.2013.92
56. Meylan F, Hawley ET, Barron L, Barlow JL, Penumetcha P, Pelletier M, et al. The TNF-family cytokine TL1A promotes allergic immunopathology through group 2 innate lymphoid cells. *Mucosal Immunol*. (2014) 7:958–68. doi: 10.1038/mi.2013.114
57. Denney L, Byrne AJ, Shea TJ, Buckley JS, Pease JE, Herledan GMF, et al. Pulmonary epithelial cell-derived cytokine TGF-β1 is a critical cofactor for enhanced innate lymphoid cell function. *Immunity*. (2015) 43:945–58. doi: 10.1016/j.immuni.2015.10.012
58. Rigas D, Lewis G, Aron JL, Wang B, Banie H, Sankaranarayanan I, et al. Type 2 innate lymphoid cell suppression by regulatory T cells attenuates airway hyperreactivity and requires inducible T-cell costimulator-inducible T-cell costimulator ligand interaction. *J Allergy Clin Immunol*. (2017) 139:1468–1477.e2. doi: 10.1016/j.jaci.2016.08.034
59. Krishnamoorthy N, Burkett PR, Dalli J, Abdulnour R-EE, Colas R, Ramon S, et al. Cutting edge: maresin-1 engages regulatory T cells to limit type 2 innate lymphoid cell activation and promote resolution of lung inflammation. *J Immunol*. (2015) 194:863–7. doi: 10.4049/jimmunol.1402534
60. Moro K, Kabata H, Tanabe M, Koga S, Takeno N, Mochizuki M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol*. (2016) 17:76–86. doi: 10.1038/ni.3309
61. Kabata H, Moro K, Koyasu S. The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms. *Immunol Rev*. (2018) 286:37–52. doi: 10.1111/imr.12706
62. Hunter CA, Kastelein R. Interleukin-27: balancing protective and pathological immunity. *Immunity*. (2012) 37:960–9. doi: 10.1016/j.immuni.2012.11.003
63. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol*. (2008) 180:5771–7. doi: 10.4049/jimmunol.180.9.5771
64. Oriss TB, McCarthy SA, Morel BF, Campana MA, Morel PA. Crossregulation between T helper cell (Th)1 and Th2: inhibition of Th2 proliferation by IFN-gamma involves interference with IL-1. *J Immunol*. (1997) 158:3666–72.
65. Duerr CU, McCarthy CDA, Mindt BC, Rubio M, Meli AP, Pothlichet J, et al. Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat Immunol*. (2016) 17:65–75. doi: 10.1038/ni.3308
66. Califano D, Furuya Y, Roberts S, Avram D, McKenzie ANJ, Metzger DW. IFN-γ increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells. *Mucosal Immunol*. (2018) 11:209–19. doi: 10.1038/mi.2017.41
67. Molofsky AB, Van Gool F, Liang H-E, Van Dyken SJ, Nussbaum JC, Lee J, et al. Interleukin-33 and interferon-γ counter-regulate group 2 innate lymphoid cell activation during immune perturbation. *Immunity*. (2015) 43:161–74. doi: 10.1016/j.immuni.2015.05.019
68. Mchedlidze T, Kindermann M, Neves AT, Voehringer D, Neurath MF, Wirtz S. IL-27 suppresses type 2 immune responses *in vivo* via direct effects on group 2 innate lymphoid cells. *Mucosal Immunology*. (2016) 9:1384–94. doi: 10.1038/mi.2016.20
69. Konya V, Mjösberg J. Lipid mediators as regulators of human ILC2 function in allergic diseases. *Immunol Lett*. (2016) 179:36–42. doi: 10.1016/j.imlet.2016.07.006
70. Shimokawa C, Kanaya T, Hachisuka M, Ishiwata K, Hisaeda H, Kurashima Y, et al. Mast cells are crucial for induction of group 2 innate lymphoid cells and clearance of helminth infections. *Immunity*. (2017) 46:863–874.e4. doi: 10.1016/j.immuni.2017.04.017
71. Tung H-Y, Plunkett B, Huang S-K, Zhou Y. Murine mast cells secrete and respond to interleukin-33. *J Interferon Cytokine Res*. (2014) 34:141–7. doi: 10.1089/jir.2012.0066
72. Furukawa S, Moriyama M, Miyake K, Nakashima H, Tanaka A, Maehara T, et al. Interleukin-33 produced by M2 macrophages and other immune cells contributes to Th2 immune reaction of IgG4-related disease. *Sci Rep*. (2017) 7:42413. doi: 10.1038/srep42413
73. Lefrançais E, Duval A, Mirey E, Roga S, Espinosa E, Cayrol C, et al. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc Natl Acad Sci USA*. (2014) 111:15502–7. doi: 10.1073/pnas.1410700111
74. Lefrançais E, Roga S, Gautier V, Gonzalez-de-Peredo A, Monsarrat B, Girard J-P, et al. IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc Natl Acad Sci USA*. (2012) 109:1673–8. doi: 10.1073/pnas.1115884109
75. Bouchery T, Harris NL. Only two can tango: mast cells displace epithelial cells to dance with ILC2s. *Immunity*. (2017) 46:766–8. doi: 10.1016/j.immuni.2017.04.020
76. Csóka B, Németh ZH, Duerr CU, Fritz JH, Pacher P, Haskó G. Adenosine receptors differentially regulate type 2 cytokine production by IL-33-activated bone marrow cells, ILC2s, and macrophages. *FASEB J*. (2018) 32:829–37. doi: 10.1096/fj.201700770R
77. Patel N, Wu W, Mishra PK, Chen F, Millman A, Csóka B, et al. A2B adenosine receptor induces protective antihelminth type 2 immune responses. *Cell Host Microbe*. (2014) 15:339–50. doi: 10.1016/j.chom.2014.02.001
78. Samuchiwal SK, Boyce JA. Role of lipid mediators and control of lymphocyte responses in type 2 immunopathology. *J Allergy Clin Immunol*. (2018) 141:1182–90. doi: 10.1016/j.jaci.2018.02.006
79. Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie ANJ, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol*. (2014) 133:1184–94. doi: 10.1016/j.jaci.2013.10.056
80. Chang JE, Doherty TA, Baum R, Broide D. Prostaglandin D2 regulates human type 2 innate lymphoid cell chemotaxis. *J Allergy Clin Immunol*. (2014) 133:899–901.e3. doi: 10.1016/j.jaci.2013.09.020
81. Trabanelli S, Gomez-Cadena A, Salomé B, Michaud K, Mavilio D, Landis BN, et al. Human innate lymphoid cells (ILCs): toward a uniform immune-phenotyping. *Cytometry B Clin Cytom*. (2018) 94:392–9. doi: 10.1002/cyto.b.21614
82. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol*. (2013) 132:205–13. doi: 10.1016/j.jaci.2013.03.048
83. von Moltke J, O'Leary CE, Barrett NA, Kanaoka Y, Austen KF, Locksley RM. Leukotrienes provide an NFAT-dependent signal that synergizes with IL-33 to activate ILC2s. *J Exp Med*. (2017) 214:27–37. doi: 10.1084/jem.20161274
84. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci Transl Med*. (2013) 5:174ra26. doi: 10.1126/scitranslmed.3004812
85. Zhou W, Toki S, Zhang J, Goleniewksa K, Newcomb DC, Cephus JY, et al. Prostaglandin I2 signaling and inhibition of group 2 innate lymphoid cell responses. *Am J Respir Crit Care Med*. (2016) 193:31–42. doi: 10.1164/rccm.201410-1793OC
86. Lund SJ, Portillo A, Cavagnero K, Baum RE, Naji LH, Badrani JH, et al. Leukotriene C4 potentiates IL-33–induced group 2 innate lymphoid cell activation and lung inflammation. *J Immunol*. (2017) 199:1096–104. doi: 10.4049/jimmunol.1601569
87. Grencis RK. Immunity to helminths: resistance, regulation, and susceptibility to gastrointestinal nematodes. *Annu Rev Immunol*. (2015) 33:201–25. doi: 10.1146/annurev-immunol-032713-120218
88. Filbey K, Bouchery T, Le Gros G. The role of ILC2 in hookworm infection. *Parasite Immunol*. (2018) 40:e12429. doi: 10.1111/pim.12429
89. Huang L, Appleton JA. Eosinophils in helminth infection: defenders and dupes. *Trends Parasitol*. (2016) 32:798–807. doi: 10.1016/j.pt.2016.05.004
90. Wilhelm C, Harrison OJ, Schmitt V, Pelletier M, Spencer SP, Urban JF, et al. Critical role of fatty acid metabolism in ILC2-mediated barrier protection

during malnutrition and helminth infection. *J Exp Med*. (2016) 213:1409–18. doi: 10.1084/jem.20151448
91. Saenz SA, Siracusa MC, Perrigoue JG, Spencer SP, Urban JF, Tocker JE, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature*. (2010) 464:1362–6. doi: 10.1038/nature08901
92. Zaiss MM, Maslowski KM, Mosconi I, Guenat N, Marsland BJ, Harris NL. IL-1β suppresses innate IL-25 and IL-33 production and maintains helminth chronicity. *PLoS Pathog*. (2013) 9:e1003531. doi: 10.1371/journal.ppat.1003531
93. Owyang AM, Zaph C, Wilson EH, Guild KJ, McClanahan T, Miller HRP, et al. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J Exp Med*. (2006) 203:843–9. doi: 10.1084/jem.20051496
94. Inclan-Rico JM, Siracusa MC. First responders: innate immunity to helminths. *Trends Parasitol*. (2018) 34:861–80. doi: 10.1016/j.pt.2018.08.007
95. Allen JE, Sutherland TE. Host protective roles of type 2 immunity: parasite killing and tissue repair, flip sides of the same coin. *Semin Immunol*. (2014) 26:329–40. doi: 10.1016/j.smim.2014.06.003
96. Zaiss DM, Yang L, Shah PR, Kobie JJ, Urban JF, Mosmann TR. Amphiregulin, a TH2 cytokine enhancing resistance to nematodes. *Science*. (2006) 314:1746. doi: 10.1126/science.1133715
97. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CGK, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol*. (2011) 12:1045–54. doi: 10.1038/ni.2131
98. Wilhelm C, Hirota K, Stieglitz B, Van Snick J, Tolaini M, Lahl K, et al. An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat Immunol*. (2011) 12:1071–7. doi: 10.1038/ni.2133
99. Monticelli LA, Osborne LC, Noti M, Tran SV, Zaiss DMW, Artis D. IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin–EGFR interactions. *Proc Natl Acad Sci USA*. (2015) 112:10762–7. doi: 10.1073/pnas.1509070112
100. Monticelli LA, Buck MD, Flamar A-L, Saenz SA, Tait Wojno ED, Yudanin NA, et al. Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation. *Nat Immunol*. (2016) 17:656–65. doi: 10.1038/ni.3421
101. Sugita K, Steer CA, Martinez-Gonzalez I, Altunbulakli C, Morita H, Castro-Giner F, et al. Type 2 innate lymphoid cells disrupt bronchial epithelial barrier integrity by targeting tight junctions through IL-13 in asthmatic patients. *J Allergy Clin Immunol*. (2018) 141:300–310.e11. doi: 10.1016/j.jaci.2017.02.038
102. Halim TYF, Steer CA, Mathä L, Gold MJ, Martinez-Gonzalez I, McNagny KM, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity*. (2014) 40:425–35. doi: 10.1016/j.immuni.2014.01.011
103. Oliphant CJ, Hwang YY, Walker JA, Salimi M, Wong SH, Brewer JM, et al. MHCII-mediated dialog between group 2 innate lymphoid cells and CD4+ T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity*. (2014) 41:283–95. doi: 10.1016/j.immuni.2014.06.016
104. Mirchandani AS, Besnard A-G, Yip E, Scott C, Bain CC, Cerovic V, et al. Type 2 innate lymphoid cells drive CD4+ Th2 cell responses. *J Immunol*. (2014) 192:2442–8. doi: 10.4049/jimmunol.1300974
105. Halim TYF, Rana BMJ, Walker JA, Kerscher B, Knolle MD, Jolin HE, et al. Tissue-restricted adaptive type 2 immunity is orchestrated by expression of the costimulatory molecule OX40L on group 2 innate lymphoid cells. *Immunity*. (2018) 48:1195–1207.e6. doi: 10.1016/j.immuni.2018.05.003
106. Mjösberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity*. (2012) 37:649–59. doi: 10.1016/j.immuni.2012.08.015
107. Roediger B, Kyle R, Tay SS, Mitchell AJ, Bolton HA, Guy TV, et al. IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation. *J Allergy Clin Immunol*. (2015) 136:1653–1663.e7. doi: 10.1016/j.jaci.2015.03.043
108. Bouchery T, Kyle R, Camberis M, Shepherd A, Filbey K, Smith A, et al. ILC2s and T cells cooperate to ensure maintenance of M2 macrophages for lung immunity against hookworms. *Nat Commun*. (2015) 6:6970. doi: 10.1038/ncomms7970
109. Halim TY, Hwang YY, Scanlon ST, Zaghouani H, Garbi N, Fallon PG, et al. Group 2 innate lymphoid cells license dendritic cells to potentiate memory T helper 2 cell responses. *Nat Immunol*. (2016) 17:57–64. doi: 10.1038/ni.3294
110. Martinez-Gonzalez I, Mathä L, Steer CA, Ghaedi M, Poon GFT, Takei F. Allergen-experienced group 2 innate lymphoid cells acquire memory-like properties and enhance allergic lung inflammation. *Immunity*. (2016) 45:198–208. doi: 10.1016/j.immuni.2016.06.017
111. Riedel J-H, Becker M, Kopp K, Düster M, Brix SR, Meyer-Schwesinger C, et al. IL-33-mediated expansion of type 2 innate lymphoid cells protects from progressive glomerulosclerosis. *J Am Soc Nephrol*. (2017) 28:2068–80. doi: 10.1681/ASN.2016080877
112. Drake LY, Iijima K, Bartemes K, Kita H. Group 2 innate lymphoid cells promote an early antibody response to a respiratory antigen in mice. *J Immunol*. (2016) 197:1335–42. doi: 10.4049/jimmunol.1502669
113. Anthony RM, Rutitzky LI, Urban JF, Stadecker MJ, Gause WC. Protective immune mechanisms in helminth infection. *Nat Rev Immunol*. (2007) 7:975–87. doi: 10.1038/nri2199
114. Esser-von Bieren J, Mosconi I, Guiet R, Piersgilli A, Volpe B, Chen F, et al. Antibodies trap tissue migrating helminth larvae and prevent tissue damage by driving IL-4Rα-independent alternative differentiation of macrophages. *PLoS Pathog*. (2013) 9:e1003771. doi: 10.1371/journal.ppat.1003771
115. Schwartz C, Turqueti-Neves A, Hartmann S, Yu P, Nimmerjahn F, Voehringer D. Basophil-mediated protection against gastrointestinal helminths requires IgE-induced cytokine secretion. *Proc Natl Acad Sci USA*. (2014) 111:E5169–77. doi: 10.1073/pnas.1412663111
116. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*. (2010) 125:S73–80. doi: 10.1016/j.jaci.2009.11.017
117. Maizels RM, McSorley HJ. Regulation of the host immune system by helminth parasites. *J Allergy Clin Immunol*. (2016) 138:666–75. doi: 10.1016/j.jaci.2016.07.007
118. Filbey KJ, Grainger JR, Smith KA, Boon L, van Rooijen N, Harcus Y, et al. Innate and adaptive type 2 immune cell responses in genetically controlled resistance to intestinal helminth infection. *Immunol Cell Biol*. (2014) 92:436–48. doi: 10.1038/icb.2013.109
119. McSorley HJ, Blair NF, Smith KA, McKenzie ANJ, Maizels RM. Blockade of IL-33 release and suppression of type 2 innate lymphoid cell responses by helminth secreted products in airway allergy. *Mucosal Immunol*. (2014) 7:1068–78. doi: 10.1038/mi.2013.123
120. Osbourn M, Soares DC, Vacca F, Cohen ES, Scott IC, Gregory WF, et al. HpARI protein secreted by a helminth parasite suppresses interleukin-33. *Immunity*. (2017) 47:739–751.e5. doi: 10.1016/j.immuni.2017.09.015
121. Zaiss MM, Rapin A, Lebon L, Dubey LK, Mosconi I, Sarter K, et al. The intestinal microbiota contributes to the ability of helminths to modulate allergic inflammation. *Immunity*. (2015) 43:998–1010. doi: 10.1016/j.immuni.2015.09.012
122. Thio CL-P, Chi P-Y, Lai AC-Y, Chang Y-J. Regulation of type 2 innate lymphoid cell-dependent airway hyperreactivity by butyrate. *J Allergy Clin Immunol*. (2018) 142:1867–1883.e12. doi: 10.1016/j.jaci.2018.02.032
123. Peebles RS. At the bedside: the emergence of group 2 innate lymphoid cells in human disease. *J Leukoc Biol*. (2015) 97:469–75. doi: 10.1189/jlb.3BT0814-383R
124. Bernink JH, Germar K, Spits H. The role of ILC2 in pathology of type 2 inflammatory diseases. *Curr Opin Immunol*. (2014) 31:115–20. doi: 10.1016/j.coi.2014.10.007
125. Jeffery HC, McDowell P, Lutz P, Wawman RE, Roberts S, Bagnall C, et al. Human intrahepatic ILC2 are IL-13positive amphiregulinpositive and their frequency correlates with model of end stage liver disease score. *PLoS ONE*. (2017) 12:e0188649. doi: 10.1371/journal.pone.0188649
126. Salimi M, Stöger L, Liu W, Go S, Pavord I, Klenerman P, et al. Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. *J Allergy Clin Immunol*. (2017) 140:1090-1100.e11. doi: 10.1016/j.jaci.2016.12.958
127. Snelgrove RJ, Lloyd CM. Tasting type 2 inflammation in the airways. *J Allergy Clin Immunol*. (2018) 142:403–4. doi: 10.1016/j.jaci.2018.06.009

128. Kohanski MA, Workman AD, Patel NN, Hung L-Y, Shtraks JP, Chen B, et al. Solitary chemosensory cells are a primary epithelial source of IL-25 in patients with chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol*. (2018) 142:460–469.e7. doi: 10.1016/j.jaci.2018.03.019
129. Shin H-W, Kim D-K, Park M-H, Eun KM, Lee M, So D, et al. IL-25 as a novel therapeutic target in nasal polyps of patients with chronic rhinosinusitis. *J Allergy Clin Immunol*. (2015) 135:1476–1485.e7. doi: 10.1016/j.jaci.2015.01.003
130. Boyd A, Ribeiro JMC, Nutman TB. Human CD117 (cKit)+ innate lymphoid cells have a discrete transcriptional profile at homeostasis and are expanded during filarial infection. *PLoS ONE*. (2014) 9:e108649. doi: 10.1371/journal.pone.0108649
131. Nausch N, Appleby LJ, Sparks AM, Midzi N, Mduluza T, Mutapi F. Group 2 innate lymphoid cell proportions are diminished in young helminth infected children and restored by curative anti-helminthic treatment. *PLoS Negl Trop Dis*. (2015) 9:e0003627. doi: 10.1371/journal.pntd.0003627
132. Smith KA, Löser S, Varyani F, Harcus Y, McSorley HJ, McKenzie AN, et al. Concerted IL-25R and IL-4Rα signaling drive innate type 2 effector immunity for optimal helminth expulsion. *eLife*. (2018) 7:e38269. doi: 10.7554/eLife.38269

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: 10 April 2019
doi: 10.3389/fimmu.2019.00620

Innate Lymphoid Cells in Helminth Infections—Obligatory or Accessory?

Stephan Löser[1], *Katherine A. Smith*[2,3] *and Rick M. Maizels*[1]*

[1] Wellcome Centre for Integrative Parasitology, Institute of Infection, Immunology and Inflammation, University of Glasgow, Glasgow, United Kingdom, [2] Cardiff Institute of Infection and Immunity, Cardiff University, Cardiff, United Kingdom, [3] Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

ILCs burst onto the immunological scene with their involvement in bacterial and helminth infections. As their influence has emerged, it has become clear that they play a fundamental role in regulating barrier tissue homeostasis and the immune response during inflammation. A subset of ILCs, ILC2s, has become the focus of attention for many helminth biologists—stepping into the limelight as both the elusive initiator and amplifier of the type-2 response. In many of the early reports, conclusions as to their function were based on experiments using unadapted parasites or immune-compromised hosts. In this review we re-examine the generation and function of type-2 ILCs in helminth infection and the extent to which their roles may be essential or redundant, in both primary and challenge infections. ILC2s will be discussed in terms of a broader innate network, which when in dialogue with adaptive immunity, allows the generation of the anti-parasite response. Finally, we will review how helminths manipulate ILC2 populations to benefit their survival, as well as dampen systemic inflammation in the host, and how this understanding may be used to improve strategies to control disease.

Keywords: parasites, immunity, alarmin, cytokines, receptors

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Thomas Nutman,
National Institutes of Health (NIH), United States
Yuefeng Huang,
Columbia University, United States

***Correspondence:**
Rick M. Maizels
rick.maizels@glasgow.ac.uk

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

Received: *20 December 2018*
Accepted: *08 March 2019*
Published: *10 April 2019*

Citation:
Löser S, Smith KA and Maizels RM (2019) Innate Lymphoid Cells in Helminth Infections—Obligatory or Accessory? Front. Immunol. 10:620. doi: 10.3389/fimmu.2019.00620

INTRODUCTION

Parasitic helminth infections by tapeworms, roundworms or flukes present an ongoing threat to human health and quality of life, but also significantly dampen agricultural productivity through infection of livestock. Worldwide, over 2 billion people are infected with helminth parasites (1), and in the absence of any vaccines, control relies on repeated drug treatment. Understanding immunity to helminths is essential to develop new vaccines to eliminate these infections.

Until about a decade ago it was suggested that resistance to helminth infection relies predominantly on T helper 2 (Th2) cells of the adaptive immune system, orchestrating nematode expulsion in an antigen-specific manner. Since then a new player has entered the game, the type-2 innate lymphoid cells (ILC2), first discovered in T- and B-cell-deficient mice as a subset rapidly releasing type-2 cytokines, in response to stimulation by the alarmin IL-25 (2, 3).

Thereafter, convergent approaches, including functional testing of new IL-17 family members (3–5), determining innate sources of type-2 cytokines (6–8) and the characterization of mesenteric lymphoid clusters (9), all led to the identification of an innate lymphocyte population, capable of producing type-2 cytokines, albeit with varied monikers such as "nuocytes" (6) or "natural helper cells" (9). Soon re-branded as ILC2s, these innate cells were found localized to differing tissues and therefore primed to act as first responders to the immune challenges such as helminth infections before adaptive immunity has developed (10).

Since the discovery of these innate Th2 cell surrogates, research delineating ILC2 functions, especially in the disease settings of helminth infection and allergic asthma, have dominated the scientific field of type-2 immunity. Wide-ranging studies have reported on the cells' origin and differentiation, plasticity, mobility, functionality and communication with various other cells of the immune system, and beyond (11, 12). Much has been learned from mouse models of intestinal helminth infection, in particular *Nippostrongylus brasiliensis,* a rat parasite which is poorly adapted (and thus readily dislodged) from mice, and 2 natural parasites of mice, *Heligmosomoides polygyrus* and *Trichuris muris* (**Figure 1**). ILC2s are also stimulated by helminths in the tissues, such as *Litomosoides sigmodontis* in the pleural cavity (13) and by the eggs of *Schistosoma mansoni* in the liver and lungs (14).

GENERATION AND REGULATION OF ILC2 ACTIVATION

In mice, the development of both ILC2s and Th2 cells starts with common lymphoid progenitors (CLPs) of the bone marrow (15, 16), but branches in two different directions. In fact, the development of all T lymphocytes begins with the differentiation of lymphoblasts from CLPs, their migration to the thymus, then if surviving both positive- and negative selection, they emerge as highly specific but antigen-naïve immune cells patrolling through the circulation, secondary lymphoid tissue and peripheral sites. Importantly, T cells only polarize into functional Th2 effector cells following specific antigen encounter in the presence of interleukin 4 (IL-4) (17).

In contrast, the development of ILC2s is rather primitive. CLPs give rise to common innate lymphoid progenitors (CILPs) dependent on the expression of various transcription factors, amongst others inhibitor of DNA binding 2 (Id2) and nuclear factor IL-3 induced (Nfil3) (9, 18, 19). Yang et al suggest that early innate lymphoid cell progenitors (EILPs) resemble an intermediate step in the differentiation of CLPs into CILPs. The development of this T cell factor-1 (TCF-1) positive population is still independent of Id2 and EILPs are progeny of both NK cells and ILCs (20). CILPs give rise to common helper innate lymphoid progenitors (CHILP) which differentiate based on promyelocytic leukemia zinc finger (Plzf) expression into ILC precursors (ILCPs) (21), able to polarize into three different innate lymphoid cell populations, ILC1s via expression of Tbx21/T-bet (22, 23), GATA3 positive ILC2s (15, 24) or, based on Roryt expression, ILC3s (25, 26).

This broad classification of 3 ILC subsets may hide important distinctions within, for example, ILC2s which have been divided into natural and inflammatory, responsive to different cytokines (27), or between cells of similar cytokine profiles but different tissue residence, location or preference for skin, lung, or gut (28). ILC2s are mainly regarded as tissue–resident, expanding upon helminth infection at the respective mucosal site (29). This model has been challenged recently in a study demonstrating the migratory capabilities of IL-25-induced "inflammatory ILC2s" of the intestine (30, 31). These, when activated by IL-25, were found to enter the lymphatics and transfer to the lung where they contribute to the anti-helminth response. Controversy also arises over whether these ILC subsets are committed lineages, or show

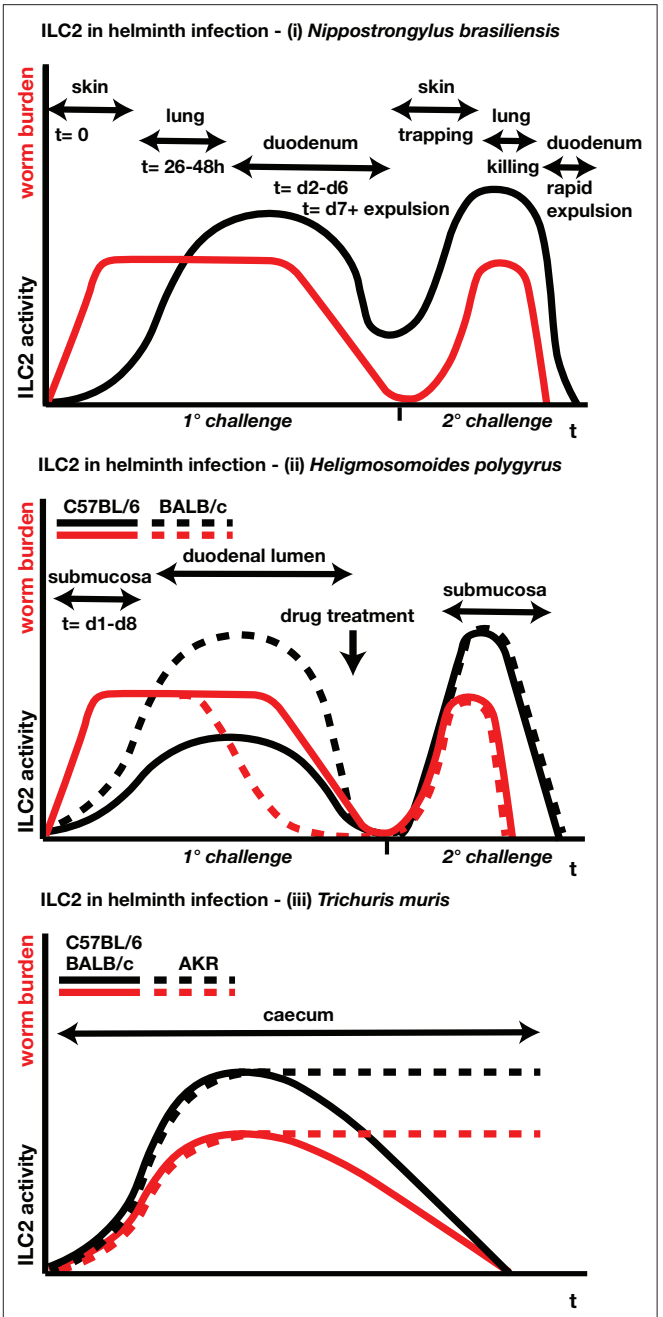

FIGURE 1 | Schematic time-lines for parasite burdens and ILC2 responses for (i) *N. brasiliensis,* (ii) *H. polygyrus,* and (iii) *T. muris*. Red lines indicate parasite burdens (larval/adult numbers, egg production); black lines indicate ILC2 activity (proliferation, cytokine release). Location and timing of different parasite stages are indicated where appropriate. For *N. brasiliensis* and *H. polygyrus*, both primary and secondary infections are shown; primary *N. brasiliensis* infections are expelled naturally, while for *H. polygyrus* anthelmintic drug treatment is required in susceptible strains to clear the primary infection prior to a challenge re-infection. For *H. polygyrus* and *T. muris*, separate lines indicate genetically resistant and susceptible strains of mice.

plasticity akin to their adaptive T cell cousins; while commitment would represent "trained immunity" in which the innate immune system is more prepared for subsequent encounters with the same pathogen, it might lock the innate response into an inappropriate and potentially pathogenic program that is difficult for the host to reverse.

HELMINTHS AND ILC2s—ANCIENT ANTAGONISTS?

ILC2s and Th2 cells share common attributes, but they also differ in the complexity of their development, the rules governing their tissue tropism, and the specificity of immune stimuli to which they respond (30). This poses the question of whether the more complex and target-specific Th2 cells developed evolutionarily from ILC2s, or if both type-2 immune cell populations share vertical descent from one ancestral population. The first would imply that ILC2s emerged prior to Th2 cells, potentially as an elementary cell type to combat helminth infection in ancient hosts.

To shed light on the evolutionary development of the three different ILC populations, including ILC2s, Vivier et al analyzed comparative expression of critical genes in different vertebrate species from the lamprey to mammals (32). Notably, a significant part of the ILC progenitor gene profile, including *Id2*, *Nfil3* and *Gata3,* as well as parts of the ILC2 profile, including *Ptpn13*, *Ar*, *Rxrg*, *Ccr8,* and *Hs3st1* are already evident in jawless vertebrates, whereas the *Gata3*-controlled type-2 cytokine genes, *Il5* and *Il13*, crucial for ILC2 and Th2 cell effector functions in mice and humans, were exclusively found in birds and mammals (32).

This initial study certainly suggests that ILC2s emerged in evolution of the earliest agnathan vertebrates, in response to helminth parasites. Even today, sea lamprey intestines contain a variety of nematode species, ingested while feeding on fish (33). A different perspective on the evolutionary development comes from a phylogenetic analysis of genes crucial for ILC2 activation in mammals. Here it has been highlighted that lampreys do express IL-17 family orthologs, amongst them IL-25 (34). Orthologs of the crucial receptor heterodimer subunit IL-17RB for IL-25 signaling in humans and mice however, have been detected only from the emergence of cartilaginous fish onwards and have not been found in jawless fish (34).

Phylogenetic analyses of genes involved in the differentiation of different immune cell populations suggest that ILC2s, as T-cells, developed in ancient mammalian ancestors. Substantial differences in the differentiation of both cell types certainly suggest that their relationship is distinct and that ILC2s are closely related to the other ILC subtypes. A different perspective might be provided through comparisons of genome-wide chromatin accessibility of both populations during helminth infection (35). Hierarchical clustering of these regulatory ensembles—termed "regulomes" within Th2 cells and ILC2s isolated from lungs at day 10 post *N. brasiliensis* infection confirmed the difference between pre-established ILC2s and naïve CD4 T cells. Regulomes of both cells during infection showed high similarity, not discounting a potential close relationship between both cell types during evolution and more ancient ILC ancestor that has preceded Th2 cells (35).

This perspective is further supported by the aforementioned study on the migration of activated iILC2s, highlighting that both T cells and iILC2s share the capability to migrate along a sphingosine-1-phosphate (S1P) gradient, a mechanism crucial for effector T cells to leave lymphoid tissues and essential for iILC2s to enter the lymphatics from the intestine (31). The report extends the parallelism of both type-2 cell types beyond the level of genomics and immune effector functions not excluding that the S1P trafficking mechanism of T cells was adopted from ancient ILCs (30).

ALARMIN ACTIVATION OF ILC2s—THE ROLE OF IL-25

As discussed above, ILC2 development requires the transcription factor GATA3 (24) and the nuclear receptor RORα (15, 36). Once differentiated, ILC2s are highly responsive to the alarmins, IL-25, IL-33 and TSLP, binding, respectively to the heterodimeric receptors IL17RA/IL17RB, ST2/IL1RacP, and TSLPR(CRLF2)/IL7Rα (37), while also requiring signaling through the common cytokine receptor γ chain (γ_c) that is shared by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (9).

IL-25 is closely linked, in presence and function, with helminth infections, and was first characterized by elevated expression in the small intestine following *N. brasiliensis* infection, leading to IL-5 production by the then unnamed ILC2s (3). IL-25$^{-/-}$ mice were then shown to be unable to expel *T. muris,* while genetically susceptible mice given exogenous IL-25 became resistant to infection (38). Similarly, IL-25$^{-/-}$ mice were used to demonstrate that IL-25-dependent activation of ILC2s is required for protective immunity to *N. brasiliensis* infection (4). Moreover, it was demonstrated that transfer of IL-25-stimulated ILCs could mediate expulsion of *N. brasiliensis* in mouse strains lacking key type-2 components, such as IL-13, IL-25, or IL-33 (6). In particular, while exogenous IL-25 induced worm clearance in RAG-deficient mice (31), it did not do so in animals lacking both adaptive immunity and ILCs (Rag-2$^{-/-}$ x $\gamma_c^{-/-}$ mice) (7). This latter study also demonstrated that transfer of ILCs alone was not able to induce worm clearance in Rag-2$^{-/-}$ x $\gamma_c^{-/-}$ mice unless exogenous IL-25 was administered to recipient animals.

Subsequently, IL-25 has been shown to activate a variety of innate cells, including multi-potent progenitor type-2 cells (39), NKT cells (40, 41) as well as monocytes and eosinophils (42). Within the ILC2 population, it has been proposed that one subset of "inflammatory ILC2s" (iILC2s) preferentially express the IL-25 receptor, and not the IL-33 receptor ST2 (27). Inflammatory ILC2s arise early in *N. brasiliensis* infection and give rise to IL-33-responsive "natural ILC2s" for worm expulsion, although it was also found that iILC2s, co-expressing GATA3, and RORγt, have the capacity to switch to IL-17 producing ILC3s in the different setting of fungal infection (27).

Recently, it was discovered that epithelial tuft cells of the small intestine detect the presence of helminths and release IL-25 (43–45), resulting in ILC2 proliferation (44) (**Figure 2A**). While the helminth derived mediator responsible for activating the tuft cell signaling cascade is still unknown, it is clear that protozoans are capable to induce tuft cells and successively ILC2 activation, through the release of succinate and stimulation of the succinate receptor GPR91 (46, 47). The activation of intestinal ILC2s by IL-25 is negatively regulated by the deubiquitinase A20 (*Tnfaip3*), which binds and inhibits the IL-25 receptor subunit IL-17RA (48). During infection with *N. brasiliensis*, A20 expression in ILC2s is downregulated, allowing increased pathogen-induced ILC2 proliferation (49).

While two other key alarmin cytokines, IL-33, discussed below, and thymic stromal lymphopoietin (TSLP) are able to induce ILC2 expansion, the responsiveness to these differs between ILC2 populations located at various mucosal sites. Small intestinal ILC2s express elevated levels of IL-17RB and are therefore responsive to tuft cell-derived IL-25, whereas pulmonary and adipose tissue ILC2s express high levels of ST2, resulting in increased IL-33 sensitivity (50). While not essential for ILC differentiation, TSLP is particularly important for activating ILC2s in the skin, for example in models of atopic dermatitis, which is abated in *Tslpr*$^{-/-}$ mice (51) and is also required for immunity to *T. muris* in the intestinal tract (52). However, TSLP also activates dendritic cells to promote adaptive Th2 responses (53), and acts on other innate populations such as basophils (54), so that it promotes type 2 responses at multiple and complementary levels.

IL-33, KEY PLAYER FOR ILC2s

IL-33, an IL-1-like cytokine that potently activates the type-2 response, is released from damaged epithelial cells (**Figure 2B**), and from innate subsets such as mast cells responding to damage signals from their environment (55–57). The role of IL-33 in ILC2 activation has been explored intensively by utilizing murine helminth models with pulmonary life cycle stages, including *N. brasiliensis* and the threadworm *Strongyloides venezuelensis*. Both induce a significant increase in lung/airway IL-33, IL-33 dependent ILC2 activation, type-2 cytokine release and eosinophilia (6, 7, 9, 58, 59). IL-33-deficient mice show a significantly elevated intestinal *N. brasiliensis* worm burden at day 6 p.i. and increased fecal egg counts at day 8 post *S. venezuelensis* infection, underlining a contribution of IL-33 activated ILC2s to the clearance of these nematodes (58, 59). Mice lacking the IL-33-specific receptor subunit ST2 are also highly susceptible to *H. polygyrus* infection (60), in addition, exogenous IL-33 confers resistance on mice genetically susceptible to *T. muris* (61).

As deficiency in either IL-25 or IL-33 results in greatly enhanced susceptibility to helminth infection, these alarmins are not generally redundant. However, it is possible to circumvent the lack of one alarmin with cell populations strongly activated by another. Thus, transfer of ILC2s cultured in the presence of IL-7 and IL-33 restored expulsion of *N. brasiliensis* in mice lacking the receptor for IL-25 (*Il17rb*$^{-/-}$) (6). Moreover, the same authors reported that transfer of IL-13-expressing ILCs was sufficient to induce expulsion in mice lacking both receptors for IL-25 and IL33 (*Il17rb/Il1rl1*$^{-/-}$) or type-2 cytokines (*Il-4/Il-13*$^{-/-}$). Here, as discussed below, the ability of ILC2s to mediate worm expulsion was dependent on dialogue with the adaptive immune system and generation of antigen-specific T cell production of IL-13.

ILC2s—OTHER SIGNALS, SMALL AND LARGE

A range of small molecules are also able to orchestrate ILC2 functions, including arachadonic acid-derived eicosanoid (20-carbon) lipids. Human ILC2s can be activated by prostaglandin D2 through the CRTH2 (DP2) receptor (**Figure 2C**) (62) and their cytokine production can be further enhanced by stimulation with leukotriene E4 (63). In mice, leukotrienes can sufficiently induce type-2 cytokine production by ILC2s, with leukotrienes C4 and D4 being most efficient in provoking this response, whereas stimulation with leukotriene B4 and E4 only resulted in marginal IL-13 production (**Figure 2C**) (64, 65). In particular, leukotriene D4 drives IL-4 production by ILCs (66, 67). The role of eicosanoid mediators in nematode infection was investigated using gene-deficient mice of the respective leukotriene receptors or synthases, highlighting that deficiency in the LTB4 high affinity receptor *Ltb4r1* did not affect ILC2 activation in the lungs of *N. brasiliensis* infected mice. Activation was reduced by *Cystlt1r* (LTD4/LTC4 receptor) or *Ltc4s* (LTC4 synthase) deletion and abrogation of ILC2 activation was achieved in mice lacking *Alox5* (5 lipoxygenase, which catalyses the initial step of leukotriene synthesis) or the combination of *Ltb4r1* and *Ltc4s* (65). Inhibitory properties with regards to ILC2 activation were reported for lipoxin A4 (**Figure 2C**) (68) and prostaglandin E2 for human (69) and prostaglandin I2 for mouse (70) ILC2 cells.

Recent studies have highlighted the sensitivity of ILC2s to dietary vitamin A deficiency (71) and related their function to a requirement for fatty acids, acquired from the environment (72). In the event of vitamin A deficiency, the subsequent absence of retinoic acid resulted in the predominance of ILC2s in the small intestine and an increased resistance to *T. muris* infection (**Figure 2A**) (71). Under these conditions, ILC2s in the mesenteric adipose tissue increased their acquisition of long-chain fatty acids and raised production of IL-5 and IL-13 (72). Thus, as a result of nutrient deprivation, enhanced ILC2 activity is allowed ensuring that helminths are discharged more efficiently.

More recently, the A2B adenosine receptor was found to promote ILC2 activation and type-2 cytokine expression, controlling the primary and secondary immune response to *H. polygyrus* infection and the primary immune response to *N. brasiliensis* infection (73). This study also revealed adenosine as a

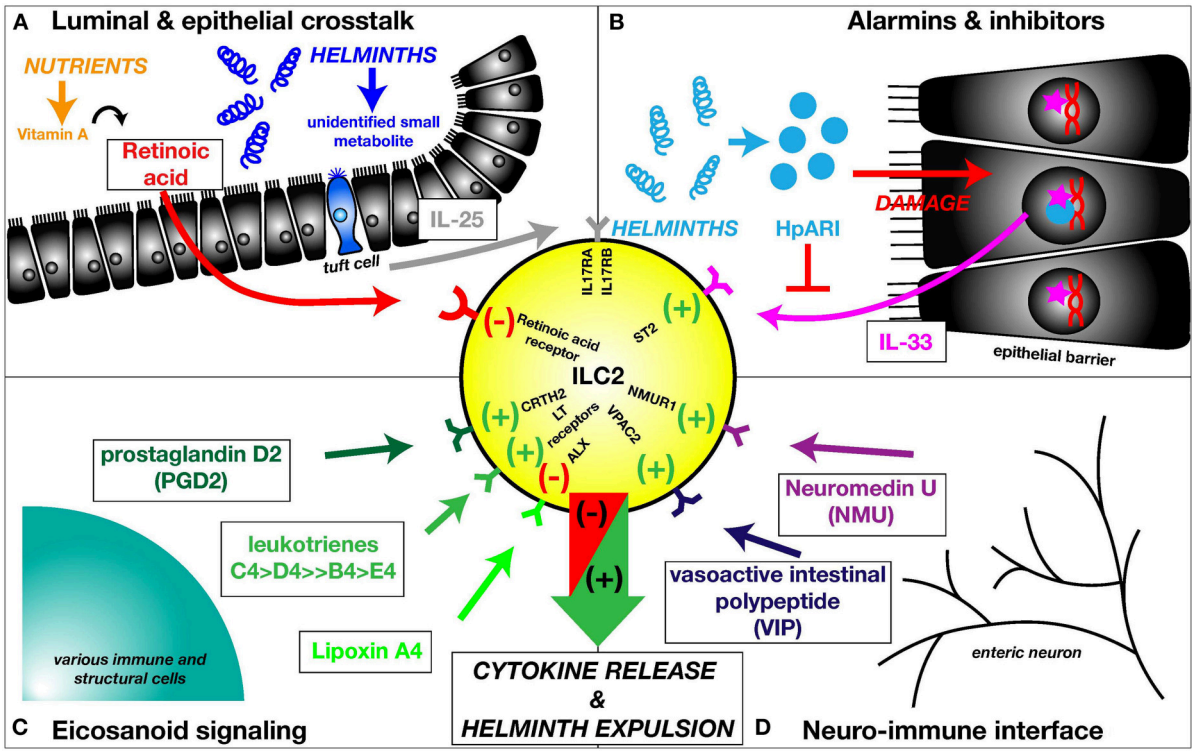

FIGURE 2 | ILC2s are embedded into a multifaceted network of barrier- and neuro-immune responses. **(A)** *Luminal & epithelial crosstalk.* Intestinal nematodes are detected by tuft cells of the small intestinal epithelium. While the helminth-derived mediator activating tuft cells is unknown, these chemosensory cells release IL-25, activating the IL-25 receptor (IL17RA/IL17RB) on ILC2s. ILC2s are negatively regulated by the vitamin A metabolite retinoic acid, signaling through retinoic acid receptors. **(B)** *Alarmins & inhibitors*. In addition to IL-25, ILC2s are induced by IL-33, an alarmin located inside the nuclei of epithelial cells. Upon damage to the epithelial lining, IL-33 is released. The nematode-derived alarmin inhibitor HpARI is able to sequester IL-33 inside the nuclei of damaged cells, attenuating the IL-33-induced ILC2 activation. **(C)** *Eicosanoid signaling.* Additionally, ILC2s are activated by various eicosanoids, including PGD2 and leukotrienes, activating the respective receptors, while negative regulation of ILC2s has been documented for lipoxin A4. **(D)** *Neuro-immune interface.* ILC2s are essential for the communication between enteric neurons and the epithelial barrier. NMU, released by neurons during helminth infections activates ILC2s via NMUR1 potentiating the epithelial response initiated to discharge the intruders. ILC2s are also stimulated by VIP, but VIP release by neurons during helminth infection has not been documented as of yet.

novel danger-associated molecular pattern (DAMP), responsible for initiating helminth-induced type-2 responses, through activation of ILC2s.

Additional receptors reported to be highly expressed by ILC2s and required for their expansion and function include the tumor necrosis factor (TNF)-receptor superfamily member DR3 (TNFRSF25) and the IL-9R. In the absence of TL1A ligation to DR3, there is significantly reduced ILC2 expansion, type-2 cytokine production and *N. brasiliensis* expulsion (74), whereas IL-9R signaling was required for ILC2 accumulation, expulsion of *N. brasiliensis* and resolution of tissue damage in the lung (75). IL-4- and IL-13-dependent expression of the acidic mammalian chitinase (AMCase) was also found to promote ILC2 expansion in the lung and expulsion of both *N. brasiliensis* and *H. polygyrus* from the gut (76).

Recently, the capacity of ILC2s to migrate between mucosal sites has become recognized with the experimental demonstration of a S1P-dependent pathway leading ILCs from the gut to the lung (30). MLN and lung iILC2s express S1P receptors and FTY720 inhibition of this pathway blocked the accumulation of intestinal iILC2s in the lung. When administered to Rag1$^{-/-}$ mice, FTY720 induced a significant increase in mortality to *N. brasiliensis* infection, which in turn can be prevented by an intravenous adoptive transfer of iILC2s prior to FTY720 administration (31). The role of chemokines in ILC2 migration and trafficking is understudied, though gene expression studies show that in comparison to other NK-cell and ILC subsets, small intestinal ILC2s express enhanced levels of *Cxcr6* and *Ccr4, 8,* and *9*, while also producing attenuated levels of the chemokine ligand *Ccl1* (77). Additionally, ILC2s express a number of surface receptors regulating cell-cell interactions. ILC2s express KLRG1, which as shown using human skin ILC2s, can interact with E-cadherin and dampen ILC2 specific type-2 cytokine expression (78). ILC2s also display ligands for OX40 and ICOS that mediate interactions with T cells as discussed below.

ILC2s at the Neuro-Immune Interface

Most recently, emphasis has been placed on delineating ILC2 function in the neuro-immune network during helminth

infection. ILC2s are located in close proximity to enteric neurons, primed to receive neuronal signals during infection. ILC2s were found to selectively express Neuromedin U receptor 1 (*Nmur1*), whereas mucosal neurons expressed the small neuropeptide neuromedin U (*Nmu*) (79, 80). Following infection with *N. brasiliensis*, *Nmu* expression was increased in both the lung and the gut, resulting in robust ILC2 responses through *Nmur1* and worm clearance (**Figure 2D**); in contrast, if adoptively transferred into *Rag2 Il2rg* deficient mice, *Nmur1* (NMU receptor 1) deficient ILC2s produce significantly reduced amounts of type-2 cytokine than their gene sufficient counterparts (79, 80). ILC2s are also activated by the neuropeptides vasoactive intestinal peptide (VIP) (81) (**Figure 2D**) and calcitonin gene-related peptide (CGRP) (82), although as yet it is unclear if these neuronal effectors play a significant role in ILC2 activation during helminth infection.

Interestingly, ILC2 activity was reported to be negatively regulated by acetylcholine through the nicotinic acetylcholine receptor α7nAChR (83), catecholamines (e.g., epinephrine) ligating the β2-adrenergic receptor (84), as well as the aryl hydrocarbon receptor (AhR) although in the latter case this may operate through preferential expansion of ILC3 cells (85).

DIALOGUE WITH ADAPTIVE IMMUNITY

An important early finding in the field was that ILCs did not prosper when transferred to RAG-deficient animals, and indeed ILC2 transfer did not succeed in conferring immunity to *N. brasiliensis* to this genotype (6). It is now well appreciated that ILC2s closely communicate with the adaptive immune system, allowing for the initiation and amplification of a robust type-2 response (86). For example, ILC2s promote Th2 immunity by enhancing $CD4^+$ T cell function through MHCII expression (87, 88). In the setting of helminth infection, ILC2s express high levels of surface MHC class II, as well as IL-25R (IL-17RB), and drive Th2 and Th9-mediated clearance of *Trichinella spiralis* (89). ILC2 "help" for Th2 cell differentiation may require IL-4, as mice lacking this gene only in the ILC compartment show attenuated Th2 cytokine production following *H. polygyrus* infection, although no increase in susceptibility was reported (67).

ILC2s have also been shown to upregulate expression of the checkpoint inhibitor PD-L1, following infection with *N. brasiliensis*. Surprisingly, expression of this molecule by ILC2s promoted production of IL-13 by Th2 cells and enhanced expulsion of the gastrointestinal nematode (90). Interestingly, ILC2s were also reported to promote regulatory T-cell expansion following *N. brasiliensis* infection. This interaction was suggested to be reliant on the expression of ICOSL by ILC2s and ICOS by Tregs (91). In the context of helminth infection with *N. brasiliensis*, lung ILC2s express OX40L in response to IL-33 stimulation allowing enhanced communication with $OX40^+$ T-cells (92). ILC-specific OX40L deficient *Il7r(Cre) Tnfsf4(fl/fl)* mice display a suppressed adaptive type-2 immune profile at d28 p.i., with significantly decreased numbers of both $GATA3^{\pm}$ Tregs and $GATA3^+$ Th2 cells, together with a mildly elevated intestinal worm burden at day 5 p.i. (92). Finally, in addition to ICOSL, ILC2s express ICOS, which can act as an inhibitory receptor in interactions with regulatory T cells (93).

ILC2s IN HELMINTH INFECTIONS—SUFFICIENCY, NECESSITY, OR REDUNDANCY?

The immune system has evolved multiple protection mechanisms against helminth infection, and the literature has documented a range of cell types, which if suitably activated and expanded, can mediate helminth expulsion (94, 95). For reasons discussed above, elimination of *N. brasiliensis* may be achieved more readily by multiple mechanisms, providing a sensitive test-bed for immunity to helminths. In this system, the principle has been established that innate sources of IL-4/IL-13 are sufficient for expulsion in animals unable to produce these cytokines within the T cell population (96). Consistent with this, adoptive transfer of ILC2s (6) induces nematode expulsion. However, transgenic mice with chronic eosinophilia [through IL-5 over-expression (97)] are also resistant to infection. The demonstration that disparate cell types are individually sufficient to combat helminth infection in mouse models is consonant with other studies showing that few are essential; demonstrating considerable redundancy.

In mouse models other than *N. brasiliensis*, with parasite species naturally adapted to the mouse, adoptive cell transfer of ILC2s has not been quite so effective, inducing partial, but not complete, clearance of *T. muris* (5) and *H. polygyrus* (67). In the case of *H. polygyrus*, Hepworth and colleagues (98) reported "negligible" MLN ILC2 numbers (0.2×10^5 cells) at day 6 of infection of C57BL/6 mice, requiring exogenous IL-25 treatment to boost the number of MLN ILC2 cells to reach levels observed during *N. brasiliensis* infection (*H. polygyrus*: 3×10^5 cells vs. *N. brasiliensis*: 5×10^5 at d5 p.i.) (99). This may reflect the poor responsiveness of this strain, as ILC2 responses are greater in more resistant mouse strains such as BALB/c and SJL (100) (**Figure 1B**). However, the theme of multiple pathways to resistance recurs with *H. polygyrus*, as both transfer of M2 macrophages (101) or transgenic amplification of mast cells (resulting in higher ILC2 levels) (57) are protective against infection.

ILC2-mediated immunity to *N. brasiliensis* infection can also be driven by other helminths, such as *S. venezuelensis*, which evokes a sufficiently strong expansion of ILCs in the lung, that a subsequent *N. brasiliensis* infection is significantly reduced in both worm number and egg output (102). Consistent with other information on the lung environment, the ILC2 response at this site required IL-33. Perhaps this finding recalls an ancient evolutionary role of ILCs in providing early chordates with generic protection against helminth infection, as discussed above.

An important point of consideration is that ILC2s are not the sole target of IL-25 activation in helminth infection, given evidence that other innate cell types such as monocytes (101),

other myeloid cells (103) and NKT cells are responsive to this cytokine. In addition, IL-25 promotes progenitor cells [mpp-type2, (5)], which can differentiate into mast cells. Many of these cell types are thought to be involved in immunity to helminth infection. Indeed, utilizing the *H. polygyrus* model of nematode infection, we were able to show that worm expulsion could be induced by IL-4 and IL-25 in RAG-deficient mice, and that this process was unimpeded by depletion of $CD90^+$ ILCs prior to cytokine administration (42). Hence in these more tenacious helminth infections, ILC2s may not be sufficient for immunity, and indeed may even be redundant.

ILCs IN HUMANS

While these studies extended our appreciation of ILC2s across a considerable number of helminth infections in mice, less information is available on their involvement in human helminth infections (104, 105). The theme of redundancy is echoed in human studies, as individuals lacking ILCs (following hematopoietic stem cell transplantation therapy) appear to lead healthy lives for many years (106, 107), although these patients are not likely to have been exposed to helminth parasite infections.

As highlighted by Nausch and Mutapi, many helminth parasites invade through, or establish a niche in, the skin of their host, and consequently are likely to encounter skin ILC2 cells (105). Parasites also frequently interact with mucosal sites, in particular the gastrointestinal tract, where ILC2s may be activated. Due to inaccessibility of these tissues in infected patients, studies delineating the immunology of human nematode infections are mostly reduced to analyses of peripheral blood, in which ILC2s may represent $< 0.05\%$ of live blood leucocytes (105). Nonetheless, helminths do cause changes in peripheral blood ILC populations. In a patient cohort of Zimbabwean children infected with *S. haematobium,* significantly fewer circulating ILCs were found in 6–13 years-olds, but normal levels observed in Schistosome-specific antibody positive infected children older than 14 years (108). Thus, there is an age-specific effect, which speaks to the increased relevance of ILC2s during early life before immunity is dominated by a mature adaptive immune response. In a second study, blood was taken from adult patients infected with the filarial nematodes *Loa loa, O. volvulus,* and *Wuchereria bancrofti*, and ILC2s and ILC3s were analyzed together as $CD127^+CD117^+$ cells (13). These authors reported increased blood ILCs in these infections, contrasting with the observations made of *S. haematobium* infection, highlighting that the ILC response might be heterogeneous in different infection settings and age groups.

The inaccessibility of ILC2s in the key tissues of humans returns the focus to events induced by human parasites in mouse models of helminth infection. Initial studies highlight an upregulation of key ILC2- inducing alarmin genes *Il1a*, *Il1b*, *Il33,* and *Tslp* shortly (6 h) after percutaneous *S. mansoni* cercariae infection of the pinna (109). Further, a study of combined IL-25, IL-33, and TSLP cytokine targeting during chronic murine *S. mansoni* infection revealed that ablation of either or both IL-25 and IL-33 is not sufficient to significantly alter *S. mansoni*-induced type-2 pathology in both the lung and the liver (110). Indeed it was needed to target all 3 alarmins utilizing IL-$33^{-/-}$ x $TSLP^{-/-}$ mice administered anti-IL-25 from week 4 to significantly reduce the liver granuloma volume at week 9, concomitant with a reduced granuloma eosinophil percentage and attenuated MLN $IL13^+$ ILC2 levels. However, by week 12 alarmin neutralization did not alter type-2 pathology, and Th2 cytokines had actually increased (110), again underlining that ILC2 effector functions may be restricted to the acute phase of type-2 immunity and that loss of ILC2 function can be compensated for by accelerated activity of Th2 cells.

CAN HELMINTHS MANIPULATE ILC2 FUNCTION?

Helminth infections can clearly stimulate type-2 responses yet have the ability to modify them, as part of their immune evasion strategy. An obvious immune cell candidate for targeting in order to avoid effective immunity is the ILC2. Following infection with the chronic helminth parasite *H. polygyrus*, there is a limited expansion of ILC2s in the mesenteric lymph nodes (MLN) (42, 98, 100). Indeed, a recent study revealed preferential trafficking of LTi-like ILC3s to the MLN following *H. polygyrus* infection (111). Infection with this chronic gastrointestinal nematode has been shown to promote the release of host-derived IL-1β, which limits IL-25 production and the subsequent activation of ILC2s (112).

Further evidence of the ability of *H. polygyrus* in adapting to the hosts' capacity to drive ILC2 activation via alarmin cytokines was recently revealed by the discovery of the IL-33 inhibitor HpARI, which is released by the helminth and sequesters IL-33 in the tissue (**Figure 2B**) (113). In addition, the same parasite releases extracellular vesicles that target expression of ST2, the IL-33 receptor, and are able to dampen the ILC2 response to *Alternaria alternata* fungal allergen challenge *in vivo* (114). Many helminths, *H. polygyrus* included, further modulate the adaptive immune system by promoting regulatory population such as regulatory T cells (115); with the recent description of IL-10-producing regulatory ILCs (116), it will be interesting to establish if some parasite species can dampen host immunity through immunosuppressive cells of the innate, as well as the adaptive, immune system.

CONCLUSION

ILC2s are clearly an inherent feature of the immune response to helminth infection, and in all probability their evolution has been driven by the threat of parasites. While in experimental model systems they are not always found to be essential, they are often center stage, particularly in the early phases of infection of each helminth system so far analyzed. They also form an important conceptual and mechanistic link with the allergic response that will allow us to understand in more detail the genesis and control of allergic disorders. In this respect, a much fuller analysis of ILC biology and function in the human type 2 response, both in

helminth infections and allergy, is eagerly awaited. Finally, the question remains to be answered of whether we can design new interventions, ideally vaccines, which take advantage of the ILC2 phenotype to promote protective immunity against helminth parasites and to control and remove the enormous burden of worm infections across the world.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. SL, KS, and RM have planned, written, and revised the article collaboratively. SL and RM have planned and created the figures.

ACKNOWLEDGMENTS

We thank the Wellcome Trust for support through a Senior Investigator Award to RM for SL (Ref 1061220), and for core support through the Wellcome Centre for Integrative Parasitology (Ref 104111), and European Commission for support through a Marie Skłodowska-Curie global fellowship to KS (Ref 657639).

REFERENCES

1. Herricks JR, Hotez PJ, Wanga V, Coffeng LE, Haagsma JA, Basanez MG, et al. The global burden of disease study 2013: What does it mean for the NTDs? *PLoS Negl Trop Dis.* (2017) 11:e0005424. doi: 10.1371/journal.pntd.0005424
2. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity.* (2001) 15:985–95. doi: 10.1016/S1074-7613(01)00243-6
3. Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, et al. New IL-17 family members promote Th1 or Th2 responses in the lung: *in vivo* function of the novel cytokine IL-25. *J Immunol.* (2002) 169:443–53. doi: 10.4049/jimmunol.169.1.443
4. Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, et al. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med.* (2006) 203:1105–16. doi: 10.1084/jem.20051615
5. Saenz SA, Siracusa MC, Perrigoue JG, Spencer SP, Urban JF Jr, Tocker JE, et al. IL25 elicits a multipotent progenitor cell population that promotes T_H2 cytokine responses. *Nature.* (2010) 464:1362–6. doi: 10.1038/nature08901
6. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* (2010) 464:1367–70. doi: 10.1038/nature08900
7. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA.* (2010) 107:11489–94. doi: 10.1073/pnas.1003988107
8. Voehringer D, Reese TA, Huang X, Shinkai K, Locksley RM. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J Exp Med.* (2006) 203:1435–46. doi: 10.1084/jem.20052448
9. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T_H2 cytokines by adipose tissue-associated c-Kit$^+$Sca-1$^+$ lymphoid cells. *Nature.* (2010) 463:540–4. doi: 10.1038/nature08636
10. Inclan-Rico JM, Siracusa MC. First responders: innate immunity to helminths. *Trends Parasitol.* (2018) 34:861–80. doi: 10.1016/j.pt.2018.08.007
11. Kabata H, Moro K, Koyasu S. The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms. *Immunol Rev.* (2018) 286:37–52. doi: 10.1111/imr.12706
12. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell.* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
13. Boyd A, Ribeiro JM, Nutman TB. Human CD117 (cKit)+ innate lymphoid cells have a discrete transcriptional profile at homeostasis and are expanded during filarial infection. *PLoS ONE.* (2014) 9:e108649. doi: 10.1371/journal.pone.0108649
14. Hams E, Armstrong ME, Barlow JL, Saunders SP, Schwartz C, Cooke G, et al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc Natl Acad Sci USA.* (2014) 111:367–72. doi: 10.1073/pnas.1315854111
15. Wong SH, Walker JA, Jolin HE, Drynan LF, Hams E, Camelo A, et al. Transcription factor RORα is critical for nuocyte development. *Nat Immunol.* (2012) 13:229–36. doi: 10.1038/ni.2208
16. Yang Q, Saenz SA, Zlotoff DA, Artis D, Bhandoola A. Cutting edge: natural helper cells derive from lymphoid progenitors. *J Immunol.* (2011) 187:5505–9. doi: 10.4049/jimmunol.1102039
17. Paul WE, Zhu J. How are T_H2-type immune responses initiated and amplified? *Nat Rev Immunol.* (2010) 10:225–35. doi: 10.1038/nri2735
18. Klose CSN, Flach M, Möhle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell.* (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
19. Yu X, Wang Y, Deng M, Li Y, Ruhn KA, Zhang CC, et al. The basic leucine zipper transcription factor NFIL3 directs the development of a common innate lymphoid cell precursor. *Elife.* (2014) 3:04406. doi: 10.7554/eLife.04406
20. Yang Q, Li F, Harly C, Xing S, Ye L, Xia X, et al. TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow. *Nat Immunol.* (2015) 16:1044–50. doi: 10.1038/ni.3248
21. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature.* (2014) 508:397–401. doi: 10.1038/nature13047
22. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol.* (2013) 14:221–9. doi: 10.1038/ni.2534
23. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, et al. Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity.* (2010) 33:736–51. doi: 10.1016/j.immuni.2010.10.017
24. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity.* (2012) 37:634–48. doi: 10.1016/j.immuni.2012.06.020
25. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol.* (2009) 10:83–91. doi: 10.1038/ni.1684
26. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity.* (2008) 29:958–70. doi: 10.1016/j.immuni.2008.11.001
27. Huang Y, Guo L, Qiu J, Chen X, Hu-Li J, Siebenlist U, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol.* (2015) 16:161–9. doi: 10.1038/ni.3078
28. Ricardo-Gonzalez RR, Van Dyken SJ, Schneider C, Lee J, Nussbaum JC, Liang HE, et al. Tissue signals imprint ILC2 identity with anticipatory function. *Nat Immunol.* (2018) 19:1093–9. doi: 10.1038/s41590-018-0201-4
29. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science.* (2015) 350:981–5. doi: 10.1126/science.aac9593
30. Germain RN, Huang Y. ILC2s - resident lymphocytes pre-adapted to a specific tissue or migratory effectors that adapt to where they move? *Curr Opin Immunol.* (2018) 56:76–81. doi: 10.1016/j.coi.2018.11.001

31. Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science.* (2018) 359:114–9. doi: 10.1126/science.aam5809
32. Vivier E, van de Pavert SA, Cooper MD, Belz GT. The evolution of innate lymphoid cells. *Nat Immunol.* (2016) 17:790–4. doi: 10.1038/ni.3459
33. Gérard C, Verrez-Bagnis V, Jérôme M, Lasne E. Petromyzon marinus (*Petromyzontidae*), an unusual host for helminth parasites in western Europe. *Dis Aquat Organ.* (2015) 113:263–7. doi: 10.3354/dao02842
34. Han Q, Das S, Hirano M, Holland SJ, McCurley N, Guo P, et al. Characterization of lamprey IL-17 family members and their receptors. *J Immunol.* (2015) 195:5440–51. doi: 10.4049/jimmunol.1500892
35. Shih HY, Sciumè G, Mikami Y, Guo L, Sun HW, Brooks SR, et al. Developmental acquisition of regulomes underlies innate lymphoid cell functionality. *Cell.* (2016) 165:1120–33. doi: 10.1016/j.cell.2016.04.029
36. Halim TY, MacLaren A, Romanish MT, Gold MJ, McNagny KM, Takei F. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity.* (2012) 37:463–74. doi: 10.1016/j.immuni.2012.06.012
37. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells - a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
38. Owyang AM, Zaph C, Wilson EH, Guild KJ, McClanahan T, Miller HR, et al. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J Exp Med.* (2006) 203:843–9. doi: 10.1084/jem.20051496
39. Saenz SA, Siracusa MC, Monticelli LA, Ziegler CG, Kim BS, Brestoff JR, et al. IL-25 simultaneously elicits distinct populations of innate lymphoid cells and multipotent progenitor type 2 (MPPtype2) cells. *J Exp Med.* (2013) 210:1823–37. doi: 10.1084/jem.20122332
40. Hams E, Locksley RM, McKenzie AN, Fallon PG. IL-25 elicits innate lymphoid type 2 and type II NKT cells that regulate obesity in mice. *J Immunol.* (2013) 191:5349–53. doi: 10.4049/jimmunol.1301176
41. Terashima A, Watarai H, Inoue S, Sekine E, Nakagawa R, Hase K, et al. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J Exp Med.* (2008) 205:2727–33. doi: 10.1084/jem.20080698
42. Smith KA, Löser S, Varyani F, Harcus Y, McSorley HJ, McKenzie AN, et al. Concerted IL-25R and IL-4Rα signaling drive innate type 2 effector immunity for optimal helminth expulsion. *eLife.* (2018) 7:e38269. doi: 10.7554/eLife.38269
43. Gerbe F, Sidot E, Smyth DJ, Ohmoto M, Matsumoto I, Dardalhon V, et al. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature.* (2016) 529:226–30. doi: 10.1038/nature16527
44. Howitt MR, Lavoie S, Michaud M, Blum AM, Tran SV, Weinstock JV, et al. Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. *Science.* (2016) 351:1329–33. doi: 10.1126/science.aaf1648
45. von Moltke J, Ji M, Liang HE, Locksley RM. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature.* (2016) 529:221–5. doi: 10.1038/nature16161
46. Lei W, Ren W, Ohmoto M, Urban JF, Matsumoto I, Margolskee RF, et al. Activation of intestinal tuft cell-expressed Sucnr1 triggers type 2 immunity in the mouse small intestine. *Proc Natl Acad Sci USA.* (2018) 115:5552–7. doi: 10.1073/pnas.1720758115
47. Nadjsombati MS, McGinty JW, Lyons-Cohen MR, Jaffe JB, DiPeso L, Schneider C, et al. Detection of succinate by intestinal tuft cells triggers a type 2 innate immune circuit. *Immunity.* (2018) 49:33–41 e37. doi: 10.1016/j.immuni.2018.06.016
48. Garg AV, Ahmed M, Vallejo AN, Ma A, Gaffen SL. The deubiquitinase A20 mediates feedback inhibition of interleukin-17 receptor signaling. *Sci Signal.* (2013) 6:ra44. doi: 10.1126/scisignal.2003699
49. Schneider C, O'Leary CE, von Moltke J, Liang HE, Ang QY, Turnbaugh PJ, et al. A Metabolite-triggered tuft cell-iLC2 circuit drives small intestinal remodeling. *Cell.* (2018) 174:271–84 e214. doi: 10.1016/j.cell.2018.05.014
50. Huang Y, Mao K, Germain RN. Thinking differently about ILCs-Not just tissue resident and not just the same as CD4(+) T-cell effectors. *Immunol Rev.* (2018) 286:160–71. doi: 10.1111/imr.12704
51. Kim BS, Siracusa MC, Saenz SA, Noti M, Monticelli LA, Sonnenberg GF, et al. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med.* (2013) 5:170ra116. doi: 10.1126/scitranslmed.3005374
52. Taylor BC, Zaph C, Troy AE, Du Y, Guild KJ, Comeau MR, et al. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med.* (2009) 206:655–67. doi: 10.1084/jem.20081499
53. Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med.* (2005) 202:1213–23. doi: 10.1084/jem.20051135
54. Siracusa MC, Saenz SA, Hill DA, Kim BS, Headley MB, Doering TA, et al. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature.* (2011) 477:229–33. doi: 10.1038/nature10329
55. Cayrol C, Girard JP. Interleukin-33 (IL-33): A nuclear cytokine from the IL-1 family. *Immunol Rev.* (2018) 281:154–68. doi: 10.1111/imr.12619
56. Molofsky AB, Savage AK, Locksley RM. Interleukin-33 in tissue homeostasis, injury, and inflammation. *Immunity.* (2015) 42:1005–19. doi: 10.1016/j.immuni.2015.06.006
57. Shimokawa C, Kanaya T, Hachisuka M, Ishiwata K, Hisaeda H, Kurashima Y, et al. Mast cells are crucial for induction of group 2 innate lymphoid cells and clearance of helminth infections. *Immunity.* (2017) 46:863–74 e864. doi: 10.1016/j.immuni.2017.04.017
58. Hung LY, Lewkowich IP, Dawson LA, Downey J, Yang Y, Smith DE, et al. IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity against hookworms. *Proc Natl Acad Sci USA.* (2013) 110:282–7. doi: 10.1073/pnas.1206587110
59. Yasuda K, Muto T, Kawagoe T, Matsumoto M, Sasaki Y, Matsushita K, et al. Contribution of IL-33-activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice. *Proc Natl Acad Sci USA.* (2012) 109:3451–6. doi: 10.1073/pnas.1201042109
60. Coakley G, McCaskill JL, Borger JG, Simbari F, Robertson E, Millar M, et al. Extracellular vesicles from a helminth parasite suppress macrophage activation and constitute an effective vaccine for protective immunity. *Cell Rep.* (2017) 19:1545–57. doi: 10.1016/j.celrep.2017.05.001
61. Humphreys NE, Xu D, Hepworth MR, Liew FY, Grencis RK. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol.* (2008) 180:2443–9. doi: 10.4049/jimmunol.180.4.2443
62. Wojno ED, Monticelli LA, Tran SV, Alenghat T, Osborne LC, Thome JJ, et al. The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol.* (2015) 8:1313–23. doi: 10.1038/mi.2015.21
63. Salimi M, Stöger L, Liu W, Go S, Pavord I, Klenerman P, et al. Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. *J Allergy Clin Immunol.* (2017) 140:1090–100 e1011. doi: 10.1016/j.jaci.2016.12.958
64. Lund SJ, Portillo A, Cavagnero K, Baum RE, Naji LH, Badrani JH, et al. Leukotriene C4 potentiates IL-33-induced group 2 innate lymphoid cell activation and lung inflammation. *J Immunol.* (2017) 199:1096–104. doi: 10.4049/jimmunol.1601569
65. von Moltke J, O'Leary CE, Barrett NA, Kanaoka Y, Austen KF, Locksley RM. Leukotrienes provide an NFAT-dependent signal that synergizes with IL-33 to activate ILC2s. *J Exp Med.* (2017) 214:27–37. doi: 10.1084/jem.20161274
66. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol.* (2013) 132:205–13. doi: 10.1016/j.jaci.2013.03.048
67. Pelly VS, Kannan Y, Coomes SM, Entwistle LJ, Rückerl D, Seddon B, et al. IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. *Mucosal Immunol.* (2016) 9:1407–17. doi: 10.1038/mi.2016.4
68. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci Transl Med.* (2013) 5:174ra126. doi: 10.1126/scitranslmed.3004812
69. Maric J, Ravindran A, Mazzurana L, Björklund ÅK, Van Acker A, Rao A, et al. Prostaglandin E2 suppresses human group 2 innate lymphoid cell function. *J Allergy Clin Immunol.* (2018) 141:1761–73 e1766. doi: 10.1016/j.jaci.2017.09.050

70. Zhou W, Toki S, Zhang J, Goleniewksa K, Newcomb DC, Cephus JY, et al. Prostaglandin I2 signaling and inhibition of group 2 innate lymphoid cell responses. *Am J Respir Crit Care Med.* (2016) 193:31–42. doi: 10.1164/rccm.201410-1793OC
71. Spencer SP, Wilhelm C, Yang Q, Hall JA, Bouladoux N, Boyd A, et al. Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science.* (2014) 343:432–7. doi: 10.1126/science.1247606
72. Wilhelm C, Harrison OJ, Schmitt V, Pelletier M, Spencer SP, Urban JF, et al. Critical role of fatty acid metabolism in ILC2-mediated barrier protection during malnutrition and helminth infection. *J Exp Med.* (2016) 213:1409–18. doi: 10.1084/jem.20151448
73. Patel N, Wu W, Mishra PK, Chen F, Millman A, Csóka B, et al. A2B adenosine receptor induces protective antihelminth type 2 immune responses. *Cell Host Microbe.* (2014) 15:339–50. doi: 10.1016/j.chom.2014.02.001
74. Yu X, Pappu R, Ramirez-Carrozzi V, Ota N, Caplazi P, Zhang J, et al. TNF superfamily member TL1A elicits type 2 innate lymphoid cells at mucosal barriers. *Mucosal Immunol.* (2014) 7:730–40. doi: 10.1038/mi.2013.92
75. Turner JE, Morrison PJ, Wilhelm C, Wilson M, Ahlfors H, Renauld JC, et al. IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J Exp Med.* (2013) 210:2951–65. doi: 10.1084/jem.20130071
76. Vannella KM, Ramalingam TR, Hart KM, de Queiroz Prado R, Sciurba J, Barron L, et al. Acidic chitinase primes the protective immune response to gastrointestinal nematodes. *Nat Immunol.* (2016) 17:538–44. doi: 10.1038/ni.3417
77. Robinette ML, Fuchs A, Cortez VS, Lee JS, Wang Y, Durum SK, et al. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nat Immunol.* (2015) 16:306–17. doi: 10.1038/ni.3094
78. Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med.* (2013) 210:2939–50. doi: 10.1084/jem.20130351
79. Cardoso V, Chesné J, Ribeiro H, García-Cassani B, Carvalho T, Bouchery T, et al. Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature.* (2017) 549:277–81. doi: 10.1038/nature23469
80. Klose CSN, Mahlakõiv T, Moeller JB, Rankin LC, Flamar AL, Kabata H, et al. The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation. *Nature.* (2017) 549:282–6. doi: 10.1038/nature23676
81. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature.* (2013) 502:245–8. doi: 10.1038/nature12526
82. Sui P, Wiesner DL, Xu J, Zhang Y, Lee J, Van Dyken S, et al. Pulmonary neuroendocrine cells amplify allergic asthma responses. *Science.* (2018) 360:eaan8546. doi: 10.1126/science.aan8546
83. Galle-Treger L, Suzuki Y, Patel N, Sankaranarayanan I, Aron JL, Maazi H, et al. Nicotinic acetylcholine receptor agonist attenuates ILC2-dependent airway hyperreactivity. *Nat Commun.* (2016) 7:13202. doi: 10.1038/ncomms13202
84. Moriyama S, Brestoff JR, Flamar AL, Moeller JB, Klose CSN, Rankin LC, et al. beta2-adrenergic receptor-mediated negative regulation of group 2 innate lymphoid cell responses. *Science.* (2018) 359:1056–61. doi: 10.1126/science.aan4829
85. Li S, Bostick JW, Ye J, Qiu J, Zhang B, Urban JF, et al. Aryl Hydrocarbon receptor signaling cell intrinsically inhibits intestinal group 2 innate lymphoid cell function. *Immunity.* (2018) 49:915–28. doi: 10.1016/j.immuni.2018.09.015
86. Mirchandani AS, Besnard AG, Yip E, Scott C, Bain CC, Cerovic V, et al. Type 2 innate lymphoid cells drive $CD4^+$ Th2 cell responses. *J Immunol.* (2014) 192:2442–8. doi: 10.4049/jimmunol.1300974
87. Liu B, Lee JB, Chen CY, Hershey GK, Wang YH. Collaborative interactions between type 2 innate lymphoid cells and antigen-specific CD4+ Th2 cells exacerbate murine allergic airway diseases with prominent eosinophilia. *J Immunol.* (2015) 194:3583–93. doi: 10.4049/jimmunol.1400951
88. Oliphant CJ, Hwang YY, Walker JA, Salimi M, Wong SH, Brewer JM, et al. MHCII-mediated dialog between Group 2 innate lymphoid cells and $CD4^+$ T cells potentiates Type 2 immunity and promotes parasitic helminth expulsion. *Immunity.* (2014) 41:283–95. doi: 10.1016/j.immuni.2014.06.016
89. Angkasekwinai P, Sodthawon W, Jeerawattanawart S, Hansakon A, Pattanapanyasat K, Wang YH. ILC2s activated by IL-25 promote antigen-specific Th2 and Th9 functions that contribute to the control of *Trichinella spiralis* infection. *PLoS ONE.* (2017) 12:e0184684. doi: 10.1371/journal.pone.0184684
90. Schwartz C, Khan AR, Floudas A, Saunders SP, Hams E, Rodewald HR, et al. ILC2s regulate adaptive Th2 cell functions via PD-L1 checkpoint control. *J Exp Med.* (2017) 214:2507–21. doi: 10.1084/jem.20170051
91. Molofsky AB, Van Gool F, Liang HE, Van Dyken SJ, Nussbaum JC, Lee J, et al. Interleukin-33 and interferon-gamma counter-regulate group 2 innate lymphoid cell activation during immune perturbation. *Immunity.* (2015) 43:161–74. doi: 10.1016/j.immuni.2015.05.019
92. Halim TYF, Rana BMJ, Walker JA, Kerscher B, Knolle MD, Jolin HE, et al. Tissue-Restricted Adaptive Type 2 Immunity Is Orchestrated by Expression of the Costimulatory Molecule OX40L on Group 2 Innate Lymphoid Cells. *Immunity.* (2018) 48:1195–207 e1196. doi: 10.1016/j.immuni.2018.05.003
93. Rigas D, Lewis G, Aron JL, Wang B, Banie H, Sankaranarayanan I, et al. Type 2 innate lymphoid cell suppression by regulatory T cells attenuates airway hyperreactivity and requires inducible T-cell costimulator-inducible T-cell costimulator ligand interaction. *J Allergy Clin Immunol.* (2017) 139:1468–77 e1462. doi: 10.1016/j.jaci.2016.08.034
94. Harris NL, Loke P. Recent advances in Type-2-cell-mediated immunity: insights from helminth infection. *Immunity.* (2017) 47:1024–36. doi: 10.1016/j.immuni.2017.11.015
95. Maizels RM, Hewitson JP, Smith KA. Susceptibility and immunity to helminth parasites. *Curr Opin Immunol.* (2012) 25:459–66. doi: 10.1016/j.coi.2012.06.003
96. Oeser K, Schwartz C, Voehringer D. Conditional IL-4/IL-13-deficient mice reveal a critical role of innate immune cells for protective immunity against gastrointestinal helminths. *Mucosal Immunol.* (2015) 8:672–82. doi: 10.1038/mi.2014.101
97. Dent LA, Daly CM, Mayrhofer G, Zimmerman T, Hallett A, Bignold LP, et al. Interleukin-5 transgenic mice show enhanced resistance to primary infections with *Nippostrongylus brasiliensis* but not primary infections with *Toxocara canis*. *Infect Immun.* (1999) 67:989–93.
98. Hepworth MR, Maurer M, Hartmann S. Regulation of type 2 immunity to helminths by mast cells. *Gut Microbes.* (2012) 3:476–81. doi: 10.4161/gmic.21507
99. Taylor S, Huang Y, Mallett G, Stathopoulou C, Felizardo TC, Sun MA, et al. PD-1 regulates KLRG1(+) group 2 innate lymphoid cells. *J Exp Med.* (2017) 214:1663–78. doi: 10.1084/jem.20161653
100. Filbey KJ, Grainger JR, Smith KA, Boon L, van Rooijen N, Harcus Y, et al. Innate and adaptive type 2 immune cell responses in genetically controlled resistance to intestinal helminth infection. *Immunol Cell Biol.* (2014) 92:436–48. doi: 10.1038/icb.2013.109
101. Yang Z, Grinchuk V, Urban JF, Bohl J, Sun R, Notari L, et al. Macrophages as IL-25/IL-33-responsive cells play an important role in the induction of type 2 immunity. *PLoS ONE.* (2013) 8:e59441. doi: 10.1371/journal.pone.0059441
102. Yasuda K, Adachi T, Koida A, Nakanishi K. Nematode-infected mice acquire resistance to subsequent infection with unrelated nematode by inducing highly responsive group 2 innate lymphoid cells in the lung. *Front Immunol.* (2018) 9:2132. doi: 10.3389/fimmu.2018.02132
103. Petersen BC, Budelsky AL, Baptist AP, Schaller MA, Lukacs NW. Interleukin-25 induces type 2 cytokine production in a steroid-resistant interleukin-17RB+ myeloid population that exacerbates asthmatic pathology. *Nat Med.* (2012) 18:751–8. doi: 10.1038/nm.2735
104. Bonne-Année S, Nutman TB. Human innate lymphoid cells (ILCs) in filarial infections. *Parasite Immunol.* (2017) 40:e12442. doi: 10.1111/pim.12442
105. Nausch N, Mutapi F. Group 2 ILCs: A way of enhancing immune protection against human helminths? *Parasite Immunol.* (2018) 40. doi: 10.1111/pim.12450
106. Vely F, Barlogis V, Vallentin B, Neven B, Piperoglou C, Ebbo M, et al. Evidence of innate lymphoid cell redundancy in humans. *Nat Immunol.* (2016) 17:1291–9. doi: 10.1038/ni.3553
107. Weinkove R, Filbey K, Le Gros G. Immunity without innate lymphoid cells. *Nat Immunol.* (2016) 17:1237–8. doi: 10.1038/ni.3567

108. Nausch N, Appleby LJ, Sparks AM, Midzi N, Mduluza T, Mutapi F. Group 2 innate lymphoid cell proportions are diminished in young helminth infected children and restored by curative anti-helminthic treatment. *PLoS Negl Trop Dis.* (2015) 9:e0003627. doi: 10.1371/journal.pntd.0003627
109. Bourke CD, Prendergast CT, Sanin DE, Oulton TE, Hall RJ, Mountford AP. Epidermal keratinocytes initiate wound healing and pro-inflammatory immune responses following percutaneous schistosome infection. *Int J Parasitol.* (2015) 45:215–24. doi: 10.1016/j.ijpara.2014.11.002
110. Vannella KM, Ramalingam TR, Borthwick LA, Barron L, Hart KM, Thompson RW, et al. Combinatorial targeting of TSLP, IL-25, and IL-33 in type 2 cytokine-driven inflammation and fibrosis. *Sci Transl Med.* (2016) 8:337ra365. doi: 10.1126/scitranslmed.aaf1938
111. Mackley EC, Houston S, Marriott CL, Halford EE, Lucas B, Cerovic V, et al. CCR7-dependent trafficking of RORgamma(+) ILCs creates a unique microenvironment within mucosal draining lymph nodes. *Nat Commun.* (2015) 6:5862. doi: 10.1038/ncomms6862
112. Zaiss MM, Maslowski KM, Mosconi I, Guenat N, Marsland BJ, Harris NL. IL-1β suppresses innate IL-25 and IL-33 production and maintains helminth chronicity. *PLoS Pathog.* (2013) 9:e1003531. doi: 10.1371/journal.ppat.1003531
113. Osbourn M, Soares DC, Vacca F, Cohen ES, Scott IC, Gregory WF, et al. HpARI Protein Secreted by a Helminth Parasite Suppresses Interleukin-33. *Immunity.* (2017) 47:739–51 e735. doi: 10.1016/j.immuni.2017.09.015
114. Buck AH, Coakley G, Simbari F, Kumar S, Lear M, Abreu-Goodget C, et al. Exosomes secreted by a nematode parasite transfer RNA to mammalian cells and regulate genes of the innate immune system. *Nat Commun.* (2014) 5:5488. doi: 10.1038/ncomms6488
115. Maizels RM, Smith KA. Regulatory T cells in infection. *Adv Immunol.* (2011) 112:73–136. doi: 10.1016/B978-0-12-387827-4.00003-6
116. Wang S, Xia P, Chen Y, Qu Y, Xiong Z, Ye B, et al. Regulatory innate lymphoid *cell*s control innate intestinal inflammation. *Cell.* (2017) 171:201–16. doi: 10.1016/j.cell.2017.07.027

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

ORIGINAL RESEARCH
published: 16 April 2019
doi: 10.3389/fimmu.2019.00826

Group 2 Innate Lymphoid Cells Are Redundant in Experimental Renal Ischemia-Reperfusion Injury

*Guy J. M. Cameron[1,2], Kelly M. Cautivo[3], Svenja Loering[1,2], Simon H. Jiang[4,5], Aniruddh V. Deshpande[1,2,6], Paul S. Foster[1,2], Andrew N. J. McKenzie[7], Ari B. Molofsky[3], Philip M. Hansbro[1,2,8,9] and Malcolm R. Starkey[1,2]**

[1] Priority Research Centre's GrowUpWell and Healthy Lungs, School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW, Australia, [2] Hunter Medical Research Institute, New Lambton Heights, NSW, Australia, [3] Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA, United States, [4] Department of Immunology and Infectious Disease, John Curtin School of Medical Research, Australia National University, Canberra, ACT, Australia, [5] Department of Renal Medicine, The Canberra Hospital, Canberra, ACT, Australia, [6] The John Hunter Children's Hospital, New Lambton Heights, NSW, Australia, [7] MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge, United Kingdom, [8] Centre for inflammation, Centenary Institute, Sydney, NSW, Australia, [9] Faculty of Science, University of Technology, Ultimo, NSW, Australia

Acute kidney injury (AKI) can be fatal and is a well-defined risk factor for the development of chronic kidney disease. Group 2 innate lymphoid cells (ILC2s) are innate producers of type-2 cytokines and are critical regulators of homeostasis in peripheral organs. However, our knowledge of their function in the kidney is relatively limited. Recent evidence suggests that increasing ILC2 numbers by systemic administration of recombinant interleukin (IL)-25 or IL-33 protects against renal injury. Whilst ILC2s can be induced to protect against ischemic- or chemical-induced AKI, the impact of ILC2 deficiency or depletion on the severity of renal injury is unknown. Firstly, the phenotype and location of ILC2s in the kidney was assessed under homeostatic conditions. Kidney ILC2s constitutively expressed high levels of IL-5 and were located in close proximity to the renal vasculature. To test the functional role of ILC2s in the kidney, an experimental model of renal ischemia-reperfusion injury (IRI) was used and the severity of injury was assessed in wild-type, ILC2-reduced, ILC2-deficient, and ILC2-depleted mice. Surprisingly, there were no differences in histopathology, collagen deposition or mRNA expression of injury-associated (*Lcn2*), inflammatory (*Cxcl1*, *Cxcl2,* and *Tnf*) or extracellular matrix (*Col1a1*, *Fn1*) factors following IRI in the absence of ILC2s. These data suggest the absence of ILC2s does not alter the severity of renal injury, suggesting possible redundancy. Therefore, other mechanisms of type 2-mediated immune cell activation likely compensate in the absence of ILC2s. Hence, a loss of ILC2s is unlikely to increase susceptibility to, or severity of AKI.

Keywords: ILC2, group 2 innate lymphoid cell, IL-5, IL-13, kidney, renal, IRI, ischemia-reperfusion injury

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Yuefeng Huang,
Columbia University, United States
Yakup Tanriver,
University of Freiburg, Germany

***Correspondence:**
Malcolm R. Starkey
malcolm.starkey@newcastle.edu.au

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

Received: *20 December 2018*
Accepted: *28 March 2019*
Published: *16 April 2019*

Citation:
Cameron GJM, Cautivo KM, Loering S, Jiang SH, Deshpande AV, Foster PS, McKenzie ANJ, Molofsky AB, Hansbro PM and Starkey MR (2019) Group 2 Innate Lymphoid Cells Are Redundant in Experimental Renal Ischemia-Reperfusion Injury. Front. Immunol. 10:826. doi: 10.3389/fimmu.2019.00826

INTRODUCTION

Acute kidney injury (AKI) and its associated pathologies have profound effects on human health (1). A common cause of AKI is renal ischemia, which primarily affects the tubular epithelium due to the high mitochondrial density and metabolic activity in these cells (2); reviewed in Devarajan (3) and Bonventre and Yang (4). Acute tubular necrosis impairs waste excretion, alters

water and electrolyte imbalance, and causes robust inflammatory responses; reviewed in Bonventre and Yang (4). Influx of neutrophils and monocytes contributes to the injury, however other innate immune cells can facilitate the return to homeostasis (5); reviewed in Friedewald and Rabb (6). Innate lymphoid cells (ILCs) are a recent addition to this family and are categorized into 3 groups based on the transcription factors that are required for their development and by the effector cytokines they produce (7). Group 2 ILCs (ILC2s) are potent producers of type 2 cytokines, predominantly interleukin (IL)-5 and IL-13 (8, 9). These cells also promote tissue recovery following insult in multiple organs and have diverse functions *in vivo* (10). For example, following respiratory viral infection with influenza virus, ILC2s co-ordinate repair of the airway epithelium by producing the growth factor amphiregulin (AREG) (11). More recently, ILC2s have been investigated in models of renal injury including ischemia-reperfusion injury (IRI), and nephrotoxic chemical-induced injury with doxorubicin or cisplatin (12–16). Renal IRI is typically achieved by temporarily restricting blood flow to the kidney for 20–30 min, modeling trauma from transplant or surgical intervention (17–21). Collectively, these studies show that *in vivo* administration of recombinant mouse cytokines that activate ILC2s, namely IL-25 or IL-33, is sufficient to reduce the severity of tubular epithelial cell injury (12–16). Similar results have been achieved with adoptive transfer of *ex vivo* activated ILC2s (12, 15). Whilst ILC2s can be artificially induced to proliferate and protect against the deleterious consequences of experimental renal injury, the impact of ILC2-deficiency remains incompletely understood. Indeed, other immune cells, which have complex interactions with the ILC2s such as regulatory T cells (T_{reg}) and alternatively activated macrophages (AAM; also known as M2) are critical for this renoprotective effect (22–25). In this study, we sought to further characterize ILC2s in the kidney, their location within this organ and determine their functional role in IRI using a loss-of-function approach. Here, we found that kidney ILC2s constitutively express IL-5 and are primarily located in close proximity to the renal vasculature, within the adventitia. Additionally, we demonstrate that a reduction, deficiency or depletion of ILC2s had minimal impact on the severity of IRI. Whilst activation of ILC2s and the associated amplification of local type 2 immunity has been previously shown to reduce the deleterious consequences of AKI, our results reveal that comparable injury occurs in the absence of ILC2s, suggesting that ILC2s may be redundant in IRI when other compensatory immune cells such as T cells are present.

MATERIALS AND METHODS

Mice

Eight to twelve week-old male wild-type (WT; C57BL/6JAusb), vehicle (saline-treated $Icos^{dtr/+}Cd4^{cre/+}$), ILC2-reduced ($Rora^{fl/+}Il7r^{cre/+}$), ILC2-deficient ($Rora^{fl/fl}Il7r^{cre/+}$), ILC2-depleted (Diptheria toxoid-treated [DTx] $Icos_{dtr/+}$ $Cd4_{cre/+}$), and IL-5/IL-13 dual reporter mice ($Il5^{venus/+}Il13^{td-tomato/+}$) mice were obtained from Australian Bioresources (Moss Vale, Australia). $Il5^{td-tomatoCre}$; Rosa26-CAG-RFP reporter-tracker mice were obtained from Jackson Labs (ref# 030926 and 007914). All mice that underwent surgery were housed under specific pathogen free, physical containment 2 conditions, in individually ventilated cages. Mice were exposed to normal room air within a sterilized environment during surgery. Mice were allowed 1 week to acclimatize before experiments were started and were maintained on a 12-h day/night cycle with access to standard laboratory chow and water *ad libitum*.

Flow Cytometry and t-SNE Analysis

Kidneys and lungs were collected from reporter mice. Single-cell suspensions were prepared as described in "Preparation of single-cell suspensions from mouse lung with Collagenase D treatment" (Miltenyi Biotec GmbH, 2008; Bergisch Gladbach, Germany). Cells were blocked with Fc block (purified anti-mouse CD16/32; Biolegend, San Diego, USA) for 30 min and stained with fluorescently-conjugated antibodies against target cell surface antigens (**Supplementary Table 1**). Staining and washing steps were performed with BSA stain buffer (BD Biosciences, North Ryde, Australia). Samples were acquired on a BD LSR Fortessa X20 flow cytometer. Flow cytometry data were analyzed using FlowJo v10.5.3 (Tree Star, Ashland, USA). The t-distributed stochastic neighbor embedding (t-SNE) was performed on ILC2s ($CD45^+Lineage^-[TCR^-[TCR\alpha\beta^-TCR\gamma\delta^-CD8^-CD4^-]CD11b^-GR\text{-}1^-B220^-TER\text{-}119^-CD3^-NK\text{-}1.1^-]IL7R\alpha^+CD90.2^+ST2^+$ $FSC^{low}SSC^{low}$ single cells). Using the random down sampling plugin, the number of events in each sample was normalized to equal the lowest, rounded down to the nearest 10 events. All populations were combined into one .fcs file by concatenating the down sampled populations. The t-SNE analysis was performed on the concatenated sample containing the gated populations from all kidney and lung samples combined. For this, the compensated channels that were not used for gating were assessed under the following t-SNE settings: Iteration 1000, Perplexity 20, Eta 224. Histograms were used to show the differential expression of cell surface antigens and cytokines in the lung and kidney.

Immunofluorescence

$Il5^{td-tomatoCre}$; Rosa26-CAG-RFP reporter-tracker mice were euthanized and perfused intracardially with phosphate buffered saline (PBS) and then 4% paraformaldehyde (PFA) in PBS. Kidneys were removed and kept in fresh 4% PFA for 24 h at 4°C and then washed in PBS. Three hundred micrometer sections were prepared using a vibratome. Subsequently, tissue sections were incubated in permeabilization buffer (PBS/0.2% Triton X-100/0.3M glycine) then blocked in PBS/0.2% Triton X-100/5% donkey serum at 4°C overnight. Samples were washed in PBS/0.2% Tween-20 once, then incubated with primary antibodies diluted in PBS/0.2% Tween-20/3% donkey serum at room temperature until the next day. Next, samples were washed in PBS/0.2% Tween-20 for 30 min, 3–4 times, then incubated with secondary antibodies diluted in PBS/0.2% Tween-20/3% donkey serum at room temperature for 6–8 h. Samples were washed in PBS/0.2% Tween-20 for 1 day and then dehydrated in an ascending ethanol series (20, 30, 50, 70, 95, 100%), 10 min each step. Finally, kidney sections were cleared by soaking in

methyl salicylate (M-2047; Sigma-Aldrich, Castle Hill, Australia) and then mounted in fresh methyl salicylate onto a concave coverslip or chamber. Images were captured with Nikon A1R laser scanning confocal including 405, 488, 561, and 650 laser lines for excitation and imaging with 16X/0.8 or 25X1.1, NA Plan Apo long working distance water immersion objectives. Z steps were acquired every 6 μm. Images were processed with ImageJ (NIH, Bethesda, MD, US) and Bitplane Imaris software v8 (Andor Technology PLC, Belfast, N. Ireland). Surface reconstructions of alpha smooth muscle actin-labeled blood vessels were performed with Imaris and pseudocolored based on their characteristic architecture and volume to visualize large arteries and veins. The following antibodies and dilutions were used: Living Colors anti-DsRed Rabbit Polyclonal Pan Antibody (1:500; TaKaRa, Mountain View, USA), goat polyclonal anti-alpha smooth muscle actin antibody (1:200; Abcam, Cambridge, UK), eFluor 660 LYVE1 monoclonal Antibody (1:300, clone ALY7, eBioscience), armenian hamster anti-mouse CD3 antibody (1:100, clone 145-2C11, Biolegend), Alexa Fluor® 488 donkey anti-goat IgG (H+L), Alexa Fluor® 555 donkey anti-rabbit IgG (H+L) Alexa Fluor® 647 goat anti-hamster IgG (H+L) cross-adsorbed (1:400, ThermoFisher Scientific).

Unilateral Ischemia-Reperfusion Injury

Surgical anesthesia was induced by 4% isoflurane inhalation and maintained with continuous 1–1.5% isoflurane inhalation through a nose cone. Throughout the procedure, mice were kept on a surgical platform maintained at 37 ± 1°C by a digital thermostat-controlled heat mat. Nephrectomy of the right kidney was performed in all animals. To induce unilateral ischemia the vessels of the left kidney were clamped near the renal pedicle for 29-min using a non-traumatic sterile vascular clamp (Coherent Scientific; Hilton, Australia) applying sufficient force to prevent blood flow to and from the kidney without irreversible damage to the vessels. Uniform and rapid reperfusion of the kidney was observed after removing the vascular clamp in all mice. The muscle and skin wounds were closed using a 5/0 vicryl suture (Johnson & Johnson Medical, North Ryde, Australia), the skin was then sealed using dermal adhesive (Provet, Charlestown, Australia) to protect the wound and prevent infection. Control animals were subjected to a sham operation with identical anesthesia time and received only a contralateral nephrectomy. For effective analgesia mice received 0.1 mg/kg of buprenorphine subcutaneously 30 min prior to the operation and then 0.05–0.1 mg/kg every 8–12 h, as required. Animals were typically weaned off analgesia by 4 days post-surgery without any signs of pain or distress as defined by facial characteristics using the standard mouse grimace scale; as described (26). All mice survived to the pre-determined endpoint.

Depletion of ILC2s *in vivo*

ILC2s were depleted in $Icos^{dtr/+}Cd4^{cre/+}$ mice by intraperitoneal injection of 0.025 mg/kg of DTx (Sigma-Aldrich) as previously described (27). DTx was administered 30 min prior to surgery and daily thereafter until the endpoint. To confirm ILC2-depletion, a subset of naïve animals received DTx for 3 days. Control $Icos^{dtr/+}Cd4^{cre/+}$ mice received intraperitoneal saline injection.

Histology

Excised kidneys were immediately sectioned in half longitudinally through the hilum. Half was fixed in 10% buffered formalin, containing 4% formaldehyde for 24 h before temporary storage in 10% ethanol, paraffin embedding, and sectioning. Four micrometer, longitudinally cut sections were stained with periodic acid-Schiff (PAS) and Masson's trichrome. Histopathology was quantified by visualizing dilation of tubules, apoptosis or cast formation by a single blinded investigator using a well-established semi-quantitative method (28), with minor alterations as described (15). An average was taken of the score from duplicate PAS stained sections from each animal. A score of 0 = 0% of tubules were affected by the above histopathological features; 1 = 1–10%; 2 = 11–25%; 3 = 26–50%; 4 = 51–75%; 5 = 76–100%. Collagen deposition was assessed using Masson's trichrome and collagen was identified as methyl blue content between adjacent tubules.

RNA Extraction, Reverse Transcription, and qPCR

Half of one kidney was retained for gene expression analysis. The tissue was homogenized in 400 μl of PBS using a TissueLyser LT™ (Qiagen, Chadstone Centre, Australia) through 50 oscillations per second for 2 min. Two hundred microliter of the sample was removed for other analyses. One milliliter of TRI Reagent® (Sigma-Aldrich) was added to the remaining 200 μl of the sample, which received a further 50 oscillations per second for 1–2 min until completely homogenized. Following 5 min of incubation at room temperature and centrifugation at 12,000x *g* for 10 min, the supernatant was extracted and vortexed with 250 μl of chloroform (Sigma-Aldrich). After incubating for 10 min at room temperature and centrifugation at 12,000x *g* for 15 min, the supernatant was extracted and vortexed with 500 μl of 70% isopropanol (Sigma-Aldrich). The sample was incubated again for 10 min at room temperature then was centrifuged at 12,000x *g* for 10 min. The supernatant was discarded without disrupting the RNA-rich pellet. The pellet was washed twice with 1 ml of 75% RNA-grade ethanol (Sigma-Aldrich), centrifuged at 10,000x *g* for 5 min and the supernatant was discarded each time. The pellet was air-dried at room-temperature briefly before re-suspending in 80–100 μl of UltraPure™ DNase/RNase-free double distilled water (ddH_2O; Thermo-Fisher Scientific) and was then stored at −80°C prior to reverse transcription. Immediately prior to reverse transcription, RNA yield was determined by spectrophotometric assessment using a Nanodrop2000™ (Thermo-Fisher Scientific). Five microliter of each sample was diluted in ddH_2O such that 1,000 ng of RNA was loaded per sample. Following DNAse (Sigma-Aldrich) treatment, cDNA was generated using the M-MLV (Thermo-Fisher Scientific, North Ryde, Australia) enzyme standard operating procedures using a T100™ thermal cycler (BioRad Laboratories, Gladesville, Australia). Using SYBR based methodology, qPCR was performed using CFX384 Touch™ (BioRad Laboratories) as per standard operating procedures. Each primer set was gradient tested to determine the optimal temperature for amplification (**Supplementary Table 2**). Cycle quantification was interpreted using regression for each target, gene expression was normalized relative to hypoxanthine

guanine phosphoribosyl transferase (*Hprt*). Melt curves were assessed for signs of primer dimerization and non-specific amplification. On every plate, at least one negative control and no-template control was used for each target to ensure contamination was not present within each master mix or the ddH_2O used.

RT² Profiler Array

RT² Profiler™ PCR Array Mouse Extracellular Matrix & Adhesion Molecules (Qiagen) was performed as per manufacturer's instructions and data was uploaded then analyzed by the online tool, available from: https://dataanalysis.qiagen.com/pcr/arrayanalysis.php. This array allows profiling of 84 genes in a maximum of 4 samples, therefore, equal amounts of cDNA for each replicate in a group were pooled from WT mice on day 1, 3, or 7 following IRI, and sham pooled from each replicate from each of these timepoints. For each target, the fold change following IRI was compared to sham expression.

Statistical Analysis

All data were analyzed with Graphpad Prism software v8.02 using non-parametric unpaired *t*-tests (Mann-Whitney *U*-test). $P < 0.05$ was set as threshold for determining statistically significant differences. $^{*}P < 0.05$, ^{ns} not significant. All data are expressed as mean ± SEM. In each analysis there were $n = 4$–8 replicates per group and results were representative of at least two experiments. Sample size for each experiment is described in the corresponding figure legend.

RESULTS

Renal ILC2s Constitutively Express IL-5 and Are Localized to the Renal Vasculature Under Homeostatic Conditions

A detailed analysis of renal ILC2 phenotype and location under homeostatic conditions was performed. There were significantly more ILC2s ($CD45^+Lineage^-$IL-$7R\alpha^+CD90.2^+ST2^+FSC^{low}SSC^{low}$ single cells), in the kidney compared to the lung when represented as a percentage of total $CD45^+$ single cells (**Figure 1A**; **Supplementary Figures 1A, B**). t-SNE analysis of kidney and lung ILC2s showed unique clustering, although these clusters were not entirely distinct from one another (**Figure 1B**). However, kidney ILC2s had markedly lower expression of CD25, consistent expression of ICOS and KLRG1, but higher expression of IL-5 compared to lung ILC2s (**Figure 1C**). Kidney ILC2s had higher constitutive expression of IL-5 than IL-13 (**Figure 1D**; **Supplementary Figures 1C, D**). ILC2s were determined to be the major IL-5 producing cell type in the kidney, with a negligible contribution from T helper type-2 (T_H2) cells (**Figure 1E**, **Supplementary Figures 1E, F**). Therefore, IL-5 was used as a surrogate marker to identify the location of ILC2s within the mouse kidney, as has been described for multiple peripheral organs (29–31). This was achieved by utilizing mice that express an IL-5 linked cre-recombinase crossed to a Rosa-tdtomato lineage tracker (30, 31). IL-5^+ cells (predominantly ILC2s) were found throughout the kidney, but were primarily localized to the major vasculature, as visualized with alpha smooth muscle actin (α-SMA) positive staining (**Figure 2A**; **Supplementary Figures 2A, B**), consistent with our recent report (29). ILC2s were found to be associated with both interlobular and arcuate renal vessels in the cortex and medullary regions, as shown by lymphatic endothelial hyaluronan receptor-1 (LYVE1) expression (**Figure 2B**). Further imaging demonstrated that very few of the $CD3^+$ cells (predominantly T_H2 cells) contributed to the endogenous IL-5^+ signal (**Figure 2C**). ILC2s were identified in the adventitia of the vessel using pseudocolored surface reconstructions of the arterial α-SMA.

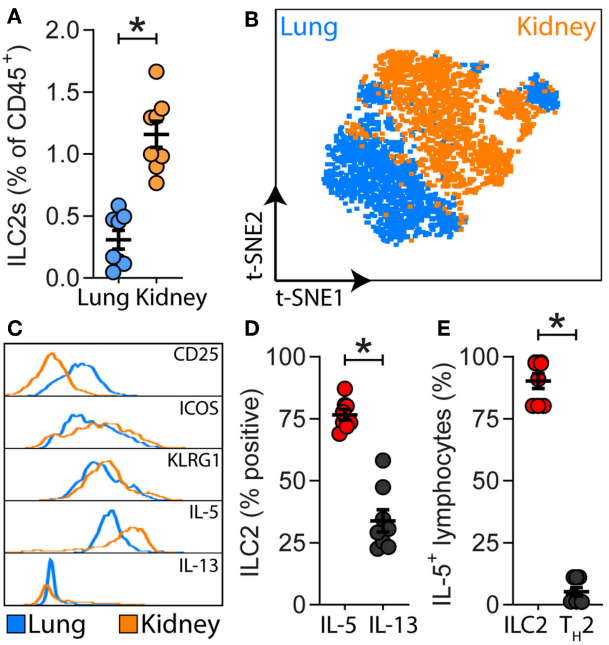

FIGURE 1 | ILC2s are present in the kidney and have a unique signature compared to lung ILC2s. **(A)** ILC2s ($CD45^+Lineage^-$[TCR^-[$TCR\alpha\beta^-$ $TCR\gamma\delta^-CD8^-CD4^-$]$CD11b^-GR\text{-}1^-B220^-TER\text{-}119^-CD3^-NK\text{-}1.1^-$]IL-$7R\alpha^+CD90.2^+ST2^+FSC^{low}SSC^{low}$ single cells) in kidney and lung single cell suspensions from naïve $Il5^{venus/+}Il13^{td-tomato/+}$ mice, $n = 8$. **(B)** Comparison of lung and kidney ILC2s by t-distributed stochastic neighbor embedding (t-SNE) analysis based on the cell surface antigens CD25, ICOS, KLRG1, and the type 2 effector cytokines IL-5 and IL-13. **(C)** Differential expression of ILC2-associated cell surface antigens and type 2 effector cytokines. **(D)** Percentage of IL-5^+ and IL-13^+ renal ILC2s. **(E)** Proportions of $CD45^+CD19^-CD11b^-CD49b^-CD90.2^+$IL-$5^+$ single cells which were consistent with ILC2s ($CD3^-CD4^-$) and T_H2s ($CD3^+CD4^+$) in kidney single cell suspensions from $Il5^{td-tomatoCre}$; Rosa-CAG-RFP mice, $n = 8$. All data are expressed as mean ±SEM. $^{*}P < 0.05$ by Mann-Whitney *U*-test.

Reduction, Deficiency, or Depletion of ILC2s Does Not Alter the Severity of Experimental Renal Ischemia-Reperfusion Injury in Mice

Given ILC2s were present in the kidney and localized to the renal vasculature, the impact of ILC2 reduction, deficiency or depletion on the severity of experimental renal IRI was assessed. This was achieved by using ILC2-reduced ($Rora^{fl/+}Il7r^{cre/+}$),

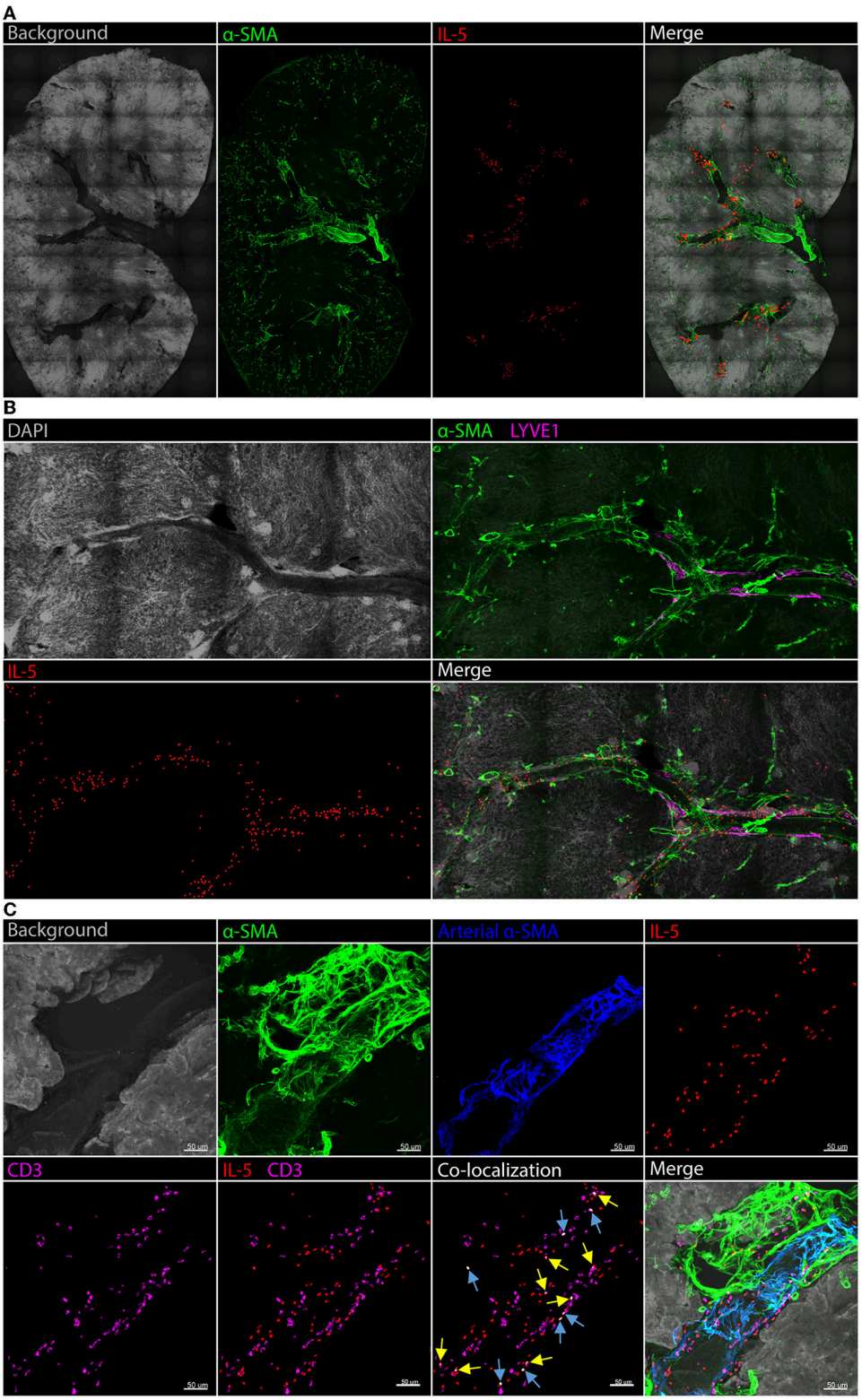

FIGURE 2 | Kidney ILC2s are localized around the vasculature under homeostatic conditions. Kidney sections from *Il5*$^{td-tomatoCre}$; Rosa-CAG-RFP mice were stained for IL-5^{+} cells (predominantly ILC2s), the IL-5^{+} pixel surface area was increased to improve clarity, tissue sections were co-stained for α-SMA for structure determination. **(A)** Gross structure of the kidney, ILC2s were located in close proximity to the renal vessels throughout the tissue. **(B)** A magnified view of a vessel

(Continued)

FIGURE 2 | spanning regions of the kidney with the addition of DAPI; LYVE1 staining indicates lymphatics which track vasculature in the cortex, ILC2s displayed no preference toward medullary or cortical vessels. **(C)** $CD3^+$ cells (predominantly T_H2 cells) contributed negligible IL-5^+ signal. Background and arterial α-SMA staining demonstrates ILC2s and T_H2 cells are located in the adventitia of the vessel. Blue arrows indicate IL-5 and CD3 co-localization in the same cell, yellow arrows indicate partial co-localization from adjacent cells.

-deficient ($Rora^{fl/fl}Il7r^{cre/+}$), and -depleted (DTx-treated $Icos^{dtr/+}Cd4^{cre/+}$) mice compared to WT and vehicle (saline-treated $Icos^{dtr/+}Cd4^{cre/+}$) controls. These mice have been previously described as appropriate tools for assessing ILC2 function *in vivo* in other organs such as the lung (27, 32). Here we show that these tools are also appropriate for assessing ILC2 function in the kidney. Indeed, kidney ILC2s were significantly lower in the ILC2-reduced, -deficient, and -depleted groups compared to WT or saline-treated controls (**Figure 3A**). Time course analysis of IRI in wild-type mice identified day 7 as the most appropriate time point for the assessment of injury severity due to the presence of histopathological features of acute tubular necrosis, collagen deposition and increased mRNA expression of extracellular matrix, injury-associated, and inflammatory factors (**Supplementary Figures 3, 4**). At day 7, 25/84 extracellular matrix factors were increased >2-fold compared to sham controls, whilst 1/84 and 6/84 were increased at day 1 and 3, respectively (**Supplementary Figure 4A**). IRI induced acute tubular necrosis characterized by dilated tubules and cast formation, compared to sham controls (**Figure 3B**, **Supplementary Figures 3A, 4B**). This was quantified as a tubular injury score and the severity of injury was similar in all genotypes (**Figure 3C**). IRI also induced collagen deposition, marked by methyl blue content between the tubules in the medulla and cortex, compared to sham controls (**Figure 3D**; **Supplementary Figure 3B**). Collagen deposition was unaffected by the absence of ILC2s. IRI also increased the mRNA expression of injury-associated factors, chemokines, and pro-inflammatory cytokines (**Figures 3E–J**, **Supplementary Figures 4C–K**). Neutrophil gelatinase-associated lipocalin, (*Lcn2*; NGAL), a biomarker of renal injury (33, 34), was increased by IRI and unaffected by ILC2 reduction, deficiency, or depletion (**Figure 3E**). C-X-C motif chemokine ligand 1 (*Cxcl1*) and *Cxcl2* were also significantly increased by IRI in all genotypes (**Figures 3F,G**). Similarly, the pro-inflammatory cytokine tumor necrosis factor (*Tnf*) was increased by IRI, compared to sham controls (**Figure 3H**). Consistent with visible collagen deposition, mRNA expression of the extracellular matrix factors collagen type I alpha 1 chain (*Col1a1*), and fibronectin 1 (*Fn1*) were increased by IRI in all genotypes (**Figures 3I,J**).

DISCUSSION

ILC2s are critical regulators of tissue homeostasis, but their role in the kidney remains to be fully elucidated. Whilst ILC2s can be induced to proliferate and protect against the deleterious consequences of experimental renal injury, it is not yet known what occurs in the absence of these cells. We first examined whether ILC2 numbers, as a proportion of $CD45^+$ hematopoietic cells, are different in lung and kidney. ILC2s accounted for a greater proportion of $CD45^+$ cells in the kidney than in the lung. t-SNE analysis grouped ILC2s from both sites differently, however the clusters were not entirely distinct between these tissues. There were differences in their cell surface antigen and type 2 cytokine expression, with kidney ILC2s expressing greater amounts of IL-5. Indeed, kidney ILC2s constitutively expressed high levels of IL-5 under homeostatic conditions. ILC2s were also the major source of IL-5 in the kidney, with negligible contribution from T_H2 cells. Therefore, IL-5 was used as a surrogate marker to determine the location of these cells in the kidney. ILC2s were identified within the kidney and were localized almost exclusively along the renal vasculature. In these studies, an IL-5 linked cre-recombinase was used in conjunction with a flox-stop-flox sequence upstream of a CAG-RFP-WPRE-cassette in the constitutively expressed ROSA26 locus to locate the ILC2s. LYVE1, which is expressed by renal lymphatic endothelial cells, was used to further define the location of these cells within the mouse kidney (35). As expected, LYVE1 staining was embedded within the connective tissue of renal arteries of the cortex, but not within the medulla since no discernable lymphatic network exists in this region of the kidney (35–37). Presumably ILC2s are situated close to the vascular as renal endothelial cells are a major source of IL-33 in the kidney (38, 39). Confirming our observation by FACS of single cell kidney suspensions, ILC2s were again found to be the major producers of IL-5 in kidney tissue sections, with minimal contribution from $CD3^+$ T cells. Whilst these studies show the location of ILC2s in the kidney using reporter mice, it remains to be determined if ILC2s are also located adjacent to the vasculature in the human kidney, and whether the location of ILC2s is altered in response to renal injury. Although multiple reports have found ILC2 numbers are unchanged by renal injury, their phenotype and activation state following injury requires further elucidation (12–16). Next, we characterized the functional role of ILC2s in the kidney using a loss-of-function approach. To achieve this, we took advantage of ILC2-reduced and -deficient mice, and mice that can be conditionally depleted of ILC2s following the administration of DTx (27). We validated the use of these tools for knockdown or ablation of ILC2s in the kidney. An experimental model of IRI was chosen, given the proximity of ILC2s to the renal vasculature. Although features of injury were visibly evident following IRI, a reduction, deficiency, or depletion in ILC2s did not alter gross histopathology in the kidney, nor did it cause mortality. Indeed, IRI-induced remodeling and collagen deposition occurred independent of ILC2s. Whilst it is possible that an earlier time point following injury may have identified differences in histopathology and gene expression, the time point in our study also allowed assessment of collagen deposition, an important feature of remodeling that is regulated by ILC2s (16, 32, 40–44). It is also plausible that a reduction in ILC2s during

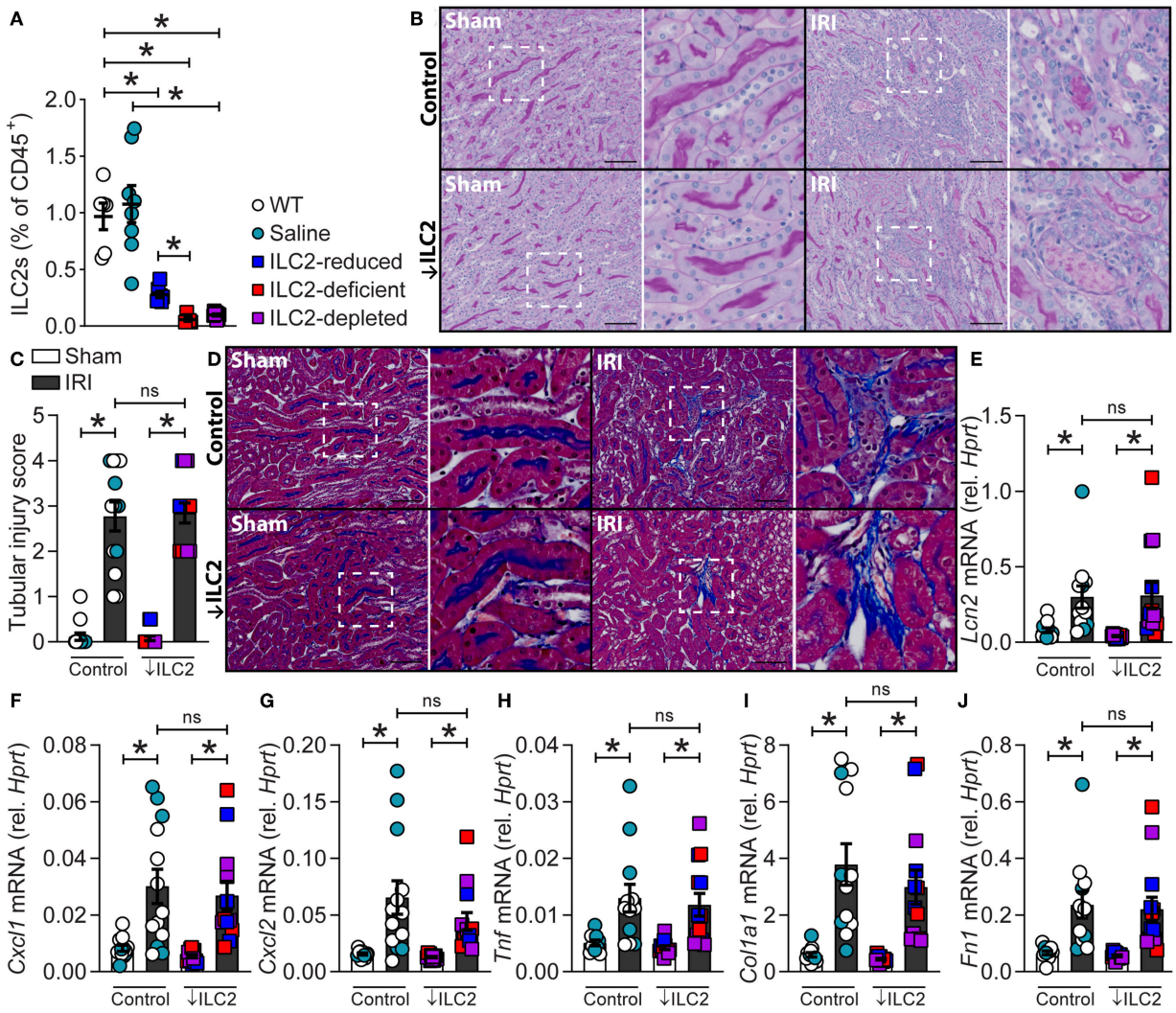

FIGURE 3 | A reduction, absence or depletion of ILC2s does not alter the severity of experimental renal ischemia-reperfusion injury. All mice were subjected to 29-min unilateral IRI with contralateral nephrectomy and were assessed compared to sham surgical controls for each genotype. All parameters were assessed 7 days after injury. **(A)** Kidney ILC2s (CD45$^+$Lineage$^-$[TCR$^-$[TCRαβ$^-$TCRγδ$^-$CD8$^-$CD4$^-$]CD11b$^-$GR-1$^-$B220$^-$TER-119$^-$CD3$^-$NK-1.1$^-$]IL-7Rα$^+$ CD90.2$^+$ST2$^+$FSC^{low}SSC^{low} single cells) as a percentage of CD45$^+$ single cells from naïve C57BL/6JAusB wild-type (WT, $n = 6$; uncolored), vehicle (saline-treated *Icos*$^{dtr/+}$*Cd4*$^{cre/+}$, $n = 8$; teal), ILC2-reduced (*Rora*$^{fl/+}$*Il7r*$^{cre/+}$, $n = 7$; blue), ILC2-deficient (*Rora*$^{fl/fl}$*Il7r*$^{cre/+}$, $n = 5$; red), and ILC2-depleted (DTx-treated-*Icos*$^{dtr/+}$*Cd4*$^{cre/+}$, $n = 8$; purple) mice. **(B)** Representative images of periodic acid-Schiff stained kidney sections from control (WT, $n = 8$; Saline, $n = 5$) and ↓ILC2 (ILC2-reduced, $n = 4$; -deficient, $n = 4$ & -depleted, $n = 5$) showing dilated tubules and cast formation. **(C)** Semi-quantitative tubular injury score indicating injury in terms of the proportion of tubules effected by casts, dilation, apoptosis, and/or loss of brush border, where a score of 5 indicates 76-100% of tubules were affected. **(D)** Representative images of Masson's trichrome stained kidney sections following IRI, blue staining indicates collagen deposition. **(E–J)** mRNA expression of injury (*Lcn2*), inflammatory (*Cxcl1*, *Cxcl2,* and *Tnf*), and extracellular matrix (*Col1a1* and *Fn1*) factors in kidney homogenates relative to *Hprt*. Scale bar in each image indicates 100 μm. All data are expressed as mean ± SEM. $^*P < 0.05$, ^{ns} not significant; by Mann-Whitney *U*-test.

AKI could be detrimental to kidney structure and function, when assessed at later time points due to potential maladaptive repair responses in the absence of ILC2s. To address this, future studies will need to investigate the role of ILC2s in the progression of AKI to chronic kidney disease (45, 46).

Collectively, this study demonstrates that a reduction, deficiency or depletion in ILC2s does not alter the severity of experimental AKI in mice. Whilst activation of ILC2s and the associated amplification of local type 2 immunity has been previously shown to reduce the deleterious consequences of AKI, comparable injury occurs when ILC2s are reduced, absent or depleted suggesting possible redundancy and compensation by other immune cells, such as T_{reg}, AAM, and T_H2 cells. This concept is supported by studies using *Rag1*$^{-/-}$ or *Rag2*$^{-/-}$ mice that lack mature T and B cells, in models of kidney injury (13, 15, 47, 48). In one study, anti-CD90.2 administration was used to deplete ILC2s, however these animals were also deficient in T_{reg} and T_H2 cells (15). In this model system, despite the depletion of ILC2s with anti-CD90.2, compensation from the T cell compartment is not possible. Our data show

that a loss of ILC2s, when the T cell compartment remains intact, has minimal effects on the severity of IRI. Our data supports the concept that the ILC2s in addition to other immune cells, such as T_{reg} and AAM, contribute to AKI. Indeed, T_{reg} depletion worsened histopathology following IRI (15). Similarly, macrophage polarization toward AAM promotes the resolution of injury (5, 12, 46, 49, 50). Further studies are required to elucidate the reason for the presence of ILC2s in the kidney, including ascending urinary tract infections and other renal insults as well as in progression to chronic disease.

ETHICS STATEMENT

All surgical procedures were approved by The University of Newcastle's Animal Care and Ethics Committee in accordance with the Australian code for the care and use of animals for scientific purposes. Procedures for immunofluorescent imaging were approved and performed in accordance with guidelines established by UCSF Animal Care and Use Committee and Laboratory Animal Resource Center.

AUTHOR CONTRIBUTIONS

The work presented was performed in collaboration with all authors. GC performed the surgical model, flow cytometry, histological assessment, gene expression analysis, and analyzed the data. KC and ABM performed the immunofluorescence experiments. SL aided with animal monitoring and flow cytometry. SJ and AD provided clinical insight for study design and surgical technique. ANM created, supplied and advised on the use of ILC2-deficient & -depleted and *Il13*$^{td-tomato}$ mice. PF supplied and advised on the use of *Il5*^{venus} reporter line. PF and PH advised on experimental design. MS designed, supervised, and facilitated all aspects of the studies. All authors participated in the interpretation of data, preparation and editing of manuscript for intellectual content.

FUNDING

MS is supported by an Australian Research Council (ARC) Discovery Early Career Researcher Award (DECRA) fellowship and grants from National Health and Medical Research Council (NHMRC), Hunter Medical Research Institute and Hunter Children's Research foundation.

ACKNOWLEDGMENTS

We acknowledge Dr. Chantal Donovan and Dr. Gang Liu for their advice on factors relating to remodeling. We also acknowledge Prof. Hans-Reimer Rodewald, Prof. Richard Locksley, Prof. Kiyoshi Takatsu, PF, and ANM for the generation or supply of the mouse lines containing the *Cd127*^{cre}, *Il5*^{cre}, *Il5*$^{td-tomato}$, *Il5*^{venus}, and *Il13*$^{td-tomato}$ modifications.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00826/full#supplementary-material

REFERENCES

1. Susantitaphong P, Cruz DN, Cerda J, Abulfaraj M, Alqahtani F, Koulouridis I, et al. World incidence of AKI: a meta-analysis. *Clin J Am Soc Nephrol.* (2013) 8:1482–93. doi: 10.2215/CJN.00710113
2. Hall AM, Unwin RJ, Parker N, Duchen MR. Multiphoton imaging reveals differences in mitochondrial function between nephron segments. *J Am Soc Nephrol.* (2009) 20:1293–302. doi: 10.1681/ASN.2008070759
3. Devarajan P. Update on mechanisms of ischemic acute kidney injury. *J Am Soc Nephrol.* (2006) 17:1503–20. doi: 10.1681/ASN.2006010017
4. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest.* (2011) 121:4210–21. doi: 10.1172/JCI45161
5. Lee S, Huen S, Nishio H, Nishio S, Lee HK, Choi B-S, et al. Distinct macrophage phenotypes contribute to kidney injury and repair. *J Am Soc Nephrol.* (2011) 22:317–26. doi: 10.1681/ASN.2009060615
6. Friedewald JJ, Rabb H. Inflammatory cells in ischemic acute renal failure. *Kidney Int.* (2004) 66:486–91. doi: 10.1111/j.1523-1755.2004.761_3.x
7. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells - a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
8. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature.* (2010) 463:540–4. doi: 10.1038/nature08636
9. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TKA, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* (2010) 464:1367–70. doi: 10.1038/nature08900
10. Loering S, Cameron GJM, Starkey MR, Hansbro PM. Lung development and emerging roles for type 2 immunity. *J Pathol.* (2018) 247:686–96. doi: 10.1002/path.5211
11. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1031/ni.2131
12. Huang Q, Niu Z, Tan J, Yang J, Liu Y, Ma H, et al. IL-25 elicits innate lymphoid cells and multipotent progenitor type 2 cells that reduce renal ischemic/reperfusion injury. *J Am Soc Nephrol.* (2015) 26:2199–211. doi: 10.1681/ASN.2014050479
13. Riedel J-H, Becker M, Kopp K, Duster M, Brix SR, Meyer-Schwesinger C, et al. IL-33-mediated expansion of type 2 innate lymphoid cells protects from progressive glomerulosclerosis. *J Am Soc Nephrol.* (2017) 28:2068–80. doi: 10.1681/ASN.2016080877
14. Stremska ME, Jose S, Sabapathy V, Huang L, Bajwa A, Kinsey GR, et al. IL233, A novel IL-2 and IL-33 hybrid cytokine, ameliorates renal injury. *J Am Soc Nephrol.* (2017) 28:2681–93. doi: 10.1681/ASN.2016121272
15. Cao Q, Wang Y, Niu Z, Wang C, Wang R, Zhang Z, et al. Potentiating tissue-resident type 2 innate lymphoid cells by IL-33 to prevent renal ischemia-reperfusion injury. *J Am Soc Nephrol.* (2018) 29:961–76. doi: 10.1681/ASN.2017070774
16. Sabapathy V, Cheru NT, Corey R, Mohammad S, Sharma R. A novel hybrid cytokine IL233 mediates regeneration following doxorubicin-induced nephrotoxic injury. *Sci Rep.* (2019) 9:3215. doi: 10.1038/s41598-019-39886-9
17. Cameron GJM, Jiang SH, Loering S, Deshpande AV, Hansbro PM, Starkey MR. Emerging therapeutic potential of group 2 innate lymphoid cells in acute kidney injury. *J Pathol.* (2019) 2019:5242. doi: 10.1002/path.5242

18. Singh AP, Junemann A, Muthuraman A, Jaggi AS, Singh N, Grover K, et al. Animal models of acute renal failure. *Pharmacol Rep.* (2012) 64:31–44. doi: 10.1016/S1734-1140(12)70728-4
19. Ramesh G, Ranganathan P. Mouse models and methods for studying human disease, acute kidney injury (AKI). *Methods Mol Biol.* (2014) 1194:421–36. doi: 10.1007/978-1-4939-1215-5_24
20. Skrypnyk NI, Harris RC, de Caestecker MP. Ischemia-reperfusion model of acute kidney injury and post injury fibrosis in mice. *J Vis Exp.* (2013) 9:78. doi: 10.3791/50495
21. Wei Q, Dong Z. Mouse model of ischemic acute kidney injury: technical notes and tricks. *Am J Physiol Renal Physiol.* (2012) 303:F1487–94. doi: 10.1152/ajprenal.00352.2012
22. Starkey MR, McKenzie ANJ, Belz GT, Hansbro PM. Pulmonary group 2 innate lymphoid cells: surprises and challenges. *Mucosal Immunol.* (2019) 12:299–311. doi: 10.1038/s41385-018-0130-4
23. Jang HR, Rabb H. Immune cells in experimental acute kidney injury. *Nat Rev Nephrol.* (2015) 11:88–101. doi: 10.1038/nrneph.2014.180
24. Turner J-E, Becker M, Mittrucker H-W, Panzer U. Tissue-resident lymphocytes in the kidney. *J Am Soc Nephrol.* (2018) 29:389–99. doi: 10.1681/ASN.2017060599
25. Jang HR, Rabb H. The innate immune response in ischemic acute kidney injury. *Clin Immunol.* (2009) 130:41–50. doi: 10.1016/j.clim.2008.08.016
26. Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, et al. Coding of facial expressions of pain in the laboratory mouse. *Nat Methods.* (2010) 7:447–9. doi: 10.1038/nmeth.1455
27. Oliphant CJ, Hwang YY, Walker JA, Salimi M, Wong SH, Brewer JM, et al. MHCII-mediated dialog between group 2 innate lymphoid cells and CD4+ T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity.* (2014) 41:283–95. doi: 10.1016/j.immuni.2014.06.016
28. Shih W, Hines WH, Neilson EG. Effects of cyclosporin A on the development of immune-mediated interstitial nephritis. *Kidney Int.* (1988) 33:1113–8.
29. Dahlgren MW, Jones SW, Cautivo KM, Dubinin A, Ortiz-Carpena JF, Farhat S, et al. Adventitial stromal cells define group 2 innate lymphoid cell tissue niches. *Immunity.* (2019) 50:707–22. doi: 10.1016/j.immuni.2019.02.002
30. Molofsky AB, Nussbaum JC, Liang H-E, Van Dyken SJ, Cheng LE, Mohapatra A, et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J Exp Med.* (2013) 210:535–49. doi: 10.1084/jem.20121964
31. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature.* (2013) 502:245–8. doi: 10.1038/nature12526
32. Donovan C, Starkey MR, Kim RY, Rana BMJ, Barlow JL, Jones B, et al. Roles for T/B lymphocytes and ILC2s in experimental chronic obstructive pulmonary disease. *J Leukoc Biol.* (2019) 105:143–50. doi: 10.1002/JLB.3AB0518-178R
33. Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol.* (2003) 14:2534–43. doi: 10.1097/01.ASN.0000088027.54400.C6
34. Supavekin S, Zhang W, Kucherlapati R, Kaskel FJ, Moore LC, Devarajan P. Differential gene expression following early renal ischemia/reperfusion. *Kidney Int.* (2003) 63:1714–24. doi: 10.1046/j.1523-1755.2003.00928.x
35. Skorecki K, Chertow GM, Marsden PA, Taal MW, Alan SL, Luyckx V. Anatomy of the kidney: Lymphatics. In: *Brenner and Rector's The Kidney E-Book*. 10th ed. Amsterdam: Elsevier (2015). p. 80–1.
36. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol.* (1999) 144:789–801. doi: 10.1083/jcb.144.4.789
37. Lee H-W, Qin Y-X, Kim Y-M, Park E-Y, Hwang J-S, Huo G-H, et al. Expression of lymphatic endothelium-specific hyaluronan receptor LYVE-1 in the developing mouse kidney. *Cell Tissue Res.* (2011) 343:429–44. doi: 10.1007/s00441-010-1098-x
38. Shelite TR, Liang Y, Wang H, Mendell NL, Trent BJ, Sun J, et al. IL-33-dependent endothelial activation contributes to apoptosis and renal injury in orientia tsutsugamushi-infected mice. *PLoS Negl Trop Dis.* (2016) 10:e0004467. doi: 10.1371/journal.pntd.0004467
39. Ferhat M, Robin A, Giraud S, Sena S, Goujon J-M, Touchard G, et al. Endogenous IL-33 contributes to kidney ischemia-reperfusion injury as an alarmin. *J Am Soc Nephrol.* (2018) 29:1272–88. doi: 10.1681/ASN.2017060650
40. Mchedlidze T, Waldner M, Zopf S, Walker J, Rankin A, Schuchmann M, et al. Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity.* (2013) 39:357–71. doi: 10.1016/j.immuni.2013.07.018
41. Moretti S, Renga G, Oikonomou V, Galosi C, Pariano M, Iannitti RG, et al. A mast cell-ILC2-Th9 pathway promotes lung inflammation in cystic fibrosis. *Nat Commun.* (2017) 8:14017. doi: 10.1038/ncomms14017
42. Hams E, Armstrong ME, Barlow JL, Saunders SP, Schwartz C, Cooke G, et al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc Natl Acad Sci USA.* (2014) 111:367–72. doi: 10.1073/pnas.1315854111
43. Wohlfahrt T, Usherenko S, Englbrecht M, Dees C, Weber S, Beyer C, et al. Type 2 innate lymphoid cell counts are increased in patients with systemic sclerosis and correlate with the extent of fibrosis. *Ann Rheum Dis.* (2016) 75:623–6. doi: 10.1136/annrheumdis-2015-207388
44. Li D, Guabiraba R, Besnard AG, Komai-Koma M, Jabir MS, Zhang L, et al. IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. *J Allergy Clin Immunol.* (2014) 134:1422–32. doi: 10.1016/j.jaci.2014.05.011
45. Sharp CN, Doll MA, Megyesi J, Oropilla GB, Beverly LJ, Siskind LJ. Subclinical kidney injury induced by repeated cisplatin administration results in progressive chronic kidney disease. *Am J Physiol Renal Physiol.* (2018) 315:F161–72. doi: 10.1152/ajprenal.00636.2017
46. Liu J, Kumar S, Dolzhenko E, Alvarado GF, Guo J, Lu C, et al. Molecular characterization of the transition from acute to chronic kidney injury following ischemia/reperfusion. *JCI Insight.* (2017) 2:94716. doi: 10.1172/jci.insight.94716
47. Li L, Huang L, Sung SJ, Lobo PI, Brown MG, Gregg RK, et al. NKT cell activation mediates neutrophil IFN-gamma production and renal ischemia-reperfusion injury. *J Immunol.* (2007) 178:5899–911. doi: 10.4049/jimmunol.178.9.5899
48. Chan AJ, Alikhan MA, Odobasic D, Gan PY, Khouri MB, Steinmetz OM, et al. Innate IL-17A-producing leukocytes promote acute kidney injury via inflammasome and Toll-like receptor activation. *Am J Pathol.* (2014) 184:1411–8. doi: 10.1016/j.ajpath.2014.01.023
49. Cao Q, Wang C, Zheng D, Wang Y, Lee VWS, Wang YM, et al. IL-25 induces M2 macrophages and reduces renal injury in proteinuric kidney disease. *J Am Soc Nephrol.* (2011) 22:1229–39. doi: 10.1681/ASN.2010070693
50. Chung S, Overstreet JM, Li Y, Wang Y, Niu A, Wang S, et al. TGF-beta promotes fibrosis after severe acute kidney injury by enhancing renal macrophage infiltration. *JCI Insight.* (2018) 3:123563. doi: 10.1172/jci.insight.123563

Conflict of Interest Statement: ANM has grant funding from GSK and AstraZeneca/MedImmune.

The remaining 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: 07 May 2019
doi: 10.3389/fimmu.2019.00861

Innate Lymphoid Cells in Mucosal Immunity

*Santosh K. Panda and Marco Colonna**

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, United States

Innate lymphoid cells (ILCs) are innate counterparts of T cells that contribute to immune responses by secreting effector cytokines and regulating the functions of other innate and adaptive immune cells. ILCs carry out some unique functions but share some tasks with T cells. ILCs are present in lymphoid and non-lymphoid organs and are particularly abundant at the mucosal barriers, where they are exposed to allergens, commensal microbes, and pathogens. The impact of ILCs in mucosal immune responses has been extensively investigated in the gastrointestinal and respiratory tracts, as well as in the oral cavity. Here we review the state-of-the-art knowledge of ILC functions in infections, allergy and autoimmune disorders of the mucosal barriers.

Keywords: ILCs, NK cells, mucosal infections, COPD, IBD – Inflammatory bowel diseases, allergy and asthma

INTRODUCTION

ILCs are a family of lymphocytes comprising the innate counterparts of T cells. They are poised to secrete cytokines that respond swiftly to pathogenic tissue damage and shape subsequent adaptive immunity (1). While lacking antigen-specific receptors, ILCs detect changes in the microenvironment through receptors for cytokines that are released upon tissue damage, as well as a broad range of receptors for nutrient components, microbial products, lipid mediators, and neuronal transmitters. Found in both lymphoid and non-lymphoid tissues, ILCs are primarily tissue resident cells and are particularly abundant at the mucosal surfaces of the intestine and lung, whereas they are extremely rare in peripheral blood (2, 3). Based on the signature cytokines they produce, their phenotype, and their developmental pathways, ILCs are divided into three major groups: ILC1s, ILC2s, and ILC3s. Two additional immune cell types, NK cells and lymphoid tissue inducer (LTi) cells, are generally included in the ILC family because their phenotypic, developmental and functional properties overlap considerably with those of ILC1s and ILC3s, respectively (4).

ILC1s secrete IFN-γ in response to IL-12, IL-15, and IL-18. IFN-γ promotes the ability of macrophages and DCs to eliminate intracellular bacteria and to present antigens by inducing expression of MHC and adhesion molecules. The features and functions typical of ILC1s largely overlap with those of NK cells, which also produce IFN-γ. In mice, ILC1s and NK cells both express NKp46 and NK1.1: in humans, both cell types express CD56 and NKp46 (5). A subset of human ILC1s that lacks CD56 and expresses CD127 has also been reported (6). In both humans and mice, ILC1s express markers of tissue residency, such as CD49a and CD103, as well as tissue retention, like CD69, whereas NK cells express markers indicative of recirculation through blood, including CD62L, CCR7, and S1PR (4, 7). Based on higher expression of perforin and granzymes, NK cells have more cytolytic potential than do ILC1s (4, 8). ILC1s can kill target cells through the cell death-inducing molecule TRAIL. In mice, the differentiation of ILC1s requires the transcription factors Hobit and T-bet, whereas NK cells rely on T-bet and Eomes (9–11). However, the expression

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Ya-Jen Chang,
Academia Sinica, Taiwan
Christoph Wilhelm,
University of Bonn, Germany

****Correspondence:***
Marco Colonna
mcolonna@wustl.edu

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

Received: *11 February 2019*
Accepted: *03 April 2019*
Published: *07 May 2019*

Citation:
Panda SK and Colonna M (2019) Innate Lymphoid Cells in Mucosal Immunity. Front. Immunol. 10:861. doi: 10.3389/fimmu.2019.00861

of these transcription factors in human ILC1s and NK cells does not follow the clear pattern seen in mice. In fact, while human intraepithelial ILC1s express Eomes (5, 12), human hepatic NK cells do not (13), Moreover, differential expression of Eomes during distinct developmental stages of NK cells has been observed (14).

ILC2s, the innate counterparts of Th2, secrete type-2 cytokines such as IL-5, IL-9, IL-13, and amphiregulin in response to TSLP, IL-25, and IL-33. IL-13 plays crucial roles in the expulsion of helminths. Moreover, IL-13 and amphiregulin help repair the tissue damage engendered by helminth and viral infections (15–17). In humans, ILC2s express CRTH2, KLRG1, ST2, and CD25 (18, 19). Although human and mouse ILC2s share many surface markers, expression of CD44 and CD161 differs between the two. Mouse ILC2s express CD44 but not CD161 whereas human ILC2s invert this phenotype and express CD161 but not CD44 (4, 20). ILC2 development depends on the transcription factors GATA3, RORα, and TCF-1 in mice (21–23). GATA3 is also required for maintenance of ILC2 number *in vivo* and its effector function in mice (22, 24). Further, it was demonstrated that GATA3 is required for activation of human ILC2s (25). Although ILC2s are considered homogenous, they are classified into two groups based on their responsiveness to IL-33 and IL-25. IL-33 responsive ILC2s present in the steady state are called natural ILC2s (nILC2), whereas IL-25 responsive ILC2s elicited upon exposure to IL-25 or helminth infection are referred to as inflammatory ILC2s (iILC2) (26, 27). nILC2s are demarcated by elevated expression of Thy1 and ST2 along with relatively little KLRG1; in comparison, iILC2s express more KLRG1, less Thy1 and almost no ST2 on the cell surface (26, 27). Another subpopulation of ILC2s that produce IL-10 ($ILC2_{10}$) has also been identified (28). $ILC2_{10}$ can be induced by IL-33 or papain and are transcriptionally distinct; moreover this population undergoes contraction when the stimulus is removed and the few remaining $ILC2_{10}$ can be promptly activated upon restimulation (28).

Because ILC3s produce IL-22 and IL-17 in response to IL-23 and IL-1β, they are considered the innate counterparts of Th17. IL-22 stimulates the secretion of antimicrobial peptides by epithelial cells and mucus production by goblet cells, thereby supporting barrier integrity in the intestine (**Figure 1**) (29–31). Moreover, it promotes the differentiation of epithelial cells from intestinal stem cells (32). IL-17 promotes granulopoiesis and the secretion of neutrophil chemoattractant (33). ILC3s also produce additional cytokines such as IL-26 (in humans), GM-CSF and TNF-α (34, 35). GM-CSF sustains the generation and survival of myeloid cells, like gut DCs, that promote generation of tolerogenic commensal-specific T cells (35). The differentiation of ILC3s is driven by the transcription factors RORγt and AHR (36, 37). Like ILC3s, LTi are dependent on RORγt and secrete IL-22 and IL-17. In the embryo, LTi drive the development of secondary lymphoid organs, including lymph nodes and Peyer's patches, through the expression of lymphotoxin (LT) that engages LTβ receptor on stromal organizer cells. In the adult, LTi cells cluster to form intestinal cryptopatches, which expand into B cell-rich isolated lymphoid follicles (37–40). In humans, ILC3s express NKp44, CD127, c-Kit, and CCR6. In mice, ILC3s express also CD127, c-Kit, but include $CCR6^{+}NKp46^{-}$ LTi as well as $CCR6^{-}NKp46^{-}$ and $CCR6^{-}NKp46^{+}$ subsets.

Since ILCs mainly populate mucosal sites, this review focuses on how these cells typically contribute to maintenance of barrier integrity and protection against various pathogenic challenges, as well as their propensity to promote allergic and autoimmune diseases when inappropriately stimulated. We individually review the impact of ILCs in the respiratory and gastrointestinal tracts as well as the oral mucosa and discuss potential targeting of ILCs for therapeutic intervention in human diseases.

ILCs IN THE RESPIRATORY TRACT MUCOSA AND LUNGS

ILCs are present throughout all segments of the respiratory tract. While ILC2s are the predominant ILC population in mice, ILC3s dominate the human respiratory tract (2, 41). However, the composition and functions of ILC subsets dynamically change during experimental mice models of pathology and human diseases.

ILCs IN RESPIRATORY INFECTIONS

Experiments in mice have shown that ILC2s promote epithelial and goblet cell proliferation and mucus production in the H1N1 influenza virus infection model through secretion of amphiregulin and IL-13 (15). These ILC2 functions contribute to restore lung epithelial integrity. Accordingly, antibody-mediated depletion of ILC2s compromised lung function during H1N1 influenza infection. However, a study by Chang et al. demonstrated that ILC2s play a pathogenic role through IL-13 production in the H3N1 influenza infection model that induces airway hyper-reactivity, a cardinal feature of asthma (42). H3N1-induced airway hyper-reactivity was attenuated in $Il13^{-/-}$ mice, whereas adoptive transfer of ILC2s restored this response (42). The reason for this discrepancy in the impact of ILC2s is yet to be deciphered; perhaps the differential virulence of the H1N1 and H3N1 viral strains used in these models is responsible. ILC2s also provide defense against helminth infection of the lung through the production of IL-9, IL-13, and amphiregulin (**Figure 1**) (16, 17, 43). A recent study by Huang et al. demonstrated that the $KLRG1^{hi}$ $ST2^{-}$ ILC2s that protect the lung from helminth-induced tissue damage originate in the intestine and migrate from the intestine to the lung during infection (17).

NK cells and ILC1s contribute to immune responses against viruses through secretion of IFN-γ (**Figure 1**). NK cells play dual roles during influenza infection. Increased virus accumulation in the lung was observed in the absence of NK cells (44–46). However, depletion of NK cells rendered mice more resistant and able to survive severe influenza infection (47). Furthermore, adoptive transfer of NK cells obtained from influenza-infected lungs into mice newly infected with influenza exacerbated the disease (47). ILC1s protect from Sendai virus and influenza infection by robust secretion of IFN-γ in experimental models (48). Beneficial roles for NK cells, ILC1s and ILC2s have been demonstrated in other mouse models of lung viral infections,

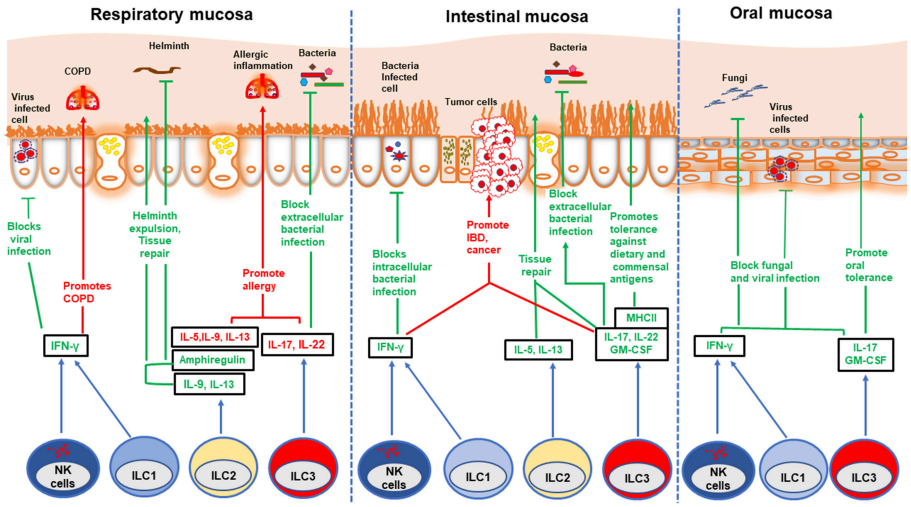

FIGURE 1 | Role of ILCs in different mucosae. Different ILC subsets play beneficial and detrimental roles in different mucosae: protection against various infections, tolerance against innocuous antigens, induction of allergy, IBD, and cancer. NK cells and ILC1s block viral and intracellular bacterial infections by secreting IFN-γ. However, ILC1s promote COPD and IBD in respiratory and intestinal mucosa, respectively. ILC2s contribute to worm expulsion and tissue repair in both respiratory and intestinal mucosa during helminth infection. Further, these cells repair viral induced lung injury by secreting amphiregulin. On the other hand, ILC2s promote allergic inflammation in lung and nasal polyps. ILC3s play dual role in both respiratory and intestinal mucosa. These cells confer protection against extracellular bacterial infection by secreting IL-22 which induces production of anti-microbial peptides by epithelial cells. ILC3s block oral fungal infection in an IL-17-dependent manner. These cells also promote tolerance against commensal and dietary antigens through MHC-II mediated antigen presentation. However, inappropriate activation of ILC3s can play a detrimental role by instigating allergy in the respiratory mucosa, as well as IBD and cancer in the intestinal mucosa.

such as rhinovirus (49, 50) and respiratory syncytial virus (RSV) (49, 51). Finally, ILC3s have also been implicated in lung infections and inflammation. Lung ILC3s play a protective role in *S. pneumonia* infected mice through secretion of IL-17 and IL-22 (**Figure 1**) (52). Despite the abundance of studies in mouse models, the role of ILCs in human respiratory infections is yet to be deciphered (**Table 1**).

ILCs IN ASTHMA

Multiple studies concur that ILC2s play a pathogenic role during lung allergy and inflammation (**Figure 1**) (73, 74). Mice lacking ILCs, T cells, and B cells developed less airway inflammation than did mice lacking only T cells and B cells in the papain-induced eosinophilic asthma model (56, 75). Furthermore, adoptive transfer of ILC2s was sufficient to induce airway inflammation in the absence of B and T cells (75). ILC2s initiate allergic lung inflammation by secreting IL-13, which promotes migration of activated DCs to the draining lymph node where they prime Th2 differentiation (56). Lung ILC2s promoted proliferation and antibody production by B1 and B2 cells *in vitro* and contributed to enhanced production of IgM *in vivo* in an experimental model of polysaccharide-mediated nasal allergy (76). Moreover, ILC2s acquired a memory-like phenotype after primary encounter with allergen and more readily promoted allergic inflammation in the lung upon secondary encounter with unrelated allergens than did naïve ILC2s (77).

In human, expansion of ILC2s and elevated type-2 cytokines have been observed in a variety of patients with allergic asthma (**Table 1**). Approximately twice as many ILC2s were found in the peripheral blood of allergic asthmatic patients than in healthy controls (60). In addition, ILC2s from allergic asthmatic patients were hyper-responsive to IL-33 and IL-25 and secreted significantly higher amounts of IL-5 and IL-13 than did ILC2s from healthy controls (60). Smith et al. reported higher percentages of ILC2s in the blood and sputum of prednisone-dependent severe eosinophilic asthma patients than in the blood of asymptomatic patients with steroid-naive mild atopic asthma or healthy controls (78). Respiratory allergen challenge of patients with allergic asthma elicited a sudden increase and rapid activation of ILC2s primed to secrete IL-5 and IL-13 (79). Further supporting a role for ILC2s in allergic asthma, the frequency of ILC2s and the levels of IL-33 in the bronchoalveolar lavage (BAL) of patients with allergic asthma were positively correlated with severity of the disease (80). Likewise, the percentage of ILC2s was higher in the peripheral blood of patients with eosinophilic asthma and positively correlated with eosinophil counts in the sputum (81). Similarly, the frequency of ILC2s was higher in the blood and BAL of children with severe therapy-resistant asthma than in children with recurrent lower respiratory tract infections (82). Finally, an increase of ILC2s in the BAL of patients with idiopathic pulmonary fibrosis has also been observed (83).

How other ILCs impact asthma is less clear. ILC3s have been suggested to play a pathogenic role in asthma patients. Numbers of IL-17-producing ILC3s in the human lung positively correlate with severity of the disease (57). IL-22 production by ILC3s has also been implicated in asthma pathogenesis, as asthma patients have increased levels of IL-22 compared to healthy controls (84). In mice, NK cells and ILC1s that produce IFN-γ inhibit ILC2 expansion and type-2 cytokine production (85, 86) and hence

TABLE 1 | Role of ILCs in different mucosa of humans and mice.

Mucosa		Host	Cells				
			NK cells	ILC1	ILC2	ILC3	References
Respiratory	Infections	Mouse	Protects from viral infections.		Repair the damage induced by viral infections	Blocks extracellular bacterial infections	(45), (48), (15, 52)
		Human	Less number of peripheral NK cells in influenza infected patients	ND	ND	ND	(53)
	COPD	Mouse	NK cells are hyperactivated during COPD	ILC2s converted Into ILC1s	ND	ND	(54, 55)
		Human		Number increased in peripheral blood and lung	ND	Accumulated in the lung tissues of COPD	(40, 41, 54)
	Allergy	Mouse	Inhibits allergic inflammation	Unclear	Promotes allergic inflammation	Induce airway hyperinflammation	(56–58)
		Human	Unclear	Unclear	Increased ILC2 numbers in patients with allergic asthma, rhinosinusitis	Increased ILC3 numbers in patients with allergic asthma	(57, 59, 60)
	Helminth infection	Mouse	Unclear	ND	Worm expulsion and tissue repair	ND	(16, 17)
		Human	Unclear	ND	ND	ND	
Intestinal	Infection	Mouse	Blocks intracellular bacterial infection		worm expulsion and tissue repair	Blocks extracellular bacterial infection	(39, 61–64)
		Human	ND	ND	ND	ND	
	Homeostasis	Mouse	ND	ND	ND	Maintain tissue homeostasis	(32, 35)
		Human	ND	ND	ND	ND	
	IBD	Mouse	Unclear	Promotes IBD	ND	Promotes IBD	(5, 65, 66)
		Human	Unclear	Accumulated in the inflamed gut	ND	Accumulated in the inflamed gut	(5, 67)
	GVHD	Mouse	Promotes/inhibits	ND	Promotes barrier integrity	Promotes barrier integrity	(68–70)
		Human	Unclear	ND	ND	Reverse correlation between the number of ILC3 and disease severity.	(70, 71)
Oral	Infection	Mouse	ND	ND	ND	Blocks fungal Infection and promote tolerance against dietary antigens	(35, 72)
		Human	ND	ND	ND	ND	

ND, Not defined.

might help control the disease. Consistent with this, depletion of NK cells during the early stages of papain-induced lung allergy in mice resulted in increased ILC2-mediated cytokine production, which exacerbated pathology (58). Furthermore, Han et al. demonstrated that IFN-γ inhibits expansion of IL-13 producing ILC2s and attenuates the development of rhinovirus-induced asthma pathology in baby mice (87). Altogether, these studies suggest that ILC1s and NK cells attenuate the initiation and progression of allergic inflammation in the lung in mouse models. However, the role of NK cells in human allergic asthma is unclear. A human study documented fewer NK cells in the peripheral blood of severe asthma patients than in healthy controls, as well as an enrichment of the $CD56^{bright}$ NK cell subset in the BAL of asthmatic patients, whereas no significant difference in the frequency of ILC2s was detected in the blood of controls, severe, and mild asthma patients (88). One report described increased frequency of an unusual population of NK cells that produces IL-4 rather than IFN-γ in the blood of asthmatic patients (89). In contrast, another study showed that heightened IFN-γ responses in the airways of patients

were associated with severe asthma, which often involves mixed granulocytic inflammation that includes neutrophils as well as eosinophils (90). Supporting a role of IFN-γ in initiation of airway hyper responsiveness these authors showed that in a mouse model mimicking human severe asthma IFN-$\gamma^{-/-}$ mice failed to exhibit the symptoms of severe asthma as compared to wild type controls (90). Further research in human patients focusing on NK cells, ILC1s, and ILC2s in both blood and inflamed tissue might decipher how the balance between different subsets of ILCs impacts asthma pathogenesis.

ILCs IN ALLERGIC RHINITIS

ILC2s have also been implicated in the pathogenesis of allergic rhinitis. The frequency of ILC2s was elevated in nasal polyps characteristic of chronic rhino-sinusitis and aspirin-exacerbated respiratory disease and positively correlated with disease severity (18, 41, 59, 91, 92). Recruitment of ILC2s to the nasal mucosa upon a nasal mucosal challenge in subjects with allergic rhinitis was demonstrated in human volunteers (93). TSLP produced by epithelial cells and IL-4 produced by eosinophils induces ILC2 activation and secretion of IL-4, IL-5, and IL-13; this activates eosinophils and thereby elicits a positive feedback loop. Disrupting this loop with anti-IL-5 antibodies was beneficial for treating chronic rhino-sinusitis in humans (94, 95). More ILC2s have been noted in the peripheral blood of allergic rhinitis patients than in blood from healthy controls (96, 97). Moreover, the response to house dust mite-specific immunotherapy negatively correlated with ILC2 numbers; patients with a good response to the allergen-specific immunotherapy had relatively few ILC2s (97). Furthermore, subcutaneous allergen therapy resulted in fewer circulating ILC2s in patients with allergic rhinitis (98). In contrast, NK cells appear to play a beneficial role in allergic rhinitis, as patients had fewer circulating $CD56^{bright}$ NK cells than did healthy controls (99).

ILCs IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE OF THE LUNG AND CANCER

Chronic obstructive pulmonary disease (COPD) is a disorder caused by cigarette smoke or long-term inhalation of noxious gases, which damage the upper and lower airways, eventually causing bronchitis and emphysema (100). Studies in mice and humans have demonstrated that lung resident ILC2s acquire an ILC1 phenotype and secrete IFN-γ during COPD (41, 54). Furthermore, ILC1 numbers were increased in the peripheral blood of patients with severe COPD (**Table 1**). Additionally, a high ILC1-to-ILC2 ratio in the blood of COPD patients positively correlated with severity of the disease (54). Shikhagaie et al. reported significantly increased numbers of neuropilin^{+} ILC3s in the lungs of patients with COPD, which were associated with formation of ectopic lymphoid aggregates (40). Further supporting a role for ILC3s in COPD, IL-17 was found to accumulate in the lungs of COPD patients (101).

In contrast to their deleterious effect in COPD, ILCs may be beneficial in lung cancer. ILC3s have been reported to congregate in human non-small cell lung carcinoma tissues where they may play a protective role by promoting the formation of tertiary lymphoid structures (102). The role of NK cells in lung cancer remains confusing, but should become more well-defined as better reagents/methods to detect NK cells and relevant activating and inhibiting receptors are developed and widely used. An early study that employed CD57, which is not specific for NK cells, found that the presence of tumor infiltrating $CD57^{+}$ cells was associated with a better prognosis in patients with primary squamous cell lung carcinoma (SCLC) (103). A more recent study suggested that infiltration of $CD16^{+}CD56^{+}$ NK cells in the tumor periphery correlated with increased survival time of patients with non-small-cell lung carcinoma (NSCLC) (104). This study also noted that intra-tumoral NK cells downregulated activating receptors, including NKG2D, and upregulated the inhibitory receptor NKG2A in a mouse model of metastatic human large cell lung cancer. Consistent with this, Platonova et al. reported that intra-tumoral NK cells in NSCLC patients have an altered phenotype, with decreased expression of activating receptors such as NKp30, NKG2D, NKp80 and increased expression of the inhibitory receptor NKG2A (105). Along these lines, Carrega et al. found that most NK cells infiltrating NSCLC were $CD56^{bright}$ $CD16^{-}$; the intra-tumoral NK cells had less cytotoxic potential than did NK cells in the peripheral blood and lung, but retained the ability to secrete IFN-γ and TNF-α (106). However, Hodge et al. reported that NK cells (as well as NKT, $CD4^{+}$ and $CD8^{+}$ T cells) isolated from lung cancer tissue secreted less perforin, granzyme B, IFN-γ, TNF-α after stimulation than did NK, NKT, and T cells isolated from healthy tissue (107). Finally, Christmas et al. found that while the frequency of peripheral NK cells did not differ between patients with NSCLC or SCLC and healthy controls, NK cells isolated from cancer patients had diminished expression of NKp46, CD25, and perforin A and were functionally impaired in terms of cytoxicity and cytokine production (108). Collectively, these studies suggest that the tumor microenvironment either recruits NK cells with limited cytotoxic potential or, more likely, directly alters the phenotype of NK cells in order to escape from NK cell-mediated anti-tumor responses. Further research in human patients is warranted to delineate the precise role of ILCs in lung cancer.

THE IMPACT OF ILCs ON INTESTINAL MUCOSA

ILC3s are the most abundant subset of ILCs in both the fetal and adult human intestine (8). In contrast, the distribution pattern of ILC1s and ILC2s changes during development. In the fetal intestine ILC2s prevail over ILC1s, whereas ILC1s assume the dominant role in the adult intestine (8, 18). ILCs are unevenly distributed throughout the segments of the gastro-intestinal (GI) tract. The upper GI tract, e.g., esophagus, is enriched with ILC1s, whereas the distal part of the ileum and colon are chiefly populated by ILC3s (109).

ILCs IN GASTROINTESTINAL TRACT INFECTIONS

The role of ILCs in intestinal infection has been demonstrated in various experimental models (**Table 1**); ILC3s participate in the immune responses to *Citrobacter rodentium, Clostridium difficile, Salmonella enterica, Listeria monocytogenes, and Toxoplasma gondii* (10, 39, 63, 110–112). Further work has shown that ILC3s restrict colonization of segmented filamentous bacteria in the gut (113) and inhibit the invasion of *Alcaligenes* species into Peyer's patches (114). ILC3s counter bacterial infections through secretion of IL-22, which stimulates epithelial cells to produce antimicrobial peptides (**Figure 1**) (31). Furthermore, IL-22 induces fucosyltransferase 2 expression in intestinal epithelial cells, which promotes fucosylation of surface proteins that are shed into the lumen. These fucosylated proteins release fucose, which is metabolized by microbes as an energy source. Fucose metabolism has been shown to suppress virulent gene expression in commensal bacteria (115), and Goto et al. demonstrated that the absence of epithelial cell fucosylation renders mice more susceptible to *Salmonella* infection (62). IL-22 production is mainly stimulated by IL-23 and IL-1β produced by inflammatory monocytes, macrophages and DCs. TNF-like ligand 1A produced by mononuclear phagocytes upon interaction with microbiota in the gut also induces IL-22 production by ILC3s and promotes healing of mucosal layer during DSS-induced acute colitis (116).

ILC1s and NK cells help combat intracellular pathogens such as *T. gondii, L. monocytogenes, Salmonella typhimurium* and viruses through IFN-γ production (10, 117, 118). ILC1s cooperate with ILC3s in providing protection against intestinal *C. difficile* infection (112); accordingly, rapid infection and death ensue in the absence of IFN-γ and IL-22. By secreting IL-5 and IL-13, ILC2s induce goblet cell differentiation and mucus production that propels worm expulsion and protects from tissue damage (61, 64, 119, 120). Finally, while most studies have focused on bacterial infections, ILCs may also impact intestinal viral infections. Studies in mice have shown a protective role for ILC3s during rotavirus infections, in which IL-22 synergizes with IFN-λ in inducing STAT1 activation (121). Recently, a cross-sectional study reported that the colons of HIV-1 infected patients are enriched with NKp44$^+$ILC1s that produce IFN-γ (122).

While the above studies support a critical role for ILCs in maintaining barrier integrity that protects mice from a variety of infections, one study has suggested that ILCs are completely redundant. Vely et al. studied SCID patients following allogeneic hematopoietic stem cell transplants that reconstituted the T cell compartment but not the ILC compartment. These patients did not manifest an increased risk of infections and other complications (123). Further research in human subjects addressing these issues is warranted.

ILCs IN AUTOIMMUNITY

ILCs have been extensively studied in intestinal autoimmune disorders, particularly inflammatory bowel disease (IBD) (**Table 1**). Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of IBD that affect distinct layers of the gut wall and colonic mucosa. Although the mechanisms underpinning IBD remain incompletely understood, immune responses against commensal microbes in the intestine have long been implicated. The role of ILC3s in IBD is unclear. Several papers have proposed that ILC3s may contribute to autoimmunity. IL-23, the key activator of ILC3s, has been shown to drive colitis (124). Accumulation of ILC3s in inflamed colons of *Helicobacter hepaticus* infected mice has been reported (65). Moreover, mice either depleted of or genetically modified to lack ILC3s were resistant to colitis (65). Similarly, IL-17-producing ILC3s have been shown to drive colitis, whereas administration of anti-IL-17 antibodies ameliorated disease (125). ILC3s also drove intestinal inflammation and pathology in the anti-CD40 model of innate colitis mice through secretion of IL-22 and GM-CSF (66). In agreement with this study, Pearson et al. demonstrated that ILC3-mediated production of GM-CSF recruits inflammatory monocytes into the intestine, which sustain intestinal inflammation (126). Corroborating these findings in humans, IL-17-producing ILC3s have been found to accumulate in the inflamed colons of patients with CD (127). Other studies have proposed a tolerogenic role for ILC3s during intestinal inflammation. ILC3s express MHC class II and can present commensal bacteria to T cells. However, because they lack costimulatory molecules, ILC3s are unable to elicit commensal-specific CD4$^+$ T cells and, in fact, induce T cell tolerance and mitigate inflammation in mouse models (128, 129). Consistent with this, unusually low expression of MHC-II has been observed on colonic ILC3s from pediatric IBD patients (128). A similar role for ILC3s in maintaining tolerance against dietary and innocuous commensal antigens has also been reported (35, 129, 130).

ILC1s have also been implicated in human IBD (**Table 1**). CD127$^+$ lamina propria ILC1s and CD127$^-$ intra-epithelial ILC1s amass in the inflamed intestine of CD patients (5, 8). Interestingly, expansion of IFN-γ producing cells with an NK cell phenotype in the lamina propria of CD patients was accompanied by a reciprocal decrease in IL-22 producing NKp44$^+$ ILC3s (131). Li et al. also observed that the frequency of ILC1s rises in the inflamed ileum of CD patients, whereas frequency of ILC3s declines (132). The increase in ILC1 frequency at the cost of ILC3s might be due to plasticity of ILC3s, which transdifferentiate into ILC1s under the influence of IL-12 and/or IL-23 produced by myeloid cells (12). Increased frequency of Lin$^-$CD127$^+$ ILC1s in the colons of patients with IBD and primary sclerosing cholangitis has also been observed. Furthermore, these authors have demonstrated that the frequency of ILC1s drops in the blood of patients, suggesting that ILC1s are recruited into the inflamed tissue (133).

ILC2s have also been implicated in intestinal inflammation. Expansion of IL-13 producing ILC2s has been shown to play a detrimental role in oxazolone-induced colitis (134). However, expansion of amphiregulin-producing ILC2s has been shown to contribute to tissue repair during DSS-induced colitis (135). Thus, ILC2s may either promote or block intestinal pathogenesis, depending on the disease setting. ILC2s have been noted

in intestinal samples from CD patients (19). Interestingly, these ILCs secreted IFN-γ, reflecting the plasticity of ILC2s during intestinal inflammation. Increased ILC2 frequency that positively correlates with severity of the disease has been observed in patients with eosinophilic oesophagitis (136). While accumulation of ILCs in the intestine of CD patients has been demonstrated, whether ILCs are involved in UC remains unclear. In fact, Gwela et al. reported no difference in ILC frequencies between UC patients and healthy controls (133).

In addition to autoimmunity, intestinal tissue damage can be induced by chemotherapy and radiotherapy. In this case, ILC3s help repair intestinal damage by secreting IL-22 (68, 137). During graft-vs.-host disease (GVHD), which can occur in leukemic patients after allogeneic hematopoietic stem cell transplant, allogeneic T cells can attack and destroy ILC3s, thereby depleting intestinal IL-22. In mice with acute GVHD, ILC3s secreted significantly less IL-22, resulting in impaired epithelial functions (**Table 1**) (68). Corroborating a role for ILC3s and ILC3-secreted IL-22 in tissue repair and resolution of inflammation during GVHD, fewer ILC3s were observed in patients with acute or chronic GVHD than in patients that did not develop GVHD, (71). Furthermore, recovery of gut homing $CD69^+$ activated ILC3s was associated with a lower incidence of GVHD (71). Finally, infusion of donor ILC2s after bone marrow transplant reduced the lethality of GVHD up to 70% and enhanced the barrier function of the GI tract in an allogenic stem cell transplant model (69). Altogether, these studies support a beneficial role for ILC2s and ILC3s in prognosis of GVHD.

ILCs IN INTESTINAL CANCER

It has been known for long time that IBD patients have an increased risk of intestinal cancer due to chronic inflammation (138). Given that ILCs accumulate in the intestinal mucosa during IBD, they could contribute to the inflammatory environment and may play a pro-tumorigenic role. Indeed, the key activator of ILC3s, IL-23, and the ILC3 effector cytokines IL-17 and IL-22 are associated with both experimental and clinical tumorigenesis (**Figure 1**) (139–142). In contrast to ILC3s, NK cells and ILC1s may have anti-tumorigenic effects through secretion of IFN-γ. A decreased frequency of NK cells expressing NKp30, NKp46, and NKG2D paralleled by an increased frequency of ILC2s was noted in the peripheral blood of gastric cancer patients in comparison to healthy controls (143). Similarly, serum levels of IFN-γ in gastric cancer patients were lower than those found in healthy donors (144, 145). Although infiltration of NK cells into gastric cancer tissues is scarce (146), the presence of intratumoral NK cells correlated with a better prognosis (147). While these studies suggest that ILCs are involved in the pathogenesis of intestinal cancer, further studies in human patients is required to address their exact role.

ILCs IN THE ORAL MUCOSA

Like the intestinal mucosa, the oral mucosa is populated with commensal microflora and exposed to dietary antigens and pathogens. Thus, ILCs present in the oral mucosa may help maintain barrier function and protect against pathogenic infections. Human ILC3s and intraepithelial ILC1s were originally identified in tonsils, which are secondary lymphoid tissues coated by the oral mucosa (5, 34). ILCs have been also found in human gingivae. Approximately 10–15% of total $CD45^+$ cells identified were ILCs and most of them were IFN-γ secreting ILC1s (148). A recent study by Brown et al. demonstrated the presence of all

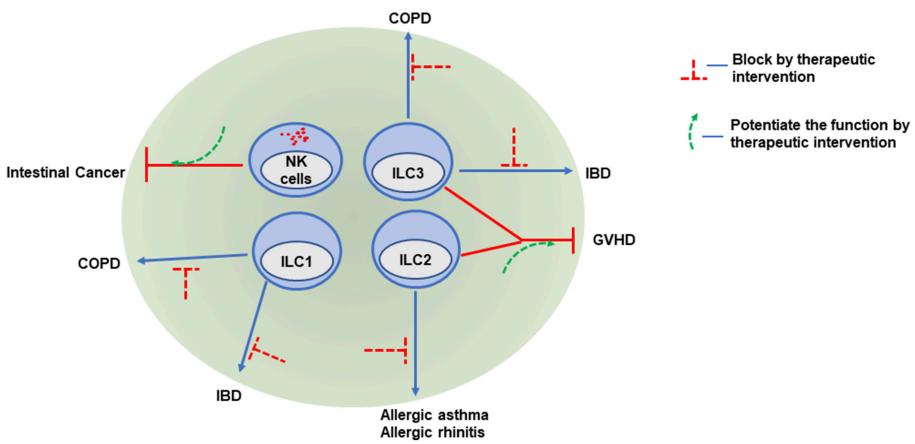

FIGURE 2 | ILCs as potential therapeutic targets. Because of their various roles in promoting or attenuating pathogenesis, ILCs may be potential therapeutic target. Infiltration of NK cells into intestinal cancer is associated with better prognosis, therefore increasing NK cell infiltration and potentiating NK cell functions in tumors might be beneficial. Hyperactivation of both ILC1 and ILC3 promote COPD and IBD. Regulation of pathogenic cytokine production or antibody mediated depletion of these cells might be a potential treatment option. Similarly, antibodies targeting type-2 cytokines produced by ILC2 may be promising in the treatment of allergy and asthma patients. Both ILC2 and ILC3 ameliorate clinical symptoms and play protective roles in GVHD. Expansion of ILC2 and ILC3 numbers by therapeutic intervention might be helpful to control GVHD.

three groups of ILCs in the murine gingivae (149). ILC3s provided protection against oropharyngeal infection with *Candida albicans* through production of IL-17A and IL-17F induced by IL-23 (**Figure 1**) (72); thus, both *Rorγt*$^{-/-}$ mice lacking ILC3s and mice depleted of ILC3s by antibody treatment suffered severe infection (72). IFN-γ-producing ILC1s and NK cells have been found in the oral mucosa of macaques. During simian immunodeficiency virus infection, expansion of ILC1s and increased IFN-γ production were noted in the oral draining lymph nodes and tonsils whereas NK cells remained unchanged (150). Future research will decipher the impact of ILCs in oral immune responses to commensals and dietary antigens.

TARGETING ILCs FOR THERAPY

A strong rationale for developing therapies specifically targeting ILCs, such as depleting or agonistic antibodies, depends on whether ILCs play unique, non-redundant roles in the context of various diseases, which is yet to be firmly established. However, evidence from both experimental mice models and humans has suggested that therapeutic targeting of ILCs might be beneficial for autoimmune disorders. Depletion of Thy-1 positive ILCs was beneficial for treating *H. hepaticus* induced colitis (65). Similarly, depletion of ILC2s blocked the development of papain-induced allergy (75). When ILCs and T cells have redundant functions, an alternative option is to neutralize the effector cytokines produced by both ILCs and T cells. Blockade of the IL-17 receptor or neutralizing IL-17 and IFN-γ in several clinical trials failed to ameliorate IBD (151, 152). However, targeting both IL-17 and IFN-γ bore promising results in preclinical models (65). Blockade of cytokines that stimulate IL-17 and IFN-γ secretion, such as IL-12 and IL-23 or IL-23 alone, has also been effective for treating CD patients (153, 154). Since GVHD is associated with fewer IL-22-producing ILC3s, enhancement of ILC3 numbers and function might be explored for the treatment of GVHD (**Figure 2**) (68, 71).

Several attempts have been made to explore the efficacy of targeting ILC2s in respiratory diseases. Anti-IL-5 and anti-IL-4 receptor α treatments have had promising effects in treating patients suffering from chronic rhinosinusitis with nasal polyps and eosinophilic asthma (94, 155, 156). Similarly, inhibiting ILC2 functions with CRTH2 antagonists can restore lung function in asthma patients (**Figure 2**) (157, 158). Further research and well-designed clinical studies should help delineate the efficacy of targeting ILCs in various mucosal diseases.

FUTURE PERSPECTIVES

ILCs appear to contribute significantly to human health and disease, playing beneficial roles in some mucosal infections and GVHD, while aggravating pathology in IBD and COPD. Are ILCs a valid therapeutic target? To address this question, it is important to understand whether ILCs play unique roles or are largely redundant with T cells. Studies in mouse models have provided limited answers, as they are mainly performed in RAG-deficient mice that lack adaptive responses. Animal models in which ILCs are selectively ablated in the presence of intact adaptive responses are underdeveloped. Most human studies describe single timepoint assays that provide a snapshot of ILCs at a particular stage of the disease. Longitudinal studies addressing multiple subsets of ILCs at different stages of disease is warranted to provide a clearer picture of their impact. For instance, while NK cells seem to control lung ILC2 expansion in asthma, it is important to understand whether NK-ILC2 cross-talk occurs at a particular stage or throughout the disease. Similarly, ILC3 secretion of IL-17 and IFN-γ seems to be detrimental in IBD, whereas IL-22 secretion may be protective. Thus, it is essential to examine the ratio of ILC3s producing IL-17/IFN-γ and IL-22 at different stages of the disease. Although ethical constraints and limitations of available tissue restrict human studies, further analysis including diverse age groups and focusing on different stages of disease would help better understand the role of ILCs as well as how they might be targeted for the therapy of mucosal diseases.

AUTHOR CONTRIBUTIONS

SP wrote the first draft. MC contributed to conceiving and writing the manuscript.

FUNDING

MC is supported by NIH grants AI095542, DE025884, AI134236, and AI134035. MC receives research support from Pfizer.

ACKNOWLEDGMENTS

We thank Susan Gilfillan for critical comments.

REFERENCES

1. Bando JK, Colonna M. Innate lymphoid cell function in the context of adaptive immunity. *Nat Immunol.* (2016) 17:783–9. doi: 10.1038/ni.3484
2. Mjosberg J, Spits H. Human innate lymphoid cells. *J Allergy Clin Immunol.* (2016) 138:1265–76. doi: 10.1016/j.jaci.2016.09.009
3. Ebbo M, Crinier A, Vely F, Vivier E. Innate lymphoid cells: major players in inflammatory diseases. *Nat Rev Immunol.* (2017) 17:665–78. doi: 10.1038/nri.2017.86
4. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell.* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
5. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity.* (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
6. Simoni Y, Fehlings M, Kloverpris HN, McGovern N, Koo SL, Loh CY, et al. Human innate lymphoid cell subsets possess tissue-type based

heterogeneity in phenotype and frequency. *Immunity.* (2017) 46:148–61. doi: 10.1016/j.immuni.2016.11.005
7. Cortez VS, Robinette ML, Colonna M. Innate lymphoid cells: new insights into function and development. *Curr Opin Immunol.* (2015) 32:71–7. doi: 10.1016/j.coi.2015.01.004
8. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol.* (2013) 14:221–9. doi: 10.1038/ni.2534
9. Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity.* (2012) 36:55–67. doi: 10.1016/j.immuni.2011.11.016
10. Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell.* (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
11. Mackay LK, Minnich M, Kragten NA, Liao Y, Nota B, Seillet C, et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science.* (2016) 352:459–63. doi: 10.1126/science.aad2035
12. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and−23 control plasticity of CD127(+) group 1 and group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity.* (2015) 43:146–60. doi: 10.1016/j.immuni.2015.06.019
13. Marquardt N, Beziat V, Nystrom S, Hengst J, Ivarsson MA, Kekalainen E, et al. Cutting edge: identification and characterization of human intrahepatic CD49a+ NK cells. *J Immunol.* (2015) 194:2467–71. doi: 10.4049/jimmunol.1402756
14. Collins A, Rothman N, Liu K, Reiner SL. Eomesodermin and T-bet mark developmentally distinct human natural killer cells. *JCI Insight.* (2017) 2:e90063. doi: 10.1172/jci.insight.90063
15. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1038/ni.2131
16. Turner JE, Morrison PJ, Wilhelm C, Wilson M, Ahlfors H, Renauld JC, et al. IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J Exp Med.* (2013) 210:2951–65. doi: 10.1084/jem.20130071
17. Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science.* (2018) 359:114–9. doi: 10.1126/science.aam5809
18. Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol.* (2011) 12:1055–62. doi: 10.1038/ni.2104
19. Lim AI, Menegatti S, Bustamante J, Le Bourhis L, Allez M, Rogge L, et al. IL-12 drives functional plasticity of human group 2 innate lymphoid cells. *J Exp Med.* (2016) 213:569–83. doi: 10.1084/jem.20151750
20. Hurrell BP, Shafiei Jahani P, Akbari O. Social networking of group two innate lymphoid cells in allergy and asthma. *Front Immunol.* (2018) 9:2694. doi: 10.3389/fimmu.2018.02694
21. Halim TY, MacLaren A, Romanish MT, Gold MJ, McNagny KM, Takei F. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity.* (2012) 37:463–74. doi: 10.1016/j.immuni.2012.06.012
22. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity.* (2012) 37:634–48. doi: 10.1016/j.immuni.2012.06.020
23. Yang Q, Monticelli LA, Saenz SA, Chi AW, Sonnenberg GF, Tang J, et al. T cell factor 1 is required for group 2 innate lymphoid cell generation. *Immunity.* (2013) 38:694–704. doi: 10.1016/j.immuni.2012.12.003
24. Yagi R, Zhong C, Northrup DL, Yu F, Bouladoux N, Spencer S, et al. The transcription factor GATA3 is critical for the development of all IL-7Ralpha-expressing innate lymphoid cells. *Immunity.* (2014) 40:378–88. doi: 10.1016/j.immuni.2014.01.012
25. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity.* (2012) 37:649–59. doi: 10.1016/j.immuni.2012.08.015
26. Huang Y, Guo L, Qiu J, Chen X, Hu-Li J, Siebenlist U, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol.* (2015) 16:161–9. doi: 10.1038/ni.3078
27. Huang Y, Paul WE. Inflammatory group 2 innate lymphoid cells. *Int Immunol.* (2016) 28:23–8. doi: 10.1093/intimm/dxv044
28. Seehus CR, Kadavallore A, Torre B, Yeckes AR, Wang Y, Tang J, et al. Alternative activation generates IL-10 producing type 2 innate lymphoid cells. *Nat Commun.* (2017) 8:1900. doi: 10.1038/s41467-017-02023-z
29. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med.* (2008) 14:275–81. doi: 10.1038/nm1710
30. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest.* (2008) 118:534–44. doi: 10.1172/JCI33194
31. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med.* (2008) 14:282–9. doi: 10.1038/nm1720
32. Lindemans CA, Calafiore M, Mertelsmann AM, O'Connor MH, Dudakov JA, Jenq RR, et al. Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. *Nature.* (2015) 528:560–4. doi: 10.1038/nature16460
33. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity.* (2005) 22:285–94. doi: 10.1016/j.immuni.2005.01.011
34. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature.* (2009) 457:722–5. doi: 10.1038/nature07537
35. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science.* (2014) 343:1249288. doi: 10.1126/science.1249288
36. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol.* (2009) 10:83–91. doi: 10.1038/ni.1684
37. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol.* (2011) 13:144–51. doi: 10.1038/ni.2187
38. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol.* (2004) 5:64–73. doi: 10.1038/ni1022
39. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature.* (2013) 494:261–5. doi: 10.1038/nature11813
40. Shikhagaie MM, Bjorklund AK, Mjosberg J, Erjefalt JS, Cornelissen AS, Ros XR, et al. Neuropilin-1 is expressed on lymphoid tissue residing lti-like group 3 innate lymphoid cells and associated with ectopic lymphoid aggregates. *Cell Rep.* (2017) 18:1761–73. doi: 10.1016/j.celrep.2017.01.063
41. Bal SM, Bernink JH, Nagasawa M, Groot J, Shikhagaie MM, Golebski K, et al. IL-1beta, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nat Immunol.* (2016) 17:636–45. doi: 10.1038/ni.3444
42. Chang YJ, Kim HY, Albacker LA, Baumgarth N, McKenzie AN, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol.* (2011). 12:631–8. doi: 10.1038/ni.2045
43. Mohapatra A, Van Dyken SJ, Schneider C, Nussbaum JC, Liang HE, Locksley RM. Group 2 innate lymphoid cells utilize the IRF4-IL-9 module to coordinate epithelial cell maintenance of lung homeostasis. *Mucosal Immunol.* (2016) 9:275–86. doi: 10.1038/mi.2015.59
44. Nogusa S, Ritz BW, Kassim SH, Jennings SR, Gardner EM. Characterization of age-related changes in natural killer cells during primary influenza infection in mice. *Mech Ageing Dev.* (2008) 129:223–30. doi: 10.1016/j.mad.2008.01.003

45. Abboud G, Tahiliani V, Desai P, Varkoly K, Driver J, Hutchinson TE, et al. Natural killer cells and innate interferon gamma participate in the host defense against respiratory vaccinia virus infection. *J Virol.* (2016) 90:129–41. doi: 10.1128/JVI.01894-15
46. Li T, Wang J, Wang Y, Chen Y, Wei H, Sun R, et al. Respiratory influenza virus infection induces memory-like liver NK cells in mice. *J Immunol.* (2017) 198:1242–52. doi: 10.4049/jimmunol.1502186
47. Zhou G, Juang SW, Kane KP. NK cells exacerbate the pathology of influenza virus infection in mice. *Eur J Immunol.* (2013) 43:929–38. doi: 10.1002/eji.201242620
48. Weizman OE, Adams NM, Schuster IS, Krishna C, Pritykin Y, Lau C, et al. ILC1 confer early host protection at initial sites of viral infection. *Cell.* (2017) 171:795–808 e712. doi: 10.1016/j.cell.2017.09.052
49. Kaiko GE, Phipps S, Angkasekwinai P, Dong C, Foster PS. NK cell deficiency predisposes to viral-induced Th2-type allergic inflammation via epithelial-derived IL-25. *J Immunol.* (2010) 185:4681–90. doi: 10.4049/jimmunol.1001758
50. Jayaraman A, Jackson DJ, Message SD, Pearson RM, Aniscenko J, Caramori G, et al. IL-15 complexes induce NK- and T-cell responses independent of type I IFN signaling during rhinovirus infection. *Mucosal Immunol.* (2014) 7:1151–64. doi: 10.1038/mi.2014.2
51. Stier MT, Bloodworth MH, Toki S, Newcomb DC, Goleniewska K, Boyd KL, et al. Respiratory syncytial virus infection activates IL-13-producing group 2 innate lymphoid cells through thymic stromal lymphopoietin. *J Allergy Clin Immunol.* (2016) 138:814–24 e811. doi: 10.1016/j.jaci.2016.01.050
52. Van Maele L, Carnoy C, Cayet D, Ivanov S, Porte R, Deruy E, et al. Activation of Type 3 innate lymphoid cells and interleukin 22 secretion in the lungs during *Streptococcus pneumoniae* infection. *J Infect Dis.* (2014) 210:493–503. doi: 10.1093/infdis/jiu106
53. Jost S, Quillay H, Reardon J, Peterson E, Simmons RP, Parry BA, et al. Changes in cytokine levels and NK cell activation associated with influenza. *PLoS ONE.* (2011) 6:e25060. doi: 10.1371/journal.pone.0025060
54. Silver JS, Kearley J, Copenhaver AM, Sanden C, Mori M, Yu L, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat Immunol.* (2016) 17:626–35. doi: 10.1038/ni.3443
55. Kim JH, Jang YJ. Role of Natural Killer Cells in Airway Inflammation. *Allergy Asthma Immunol Res.* (2018) 10:448–56. doi: 10.4168/aair.2018.10.5.448
56. Halim TY, Steer CA, Matha L, Gold MJ, Martinez-Gonzalez I, McNagny KM, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity.* (2014) 40:425–35. doi: 10.1016/j.immuni.2014.01.011
57. Kim HY, Lee HJ, Chang YJ, Pichavant M, Shore SA, Fitzgerald KA, et al. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med.* (2014) 20:54–61. doi: 10.1038/nm.3423
58. Bi J, Cui L, Yu G, Yang X, Chen Y, Wan X. NK cells alleviate lung inflammation by negatively regulating group 2 innate lymphoid cells. *J Immunol.* (2017) 198:3336–44. doi: 10.4049/jimmunol.1601830
59. Shaw JL, Fakhri S, Citardi MJ, Porter PC, Corry DB, Kheradmand F, et al. IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps. *Am J Respir Crit Care Med.* (2013) 188:432–9. doi: 10.1164/rccm.201212-2227OC
60. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol.* (2014) 134:671–8 e674. doi: 10.1016/j.jaci.2014.06.024
61. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA.* (2011) 107:11489–94. doi: 10.1073/pnas.1003988107
62. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. *Science.* (2014) 345:1254009. doi: 10.1126/science.1254009
63. Guo X, Qiu J, Tu T, Yang X, Deng L, Anders RA, et al. Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection. *Immunity.* (2014) 40:25–39. doi: 10.1016/j.immuni.2013.10.021
64. Waddell A, Vallance JE, Hummel A, Alenghat T, Rosen MJ. IL-33 induces murine intestinal goblet cell differentiation indirectly via innate lymphoid cell IL-13 secretion. *J Immunol.* (2018) 202:598–607. doi: 10.4049/jimmunol.1800292
65. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature.* (2010) 464:1371–5. doi: 10.1038/nature08949
66. Song C, Lee JS, Gilfillan S, Robinette ML, Newberry RD, Stappenbeck TS, et al. Unique and redundant functions of NKp46+ ILC3s in models of intestinal inflammation. *J Exp Med.* (2015) 212:1869–82. doi: 10.1084/jem.20151403
67. Geremia A, Jewell DP. The IL-23/IL-17 pathway in inflammatory bowel disease. *Exp Rev Gastroenterol Hepatol.* (2012) 6:223–37. doi: 10.1586/egh.11.107
68. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft vs. host disease. *Immunity.* (2012) 37:339–50. doi: 10.1016/j.immuni.2012.05.028
69. Bruce DW, Stefanski HE, Vincent BG, Dant TA, Reisdorf S, Bommiasamy H, et al. Type 2 innate lymphoid cells treat and prevent acute gastrointestinal graft-vs.-host disease. *J Clin Invest.* (2017) 127:1813–25. doi: 10.1172/JCI91816
70. Simonetta F, Alvarez M, Negrin RS. Natural killer cells in graft-vs.-host-disease after allogeneic hematopoietic cell transplantation. *Front Immunol.* (2017) 8:465. doi: 10.3389/fimmu.2017.00465
71. Munneke JM, Bjorklund AT, Mjosberg JM, Garming-Legert K, Bernink JH, Blom B, et al. Activated innate lymphoid cells are associated with a reduced susceptibility to graft-vs.-host disease. *Blood.* (2014) 124:812–21. doi: 10.1182/blood-2013-11-536888
72. Gladiator A, Wangler N, Trautwein-Weidner K, LeibundGut-Landmann S. Cutting edge: IL-17-secreting innate lymphoid cells are essential for host defense against fungal infection. *J Immunol.* (2013) 190:521–5. doi: 10.4049/jimmunol.1202924
73. Martinez-Gonzalez I, Steer CA, Takei F. Lung ILC2s link innate and adaptive responses in allergic inflammation. *Trends Immunol.* (2015) 36:189–95. doi: 10.1016/j.it.2015.01.005
74. Cheng H, Jin C, Wu J, Zhu S, Liu YJ, Chen J. Guards at the gate: physiological and pathological roles of tissue-resident innate lymphoid cells in the lung. *Protein Cell.* (2017) 8:878–95. doi: 10.1007/s13238-017-0379-5
75. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity.* (2012) 36:451–63. doi: 10.1016/j.immuni.2011.12.020
76. Drake LY, Iijima K, Bartemes K, Kita H. Group 2 innate lymphoid cells promote an early antibody response to a respiratory antigen in mice. *J Immunol.* (2016) 197:1335–42. doi: 10.4049/jimmunol.1502669
77. Martinez-Gonzalez I, Matha L, Steer CA, Ghaedi M, Poon GF, Takei F. Allergen-experienced group 2 innate lymphoid cells acquire memory-like properties and enhance allergic lung inflammation. *Immunity.* (2016) 45:198–208. doi: 10.1016/j.immuni.2016.06.017
78. Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol.* (2016) 137:75–86 e78. doi: 10.1016/j.jaci.2015.05.037
79. Chen R, Smith SG, Salter B, El-Gammal A, Oliveria JP, Obminski C, et al. Allergen-induced Increases in Sputum Levels of Group 2 Innate Lymphoid Cells in Subjects with Asthma. *Am J Respir Crit Care Med.* (2017) 196:700–12. doi: 10.1164/rccm.201612-2427OC
80. Christianson CA, Goplen NP, Zafar I, Irvin C, Good JT Jr, Rollins DR, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J Allergy Clin Immunol.* (2015) 136:59–68 e14. doi: 10.1016/j.jaci.2014.11.037
81. Liu T, Wu J, Zhao J, Wang J, Zhang Y, Liu L, et al. Type 2 innate lymphoid cells: a novel biomarker of eosinophilic airway inflammation in patients with mild to moderate asthma. *Respir Med.* (2015) 109:1391–6. doi: 10.1016/j.rmed.2015.09.016
82. Nagakumar P, Denney L, Fleming L, Bush A, Lloyd CM, Saglani S. Type 2 innate lymphoid cells in induced sputum from children with severe

asthma. *J Allergy Clin Immunol.* (2016) 137:624–6 e626. doi: 10.1016/j.jaci.2015.06.038
83. Hams E, Armstrong ME, Barlow JL, Saunders SP, Schwartz C, Cooke G, et al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc Natl Acad Sci USA.* (2014) 111:367–72. doi: 10.1073/pnas.1315854111
84. Besnard AG, Sabat R, Dumoutier L, Renauld JC, Willart M, Lambrecht B, et al. Dual role of IL-22 in allergic airway inflammation and its cross-talk with IL-17A. *Am J Respir Crit Care Med.* (2011) 183:1153–63. doi: 10.1164/rccm.201008-1383OC
85. Molofsky AB, Van Gool F, Liang HE, Van Dyken SJ, Nussbaum JC, Lee J, et al. Interleukin-33 and interferon-gamma counter-regulate group 2 innate lymphoid cell activation during immune perturbation. *Immunity.* (2015) 43:161–74. doi: 10.1016/j.immuni.2015.05.019
86. Moro K, Kabata H, Tanabe M, Koga S, Takeno N, Mochizuki M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol.* (2016) 17:76–86. doi: 10.1038/ni.3309
87. Han M, Hong JY, Jaipalli S, Rajput C, Lei J, Hinde JL, et al. IFN-gamma blocks development of an asthma phenotype in rhinovirus-infected baby mice by inhibiting type 2 innate lymphoid cells. *Am J Respir Cell Mol Biol.* (2017) 56:242–51. doi: 10.1165/rcmb.2016-0056OC
88. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci Transl Med.* (2013) 5:174ra126. doi: 10.1126/scitranslmed.3004812
89. Wei H, Zhang J, Xiao W, Feng J, Sun R, Tian Z. Involvement of human natural killer cells in asthma pathogenesis: natural killer 2 cells in type 2 cytokine predominance. *J Allergy Clin Immunol.* (2005) 115:841–7. doi: 10.1016/j.jaci.2004.11.026
90. Raundhal M, Morse C, Khare A, Oriss TB, Milosevic J, Trudeau J, et al. High IFN-gamma and low SLPI mark severe asthma in mice and humans. *J Clin Invest.* (2015) 125:3037–50. doi: 10.1172/JCI80911
91. Eastman JJ, Cavagnero KJ, Deconde AS, Kim AS, Karta MR, Broide DH, et al. Group 2 innate lymphoid cells are recruited to the nasal mucosa in patients with aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol.* (2017) 140:101–8 e103. doi: 10.1016/j.jaci.2016.11.023
92. Poposki JA, Klingler AI, Tan BK, Soroosh P, Banie H, Lewis G, et al. Group 2 innate lymphoid cells are elevated and activated in chronic rhinosinusitis with nasal polyps. *Immun Inflamm Dis.* (2017) 5:233–43. doi: 10.1002/iid3.161
93. Dhariwal J, Cameron A, Trujillo-Torralbo MB, Del Rosario A, Bakhsoliani E, Paulsen M, et al. Mucosal type 2 innate lymphoid cells are a key component of the allergic response to aeroallergens. *Am J Respir Crit Care Med.* (2017) 195:1586–96. doi: 10.1164/rccm.201609-1846OC
94. Gevaert P, Van Bruaene N, Cattaert T, Van Steen K, Van Zele T, Acke F, et al. Mepolizumab, a humanized anti-IL-5 mAb, as a treatment option for severe nasal polyposis. *J Allergy Clin Immunol.* (2011) 128:989–95 e981–988. doi: 10.1016/j.jaci.2011.07.056
95. Rivero A, Liang J. Anti-IgE and anti-il5 biologic therapy in the treatment of nasal polyposis: a systematic review and meta-analysis. *Ann Otol Rhinol Laryngol.* (2017) 126:739–47. doi: 10.1177/0003489417731782
96. Fan D, Wang X, Wang M, Wang Y, Zhang L, Li Y, et al. Allergen-dependent differences in ilc2s frequencies in patients with allergic rhinitis. *Allergy Asthma Immunol Res.* (2016) 8:216–22. doi: 10.4168/aair.2016.8.3.216
97. Mitthamsiri W, Pradubpongsa P, Sangasapaviliya A, Boonpiyathad T. Decreased CRTH2 expression and response to allergen re-stimulation on innate lymphoid cells in patients with allergen-specific immunotherapy. *Allergy Asthma Immunol Res.* (2018) 10:662–74. doi: 10.4168/aair.2018.10.6.662
98. Lao-Araya M, Steveling E, Scadding GW, Durham SR, Shamji MH. Seasonal increases in peripheral innate lymphoid type 2 cells are inhibited by subcutaneous grass pollen immunotherapy. *J Allergy Clin Immunol.* (2014) 134:1193–5 e1194. doi: 10.1016/j.jaci.2014.07.029
99. Scordamaglia F, Balsamo M, Scordamaglia A, Moretta A, Mingari MC, Canonica GW, et al. Perturbations of natural killer cell regulatory functions in respiratory allergic diseases. *J Allergy Clin Immunol.*(2008) 121:479–85. doi: 10.1016/j.jaci.2007.09.047
100. Brusselle GG, Joos GF, Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet.* (2011) 378:1015–26. doi: 10.1016/S0140-6736(11)60988-4
101. Roos AB, Sanden C, Mori M, Bjermer L, Stampfli MR, Erjefalt JS. IL-17A is elevated in end-stage chronic obstructive pulmonary disease and contributes to cigarette smoke-induced lymphoid neogenesis. *Am J Respir Crit Care Med.* (2015) 191:1232–41. doi: 10.1164/rccm.201410-1861OC
102. Carrega P, Loiacono F, Di Carlo E, Scaramuccia A, Mora M, Conte R, et al. NCR(+)ILC3 concentrate in human lung cancer and associate with intratumoral lymphoid structures. *Nat Commun.* (2015) 6:8280. doi: 10.1038/ncomms9280
103. Villegas FR, Coca S, Villarrubia VG, Jimenez R, Chillon MJ, Jareno J, et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer.* (2002) 35:23–8.
104. Jin S, Deng Y, Hao JW, Li Y, Liu B, Yu Y, et al. NK cell phenotypic modulation in lung cancer environment. *PLoS ONE.* (2014) 9:e109976. doi: 10.1371/journal.pone.0109976
105. Platonova S, Cherfils-Vicini J, Damotte D, Crozet L, Vieillard V, Validire P, et al. Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. *Cancer Res.* (2011) 71:5412–22. doi: 10.1158/0008-5472.CAN-10-4179
106. Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, et al. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells. *Cancer.* (2008) 112:863–75. doi: 10.1002/cncr.23239
107. Hodge G, Barnawi J, Jurisevic C, Moffat D, Holmes M, Reynolds PN, et al. Lung cancer is associated with decreased expression of perforin, granzyme B and interferon (IFN)-gamma by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells. *Clin Exp Immunol.* (2014) 178:79–85. doi: 10.1111/cei.12392
108. Al Omar SY, Marshall E, Middleton D, Christmas SE. Increased killer immunoglobulin-like receptor expression and functional defects in natural killer cells in lung cancer. *Immunology.* (2011) 133:94–104. doi: 10.1111/j.1365-2567.2011.03415.x
109. Kramer B, Goeser F, Lutz P, Glassner A, Boesecke C, Schwarze-Zander C, et al. Compartment-specific distribution of human intestinal innate lymphoid cells is altered in HIV patients under effective therapy. *PLoS Pathog.* (2017) 13:e1006373. doi: 10.1371/journal.ppat.1006373
110. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity.* (2008) 29:958–70. doi: 10.1016/j.immuni.2008.11.001
111. Reynders A, Yessaad N, Vu Manh TP, Dalod M, Fenis A, Aubry C, et al. Identity, regulation and *in vivo* function of gut NKp46+RORgammat+ and NKp46+RORgammat- lymphoid cells. *EMBO J.* (2011) 30:2934–47. doi: 10.1038/emboj.2011.201
112. Abt MC, Lewis BB, Caballero S, Xiong H, Carter RA, Susac B, et al. Innate immune defenses mediated by Two ILC subsets are critical for protection against acute clostridium difficile infection. *Cell Host Microbe.* (2015) 18:27–37. doi: 10.1016/j.chom.2015.06.011
113. Qiu J, Guo X, Chen ZM, He L, Sonnenberg GF, Artis D, et al. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity.* (2013) 39:386–99. doi: 10.1016/j.immuni.2013.08.002
114. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science.* (2012) 336:1321–5. doi: 10.1126/science.1222551
115. Pickard JM, Maurice CF, Kinnebrew MA, Abt MC, Schenten D, Golovkina TV, et al. Rapid fucosylation of intestinal epithelium sustains host-commensal symbiosis in sickness. *Nature.* (2014) 514:638–41. doi: 10.1038/nature13823
116. Castellanos JG, Woo V, Viladomiu M, Putzel G, Lima S, Diehl GE, et al. Microbiota-Induced TNF-like Ligand 1A drives group 3 innate lymphoid cell-mediated barrier protection and intestinal t cell activation during colitis. *Immunity.* (2018) 49:1077–89 e1075. doi: 10.1016/j.immuni.2018.10.014
117. Bao S, Beagley KW, France MP, Shen J, Husband AJ. Interferon-gamma plays a critical role in intestinal immunity against

Salmonella typhimurium infection. *Immunology.* (2000) 99:464–72. doi: 10.1046/j.1365-2567.2000.00955.x
118. Nguyen TT, Baumgarth N. Innate B cells tell ilc how it's done. *Immunity.* (2016) 45:8–10. doi: 10.1016/j.immuni.2016.06.030
119. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature.* (2010) 463:540–4. doi: 10.1038/nature08636
120. Schneider C, O'Leary CE, von Moltke J, Liang HE, Ang QY, Turnbaugh PJ, et al. A metabolite-triggered tuft cell-ILC2 circuit drives small intestinal remodeling. *Cell.* (2018) 174:271–84 e214. doi: 10.1016/j.cell.2018.05.014
121. Hernandez PP, Mahlakoiv T, Yang I, Schwierzeck V, Nguyen N, Guendel F, et al. Interferon-lambda and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection. *Nat Immunol.* (2015) 16:698–707. doi: 10.1038/ni.3180
122. Dillon SM, Castleman MJ, Frank DN, Austin GL, Gianella S, Cogswell AC, et al. Brief report: inflammatory colonic innate lymphoid cells are increased during untreated HIV-1 infection and associated with markers of gut dysbiosis and mucosal immune activation. *J Acquir Immune Defic Syndr.* (2017) 76:431–7. doi: 10.1097/QAI.0000000000001523
123. Vely F, Barlogis V, Vallentin B, Neven B, Piperoglou C, Ebbo M, et al. Evidence of innate lymphoid cell redundancy in humans. *Nat Immunol.* (2016) 17:1291–9. doi: 10.1038/ni.3553
124. Ahern PP, Schiering C, Buonocore S, McGeachy MJ, Cua DJ, Maloy KJ, et al. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity.* (2010) 33:279–88. doi: 10.1016/j.immuni.2010.08.010
125. Powell N, Walker AW, Stolarczyk E, Canavan JB, Gokmen MR, Marks E, et al. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. *Immunity.* (2012) 37:674–84. doi: 10.1016/j.immuni.2012.09.008
126. Pearson C, Thornton EE, McKenzie B, Schaupp AL, Huskens N, Griseri T, et al. ILC3 GM-CSF production and mobilisation orchestrate acute intestinal inflammation. *Elife.* (2016) 5:e10066. doi: 10.7554/eLife.10066
127. Geremia A, Arancibia-Carcamo CV, Fleming MP, Rust N, Singh B, Mortensen NJ, et al. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med.* (2011) 208:1127–33. doi: 10.1084/jem.20101712
128. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature.* (2013) 498:113–7. doi: 10.1038/nature12240
129. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells. *Science.* (2015) 348:1031–5. doi: 10.1126/science.aaa4812
130. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, et al. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci USA.* (2014) 111:13145–50. doi: 10.1073/pnas.1412008111
131. Takayama T, Kamada N, Chinen H, Okamoto S, Kitazume MT, Chang J, et al. Imbalance of NKp44(+)NKp46(-) and NKp44(-)NKp46(+) natural killer cells in the intestinal mucosa of patients with Crohn's disease. *Gastroenterology.* (2010) 139:882–92 e881–883. doi: 10.1053/j.gastro.2010.05.040
132. Li J, Doty AL, Iqbal A, Glover SC. The differential frequency of Lineage(-)CRTH2(-)CD45(+)NKp44(-)CD117(-)CD127(+)ILC subset in the inflamed terminal ileum of patients with Crohn's disease. *Cell Immunol.* (2016) 304–5, 63–8. doi: 10.1016/j.cellimm.2016.05.001
133. Gwela A, Siddhanathi P, Chapman RW, Travis S, Powrie F, Arancibia-Carcamo CV, et al. Th1 and innate lymphoid cells accumulate in primary sclerosing cholangitis-associated inflammatory bowel disease. *J Crohns Colitis.* (2017) 11:1124–34. doi: 10.1093/ecco-jcc/jjx050
134. Camelo A, Barlow JL, Drynan LF, Neill DR, Ballantyne SJ, Wong SH, et al. Blocking IL-25 signalling protects against gut inflammation in a type-2 model of colitis by suppressing nuocyte and NKT derived IL-13. *J Gastroenterol.* (2012) 47:1198–211. doi: 10.1007/s00535-012-0591-2
135. Monticelli LA, Osborne LC, Noti M, Tran SV, Zaiss DM, Artis D. IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin-EGFR interactions. *Proc Natl Acad Sci USA.* (2015) 112:10762–7. doi: 10.1073/pnas.1509070112
136. Doherty TA, Baum R, Newbury RO, Yang T, Dohil R, Aquino M, et al. Group 2 innate lymphocytes (ILC2) are enriched in active eosinophilic esophagitis. *J Allergy Clin Immunol.* (2015) 136:792–4 e793. doi: 10.1016/j.jaci.2015.05.048
137. Aparicio-Domingo P, Romera-Hernandez M, Karrich JJ, Cornelissen F, Papazian N, Lindenbergh-Kortleve DJ, et al. Type 3 innate lymphoid cells maintain intestinal epithelial stem cells after tissue damage. *J Exp Med.* (2015) 212:1783–91. doi: 10.1084/jem.20150318
138. Kappelman MD, Farkas DK, Long MD, Erichsen R, Sandler RS, Sorensen HT, et al. Risk of cancer in patients with inflammatory bowel diseases: a nationwide population-based cohort study with 30 years of follow-up evaluation. *Clin Gastroenterol Hepatol.* (2014) 12:265–73 e261. doi: 10.1016/j.cgh.2013.03.034
139. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. IL-23 promotes tumour incidence and growth. *Nature.* (2006) 442:461–5. doi: 10.1038/nature04808
140. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med.* (2009) 15:1016–22. doi: 10.1038/nm.2015
141. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature.* (2012) 491:254–8. doi: 10.1038/nature11465
142. Kirchberger S, Royston DJ, Boulard O, Thornton E, Franchini F, Szabady RL, et al. Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J Exp Med.* (2013) 210:917–31. doi: 10.1084/jem.20122308
143. Bie Q, Zhang P, Su Z, Zheng D, Ying X, Wu Y, et al. Polarization of ILC2s in peripheral blood might contribute to immunosuppressive microenvironment in patients with gastric cancer. *J Immunol Res.* (2014) 2014:923135. doi: 10.1155/2014/923135
144. Lee J, Park KH, Ryu JH, Bae HJ, Choi A, Lee H, et al. Natural killer cell activity for IFN-gamma production as a supportive diagnostic marker for gastric cancer. *Oncotarget.* (2017) 8:70431–40. doi: 10.18632/oncotarget.19712
145. Han B, Mao FY, Zhao YL, Lv YP, Teng YS, Duan M, et al. Altered NKp30, NKp46, NKG2D, and DNAM-1 expression on circulating nk cells is associated with tumor progression in human gastric cancer. *J Immunol Res.* (2018) 2018:6248590. doi: 10.1155/2018/6248590
146. Halama N, Braun M, Kahlert C, Spille A, Quack C, Rahbari N, et al. Natural killer cells are scarce in colorectal carcinoma tissue despite high levels of chemokines and cytokines. *Clin Cancer Res.* (2011) 17:678–89. doi: 10.1158/1078-0432.CCR-10-2173
147. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer.* (2000) 88:577–83. doi: 10.1002/(SICI)1097-0142(20000201)88:3<577::AID-CNCR13>3.0.CO;2-V
148. Dutzan N, Konkel JE, Greenwell-Wild T, Moutsopoulos NM. Characterization of the human immune cell network at the gingival barrier. *Mucosal Immunol.* (2016) 9:1163–72. doi: 10.1038/mi.2015.136
149. Brown JL, Campbell L, Malcolm J, Adrados Planell A, Butcher JP, Culshaw S. Enrichment of innate lymphoid cell populations in gingival tissue. *J Dent Res.* (2018) 97:1399–405. doi: 10.1177/0022034518782141
150. Li H, Reeves RK. Functional perturbation of classical natural killer and innate lymphoid cells in the oral mucosa during SIV infection. *Front Immunol.* (2012) 3:417. doi: 10.3389/fimmu.2012.00417
151. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut.* (2012) 61:1693–700. doi: 10.1136/gutjnl-2011-301668

152. Kaser A. Not all monoclonals are created equal - lessons from failed drug trials in Crohn's disease. *Best Pract Res Clin Gastroenterol.* (2014) 28:437–49. doi: 10.1016/j.bpg.2014.04.005
153. Sands BE, Chen J, Feagan BG, Penney M, Rees WA, Danese S, et al. Efficacy and safety of MEDI2070, an antibody against interleukin 23, in patients with moderate to severe crohn's disease: a phase 2a study. *Gastroenterology.* (2017) 153:77–86 e76. doi: 10.1053/j.gastro.2017.03.049
154. Rutgeerts P, Gasink C, Chan D, Lang Y, Pollack P, Colombel JF, et al. Efficacy of ustekinumab for inducing endoscopic healing in patients with crohn's disease. *Gastroenterology.* (2018) 155:1045–58. doi: 10.1053/j.gastro.2018.06.035
155. Bagnasco D, Ferrando M, Varricchi G, Passalacqua G, Canonica GW. A critical evaluation of anti-il-13 and anti-il-4 strategies in severe asthma. *Int Arch Allergy Immunol.* (2016) 170:122–31. doi: 10.1159/000447692
156. Wenzel S, Castro M, Corren J, Maspero J, Wang L, Zhang B, et al. Dupilumab efficacy and safety in adults with uncontrolled persistent asthma despite use of medium-to-high-dose inhaled corticosteroids plus a long-acting beta2 agonist: a randomised double-blind placebo-controlled pivotal phase 2b dose-ranging trial. *Lancet.* (2016) 388:31–44. doi: 10.1016/S0140-6736(16)30307-5
157. Okubo K, Hashiguchi K, Takeda T, Baba K, Kitagoh H, Miho H, et al. A randomized controlled phase II clinical trial comparing ONO-4053, a novel DP1 antagonist, with a leukotriene receptor antagonist pranlukast in patients with seasonal allergic rhinitis. *Allergy.* (2017) 72:1565–75. doi: 10.1111/all.13174
158. Singh D, Ravi A, Southworth T. CRTH2 antagonists in asthma: current perspectives. *Clin Pharmacol.* (2017) 9:165–73. doi: 10.2147/CPAA.S119295

Conflict of Interest Statement: MC receives research support from Pfizer.

The remaining author declares 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: 07 June 2019
doi: 10.3389/fimmu.2019.00930

Group 2 Innate Lymphoid Cells in Respiratory Allergic Inflammation

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

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

Millions of people worldwide are suffering from allergic inflammatory airway disorders. These conditions are regarded as a consequence of multiple imbalanced immune events resulting in an inadequate response with the exact underlying mechanisms still being a subject of ongoing research. Several cell populations have been proposed to be involved but it is becoming increasingly evident that group 2 innate lymphoid cells (ILC2s) play a key role in the initiation and orchestration of respiratory allergic inflammation. ILC2s are important mediators of inflammation but also tissue remodeling by secreting large amounts of signature cytokines within a short time period. Thereby, ILC2s instruct innate but also adaptive immune responses. Here, we will discuss the recent literature on allergic inflammation of the respiratory tract with a focus on ILC2 biology. Furthermore, we will highlight different therapeutic strategies to treat pulmonary allergic inflammation and their potential influence on ILC2 function as well as discuss the perspective of using human ILC2s for diagnostic purposes.

Keywords: group 2 innate lymphoid cells (ILC2s), mucosal immunity, respiratory tract, allergic inflammation, therapeutic strategies

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Rudi W. Hendriks,
Erasmus University Rotterdam, Netherlands
Shinji Toki,
Vanderbilt University Medical Center, United States
Ari B. Molofsky,
University of California, San Francisco, United States
Padraic Fallon,
Trinity College Dublin, Ireland

***Correspondence:**
Claudia U. Duerr
claudia.duerr@charite.de

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

Received: *07 January 2019*
Accepted: *11 April 2019*
Published: *07 June 2019*

Citation:
Helfrich S, Mindt BC, Fritz JH and Duerr CU (2019) Group 2 Innate Lymphoid Cells in Respiratory Allergic Inflammation. Front. Immunol. 10:930. doi: 10.3389/fimmu.2019.00930

INTRODUCTION

Respiratory allergic inflammatory conditions such as asthma and allergic rhinitis (hay fever) are affecting millions of people globally (1, 2). Importantly, the prevalence of these non-communicable disorders is rapidly increasing in contrast to communicable diseases of the respiratory tract, which are on the decline (1). Due to their widespread morbidity, they represent a substantial social as well as economic burden (3). The skew toward a type 2 immune response is often an important characteristic of these chronic conditions that involve both innate and adaptive branches of the immune system (4). In addition to T helper 2 (T_H2) cells of the adaptive immune system, group 2 innate lymphoid cells (ILC2s) are critical in instructing a strong type 2 immune response (5). ILC2s belong to the group of innate lymphoid cells (ILCs) that provide host defense against infectious agents, participate in inflammatory responses and mediate lymphoid organogenesis and tissue repair, particularly at mucosal barriers. Taking the newest nomenclature of ILCs into account, the ILC family is comprised of five subsets including Natural Killer (NK) cells, lymphoid tissue inducer cells (LTi cells) as well as the three helper ILC members. While LTi cells are key drivers of lymphoid organogenesis and NK cells are important to fight off viral infections, helper ILCs are regarded as the innate counterpart of T_H cells but lack the surface expression of common adaptive lineage markers as well as specific antigen receptors (6, 7). These helper ILCs, namely, ILC1s, ILC2s, and ILC3s are defined by their effector cytokine profile and transcription factor expression (8).

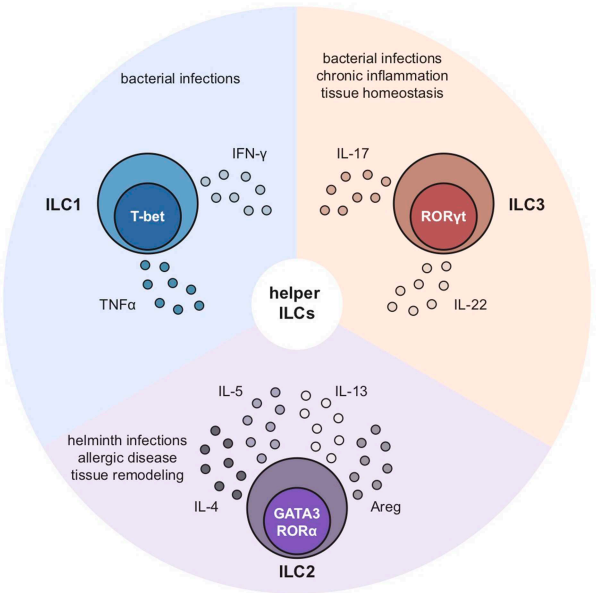

FIGURE 1 | Characteristics of ILC1s, ILC2s, and ILC3s. ILC1s, ILC2s, and ILC3s are characterized by expression of their key transcription factors and signature cytokines: ILC1s depend on T-bet and produce IFNγ, ILC2s on Gata3 and Rorα and secrete IL-4, IL-5, IL-13 as well as amphiregulin while ILC3s depend on Rorγt and release IL-17 and IL-22 upon activation.

The master transcription factors for the different helper subsets are T-bet for ILC1, Gata3 and Rorα for ILC2s and RORγt for ILC3s. Helper ILCs are important sources of innate effector cytokines such as ILC1-derived IFNγ, ILC2-derived IL-4, IL-5, IL-13, and amphiregulin (Areg) as well as ILC3-derived IL-17 and IL-22 (**Figure 1**). ILC2s are innately committed to type 2 immunity which consequently puts them in the spotlight during the onset of an allergic immune response. ILC2s are activated by local immune mediators, typically alarmins, and are able to produce large amounts of signature cytokines within a short period of time (9). Thereby, ILC2s can initiate and amplify immune responses and are able to influence innate as well as adaptive immunity by both their secreted cytokines and through cell-cell interactions. Hence, ILC2s serve as an important link between innate and adaptive effector branches of type 2 immunity. Depending on the tissue they reside in, ILC2s exhibit slightly diverse profiles shaped by their microenvironment (10). However, their specific characteristics such as their ability to produce type 2 signature cytokines in a stark and fast fashion remains unchanged. Since their detailed description in 2010 (11–13), our knowledge about this fascinating immune population has steadily increased both in mouse models but also, through applied research, in humans. In this review we will provide a brief snapshot on our current knowledge of ILC2s in mouse and human allergic respiratory inflammation. Moreover, we will summarize experimental mouse models and discuss how recent reports led to an improved understanding of therapeutic strategies in human allergic respiratory diseases with the focus on asthma.

ASTHMA – AN ALLERGIC RESPIRATORY DISEASE

One of the most common human allergic diseases in the respiratory tract is asthma. Importantly, asthma is no longer considered to be one specific disease but more of an umbrella term for chronic inflammation of the lower airways with characteristics such as wheezing, bronchoconstriction and shortness of breath (4, 14). The heterogeneity of asthma is mirrored by different immune profiles. In general, asthma is subdivided into type 2 and non-type 2 with further separation of type 2 asthma in allergic or non-allergic asthma accompanied with eosinophilia (14, 15). Non-type 2 (low) asthma is defined as asthma without eosinophilia and with increased presence of neutrophils and/or IL-17 producing cells. These different asthma subtypes are termed endotypes for better classification (16). Interestingly, although there are cases of asthma onset in adulthood in a cohort of patients, most asthmatic individuals develop the disease during childhood (17). However, the exact mechanisms are still not completely understood. Different secondary diseases are correlated with asthma such as allergic rhinitis, chronic rhinosinusitis, and the development of nasal polyps. To provide optimal care to asthmatic patients of all different subgroups, a shift to a more personalized treatment approach is in the focus of discussion. In this context, mouse models of human allergic airway inflammation are an invaluable tool to understand underlying mechanisms of disease. Common allergens, including house dust mite (HDM) and papain can trigger respiratory allergic inflammation in humans as well as mice (18, 19), and mouse models can therefore be used to recapitulate these immune responses in an experimental system and thus aid in deciphering the underlying mechanism and processes of allergic respiratory diseases. To increase our knowledge of allergic lung inflammation, different experimental mouse models can be applied. Allergens such as ovalbumin (OVA), HDM, papain, fungal extracts, ILC2-eliciting cytokines and combinations thereof are used to induce and mimic allergic inflammation in the respiratory tract. The importance of ILC2s in the onset of allergic airway inflammation is highlighted by their detection in these experiments and thoroughly stratified in **Table 1**.

ILC2S IN ALLERGIC INFLAMMATION OF THE RESPIRATORY TRACT

Allergic respiratory diseases are characterized in general by a dysregulated immune response targeting a harmless and non-pathogenic allergen. Several immune populations participate in an allergic respiratory disease including populations of the innate as well as the adaptive immune system. Different subgroups of T and B cells as well as eosinophils, basophils, NK cells and, last but not least, ILC2s are major players. Although a direct mechanistical link of ILC2s to asthma pathogenesis still needs to be established in humans, an increasing body of evidence supports an association of ILC2s with disease in asthmatic patients. These include GWAS studies revealing several genes

TABLE 1 | ILC2s in experimental mouse models of allergic airway inflammation[a].

	Model	Strain background	Route			ILC2 detection method					Observations				References
			i.n. i.t.	i.p.	Aerosol	Flow cytometry B and/or L and/or mLN			Histology L and/or mLN		ILC2↑	EOS and/or Mucus and/or AHR			
Aeroallergen exposure	House Dust Mite (HDM) *D. pteronyssinus*	C57BL/6	✓	✗	✗	✓	✓	✗	✗	✗	Very mild inflammation				(20–22)
		C57BL/6 BALB/c	✓	✗	✗	✓	✓	✓	✓	✗	✓	✓	✗	✓	(22–27)
		C57BL/6 BALB/c	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓	✗	✗	(21, 28)
	Ovalbumin (OVA)	C57BL/6 BALB/c	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	(21, 27, 29, 30)
	Fungi *Aspergillus*	C57BL/6	✓	✗	✗	✓	✓	✓	✓	✗	✓	✓	✓	✗	(31)
	Ragweed Pollen	BALB/c	✓	✗	✗	✓	✓	✗	✓	✗	✓	✓	✓	✓	(30, 32)
	Chitin	C57BL/6J BALB/c	✓	✗	✗	✓	✓	✗	✓	✗	✓	✓	✗	✗	(33, 34)
	Fungi *Alternaria*	C57BL/6 BALB/c BALB/cByJ	✓	✗	✗	✓	✓	✗	✓	✗	✓	✓	✓	✓	(20, 35–48)
	Papain protease	C57BL/6 NOD-SCID	✓	✗	✗	✓	✓	✓	✓	✗	✓	✓	✓	✗	(24, 31, 49–53)
Cytokine exposure	rIL-25	BALB/c C57BL/6	✓	✗	✗	✓	✓	✗	✓	✗	Very mild inflammation				(30)
		BALB/c	✓	✗	✗	✓	✓	✓	✗	✗	✓	✓	✗	✓	(21, 27, 29)
		C57BL/c	✓	✗	✗	✓	✓	✓	✗	✗	✓	✓	✗	✗	(23, 24, 45)
		BALB/c C57BL/6	✗	✓	✗	✓	✓	✗	✗	✗	✓	✗	✗	✗	(54, 55)
	rIL-33	BALB/c C57BL/6	✓	✓	✗	✓	✓	✗	✓	✗	✓	✓	✓	✓	(30, 33, 35, 38, 45, 56)
		C57BL/6 BALB/c BALB/cByJ	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	(21, 25, 27–29, 31, 36, 37, 39, 41, 45, 49–51, 57, 58)
		BALB/c C57BL/6 C57BL/6JRJ	✗	✓	✗	✓	✓	✗	✓	✗	✓	✓	✗	✗	(52, 54, 55, 58, 59)

• Models dependent on T_H2 cell response: *Aspergillus*, HDM, OVA (22)

• Models independent of T_H2 cell response: Chitin, Papain, *Alternaria*, cytokine exposure (22)

[a]*AHR, airway hyper–reactivity; BAL, bronchoalveolar lavage fluid; EOS, eosinophilia; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; mLN, mediastinal lymph node; r, recombinant.*

essential in ILC2 biology as susceptibility markers for human asthma such as the ILC2-activating alarmin IL-33 and its cognate receptor ST2 (IL1RL1) as well as the transcription factors GATA3 and RORα (57, 60, 61).

ILC2s are the predominant ILC population in the lung, in contrast to other mucosal surfaces such as small intestine and colon. However, the reason for this skewed distribution is still not clear. ILC2s are activated mainly by the cytokines interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) in the lungs, however, ILC2s accumulate in tissues independent of these signals and pulmonary ILC2s can also be detected in triple knockout mice deficient in the TSLP receptor, ST2, and IL-25 (10, 62). However, ILC2s show reduced IL-5 reporter expression in lung, fat, and gut tissue, but not in the skin indicating that TSLP, ST2, and IL-25 signaling is indeed needed for the functional maintenance of ILC2s in the local microenvironment of the lung (10). In addition to IL-5, ILC2s commonly secrete IL-4, IL-9, IL-13, and amphiregulin. IL-4 triggers the differentiation of T_H2 cells and induces class switching of B cells to IgE. IL-5 activates B cells and plays a role in eosinophil homeostasis (63). IL-9 is needed by ILC2s for their survival and maintenance (64, 65), and IL-13 induces goblet cell hyperplasia and mucus secretion but can also act on alveolar macrophages (13, 66) and initiate the migration of dendritic cells to the mediastinal lymph nodes (49). Moreover, ILC2s are able to serve as antigen presenting cells for T cells by expressing MHCII (67), although this appears to be less pronounced in the lungs compared to the gut (67). Additional functional characteristics of pulmonary ILC2s include their expression of *Il5* as well as ST2 (IL-33R) at steady state in contrast to intestinal ILC2s which express both IL-5 and IL-13 mRNA and mainly the IL-25 receptor chain (IL-25RB) further demonstrating that ILC2s are imprinted by their microenvironment (10, 63).

Furthermore, ILC2s get support from basophil-derived IL-4 (68), and T cell-derived IL-2 (56), thus, establishing a quick but robust allergic reaction (**Figure 2**). In addition to the typical type 2 cytokines, IL-17 secreting ILC2s have been described, a cytokine known to be regulated by RORγt that has been correlated to severe asthma phenotypes (69). However, while one group reported IL-17 expression by KLRG1^{hi}ST2^{-} inflammatory ILC2s (iILC2s) in the lungs, which correlated with their expression of RORγt (70) a more recent report showed increased IL-17^{+}ST2^{+} ILC2s (ILC2$_{17}$s) upon IL-33 or allergen challenge, independently of RORγt expression (52, 70). Finally, in line with their immunomodulatory potential, ILC2s can also acquire a regulatory phenotype and memory-like properties upon IL-33 and IL-2 stimulation *in vivo*, decreasing the expression of their pro-inflammatory repertoire as well as eosinophilic recruitment and accumulation (71). Further studies on this subset could certainly open up new venues in allergic disease therapies. Overall, ILC2s are key in the initiation, amplification, and modulation of type 2 immune responses in the respiratory tract by exhibiting these fundamental characteristics.

Due to their scarcity and phenotypical heterogeneity in mice and humans, the detection of ILC2s by flow cytometry requires some considerations. ILC2s are characterized by the absence of known lineage markers and antigen receptors as well as the expression of surface markers CD45, CD25 (IL-2Rα), CD127 (IL-7Rα), and ST2 (IL-33R) in human asthma as well as in rodent models of respiratory allergic inflammation. Interestingly, CD127 low/negative ILC2s have also been reported in asthmatic patients as well as in an allergic experimental mouse model (72, 73). Of note, iILC2s show reduced CD127 expression upon systemic challenge with IL-25 (70). Whether these ILC2s have just downregulated the expression or endocytosed CD127 due to strong activity still needs to be investigated. In addition, mouse ILC2s are further identified by the surface expression of Thy1, a marker that is not present on human ILC2s. Another difference is the expression of CD161 by human ILC2s. The functions of these two glycoproteins are still not completely understood. Both mouse and human ILC2s can express the prostaglandin receptor family member CRTH2. CRTH2 is important in the migration of ILC2s and thereby expressed to a different extent

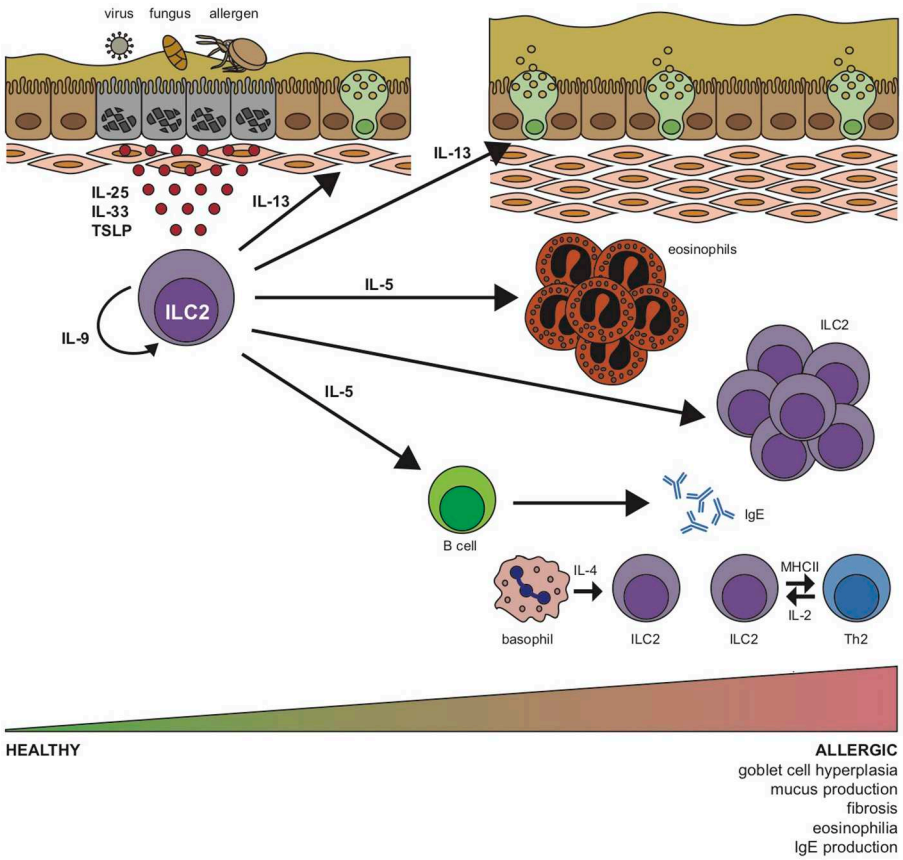

FIGURE 2 | Group 2 innate lymphoid cell (ILC2) stimulation by airway pathogens drives allergic respiratory inflammation. In response to allergens, fungi or viruses, lung epithelial cells release alarmins IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) that induce expansion and cytokine production by ILC2s. Activated ILC2s initiate an innate type 2 inflammatory response through production of IL-5, which induces eosinophilia, and IL-13, which promotes airway hyper-reactivity, goblet cell hyperplasia, mucus production and fibrosis. B cells and basophils are stimulated to release IgE and IL-4, respectively, and ILC2-derived IL-9 acts in an autocrine manner to prolong survival of ILC2s in the lung. Expression of MHCII enables ILC2 interaction with T_H2 cells, which subsequently promote ILC2 function via T cell-derived IL-2.

depending on location (74). However, the detection of mouse CRTH2 is limited by the availability of a specific detection antibody (74, 75). Our current strategies to detect ILC2s are largely based on surface receptors, which are actively used in an immune response by the cell to sense its environment and act accordingly. In addition, surface receptors and Gata3 are often used in combination to detect human and murine ILC2s (76, 77). The caveat with Gata3 is that ILC2s in the lungs are able to downregulate Gata3 expression under specific conditions such as viral (78), or helminth infections (70), however, under steady state conditions Gata3 is a reliable marker for pulmonary ILC2s.

Interestingly, ILC2s are not a homogenous population and several different subtypes of ILC2s have been reported at mucosal surfaces (79), including the lungs (31, 70). Memory ILC2s can be elicited upon repeated challenge of mice with IL-33, or when using papain as an allergen (31), thereby intensifying the immune response. Thus, ILC2s are specifically shaped by their local microenvironment and are reported to exhibit a sedentary lifestyle (45, 80). However, a subgroup of murine ILC2s, intestinally-derived inflammatory ILC2s (iILC2s) are able to traffic through the lymphatics and blood toward the lung to aid in the local response to helminth infection or systemic challenge with IL-25 (81). Moreover, the identification of circulating multipotent ILC progenitors in peripheral blood in humans indicates that ILC2 progenitors may be able to traffic to local pools of tissue-resident cells and replenish them (82). Since iILC2s are able to further differentiate into natural ILC2s (nILCs), the ability to traffic might be limited to specific circumstances and confined to not yet fully matured or differentiated ILC2 populations. However, additional studies are needed to pinpoint the potential of ILC2s to migrate within the body. nILC2s, which are present at steady state in the lungs and characterized by their ST2 expression, are typically increased upon stimulation with IL-33 (70). Both populations, iILC2s and memory ILC2s are characterized by elevated expression of the IL-25 receptor in the lungs (31, 70), however, it is still not completely understood how similar the biology and function of both populations is. So far, IL-25 receptor-expressing ILC2s have only been described in the human skin (83), and it still needs to be determined whether IL-25 receptor expression is present and regulated in human ILC2s during respiratory inflammation. KLRG1 is another commonly used marker to identify mature ILC2s in both human and mouse lungs. However, its expression is greatly influenced by androgens with ILC2s from male mice exhibiting higher expression of KLRG1 while female lungs harbor significantly higher numbers of KLRG1$^-$ ILC2s promoting lung inflammation (59, 84). The ligand for KLRG1 is E-cadherin and ILC2s isolated from human skin were shown to be restrained by this interaction (83). Testosterone can additionally regulate ILC2s by controlling their response to IL-2 as well as by the restraint of IL-5 and IL-13 production and thus resulting in decreased pulmonary pathology upon *Alternaria* challenge (42). In the same study, elevated levels of ILC2s in peripheral blood of female asthma patients in comparison to male patients have been reported which is especially interesting in the light of the increased prevalence of asthma in women. The exact role of androgens such as testosterone in asthma still needs further investigation since testosterone is able to induce IL-33 mRNA in mast cells (85), however, lower levels of IL-33 and TSLP have been detected in the bronchoalveolar lavage of *Alternaria* challenged mice.

Insights into the function of ILC2s in allergic inflammation has mainly been generated using experimental mouse models. However, a substantial amount of reports provide evidence that ILC2s are also key in human allergic respiratory inflammation. Of note, the first reports on human ILC2s provided a detailed description of ILC2s in polyp tissue of chronic rhinosinusitis patients (75, 77). Moreover, increased levels and activity of ILC2s have also been reported in asthmatic patients. ILC2s could be detected in bronchoalveolar lavage, lung tissue, sputum and blood of patients with respiratory inflammation. Although the gating strategies of ILC2s slightly vary between the distinct reports, all studies demonstrate expression of CD127 in combination with CRTH2 and/or CD44 and ST2 on ILC2s. A positive correlation of eosinophilia and ILC2s levels has been further reported in human patients similar to the observation in mouse respiratory inflammation (86, 87). Recent work opened the discussion of functional redundancy of T_H2 cells and ILC2s in humans (88). However, even if this is the case, pulmonary ILC2s have a critical function in the development of allergic diseases being innately committed to type 2 immunity and strong and immediate amplifiers of initial responses.

OBESITY-ASSOCIATED ASTHMA

The prevalence of both obesity and asthma has increased drastically in recent years. Although asthma in obese patients is characterized mainly as non-allergic with an increase in neutrophils, eosinophils have been reported to be present in elevated numbers in the lung tissue of obese asthma patients as well (89). Of note, in addition to their mucosal location, ILC2s were originally identified as fat-associated lymphoid cluster (FALC) Lineage$^-$ckit$^+$Sca-1$^+$ cells in the mesentery (12). Here, adipocytes and endothelial cells within the adipose tissue are sources of ILC2-activating IL-25 and IL-33 (90, 91). ILC2s are able to maintain the metabolic status of healthy adipose tissue by secreting IL-5 for eosinophil homeostasis, IL-13 to trigger alternative macrophage differentiation and methionine-enkephalin which directly acts on adipocytes and induces beiging of fat (92). However, in obesity ILC2s are decreased in adipose tissue and in ILC2-deficient mice, a high-fat diet accelerates obesity and insulin resistance indicating that ILC2s in adipose tissue are important for homeostasis. It thus seems contrary at first to link obesity and asthma. However, although obese mice have lower ILC2s and eosinophils in their adipose tissue, the levels of these populations are increased in the lungs in obese mice at steady state and upon allergen challenge such as with HDM. Not only ILC2s but also ILC3s are increased in the lungs of obese mice which can be enhanced by ozone triggered IL-33 or Nlrp3 inflammasome induced IL-1β, respectively (93, 94). It has been suggested that ILC2s and eosinophils might migrate from adipose tissue into lung tissue during obesity and thereby influence pulmonary immunity and

may trigger asthma. This represents an interesting potential mechanism but further research will be needed to fully support this idea.

BIOMARKERS AND ASSESSMENT OF SEVERITY OF ALLERGIC RESPIRATORY DISEASES

Interestingly, in humans, an allergic response is provoked in the skin as a first assessment of an allergic response in general but also as a first evaluation of asthma. Different tests can be used depending on the way of application using subcutaneous injections or exposure to allergen by topical application with the most common test in clinical practice still being the prick test. It still needs to be determined to what extent and how ILC2s directly contribute to the assessment of allergy via the prick test. Upon external stimuli or cellular damage, IL-33 is released from cells and engages ST2 on ILC2s but also on T_H2 cells, eosinophils, mast cells, and basophils, contributing to cutaneous allergic inflammation with increased levels of local and peripheral blood ILC2s, eosinophils, IgE, and histamine (95). IL-33 can be released locally and systemically after mechanical skin injury (i.e., scratching), promoting IgE-mediated degranulation (96). Of note, ILC2s present in the skin can control mast cell (MC) activity by direct interaction (97). Conversely, MC are an important source of IL-33 *in vivo*, contributing to ILC2 activation and type 2 immune response in disease models of multiple sclerosis (85), and helminth infection (98), a MC-ILC2 crosstalk also occurs in the lung (99). It is not yet fully understood how cutaneous antigen exposure could activate ILC2s but it is conceivable that dysregulation between MC and ILC2s could exacerbate the immune response during allergic airway inflammation and anaphylactic reaction. Moreover, the evaluation of the eosinophil count, as well as the level of IgE, including allergen-specific IgE in blood, or less common in sputum, is used to assess the grade of the allergic response. Type 2 signature cytokines (IL-4, IL-5, and IL-13) are used in addition as biomarkers. However, to assess the severity of asthma, lung function tests are routinely carried out in humans. Sequential examinations are performed on the patient such as allergy tests to pinpoint the responsible allergen(s), bronchial provocation or exhaled nitric oxide tests; before formulating any therapeutic recommendation (100). Since asthma and allergic inflammation of the respiratory tract can have multiple underlying causes, the aim is to personalize the treatment as much as possible depending on the results of the examinations. Thus, achieving control is the main objective currently proposed in asthma management where pharmacological and non-pharmacological treatment is adjusted in a continuous cycle that involves assessment, treatment and review. In mouse models, experimentally-induced airway hyper-reactivity is usually analyzed upon challenge with increasing doses of inhaled methacholine. The concept of using ILC2 prevalence as a biomarker for diagnostic purposes in lung disease is appealing. Screening of ILC2s as an early hierarchical population might already indicate asthma susceptibility before the start of symptoms or pathology in the lungs. However, due to their high phenotypic diversity, ILC2 characterization in different asthmatic subgroups and their comparison is necessary, and even then, ILC2 level and functionality should be carefully evaluated for each subgroup. Nevertheless, biomarkers and the assessment of allergic respiratory diseases show overall important similarities between rodent models of disease and clinical practice.

EXPERIMENTAL AND THERAPEUTIC STRATEGIES TO AMELIORATE RESPIRATORY INFLAMMATION

Corticosteroids

In humans, as a first and often immediate treatment inhaled corticosteroids are commonly used in both allergic and non-allergic respiratory inflammation. Corticosteroids reduce the general inflammation of the lung and provide relief of symptoms for the patient (101), but can also cause adverse side effects especially when given systemically in high doses and during long-term treatment. Importantly, corticosteroids dampen the activity of both mouse and human ILC2s (102). However, under specific conditions, corticosteroids are less able to act on ILC2 activity. This has been reported to be the case in situations of enhanced STAT5 activation upon TSLP stimulation. This increased activation of STAT5 by TSLP has been identified as a regulatory mechanism in mice (102), was further confirmed in human ILC2s (103) and helps to explain why not all asthma patients respond to corticosteroid therapy. Corticosteroid resistance can also occur in neutrophilic asthma. IL-17 contributes to neutrophil accumulation in the lungs but also increases the expression of the glucocorticoid receptor beta (GRβ) (104). GRβ inhibits the activity of GRα by direct competition for glucocorticoids. Increased expression of GRβ has been reported on cell populations in glucocorticoid-resistant patients (105, 106), and is discussed to contribute to steroid resistance in neutrophilic asthma (104). IL-17 can also be derived from pulmonary iILC2s (70) as well as $ILC2_{17}$s, which were described to be the main source of IL-17 in the lung after IL-33-induced lung inflammation (52). However, if and which role ILC2s may have in neutrophilic asthma still needs to be investigated.

Adrenergic Agonists (β_2-Agonists)

Like corticosteroids, β_2-agonists that act on β_2-adrenergic receptors are frequently used to treat asthmatic patients. A recent report showed that both human and mouse ILC2s express the β2AR (β_2-adrenergic receptor) for epinephrine and norepinephrine and that the use of an agonist during lung inflammation in mice impaired ILC2 proliferation, cytokine production and effector function (107). These findings highlight the importance of ILC2s in integrating neuroimmune signals. In humans, short acting β-agonists (SABA, e.g., Salbutamol) and long acting β-agonists (LABA, e.g., Salmeterol) are routinely used for asthma treatment. LABA is used in combination with inhaled corticosteroids whereas SABA is also approved as a monotherapy in mild asthma (108). Adverse effects of β_2-agonists have been reported and the discussion was stirred up

upon the report of detrimental effects upon excessive Fenoterol (SABA) treatment (long time and high dose) (109, 110). The underlying mechanisms of these severe consequences are only incompletely understood. However, the use of β_2-agonists for asthmatic patients under treatment is generally regarded as safe and successful (101).

Lipid Mediators: Leukotrienes & Prostaglandins

Bioactive lipid mediators are important regulators of ILC2s (111). Indeed, leukotrienes (LTs) including cysteinyl LTs (CysLTs) are generated by arachidonic acid metabolism and CysLTs have been linked to the initiation of asthma and bronchoconstriction since a long time (112). Leukotriene receptor antagonists such as Montelukast, a $CysLT_1$ receptor antagonist, are commonly prescribed to improve asthma symptoms in humans (113). Both mouse and human ILC2s have been reported to express CysLT receptors and it was shown that CysLTs positively regulate ILC2 activation (47, 114–116).

In addition, Leukotriene B4 (LTB4) has also been linked to asthma. LTB4 is a neutrophil chemoattractant in pulmonary inflammation (117), and neutrophils can be increased during the exacerbation phase of asthma (118). However, sputum samples of asthmatic patients showed an increased level of LTB4 which strikingly did not correlate with neutrophil levels in the samples of the analyzed patients (119). The high affinity receptor for LTB4, LTB4R1, has been reported on mouse ILC2s but its presence and role in human ILC2s still needs to be elucidated. Interestingly, LTB4 also plays an important role in the development of insulin resistance in obese mice (120). However, how and if ILC2s are involved in this context still needs to be addressed.

Similar to LTs, prostaglandins are products of arachidonic acid metabolism. As mentioned previously, human and mouse ILC2s express the receptor for prostaglandin D2, CRTH2 (74, 75). CRTH2 plays a potent role in activation, migration and cytokine release of T_H2 cells and eosinophils. Curiously, accumulation of pulmonary ILC2s is regulated via CRTH2 and differences in its expression on ILC2s have been reported in inflamed pulmonary tissue (121). Use of monoclonal antibodies against CRTH2 resulted in a reduction of CRTH2 expressing cells including ILC2s in mice (122). In addition to its role in migration of ILC2s, PGD_2 has been reported to potentiate the action of ILC2s eliciting cytokines leading to an increase in effector cytokine expression. Consequently, small-molecules antagonists of the CRTH2 receptor, have been promising in human trials for asthma patients (123–126). One example is OC000459 which was reported to be a safe and effective alternative treatment of eosinophilic asthma improving lung function and asthma symptoms (127, 128).

Signature Cytokines and IgE

Monoclonal antibodies are used to treat allergic respiratory diseases such as asthma in humans. These antibodies are directed against either type 2 signature cytokines IL-4, IL-5, and IL-13, their respective surface receptors or against IgE. Both T_H2 cells and ILC2s are potential sources of type 2 signature cytokines. Moreover, ILC2s have been reported to enhance the adaptive immune response and thereby influence IgE production. The following monoclonal antibodies to treat asthma are currently used in clinical practice: Dupilumab (moderate to severe asthma and severe asthma) targets IL-4 and IL-13 by binding to the IL-4Rα subunit thereby acting as a blocking antibody for these signaling pathways (129, 130). Mepolizumab and Reslizumab (both eosinophilic asthma) target IL-5 directly and thereby neutralize this signature type 2 cytokine, reducing the rate of exacerbations (131, 132). Benralizumab (severe eosinophilic asthma) is directed against the IL-5Rα subunit (133). Lebrikizumab (severe asthma) and Tralokinumab (moderate to severe asthma) are directed against IL-13 (134, 135), and Omalizumab (severe allergic asthma in adults and children) targets and neutralizes IgE (136, 137). The ILC2-eliciting cytokine TSLP plays a critical role in human asthma and antibodies to neutralize TSLP (Tezepelumab) have been tested to treat allergen-induced asthma (138, 139). Overall, these antibodies block and thereby neutralize important immune mediators secreted by ILC2s as well as T_H2 cells.

Neuropeptides

ILC2s in mouse and human can sense and respond to neuropeptides such as neuromedin U (25, 140, 141). Although pulmonary ILC2s exhibit a more moderate response to neuromedin U when compared to intestinal ILC2s under the tested conditions (140), several different neuropeptides are present in the lungs (142), including vasoactive intestinal peptide (VIP). VIP can induce cytokine stimulation of intestinal but also pulmonary ILC2s (63). Inhalation of a VIP agonist (Ro 25-1553) resulted in a short but significant bronchodilatory effect (143), however the exact mechanism and a possible link to pulmonary ILC2s is unclear. In contrast to VIP, calcitonin gene-related peptide (CGRP) is elevated in some asthmatic patients (144), and neuroendocrine cells, which co-localize in the airways with ILC2s are an important source thereof (145). ILC2s respond to CGRP by secreting more IL-5, however, targeting CGRP and thereby ILC2s still needs to be evaluated in asthma patients. Targeting neuropeptides and their receptors may be a promising concept for future therapy but still requires further investigation.

Transcription Factors

GATA3 is the master transcription factor of ILC2s and critical in regulating asthmatic responses in patients with a predominant T_H2 phenotype. Targeting GATA3 is complex due to its intranuclear location but would enable to already intervene at a very early stage in the disease formation process. Novel approaches to antagonize GATA3 using antisense molecules (DNAzymes) overcome this challenge by cleaving and inactivating GATA3 messenger RNA (mRNA). GATA3-specific DNAzyme SB010 has shown to significantly attenuate both early and late-phase asthmatic responses after allergen exposure in a phase IIa proof-of-concept trial (146).

CONCLUDING REMARKS & OUTLOOK

Since allergic respiratory diseases are rapidly increasing worldwide, there is a critical need to optimize current and develop novel therapeutic strategies. Reports in recent years have shown that ILC2s are important players in experimental mouse models of allergic airway inflammation and their role in asthmatic patients is just starting to unveil itself. Although neutralizing ILC2-elicited immune mediators or blocking respective signaling pathways are currently used to treat asthma, it is of great interest to develop alternative strategies to target not only the consequence but the cause of respiratory inflammation. This may be achieved by blocking ILC2s fairly early in disease and re-directing their activity. Moreover, the merge of immunology with other fields such as neurobiology opens new concepts and will reveal novel targets of translational interest. The unique microenvironment of the respiratory tract with its diversity of non-hematopoietic cells and their close proximity to ILC2s will as well unfold thrilling answers of ILC2 maintenance and activation in the future. We are excited to see further research on ILC2 biology in respiratory allergic inflammation which will surely provide essential knowledge to develop novel concepts and strategies for asthma treatment and improving overall respiratory health.

AUTHOR CONTRIBUTIONS

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

FUNDING

CD and SH acknowledge support by a Rahel Hirsch Habilitationsstipendium (CD) and by a Boehringer Ingelheim Fonds travel grant (SH).

ACKNOWLEDGMENTS

We acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of Charité – Universitätsmedizin Berlin.

REFERENCES

1. Yach D, Hawkes C, Gould CL, Hofman KJ. The global burden of chronic diseases: overcoming impediments to prevention and control. *JAMA*. (2004) 291:2616–22. doi: 10.1001/jama.291.21.2616
2. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature*. (2008) 454:445–54. doi: 10.1038/nature07204
3. Nunes C, Pereira AM, Morais-Almeida M. Asthma costs and social impact. *Asthma Res Pract*. (2017) 3:1. doi: 10.1186/s40733-016-0029-3
4. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol*. (2015) 16:45–56. doi: 10.1038/ni.3049
5. Licona-Limon P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. *Nat Immunol*. (2013) 14:536–42. doi: 10.1038/ni.2617
6. Artis D, Spits H. The biology of innate lymphoid cells. *Nature*. (2015) 517:293–301. doi: 10.1038/nature14189
7. Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity*. (2014) 41:354–65. doi: 10.1016/j.immuni.2014.09.005
8. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell*. (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
9. Duerr CU, Fritz JH. Regulation of group 2 innate lymphoid cells. *Cytokine*. (2016) 87:1–8. doi: 10.1016/j.cyto.2016.01.018
10. Ricardo-Gonzalez RR, Van Dyken SJ, Schneider C, Lee J, Nussbaum JC, Liang HE, et al. Tissue signals imprint ILC2 identity with anticipatory function. *Nat Immunol*. (2018) 19:1093–99. doi: 10.1038/s41590-018-0201-4
11. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA*. (2010) 107:11489–94. doi: 10.1073/pnas.1003988107
12. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit^{+}Sca-1^{+} lymphoid cells. *Nature*. (2010) 463:540–4. doi: 10.1038/nature08636
13. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. (2010) 464:1367–70. doi: 10.1038/nature08900
14. Fahy JV. Type 2 inflammation in asthma–present in most, absent in many. *Nat Rev Immunol*. (2015) 15:57–65. doi: 10.1038/nri3786
15. Koczulla AR, Vogelmeier CF, Garn H, Renz, H. New concepts in asthma: clinical phenotypes and pathophysiological mechanisms. *Drug Discov Today*. (2017) 22:388–96. doi: 10.1016/j.drudis.2016.11.008
16. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. *J Allergy Clin Immunol*. (2011) 127:355–60. doi: 10.1016/j.jaci.2010.11.037
17. van Aalderen WM. Childhood asthma: diagnosis and treatment. *Scientifica*. (2012) 2012:674204. doi: 10.6064/2012/674204
18. Novey HS, Marchioli LE, Sokol WN, Wells ID. Papain-induced asthma–physiological and immunological features. *J Allergy Clin Immunol*. (1979) 63:98–103.
19. Calderon MA, Linneberg A, Kleine-Tebbe J, De Blay F, Hernandez Fernandez de Rojas D, Virchow JC, et al. Respiratory allergy caused by house dust mites: what do we really know? *J Allergy Clin Immunol*. (2015) 136:38–48. doi: 10.1016/j.jaci.2014.10.012
20. Doherty TA, Khorram N, Chang JE, Kim HK, Rosenthal P, Croft M, et al. STAT6 regulates natural helper cell proliferation during lung inflammation initiated by Alternaria. *Am J Physiol Lung Cell Mol Physiol*. (2012) 303:L577–88. doi: 10.1152/ajplung.00174.2012
21. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol*. (2012) 42:1106–16. doi: 10.1002/eji.201142018
22. Li BWS, de Bruijn MJW, Tindemans I, Lukkes M, KleinJan A, Hoogsteden HC, et al. T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. *Eur J Immunol*. (2016) 46:1392–403. doi: 10.1002/eji.201546119
23. Coquet JM, Schuijs MJ, Smyth MJ, Deswarte K, Beyaert R, Braun H, et al. Interleukin-21-producing CD4^{+} T cells promote type 2 immunity to house dust mites. *Immunity*. (2015) 43:318–30. doi: 10.1016/j.immuni.2015.07.015
24. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity*. (2012) 36:451–63. doi: 10.1016/j.immuni.2011.12.020
25. Wallrapp A, Riesenfeld SJ, Burkett PR, Abdulnour R-EE., Nyman J, Dionne D, et al. The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation. *Nature*. (2017) 549:351–56. doi: 10.1038/nature24029
26. Gold MJ, Antignano F, Halim TY, Hirota JA, Blanchet MR, Zaph C, et al. Group 2 innate lymphoid cells facilitate sensitization to local, but not

systemic, TH2-inducing allergen exposures. *J Allergy Clin Immunol*. (2014) 133:1142–8. doi: 10.1016/j.jaci.2014.02.033

27. Li Y, Chen S, Chi Y, Yang Y, Chen X, Wang H, et al. Kinetics of the accumulation of group 2 innate lymphoid cells in IL-33-induced and IL-25-induced murine models of asthma: a potential role for the chemokine CXCL16. *Cell Mol Immunol*. (2019) 16:75–86. doi: 10.1038/s41423-018-0182-0
28. Li BWS, Stadhouders R, de Bruijn MJW, Lukkes M, Beerens D, Brem MD, et al. Group 2 innate lymphoid cells exhibit a dynamic phenotype in allergic airway inflammation. *Front Immunol*. (2017) 8:1684. doi: 10.3389/fimmu.2017.01684
29. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol*. (2012) 129:191–8.e1–4. doi: 10.1016/j.jaci.2011.09.041
30. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J Allergy Clin Immunol*. (2013) 132:933–41. doi: 10.1016/j.jaci.2013.05.012
31. Martinez-Gonzalez I, Matha L, Steer CA, Ghaedi M, Poon GF, Takei, F. Allergen-experienced group 2 innate lymphoid cells acquire memory-like properties and enhance allergic lung inflammation. *Immunity*. (2016) 45:198–208. doi: 10.1016/j.immuni.2016.06.017
32. Kato Y, Akasaki S, Muto-Haenuki Y, Fujieda S, Matsushita K, Yoshimoto T. Nasal sensitization with ragweed pollen induces local-allergic-rhinitis-like symptoms in mice. *PLoS One*. (2014) 9:e103540. doi: 10.1371/journal.pone.0103540
33. Mohapatra A, Van Dyken SJ, Schneider C, Nussbaum JC, Liang HE, Locksley RM. Group 2 innate lymphoid cells utilize the IRF4-IL-9 module to coordinate epithelial cell maintenance of lung homeostasis. *Mucosal Immunol*. (2016) 9:275–86. doi: 10.1038/mi.2015.59
34. Van Dyken SJ, Mohapatra A, Nussbaum JC, Molofsky AB, Thornton EE, Ziegler SF, et al. Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and gammadelta T cells. *Immunity*. (2014) 40:414–24. doi: 10.1016/j.immuni.2014.02.003
35. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- $CD25^{+}$ $CD44^{hi}$ lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol*. (2012) 188:1503–13. doi: 10.4049/jimmunol.1102832
36. Maazi H, Patel N, Sankaranarayanan I, Suzuki Y, Rigas D, Soroosh P, et al. ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity*. (2015) 42:538–51. doi: 10.1016/j.immuni.2015.02.007
37. Rigas D, Lewis G, Aron JL, Wang B, Banie H, Sankaranarayanan I, et al. Type 2 innate lymphoid cell suppression by regulatory T cells attenuates airway hyperreactivity and requires inducible T-cell costimulator-inducible T-cell costimulator ligand interaction. *J Allergy Clin Immunol*. (2017) 139:1468–77.e2. doi: 10.1016/j.jaci.2016.08.034
38. Shen X, Pasha MA, Hidde K, Khan A, Liang M, Guan W, et al. Group 2 innate lymphoid cells promote airway hyperresponsiveness through production of VEGFA. *J Allergy Clin Immunol*. (2018) 141:1929–31.e4. doi: 10.1016/j.jaci.2018.01.005
39. Toki S, Goleniewska K, Reiss S, Zhou W, Newcomb DC, Bloodworth MH, et al. The histone deacetylase inhibitor trichostatin A suppresses murine innate allergic inflammation by blocking group 2 innate lymphoid cell (ILC2) activation. *Thorax*. (2016) 71:633–45. doi: 10.1136/thoraxjnl-2015-207728
40. Zhou W, Toki S, Zhang J, Goleniewksa K, Newcomb DC, Cephus JY, et al. Prostaglandin I2 signaling and inhibition of group 2 innate lymphoid cell responses. *Am J Respir Crit Care Med*. (2016) 193:31–42. doi: 10.1164/rccm.201410-1793OC
41. Zhou Y, Wang W, Zhao C, Wang Y, Wu H, Sun X, et al. Prostaglandin E2 inhibits group 2 innate lymphoid cell activation and allergic airway inflammation through E-prostanoid 4-cyclic adenosine monophosphate signaling. *Front Immunol*. (2018) 9:501. doi: 10.3389/fimmu.2018.00501
42. Cephus JY, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, et al. Testosterone attenuates group 2 innate lymphoid cell-mediated airway inflammation. *Cell Rep*. (2017) 21:2487–99. doi: 10.1016/j.celrep.2017.10.110
43. Doherty TA, Khorram N, Sugimoto K, Sheppard D, Rosenthal P, Cho JY, et al. Alternaria induces STAT6-dependent acute airway eosinophilia and epithelial FIZZ1 expression that promotes airway fibrosis and epithelial thickness. *J Immunol*. (2012) 188:2622–9. doi: 10.4049/jimmunol.1101632
44. Kim HK, Lund S, Baum R, Rosenthal P, Khorram N, Doherty TA. Innate type 2 response to Alternaria extract enhances ryegrass-induced lung inflammation. *Int Arch Allergy Immunol*. (2014) 163:92–105. doi: 10.1159/000356341
45. Moro K, Kabata H, Tanabe M, Koga S, Takeno N, Mochizuki M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol*. (2016) 17:76–86. doi: 10.1038/ni.3309
46. Karta MR, Rosenthal PS, Beppu A, Vuong CY, Miller M, Das S, et al. beta2 integrins rather than beta1 integrins mediate Alternaria-induced group 2 innate lymphoid cell trafficking to the lung. *J Allergy Clin Immunol*. (2018) 141:329–38.e12. doi: 10.1016/j.jaci.2017.03.010
47. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol*. (2013) 132:205–13. doi: 10.1016/j.jaci.2013.03.048
48. Stier MT, Zhang J, Goleniewska K, Cephus JY, Rusznak M, Wu L, et al. IL-33 promotes the egress of group 2 innate lymphoid cells from the bone marrow. *J Exp Med*. (2018) 215:263–81. doi: 10.1084/jem.20170449
49. Halim TY, Steer CA, Matha L, Gold MJ, Martinez-Gonzalez I, McNagny KM, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity*. (2014) 40:425–35. doi: 10.1016/j.immuni.2014.01.011
50. Cao Y, He Y, Wang X, Liu Y, Shi K, Zheng Z, et al. Polymorphonuclear myeloid-derived suppressor cells attenuate allergic airway inflammation by negatively regulating group 2 innate lymphoid cells. *Immunology*. (2019) 156:402–12. doi: 10.1111/imm.13040
51. Xiong Y, Cui X, Li W, Lv J, Du L, Mi W, et al. BLT1 signaling in epithelial cells mediates allergic sensitization via promotion of IL-33 production. *Allergy*. (2019) 74:495–506. doi: 10.1111/all.13656
52. Cai T, Qiu J, Ji Y, Li W, Ding Z, Suo C, et al. IL-17-producing $ST2^{+}$ group 2 innate lymphoid cells play a pathogenic role in lung inflammation. *J Allergy Clin Immunol*. (2019) 143:229–44.e9. doi: 10.1016/j.jaci.2018.03.007
53. Kamachi F, Isshiki T, Harada N, Akiba H, Miyake S. ICOS promotes group 2 innate lymphoid cell activation in lungs. *Biochem Biophys Res Commun*. (2015) 463:739–45. doi: 10.1016/j.bbrc.2015.06.005
54. Drake LY, Iijima K, Bartemes K, Kita H. Group 2 innate lymphoid cells promote an early antibody response to a respiratory antigen in mice. *J Immunol*. (2016) 197:1335–42. doi: 10.4049/jimmunol.1502669
55. Drake LY, Iijima K, Kita, H. Group 2 innate lymphoid cells and $CD4^{+}$ T cells cooperate to mediate type 2 immune response in mice. *Allergy*. (2014) 69:1300–7. doi: 10.1111/all.12446
56. Roediger B, Kyle R, Tay SS, Mitchell AJ, Bolton HA, Guy TV, et al. IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation. *J Allergy Clin Immunol*. (2015) 136:1653–63.e7. doi: 10.1016/j.jaci.2015.03.043
57. Stadhouders R, Li BWS, de Bruijn MJW, Gomez A, Rao TN, Fehling HJ, et al. Epigenome analysis links gene regulatory elements in group 2 innate lymphocytes to asthma susceptibility. *J Allergy Clin Immunol*. (2018) 142:1793–807. doi: 10.1016/j.jaci.2017.12.1006
58. Paclik D, Stehle C, Lahmann A, Hutloff A, Romagnani C. ICOS regulates the pool of group 2 innate lymphoid cells under homeostatic and inflammatory conditions in mice. *Eur J Immunol*. (2015) 45:2766–72. doi: 10.1002/eji.201545635
59. Laffont S, Blanquart E, Savignac M, Cenac C, Laverny G, Metzger D, et al. Androgen signaling negatively controls group 2 innate lymphoid cells. *J Exp Med*. (2017) 214:1581–92. doi: 10.1084/jem.20161807
60. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med*. (2010) 363:1211–21. doi: 10.1056/NEJMoa0906312
61. Vicente CT, Revez JA, Ferreira MAR. Lessons from ten years of genome-wide association studies of asthma. *Clin Transl Immunol*. (2017) 6:e165. doi: 10.1038/cti.2017.54

62. Van Dyken SJ, Nussbaum JC, Lee J, Molofsky AB, Liang HE, Pollack JL, et al. A tissue checkpoint regulates type 2 immunity. *Nat Immunol*. (2016) 17:1381–87. doi: 10.1038/ni.3582
63. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature*. (2013) 502:245–8. doi: 10.1038/nature12526
64. Turner JE, Morrison PJ, Wilhelm C, Wilson M, Ahlfors H, Renauld JC, et al. IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J Exp Med*. (2013) 210:2951–65. doi: 10.1084/jem.20130071
65. Wilhelm C, Hirota K, Stieglitz B, Van Snick J, Tolaini M, Lahl K, et al. An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat Immunol*. (2011) 12:1071–7. doi: 10.1038/ni.2133
66. Saluzzo S, Gorki AD, Rana BM, Martins R, Scanlon S, Starkl P, et al. First-breath-induced type 2 pathways shape the lung immune environment. *Cell Rep*. (2017) 18:1893–905. doi: 10.1016/j.celrep.2017.01.071
67. Oliphant CJ, Hwang YY, Walker JA, Salimi M, Wong SH, Brewer JM, et al. MHCII-mediated dialog between group 2 innate lymphoid cells and $CD4^+$ T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity*. (2014) 41:283–95. doi: 10.1016/j.immuni.2014.06.016
68. Motomura Y, Morita H, Moro K, Nakae S, Artis D, Endo TA, et al. Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation. *Immunity*. (2014) 40:758–71. doi: 10.1016/j.immuni.2014.04.013
69. Chesne J, Braza F, Mahay G, Brouard S, Aronica M, Magnan, A. IL-17 in severe asthma. Where do we stand? *Am J Respir Crit Care Med*. (2014) 190:1094–101. doi: 10.1164/rccm.201405-0859PP
70. Huang Y, Guo L, Qiu J, Chen X, Hu-Li J, Siebenlist U, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol*. (2015) 16:161–9. doi: 10.1038/ni.3078
71. Seehus CR, Kadavallore A, Torre BDl, Yeckes AR, Wang Y, Tang J, et al. Alternative activation generates IL-10 producing type 2 innate lymphoid cells. *Nat Commun*. (2017) 8:1900. doi: 10.1038/s41467-017-02023-z
72. Saglani S, Gregory LG, Manghera AK, Branchett WJ, Uwadiae F, Entwistle LJ, et al. Inception of early-life allergen-induced airway hyperresponsiveness is reliant on IL-13^+CD4^+ T cells. *Sci Immunol*. (2018) 3:eaan4128. doi: 10.1126/sciimmunol.aan4128
73. Nagakumar P, Denney L, Fleming L, Bush A, Lloyd CM, Saglani, S. Type 2 innate lymphoid cells in induced sputum from children with severe asthma. *J Allergy Clin Immunol*. (2016) 137:624–26.e6. doi: 10.1016/j.jaci.2015.06.038
74. Tait Wojno ED, Monticelli LA, Tran SV, Alenghat T, Osborne LC, Thome JJ, et al. The prostaglandin D receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol*. (2015) 8:1313–23. doi: 10.1038/mi.2015.21
75. Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol*. (2011) 12:1055–62. doi: 10.1038/ni.2104
76. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity*. (2012) 37:634–48. doi: 10.1016/j.immuni.2012.06.020
77. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity*. (2012) 37:649–59. doi: 10.1016/j.immuni.2012.08.015
78. Silver JS, Kearley J, Copenhaver AM, Sanden C, Mori M, Yu L, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat Immunol*. (2016) 17:626–35. doi: 10.1038/ni.3443
79. Gury-BenAri M, Thaiss CA, Serafini N, Winter DR, Giladi A, Lara-Astiaso D, et al. The spectrum and regulatory landscape of intestinal innate lymphoid cells are shaped by the microbiome. *Cell*. (2016) 166:1231–46.e13. doi: 10.1016/j.cell.2016.07.043
80. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science*. (2015) 350:981–5. doi: 10.1126/science.aac9593
81. Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science*. (2018) 359:114–19. doi: 10.1126/science.aam5809
82. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell*. (2017) 168:1086–100.e10. doi: 10.1016/j.cell.2017.02.021
83. Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med*. (2013) 210:2939–50. doi: 10.1084/jem.20130351
84. Kadel S, Ainsua-Enrich E, Hatipoglu I, Turner S, Singh S, Khan S, et al. A major population of functional $KLRG1^-$ ILC2s in female lungs contributes to a sex bias in ILC2 numbers. *Immunohorizons*. (2018) 2:74–86. doi: 10.4049/immunohorizons.1800008
85. Russi AE, Ebel ME, Yang Y, Brown MA. Male-specific IL-33 expression regulates sex-dimorphic EAE susceptibility. *Proc Natl Acad Sci USA. (*2018) 115:E1520-E29. doi: 10.1073/pnas.1710401115
86. Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol*. (2016) 137:75–86.e8. doi: 10.1016/j.jaci.2015.05.037
87. Liu T, Wu J, Zhao J, Wang J, Zhang Y, Liu L, et al. Type 2 innate lymphoid cells: a novel biomarker of eosinophilic airway inflammation in patients with mild to moderate asthma. *Respir Med*. (2015) 109:1391–6. doi: 10.1016/j.rmed.2015.09.016
88. Vely F, Barlogis V, Vallentin B, Neven B, Piperoglou C, Ebbo M, et al. Evidence of innate lymphoid cell redundancy in humans. *Nat Immunol*. (2016) 17:1291–9. doi: 10.1038/ni.3553
89. Desai D, Newby C, Symon FA, Haldar P, Shah S, Gupta S, et al. Elevated sputum interleukin-5 and submucosal eosinophilia in obese individuals with severe asthma. *Am J Respir Crit Care Med*. (2013) 188:657–63. doi: 10.1164/rccm.201208-1470OC
90. Feng J, Li L, Ou Z, Li Q, Gong B, Zhao Z, et al. IL-25 stimulates M2 macrophage polarization and thereby promotes mitochondrial respiratory capacity and lipolysis in adipose tissues against obesity. *Cell Mol Immunol*. (2018) 15:493–505. doi: 10.1038/cmi.2016.71
91. Zeyda M, Wernly B, Demyanets S, Kaun C, Hammerle M, Hantusch B, et al. Severe obesity increases adipose tissue expression of interleukin-33 and its receptor ST2, both predominantly detectable in endothelial cells of human adipose tissue. *Int J Obes*. (2013) 37:658–65. doi: 10.1038/ijo.2012.118
92. Cautivo KM, Molofsky AB. Regulation of metabolic health and adipose tissue function by group 2 innate lymphoid cells. *Eur J Immunol*. (2016) 46:1315–25. doi: 10.1002/eji.201545562
93. Mathews JA, Krishnamoorthy N, Kasahara DI, Cho Y, Wurmbrand AP, Ribeiro L, et al. IL-33 drives augmented responses to ozone in obese mice. *Environ Health Perspect*. (2017) 125:246–53. doi: 10.1289/EHP272
94. Kim HY, Lee HJ, Chang YJ, Pichavant M, Shore SA, Fitzgerald KA, et al. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med*. (2014) 20:54–61. doi: 10.1038/nm.3423
95. Imai Y, Yasuda K, Sakaguchi Y, Haneda T, Mizutani H, Yoshimoto T, et al. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci USA. (*2013) 110:13921–6. doi: 10.1073/pnas.1307321110
96. Galand C, Leyva-Castillo JM, Yoon J, Han A, Lee MS, McKenzie ANJ, et al. IL-33 promotes food anaphylaxis in epicutaneously sensitized mice by targeting mast cells. *J Allergy Clin Immunol*. (2016) 138:1356–66. doi: 10.1016/j.jaci.2016.03.056
97. Roediger B, Kyle R, Yip KH, Sumaria N, Guy TV, Kim BS, et al. Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol*. (2013) 14:564–73. doi: 10.1038/ni.2584
98. Shimokawa C, Kanaya T, Hachisuka M, Ishiwata K, Hisaeda H, Kurashima Y, et al. Mast cells are crucial for induction of group 2 innate lymphoid cells and clearance of helminth infections. *Immunity*. (2017) 46:863–74.e4. doi: 10.1016/j.immuni.2017.04.017
99. Mortaz E, Amani S, Mumby S, Adcock IM, Movassaghi M, Folkerts J, et al. Role of mast cells and type 2 innate lymphoid

(ILC2) cells in lung transplantation. *J Immunol Res*. (2018) 2018:2785971. doi: 10.1155/2018/2785971
100. Global Initiative for Asthma. *Global Strategy for Asthma Management and Prevention*. Vancouver, WA: NIH Publication (2017). Available online at: www.ginasthma.org
101. Crompton G. A brief history of inhaled asthma therapy over the last fifty years. *Prim Care Respir J. (*2006) 15:326–31. doi: 10.1016/j.pcrj.2006.09.002
102. Kabata H, Moro K, Fukunaga K, Suzuki Y, Miyata J, Masaki K, et al. Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation. *Nat Commun*. (2013) 4:2675. doi: 10.1038/ncomms3675
103. Liu S, Verma M, Michalec L, Liu W, Sripada A, Rollins D, et al. Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: the role of thymic stromal lymphopoietin. *J Allergy Clin Immunol*. (2018) 141:257–68.e6. doi: 10.1016/j.jaci.2017.03.032
104. Vazquez-Tello A, Halwani R, Hamid Q, Al-Muhsen, S. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J Clin Immunol*. (2013) 33:466–78. doi: 10.1007/s10875-012-9828-3
105. Goleva E, Li LB, Eves PT, Strand MJ, Martin RJ, Leung, D.Y. Increased glucocorticoid receptor beta alters steroid response in glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med*. (2006) 173:607–16. doi: 10.1164/rccm.200507-1046OC
106. Hamid QA, Wenzel SE, Hauk PJ, Tsicopoulos A, Wallaert B, Lafitte JJ, et al. Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med*. (1999) 159 (5 Pt 1):1600–4. doi: 10.1164/ajrccm.159.5.9804131
107. Moriyama S, Brestoff JR, Flamar AL, Moeller JB, Klose CSN, Rankin LC, et al. beta2-adrenergic receptor-mediated negative regulation of group 2 innate lymphoid cell responses. *Science*. (2018) 359:1056–61. doi: 10.1126/science.aan4829
108. Johnston SL, Edwards MR. Mechanisms of adverse effects of {beta}-agonists in asthma. *Thorax*. (2009) 64:739–41. doi: 10.1136/thx.2009.119230
109. Crane J, Pearce N, Flatt A, Burgess C, Jackson R, Kwong T, et al. Prescribed fenoterol and death from asthma in New Zealand, 1981–83: case-control study. *Lancet*. (1989) 1:917–22.
110. Beasley R, Nishima S, Pearce N, Crane J. Beta-agonist therapy and asthma mortality in Japan. *Lancet*. (1998) 351:1406–7. doi: 10.1016/S0140-6736(05)79446-0
111. Kabata H, Moro K, Koyasu S. The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms. *Immunol Rev*. (2018) 286:37–52. doi: 10.1111/imr.12706
112. Hallstrand TS, Henderson WR Jr. An update on the role of leukotrienes in asthma. *Curr Opin Allergy Clin Immunol*. (2010) 10:60–6. doi: 10.1097/ACI.0b013e32833489c3
113. Kittana N, Hattab S, Ziyadeh-Isleem A, Jaradat N, Zaid AN. Montelukast, current indications and prospective future applications. *Expert Rev Respir Med*. (2016) 10:943–56. doi: 10.1080/17476348.2016.1207533
114. Xue L, Salimi M, Panse I, Mjosberg JM, McKenzie AN, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol*. (2014) 133:1184–94. doi: 10.1016/j.jaci.2013.10.056
115. von Moltke J, O'Leary CE, Barrett NA, Kanaoka Y, Austen KF, Locksley RM. Leukotrienes provide an NFAT-dependent signal that synergizes with IL-33 to activate ILC2s. *J Exp Med*. (2017) 214:27–37. doi: 10.1084/jem.20161274
116. Salimi M, Stoger L, Liu W, Go S, Pavord I, Klenerman P, et al. Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. *J Allergy Clin Immunol*. (2017) 140:1090–100.e11. doi: 10.1016/j.jaci.2016.12.958
117. Corhay JL, Henket M, Nguyen D, Duysinx B, Sele J, Louis R. Leukotriene B4 contributes to exhaled breath condensate and sputum neutrophil chemotaxis in COPD. *Chest*. (2009) 136:1047–54. doi: 10.1378/chest.08-2782
118. Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol*. (1995) 95:843–52.
119. Higham A, Cadden P, Southworth T, Rossall M, Kolsum U, Lea S, et al. Leukotriene B4 levels in sputum from asthma patients. *ERJ Open Res*. (2016) 2:00088-2015. doi: 10.1183/23120541.00088-2015
120. Li P, Oh DY, Bandyopadhyay G, Lagakos WS, Talukdar S, Osborn O, et al. LTB4 promotes insulin resistance in obese mice by acting on macrophages, hepatocytes and myocytes. *Nat Med*. (2015) 21:239–47. doi: 10.1038/nm.3800
121. Monticelli LA, Buck MD, Flamar AL, Saenz SA, Tait Wojno ED, Yudanin NA, et al. Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation. *Nat Immunol*. (2016) 17:656–65. doi: 10.1038/ni.3421
122. Huang T, Hazen M, Shang Y, Zhou M, Wu X, Yan D, et al. Depletion of major pathogenic cells in asthma by targeting CRTh2. *JCI Insight*. (2016) 1:e86689. doi: 10.1172/jci.insight.86689
123. Singh D, Ravi A, Southworth T. CRTH2 antagonists in asthma: current perspectives. *Clin Pharmacol*. (2017) 9:165–73. doi: 10.2147/CPAA.S119295
124. Hall IP, Fowler AV, Gupta A, Tetzlaff K, Nivens MC, Sarno M, et al. Efficacy of BI 671800, an oral CRTH2 antagonist, in poorly controlled asthma as sole controller and in the presence of inhaled corticosteroid treatment. *Pulm Pharmacol Ther*. (2015) 32:37–44. doi: 10.1016/j.pupt.2015.03.003
125. Kuna P, Bjermer L, Tornling G. Two Phase II randomized trials on the CRTh2 antagonist AZD1981 in adults with asthma. *Drug Des Devel Ther*. (2016) 10:2759–70. doi: 10.2147/DDDT.S105142
126. Gonem S, Berair R, Singapuri A, Hartley R, Laurencin MFM, Bacher G, et al. Fevipiprant, a prostaglandin D 2 receptor 2 antagonist, in patients with persistent eosinophilic asthma: a single-centre, randomised, double-blind, parallel-group, placebo-controlled trial. *Lancet Respir Med*. (2016) 4:699–707. doi: 10.1016/s2213-2600(16)30179-5
127. Pettipher R, Hunter MG, Perkins CM, Collins LP, Lewis T, Baillet M, et al. Heightened response of eosinophilic asthmatic patients to the CRTH2 antagonist OC000459. *Allergy*. (2014) 69:1223–32. doi: 10.1111/all.12451
128. Barnes N, Pavord I, Chuchalin A, Bell J, Hunter M, Lewis T, et al. A randomized, double-blind, placebo-controlled study of the CRTH2 antagonist OC000459 in moderate persistent asthma. *Clin Exp Allergy*. (2012) 42:38–48. doi: 10.1111/j.1365-2222.2011.03813.x
129. Rabe KF, Nair P, Brusselle G, Maspero JF, Castro M, Sher L, et al. Efficacy and safety of dupilumab in glucocorticoid-dependent severe asthma. *N Engl J Med*. (2018) 378:2475–85. doi: 10.1056/NEJMoa1804093
130. Castro M, Corren J, Pavord ID, Maspero J, Wenzel S, Rabe KF, et al. Dupilumab efficacy and safety in moderate-to-severe uncontrolled asthma. *N Engl J Med*. (2018) 378:2486–96. doi: 10.1056/NEJMoa1804092
131. Bleecker ER, FitzGerald JM, Chanez P, Papi A, Weinstein SF, Barker P, et al. Efficacy and safety of benralizumab for patients with severe asthma uncontrolled with high-dosage inhaled corticosteroids and long-acting beta2-agonists (SIROCCO): a randomised, multicentre, placebo-controlled phase 3 trial. *Lancet*. (2016) 388:2115–27. doi: 10.1016/S0140-6736(16)31324-1
132. Castro M, Bacharier LB. Treatment for severe eosinophilic asthma-consistent effect of anti-interleukin-5 antibodies? *Lancet*. (2016) 388:2059–60. doi: 10.1016/S0140-6736(16)31537-9
133. FitzGerald JM, Bleecker ER, Nair P, Korn S, Ohta K, Lommatzsch M, et al. Benralizumab, an anti-interleukin-5 receptor alpha monoclonal antibody, as add-on treatment for patients with severe, uncontrolled, eosinophilic asthma (CALIMA): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet*. (2016) 388:2128–41. doi: 10.1016/S0140-6736(16)31322-8
134. Hanania NA, Korenblat P, Chapman KR, Bateman ED, Kopecky P, Paggiaro P, et al. Efficacy and safety of lebrikizumab in patients with uncontrolled asthma (LAVOLTA I and LAVOLTA II): replicate, phase 3, randomised, double-blind, placebo-controlled trials. *Lancet Respir Med*. (2016) 4:781–96. doi: 10.1016/S2213-2600(16)30265-X
135. Piper E, Brightling C, Niven R, Oh C, Faggioni R, Poon K, et al. A phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma. *Eur Respir J. (*2013) 41:330–8. doi: 10.1183/09031936.00223411
136. Lanier B, Bridges T, Kulus M, Taylor AF, Berhane I, Vidaurre CF. Omalizumab for the treatment of exacerbations in children with inadequately controlled allergic (IgE-mediated) asthma. *J Allergy Clin Immunol*. (2009) 124:1210–6. doi: 10.1016/j.jaci.2009.09.021
137. Berger W, Gupta N, McAlary M, Fowler-Taylor A. Evaluation of long-term safety of the anti-IgE antibody, omalizumab, in children

with allergic asthma. *Ann Allergy Asthma Immunol*. (2003) 91:182–8. doi: 10.1016/S1081-1206(10)62175-8

138. Corren J, Parnes JR, Wang L, Mo M, Roseti SL, Griffiths JM, et al. Tezepelumab in adults with uncontrolled asthma. *N Engl J Med*. (2017) 377:936–46. doi: 10.1056/NEJMoa1704064
139. Gauvreau GM, O'Byrne PM, Boulet LP, Wang Y, Cockcroft D, Bigler J et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *N Engl J Med*. (2014) 370:2102–10. doi: 10.1056/NEJMoa 1402895
140. Cardoso V, Chesne J, Ribeiro H, Garcia-Cassani B, Carvalho T, Bouchery T, et al. Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature*. (2017) 549:277–81. doi: 10.1038/nature23469
141. Klose CSN, Mahlakoiv T, Moeller JB, Rankin LC, Flamar AL, Kabata H, et al. The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation. *Nature*. (2017) 549:282–86. doi: 10.1038/nature23676
142. Barnes PJ. Neuropeptides and asthma. *Am Rev Respir Dis*. (1991) 143 (3 Pt 2):S28–32. doi: 10.1164/ajrccm/143.3_Pt_2.S28
143. Linden A, Hansson L, Andersson A, Palmqvist M, Arvidsson P, Lofdahl CG, et al. Bronchodilation by an inhaled VPAC(2) receptor agonist in patients with stable asthma. *Thorax*. (2003) 58:217–21. doi: 10.1136/thorax.58.3.217
144. Springer J, Geppetti P, Fischer A, Groneberg DA. Calcitonin gene-related peptide as inflammatory mediator. *Pulmonary Pharmacol Therapeut*. (2003) 16:121–30. doi: 10.1016/s1094-5539(03)00049-x
145. Sui P, Wiesner DL, Xu J, Zhang Y, Lee J, Van Dyken S, et al. Pulmonary neuroendocrine cells amplify allergic asthma responses. *Science*. (2018) 360:eaan8546. doi: 10.1126/science.aan8546
146. Krug N, Hohlfeld JM, Kirsten AM, Kornmann O, Beeh KM, Kappeler D, et al. Allergen-induced asthmatic responses modified by a GATA3-specific DNAzyme. *N Engl J Med*. (2015) 372:1987–95. doi: 10.1056/NEJMoa1411776

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: 11 July 2019
doi: 10.3389/fimmu.2019.01597

The Influence of Innate Lymphoid Cells and Unconventional T Cells in Chronic Inflammatory Lung Disease

Jessica G. Borger[1]*, *Maverick Lau*[1,2†] and *Margaret L. Hibbs*[1†]

[1] Department of Immunology and Pathology, Central Clinical School, Monash University, Melbourne, VIC, Australia,
[2] Department of Pharmacology and Therapeutics, Lung Health Research Centre, University of Melbourne, Melbourne, VIC, Australia

The lungs are continuously subjected to environmental insults making them susceptible to infection and injury. They are protected by the respiratory epithelium, which not only serves as a physical barrier but also a reactive one that can release cytokines, chemokines, and other defense proteins in response to danger signals, and can undergo conversion to protective mucus-producing goblet cells. The lungs are also guarded by a complex network of highly specialized immune cells and their mediators to support tissue homeostasis and resolve integrity deviation. This review focuses on specialized innate-like lymphocytes present in the lung that act as key sensors of lung insults and direct the pulmonary immune response. Included amongst these tissue-resident lymphocytes are innate lymphoid cells (ILCs), which are classified into five distinct subsets (natural killer, ILC1, ILC2, ILC3, lymphoid tissue-inducer cells), and unconventional T cells including natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, and γδ-T cells. While ILCs and unconventional T cells together comprise only a small proportion of the total immune cells in the lung, they have been found to promote lung homeostasis and are emerging as contributors to a variety of chronic lung diseases including pulmonary fibrosis, allergic airway inflammation, and chronic obstructive pulmonary disease (COPD). A particularly intriguing trait of ILCs that has recently emerged is their plasticity and ability to alter their gene expression profiles and adapt their function in response to environmental cues. The malleable nature of these cells may aid in rapid responses to pathogen but may also have downstream pathological consequences. The role of ILC2s in Th2 allergic airway responses is becoming apparent but the contribution of other ILCs and unconventional T cells during chronic lung inflammation is poorly described. This review presents an overview of our current understanding of the involvement of ILCs and unconventional T cells in chronic pulmonary diseases.

Keywords: ILC, MAIT, NK cell, NKT cells, γδ-T cell, lung, chronic lung disease

OPEN ACCESS

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

Reviewed by:
Claudia U. Duerr, McGill University, Canada
Hirohito Kita, Mayo Clinic, United States

***Correspondence:**
Jessica G. Borger
jessica.borger@monash.edu
orcid.org/0000-0003-0704-1254

†Maverick Lau
orcid.org/0000-0002-4775-2600
Margaret L. Hibbs
orcid.org/0000-0002-3751-6751

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

Received: *15 March 2019*
Accepted: *26 June 2019*
Published: *11 July 2019*

Citation:
Borger JG, Lau M and Hibbs ML (2019) The Influence of Innate Lymphoid Cells and Unconventional T Cells in Chronic Inflammatory Lung Disease. Front. Immunol. 10:1597. doi: 10.3389/fimmu.2019.01597

INTRODUCTION

The lungs are constantly exposed to particulates from the environment. This inhaled matter includes harmless aeroallergens, airborne pathogens which can cause infection, and noxious agents including dust, smoke, and other environmental pollutants that can induce lung tissue damage. The conducting airways of the respiratory system are comprised of ciliated epithelium interspersed

with mucus-producing goblet cells. This protects against particulates and infectious agents, which adhere to the mucus, and through the actions of the cilia, are cleared from the airways. This barrier is also supported by the presence of tissue-resident immune cells that provide cellular and humoral host defense. The resting lung harbors a plethora of leukocytes including B and T cells and myeloid cells (alveolar macrophages, interstitial macrophages, monocytes, dendritic cell subsets, neutrophils, eosinophils, mast cells), with their numbers and proportions changing dramatically during infection and inflammation. In response to tissue damage, stress or infection, the respiratory epithelium secretes proteins important in inflammation and host defense such as surfactant, anti-microbial peptides (β-defensins, cathelicidins), danger signals such as alarmins, chemokines, and cytokines to recruit and activate immune cells. While this normally results in the clearance of the pathogenic agent, in certain situations the inflammatory response becomes chronic leading to progressive lung tissue damage. Defining the cellular and molecular pathways that are altered during chronic disease transition may reveal new lung disease targets for therapy.

Recent studies have revealed the presence of small populations of other distinct immune cells in the lung including innate lymphoid cells (ILCs) and unconventional T lymphocytes (CD1-restricted NKT, MAIT cells, and γδ-T cells) and described their contribution to lung homeostasis, however these innate-like cells are now also emerging as pathogenic mediators in lung disease. ILCs show transcriptional and functional parallels to the conventional polarized T helper cell subsets (Th1, Th2, Th17), with the critical difference that ILCs lack clonally distributed antigen-specific receptors, responding instead to danger and stress signals derived from mucosal epithelium, stroma, and myeloid-lineage cells. In contrast, γδ-T cells, MAIT cells, and NKT cells possess semi-variant antigen-specific receptors but likewise respond to similar mediators, although these are more restricted dependent on cell type, and in some cases, are still poorly defined. Here we will summarize how these relatively new players participate in immune-regulation and homeostasis in the lung, and elaborate on how they can become dysregulated and contribute to chronic lung diseases.

ILCs IN LUNG HOMEOSTASIS

The ILC family is comprised of several phenotypically distinct subsets that are derived from a common lymphoid precursor, which unlike conventional T lymphocytes, do not express antigen-specific receptors nor mediate antigen-specific immune responses (1). ILCs are classified into five distinct subsets based on their development, transcription factor expression and effector function (2). Conventional NK cells, which are dedicated cytotoxic effectors that kill virus-infected cells and tumor cells and require the transcription factor T-bet for their function, are now regarded as a distinct ILC subset. A second subset, lymphoid tissue-inducer (LTi) cells, are responsible for secondary lymphoid organogenesis, while the remaining three subsets of ILCs (ILC1, ILC2, ILC3) play a role akin to helper T cells. Herein, we will use the abbreviation ILCs to refer to the ILC1, 2, and 3 subsets. Much like Th1, Th2, and Th17 cells, ILC1 express T-bet (encoding for their cytotoxic potential), ILC2 express GATA-3 while $CD4^-$ ILC3 and $CD4^+$ LTi cells express retinoic acid-related orphan receptor (ROR)γt (**Figure 1**). Although distributed in both lymphoid and non-lymphoid organs throughout the body, ILCs populate barrier surfaces, notably the skin, lung, and other mucosal sites, in much greater numbers.

Under homeostatic conditions, the immune cell make-up of the mouse lung is predominantly conventional lymphocytes and myeloid cells, although NK cells and all three ILC subsets have also been identified in the airways, albeit at much lower frequencies (3, 4). The ILC2 population is thought to be tissue-resident and thus are the predominant helper-type ILCs in the steady-state mouse lung (3, 5). Indeed, in a parabiotic experiment, ILC2s found in the lung were shown to be of host origin and strikingly, very few ILC2s were found in the circulation with the vast majority residing within the lung parenchyma (6). Furthermore, during homeostatic conditions, self-renewal of ILC2s maintained the tissue-resident population (6). Even during acute infection with the helminth *Nippostrongylus brasiliensis*, the ILC2 population in the lung remained host-derived and it was not until the expulsion of the larvae, which is associated with inflammation and tissue repair, that an increase in donor-derived ILC2s was observed (6). Similarly, in another parabiosis study, after 1 month of shared circulation, T cells and eosinophils were present from both host and donor, while ILC2s of the parabiotic donor could not be detected, even 1 week after intra-tracheal IL-33 challenge (7).

Unconventional T cells are similarly thought to be tissue-resident and arise from local expansion. Indeed, γδ-T cells, with a restricted Vγ4 or Vγ6 T cell receptor (TCR), mature in the thymus as $CD44^+CD27^-$ with a defined effector phenotype of IL-17 production (γδ-T17 cells) and specifically populate lung tissue during fetal development (8). CCR6/CCR2 co-expression identifies tissue-residency of γδ-T cells including γδ-T17 cells, but a recent study has identified populations of γδ-T17 cells lacking CCR6 expression in lung tissue (9) suggesting that under specific conditions, other γδ-T cell subsets are able to migrate and populate the lungs. Evidence now suggests that inflammatory ILC2s from the intestine are also able to migrate to the lung under specific conditions (10, 11). As both the lung and gut associated tissues exhibit tonic immune activation due to interactions between the mucosa and microbiota, further evidence is required to understand the tissue and context-specific regulation of ILC and unconventional T cell responses.

Maintenance of the epithelial barrier of the respiratory tract is critical to limit exposure to pathological and immunological stimuli and ILC2s are thought to have a key role in this process. ILC2 mediators including IL-4, IL-5, IL-9, IL-13, and the epidermal growth factor-like molecule amphiregulin are critical for maintaining airway barrier integrity and tissue homeostasis. In the lung, ILCs have been shown to interact with local epithelial cells and myeloid cells. ILC2 depletion impaired airway epithelial barrier integrity following influenza virus infection through a failure to generate hyper-plastic epithelial cells, an epithelial repair response, causing epithelial cells to undergo necrosis leading to deterioration of the epithelial lining (5). To modulate

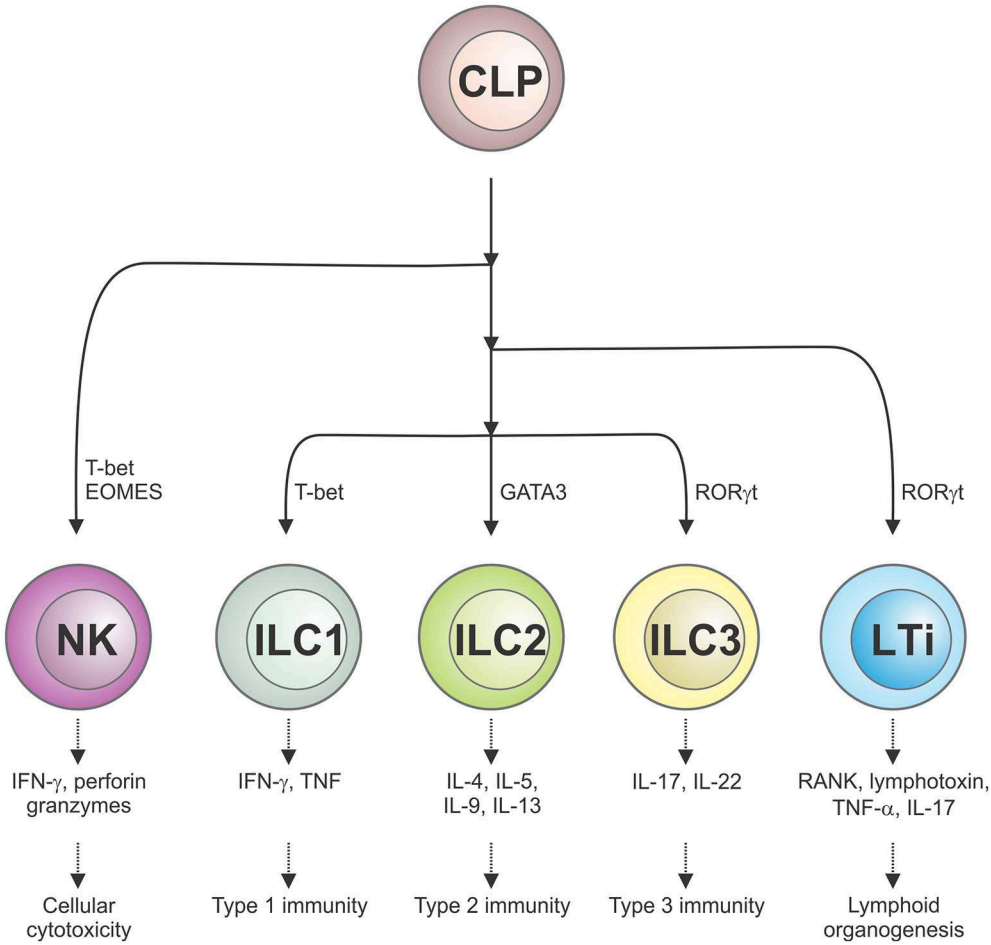

FIGURE 1 | Schematic representation of the five different ILC subsets and their roles in cellular immunity. ILCs, which are derived from a common lymphoid progenitor (CLP), are classified into five distinct subsets on the basis of their transcription factor usage and unique spectrum of cytokines that they produce. This confers their distinct roles in immune responses and lymphoid organ development.

epithelial cell responses in the airways, ILC2s may use autocrine IL-9 to promote IL-5 and IL-13 production. Exposure to chitin, which is associated with allergic responses, was shown to drive IL-33 and thymic stromal lymphopoietin (TSLP) from resident alveolar type II cells, inducing the IRF4-IL-9 module in ILC2s, highlighting a critical interaction between resident ILCs and structural cells (12). Moreover, IL-5 and IL-13 have been shown to promote mucus hyper-production and tissue repair in type 2 immune responses such as asthma and helminth infection [reviewed in (13)]. Interestingly in the human lung under homeostatic conditions, it was reported that ILC3s are the most abundant ILC population in healthy older individuals, although in this study the confounding effects of smoking (two out of five subjects sampled) and elevated BMI necessitates further investigation (14). In the lung, IL-22 is mainly produced by ILC3, in addition to γδ-T cells, and Th17 cells, and has also been shown to be involved in the maintenance of epithelial barrier function, mucus production and tissue repair [reviewed in (15)]. Thus, ILCs contribute to barrier surveillance and epithelial protection and repair through coordinated interactions with other cells in the lung.

Epithelial cell-derived mediators are critical in the regulation of ILC responses. Epithelial cell or myeloid cell derived IL-25, IL-33, and TSLP can promote an ILC2 response after allergen challenge, helminth exposure or influenza infection. IL-33, an alarmin that is highly expressed in airway epithelial cells, has been shown to be overexpressed in lungs of patients with pulmonary fibrosis, asthma, and COPD and has been shown to be a main driver of ILC2 expansion (16–18). In mice following challenge with influenza virus, release of IL-33 corresponded with elevated IL-5 production by ILC2s and the recruitment of eosinophils to the lung during the recovery phase of influenza infection (19).

Intriguingly, IL-17 production from γδ-T cells was shown during neonatal influenza to augment IL-33 production from the mucosa, generating a type 2 response and the production of amphiregulin by ILC2s (20). The authors found a similar correlation of IL-17, IL-33, and amphiregulin in the nasal wash of human infants infected with influenza, identifying an axis of

IL-17 production from γδ-T cells in the production of IL-33 for ILC2 repair responses in the lung (20).

ILC2 cells also express the receptor for TSLP, and together with IL-25 and IL-33, TSLP has been shown to induce ILC2s following rhinovirus and respiratory syncytial virus infection (21–23). A suggestive association with a SNP in the TSLP gene and severe asthma has been identified in genome-wide analysis of severe asthmatics in one study (24), and TSLP has been shown in a papain-model of asthma to illicit a type 2 response for which ILC2s were the dominant source of IL-13 and IL-5 (25). Similarly, TSLP has been shown to play an important role in iNKT cell-dependent asthma, enhancing AHR expression by increasing iNKT cell production of IL-13 (26). TGF-β, also secreted by lung epithelial cells, has been shown to skew ILC responses causing the upregulation of the TGF-β receptor (TGF-βRII) and enhancement of ILC2 activity, demonstrating that ILCs take direct cues from the lung mucosa and epithelium (27). This would suggest that the particular location of ILCs within the lung and the involvement of unconventional T cells is responsible for directing the nature and magnitude of the specific immune response.

ILCs themselves have been reported to antagonize each other, with IL-27 and interferon-γ (IFN-γ) released by ILC1s shown to inhibit ILC2s and type 2 responses in the lung (28). In addition and more fully discussed below, ILCs are highly plastic, being able to readily change phenotype and function depending on their microenvironment. The contribution of other innate-like cells such as γδ-T cells, MAIT cells, and NKT cells in directing ILC traits and functionality in the lung may be of great importance in understanding the role of ILCs in chronic lung disease, in particular when compounded by viral, bacterial, and other disease exacerbations.

PLASTICITY OF ILCs DURING INFLAMMATION

Cellular plasticity is the ability of a differentiated effector cell to adapt their function in response to altered situations and is a particular feature of $CD4^+$ T cells (29). Emerging evidence indicates that ILC subsets also demonstrate considerable plasticity in response to the inflammatory milieu as well as viral and bacterial challenge and tissue site residency. This is potentially due to the promiscuous co-expression of multiple transcription factors driving differential cytokine expression as well as an ability to alter cell surface marker expression in the face of a changing environment.

ILC1 AND NK CELL PLASTICITY

Historically, NK cells were categorized as cytolytic ILC1s but more recently have been identified as one of the five bona fide ILC populations (2). At steady state, ILC1 and NK cells can be identified by differential expression of TRAIL, CD49a, and CXCR6 and the transcription factors Eomes and promyelocytic leukemia zinc finger protein (PLZF), however during inflammation these surface markers and transcription factors can become altered which impedes the ability to track bona fide ILC1s. The cytokine milieu is largely responsible for diverting ILC identity as has been shown for ILC2s and ILC3s, which are capable of expressing T-bet and other T-bet related gene signatures as well as secreting IFN-γ. Although NK cells in the bone marrow are critically reliant on T-bet, mature cell fate is regulated by additional expression of Eomes. Indeed, deletion of Eomes at the onset of ILC1 maturation substantially blocked cytolytic NK cell development, shifting the balance toward helper-like ILC1s (30). TGF-β has been shown to suppress Eomes in a tissue-specific population of ILC1s within the salivary glands, with genetic abrogation of TGF-βRII in $NKp46^+$ cells significantly reducing ILC1 numbers, expression of CD49a, CD103, CD69a, TRAIL, and CD73 (31). Similarly, NK cells can convert into cells that resemble ILC1s due to the presence of TGF-β in a tumor microenvironment (32, 33). These ILC1s were devoid of cytotoxic activity and expressed Hobit and TIGIT, transcription factors known to suppress NK cell function.

In humans, $CD127^+$ ILC1 have been shown to differentiate into ILC3-like cells in the presence of $CD103^+$ dendritic cells secreting IL-2, IL-23, and IL-1β, which was enhanced in the presence of retinoic acid and dependent on the transcription factor RORγt; such reprogramming was fully reversible in the presence of IL-12 and IL-18 (34). Studies into the plasticity of ILC1s have been confounded since an extensive mass cytometry study of healthy and inflamed tissues revealed that whereas human ILC2 and ILC3 populations could be phenotypically delineated into separate clusters, ILC1s were identified throughout other cell populations (35, 36). This was compounded by the fact that ILC1 inherently lack a specific lineage marker (35, 36). ILC1s are particularly prominent during inflammatory conditions but whether they are true ILC1 or arise predominantly from ILC3 under the influence of IL-12 or ILC2 exposed to IL-1β still remains to be fully resolved.

ILC2 PLASTICITY

ILC2s are in general defined as lineage negative cells, which are positive for Thy1, Sca-1, GATA-3, ST2 (IL-33 receptor), ICOS, CD44, CD25, CD127, KLRG1, and c-kit low to positive (37). ILC2 cells display great heterogeneity, with single-cell sequencing of intestinal ILC2s at steady state identifying several subgroups displaying differential gene expression patterns that differed to lung ILC2s (38, 39). Whereas, natural ILC2s produce a type-2 cytokine profile, plastic inflammatory ILC2s can coproduce both type 2 and the ILC3-characteristic cytokine IL-17. Interestingly, inflammatory $KLRG1^{hi}$ ILC2s, which migrated to the lungs upon IL-25 challenge or following infection with the hookworm *Nippostrongylus brasiliensis* or the fungus *Candida albicans*, expressed both GATA3 and RORγt and could produce IL-17 as well as IL-13 *in vitro* (10). Notch signaling has been shown to induce *RORC* expression and drive IL-13/IL-17 co-producing ILC2 cells during house dust mite induced airway inflammation in mice (40). ILC2s from healthy human donors also express low amounts of RORγt and can co-produce IL-13 and IL-22 demonstrating that key functions of ILC2 and ILC3 subsets can

co-exist in one cell but appear to be exquisitely balanced by the inflammatory milieu.

Human ILC2s activated by IL-1β have been shown to convert into IFN-γ producing ILC1s by induction of low levels of T-bet and IL-12RβII expression (41, 42). IL-12 stimulation appears to act as a rheostat in directing the ILC1 or ILC2 response, although IL-12 alone is not enough to induce this functional plasticity, a process that can be reversed by exposure to IL-4 (41, 42). In patients with severe COPD, there was elevated IL-12 and an accumulation of IFN-γ^+ ILC2s (43). ILC2 were also shown to upregulate T-bet expression and acquire an ILC1 phenotype in intestinal samples from Crohn's disease patients (44). Even in healthy human donors, a small subset of ILC2 cells have the capacity to co-produce IL-13, IFN-γ, and IL-22 (45, 46).

ILC3 AND LTI PLASTICITY

Previously, LTi cells were categorized as a subset of ILC3s, although more recent studies have resolved that they are separate populations. Plasticity between the two populations, with the identification of LTi-like ILC3s and the lack of NCR expression on LTi cells that is confounded by its heterogeneous expression on ILC3s, supports the collective discussion of their plasticity. RORγt$^+$ ILC3 cells have been shown to co-express T-bet, produce IFN-γ and differentiate into ILC1 cells in response to inflammation. Purified NKp44$^+$ ILC3s from the murine fetal intestine when cultured with IL-2, IL-23, and IL-1β differentiate into ILC3, however when exposed to IL-2 and IL-12 they acquire the ILC1 phenotype, losing expression of NKp44 and c-kit (47). The switch also appears bi-directional, as IL-2 and IL-23 stimulation of these ILC1 cells, although maintaining *RORC* expression, caused a significant reduction in the Th1-specific transcription factor T-bet encoded by *TBX21* (47). Human natural killer 22 (NK-22) cells that express IL-22, now defined as ILC3 cells by current nomenclature, have demonstrated similar loss of IL-22 production and acquisition of IFN-γ expression (48–50). Culturing of tonsillar NK-22 cells, in the presence of IL-2 considerably modified the NK-22 cell cytokine profiles, with IL-2 promoting IFN-γ secretion and reducing secretion of IL-17 and IL-22 (49). One mechanism driving ILC3 plasticity derives from the levels and availability of the transcription factor T-bet, a critical mediator in lineage commitment of CCR6$^-$ RORγt$^+$ ILCs. A distinct subset of IL-22 producing ILC3s, which also express NKp46, reside in the gut and develop through T-bet regulation (12, 51). Mice exhibiting loss of T-bet expression through genetic ablation developed CCR6$^-$ RORγt$^+$ ILC3s but failed to develop NKp46-expressing RORγt$^+$ ILCs (NK-22 cells) and could not produce IFN-γ (51).

Environmental cues from commensal microbiota have been shown to be critical in upregulating T-bet expression. Indeed, specific pathogen free (SPF) mice were shown to have greater numbers of T-bet$^+$ NKp46$^-$ RORγt$^+$ ILC numbers compared to germ-free mice, with a corresponding decrease in NKp46$^-$CCR6$^-$T-bet$^-$RORγt$^+$ ILCs (51). Although studies in the lung during either homeostatic or inflammatory conditions remain to be investigated, gut microbiota has been shown to stabilize RORγt expression in LTi cells in mice treated with antibiotics, with IL-12 and IL-15 identified as the main drivers of RORγt loss (52). Similar to the lung, in the intestine, the process of transdifferentiation appears to be a requirement of ILC function during inflammation. Indeed the production of IFN-γ by T-bet-expressing CCR6$^-$ RORγt$^+$ ILCs was shown to be essential for the release of mucus-forming glycoproteins to protect the epithelial barrier during *Salmonella enterica* infection in the intestine and in Crohn's disease patients which display higher frequencies of ILC1s (34, 51). Although loss of RORγt from LTi cells and IFN-γ release leads to potent induction of colitis (52). These results reveal how intestinal microbiota can impact upon transcription factor gradients and act as rheostats for ILC functional programming, identifying how critical the tissue microenvironment is for shaping the immune response. These studies raise the exciting possibility that the lung microbiota may similarly affect ILC transdifferentiation in lung homeostasis and inflammation, which now needs investigation in the chronic disease setting, especially to further our understanding of the pathology underlying exacerbations.

ILCs IN CHRONIC LUNG DISEASE

ILCs only represent a small fraction of the total immune cells in the lung, however it is clear that they play key roles in protection of the pulmonary system against a diverse array of microbes. While critical for respiratory immunity, there is now increasing evidence that these cells are implicated in chronic lung diseases and may represent disease biomarkers or targets for therapeutic intervention. The following sections will describe what is currently known about the role of these cells in chronic lung diseases.

Asthma

Asthma is a chronic disease of the airways characterized by airway hyper-reactivity, bronchoconstriction and mucus over-production, and is classically associated with type 2 inflammation with Th2 cells thought to be the predominant source of these cytokines. Asthma is a highly heterogeneous disease with numerous endotypes including allergic and non-allergic disease (53) and it can be stimulated by many different triggers including airborne allergens and irritants, respiratory infection, cold air, and exercise. It is now appreciated that the disorder involves more than the adaptive arm of the immune system, with ILC2s, which are known to produce type 2 cytokines, being highly implicated as key players in asthmatic lung inflammation. Several recent reviews have highlighted the roles of ILCs in allergic asthma (13, 54); thus we will only touch on this briefly in this review, and instead focus on the contribution of ILCs in human studies of chronic asthma.

Several studies have identified a critical role for ILC2s in allergic asthma. ILC2 express IL17RB (IL25R) and ST2 (IL33R) and respond to intranasal administration of IL-25 and IL-33 producing IL-13 and IL-5, which contribute to the development of airway hyper-reactivity, eosinophilia, and airway inflammation (55–58). Both IL-25 and IL-33 are involved in type 2 immunity and are produced by airway epithelial cells. Allergen challenge

greatly elevated IL-33 production, eliciting IL-13-expressing ILC2s in the lung and airways to induce airway hyper-reactivity (59). IL-25 or IL-33 challenge can induce the activation and accumulation of ILC2s within the lung-draining mediastinal lymph nodes, although in lung tissue, IL-33 administration induced a more sustained response. Indeed, only IL-33 could exert a direct chemotactic effect on ILC2s, which was mediated through the activation of ERK1/2, p38, Akt, JNK, and NF-κB (60), although IL-25 also stimulates several other cell types including Th2 cells and iNKT cells (61) which together would further amplify lung inflammation. In studies investigating the steroid responsiveness of ILC2s in allergen-challenged mice, reduced numbers, and increased cell death following dexamethasone treatment was observed suggesting that they are sensitive to glucocorticosteroids in eosinophilic asthma (62).

Most studies on the role of ILCs in asthma utilize experimental models that are challenged by short-term exposure to allergens, therefore the role of ILC2s in chronic asthma remains largely unknown. Although, in line with the discovery of a pathogenic role of ILC2s in experimental asthma, ILC2s are also elevated in number and activation status in patients with chronic asthma. ILC2s accumulate in the lung tissue, airway mucosa and the sputum of asthmatic patients and the numbers are further increased when challenged with allergen (63, 64). Indeed, PBMCs isolated and exposed to IL-25 and IL-33 produced a greater amount of IL-5 and IL-13 than healthy controls (65). Increased numbers of ILC2s in asthmatics correlate with eosinophilia and asthma severity, suggesting a link to disease pathogenesis (65, 66). A transcriptomic study recently revealed a link between expression profiles of ILC2s and allergic asthma susceptibility genes in mice and humans including RORA, SMAD3, GATA3, IL13, IL18R1, and IL1RL1, suggesting a role for ILC2 in regulating susceptibility gene expression (67).

The role for ILC3s still requires further investigation, but IL-17$^+$ILC3s were found to be elevated within the BAL of asthma patients compared to healthy subjects (68). In line with this finding, genetic profiling of patients with adult-onset asthma, which is often more severe and associated with a poorer prognosis than childhood-onset asthma, revealed that ILC3 gene signatures, along with pathways involving eosinophilia and mast cells, were highly enriched in nasal brushings, sputum, and endobronchial brushings (69). IL-22 production is elevated in allergen-challenged mice and associated with airway hyper-responsiveness (70), and increased IL-22 expression has also been detected in the serum of asthma patients (71). A single study has identified Lin$^-$CD90$^+$Sca-1$^+$ILCs to be the producers of IL-22 but further studies are required to determine if ILC3s also contribute to its production (72). Overall, ILC2s are likely to play a key role in the initiation and propagation of type 2 responses in the lung which may involve crosstalk with conventional T cells. Further evidence has demonstrated crosstalk between commensal bacteria, intestinal mucosal dendritic cells and IL-22-producing ILC3s in establishing the pulmonary immune system of newborn mice and promoting their resistance to pulmonary infections and suggesting that they may play a protective role, preventing the development of lung disorders such as asthma (73). It seems likely that a more intricate cellular network involving IL-17 and IL-22 producing ILC3s and γδ-T cells as well as NKT cells may also exist in the asthmatic lung, as these cells are known to induce airway hyperresponsiveness in the absence of adaptive immunity.

Chronic Rhinosinusitis

Chronic rhinosinusitis is a persistent inflammatory disease of the nasal passages and sinuses that arises through an abnormal host response to environmental stimuli at the nasal and sinus mucosa and in its most severe form, is associated with the development of nasal polyps. Since cytokines such as IL-25, IL-33, and TSLP and their receptors are involved in the disease (74), by inference, this implicates ILCs in its pathogenesis. While studies in this area are limited, an increased proportion of ST2$^+$ ILCs have been observed in the sinonasal mucosa from patients presenting with chronic rhinosinusitis and nasal polyps (75). ST2$^+$ ILCs, now identified as ILC2s (76), have been shown to be increased in number and correlate with worsening nasal symptoms (77). A recent study found that ILC2s are not only increased in number, but they also exhibit an activated phenotype in chronic rhinosinusitis patients (78), suggesting that ILCs play a significant role in disease manifestation.

COPD

COPD is a chronic inflammatory lung disease that is characterized by airflow limitation and triggered by an exaggerated inflammatory response to noxious stimuli such as cigarette smoke. ILCs can drive disease in the lung through accumulation and/or an alteration in their subset composition. In COPD patients, IL-12 signatures and the accumulation of ILC1s are elevated. IL-12 induces the conversion of ILC2s into IFN-γ-producing ILC1s thus contributing to the type 1 inflammation associated with COPD (43). The increase in ratio of ILC1:ILC2 has also been shown to correlate with lung function decline and increased disease severity (79). Gene expression profiling studies on a small subset of samples from patients with centrilobular emphysema showed that genes expressed by NK, LTi, and ILC1 cells were enriched in the inflammatory cell infiltrate, suggesting that emphysematous destruction is driven by a Th1-type response (80).

Recent studies on experimental mouse models have provided the first evidence that ILC2s may participate in COPD pathogenesis. Following cigarette smoke exposure, ILC2-deficient mice (*Rora*$^{fl/fl}$*Il7r*^{Cre}) developed similar levels of airway inflammation to wild type mice although the loss of ILC2s appeared to protect from cigarette smoke-induced emphysema (81). However, ILC2-deficient mice had increased IL-33 and IL-13 expression and substantial collagen deposition identifying a role for ILC2s in airway fibrosis and lung remodeling processes (81).

The release of the major stimulators of ILC2s, TSLP, IL-25, and IL-33, as a result of epithelial cell injury in both asthma and COPD, provide evidence of ILC2s in airway remodeling. IL-33 expression increases in basal epithelial progenitor cells in patients with COPD, and has been linked to increased IL-13 and mucin gene 5AC expression (17), suggesting a role for

ILC2s in the airway disease components of COPD such as mucus hypersecretion, airway hyper-responsiveness and fibrosis.

When considering cell-intrinsic mechanisms regulating ILC2 function, the enzyme arginase-1 (Arg1), which is considered a classic alternatively activated macrophage marker, was recently identified as a critical mediator in the control of a metabolic program within the ILC2 subset in the lung and the development of type 2 inflammation. Arg1 is known to promote collagen synthesis and fibrosis to support wound healing in the lung. In lung tissue from patients with COPD and idiopathic pulmonary fibrosis (IPF), Arg1 was found to be elevated in ILC2s although levels between these two groups did not differ significantly, suggesting Arg1 expression is a general inflammatory signature of these cells (82). Loss of ILC-intrinsic Arg1 activity prevented a robust ILC2 response and reduced the emphysematous phenotype of COPD, identifying a role for Arg1 and its control of ILC2 responses in chronic lung inflammation (82).

A role for ILC3s in COPD has been implicated as IL-17 is a key mediator of neutrophilia in COPD. Indeed, COPD patients have a greater number of IL-17- and IL-22-expressing cells in bronchial biopsies (83) and a recent study identified the presence of both natural cytotoxicity receptor $(NCR)^{+}$ and NCR^{-} ILC3 subsets in the lungs of COPD patients (14). However, a higher frequency of NCR^{-} ILC3s were found compared to healthy controls, as well as elevated IL-17 and IL-22 production by ILC3s. Furthermore, the recent identification of $NRP1^{+}$ as a marker of LTi-like ILC3 cells in the lungs of smokers and COPD patients, suggests these cells may play a key role in the formation of ectopic pulmonary lymphoid aggregates and the promotion of airway angiogenesis (84).

The lung tissue destruction that occurs in COPD highly implicates cytotoxic lymphocytes such as NK cells in this damaging process. While early studies in COPD patients, suggested that NK cells in the circulation were reduced in number and had compromised phagocytic activity (85, 86), further studies showed that there was an increase in the proportion and cytotoxic activity of NK cells in the BAL or induced sputum from smokers with COPD compared to healthy smokers (86, 87). Moreover, NK cells isolated from the lung tissue of patients with severe COPD had increased cytotoxicity which correlated with decreased pulmonary function (88). Mouse studies confirmed these findings, with cigarette smoke exposure increasing the number of NK cells within the lung, which also displayed an activated phenotype (89–91). The upregulation of epithelial-derived IL-33 by cigarette smoke exposure during a viral exacerbation was shown to increase both NK cell recruitment and effector cytokine responses through the upregulation of ST2, although interestingly, ILC2 cells conversely downregulated ST2 expression (92). Ligands for NKG2D activatory receptors on NK cells have been found to be induced on stressed lung epithelium, which may provide a mechanism to promote NK cell activation in lung disease (93), and in support of this, sustained NKG2D activation in a transgenic mouse model was sufficient to cause pulmonary emphysema (94). This finding was reinforced by the demonstration that cigarette smoke induced the sustained expression of NKG2D ligands on mouse epithelium and strengthened by the same observation in patients with COPD (94).

Exacerbations of COPD and Asthma

Asthma and COPD are the most common chronic airway diseases, and exacerbations, which are episodes of acute worsenings of symptoms and airflow obstruction, contribute greatly to disease morbidity and mortality. The major cause of disease exacerbations are viral infections of the respiratory tract, particularly those involving rhinovirus. In respiratory viral infection, ILCs accumulate in lung tissue and play important roles in host defense, tissue integrity and the maintenance of homeostasis in the lung (5). However, they are also implicated in asthma and COPD exacerbations following lung infection (43, 95). It has been well-characterized that ILC2-activating cytokines, IL-25, and IL-33, are released by airway epithelial cells upon viral infection, where they play key roles in disease exacerbations through enhancement of type 2 inflammation. These cytokines induce ILC2 accumulation in the lung, which in turn release IL-5 and IL-13, leading to eosinophil recruitment, mucus production and macrophage polarization (96). BAL eosinophil numbers and IL-5 and IL-13 mRNA expression in lung tissue were greatly reduced when ILCs were depleted by anti-CD90.2 antibody (97). Thus, intervening in the action of ILC2s and their associated type 2 cytokines during a viral infection may be an effective strategy to manage viral exacerbations.

Cigarette smoking, which is one of the major risk factors for COPD, is known to alter the lung immune response to infection (98). Smoke-exposed mice, which show an upregulation of IL-33 expression in the lung epithelium, are completely protected from influenza virus-induced exacerbation when deficient in either IL-33 or its receptor ST2 (92). Prior exposure to smoke was shown to compromise the ability of the lung to mount a Th2 response and subsequent production of IL-13 from ILC2 cells, which led to an exacerbated Th1 proinflammatory response. In support, a study of pulmonary biopsies from patients revealed that elevated IL-33 in lung tissue sections correlated with severity of COPD (92).

Trans-differentiation of ILCs is also linked to lung disease exacerbations. In response to pathogens that trigger COPD exacerbations (influenza A virus, respiratory syncytial virus, *Staphylococcus aureus*, non-typeable *Haemophilus influenzae*), mouse ILC2s substantially lowered their expression of the transcription factor GATA-3, exhibited an increase in expression of T-bet and switched to interferon-γ-producing ILC1s (36, 43).

In mice, IL-12 and IL-18 were involved in the trans-differentiation program which occurred during infections where ILC2s were observed to accumulate and acquire ILC1 effector functions (99). Extending these studies to human samples, IL-12 could also induce the plasticity of ILC2 sorted from peripheral blood, reprogramming them into ILC1 cells (41). Interestingly, the frequency of ILC1 cells in patients with COPD was linked with disease severity and exacerbation susceptibility (43). These studies highlight the unfavorable effect of ILC2 plasticity on anti-viral immunity and its adverse effect on COPD during an exacerbation. In this context, it is critical we now learn how to control the plasticity of lung ILC2s, which may help to manage exacerbations and slow the progression of chronic lung disease.

Pulmonary Fibrosis

Pulmonary fibrosis is a feature of lung diseases such as asthma, COPD, cystic fibrosis, and idiopathic pulmonary fibrosis (IPF). IPF is a progressive, fibrotic disease of the lung with an unknown etiology and an extremely poor prognosis. It is characterized by a dysregulated wound healing response, the over-production of profibrotic factors such as IL-13 and TGF-β and the activation of myofibroblasts that leads to extracellular matrix accumulation. While this is an understudied area, type 2 responses have been implicated in the pulmonary fibrosis phenotype (100) and therefore by association, ILC2 cells may play a significant role. Levels of the cytokines IL-33 and TSLP, which are inducers of ILC2s, have been found to be significantly elevated in the BAL of patients with IPF relative to normal control subjects (18). IL-25 has also been shown to be elevated in the BAL fluid of IPF patients with a corresponding increase in numbers of ILC2 cells compared to healthy subjects (101).

The role of ILC2s in pulmonary fibrosis and airway inflammation is supported in animal models where the production of IL-13 by IL-25-elicited ILC2s was sufficient to drive collagen deposition in the lungs of bleomycin-challenged mice (101). Likewise, intranasal delivery of recombinant IL-25 to mice caused airway inflammation, connective tissue growth factor (CTGF) and TGF-β1 production and subsequent pulmonary fibrosis (102). Similar observations have been made for IL-33, with elevated levels of IL-33 detected in lung tissues during fibrosis or intestinal epithelium of patients with pulmonary fibrosis or fibrotic colitis, respectively, and in the liver of mice with hepatic fibrosis (103). Mechanistically, it has been proposed that IL-33 drives fibrosis by inducing the production of the profibrotic cytokine IL-13 in ILC2s, macrophages, and eosinophils.

NK cell activation may protect against lung fibrosis through the production of IFN-γ. In murine models of bleomycin-induced pulmonary fibrosis, CXCR3 deficiency resulted in loss of NK cell recruitment to the lung and subsequent IFN-γ production resulting in increased pulmonary fibrosis (104). The expression of NKG2D, an activatory receptor on NK cells, was shown to be reduced on NK, NKT, and γδ-T cells isolated from the BAL of patients with IPF, suggesting NK function may be impaired in pulmonary fibrosis (105, 106). Interestingly, the expression of the NKG2D ligand, MICA, is upregulated on fibroblasts and the epithelial cells within the lung of IPF patients suggesting ligand concentrations, in addition to cytokine milieu, may play a role in directing NK cell function and the concerted modulation of NKT and γδ-T cells during progression of chronic lung disease (106).

ILCs, in addition to participating in lung homeostasis, have now been shown to also contribute to a number of lung pathologies. ILCs become dysregulated in chronic lung disorders including asthma, COPD, chronic rhinosinusitis, and pulmonary fibrosis, which in part may be due to the highly heterogeneous nature of ILCs, and their ability to respond to changing local tissue environmental conditions by altering their traits and functional attributes (**Figure 2**). Further studies are now required to fully appreciate how ILCs contribute to the immunopathology of chronic lung disease.

ROLE OF UNCONVENTIONAL T CELLS IN CHRONIC LUNG DISORDERS

In addition to ILCs, other innate-like unconventional αβ- and γδ-T cells have recently emerged as central players in pulmonary immunity. Both MR1-restricted MAIT cells and CD1d-restricted NKT cells express a surface receptor comprised of a semi-variant TCR-α chain complexed with a TCR-β chain of limited repertoire. MAIT cells share some similarities with NKT cells including restriction by non-classical MHC molecules and expression the transcription factor PLZF. Unconventional γδ-T cells differ from conventional T cells due to the expression of a γδ TCR of limited TCR diversity. Unlike conventional T cells that recognize antigens complexed with MHC class I and II molecules, unconventional γδ-T cells recognize lipids, metabolites, and modified peptides presented by MHC class Ib and MHC class-I-like molecules (107). The innate-like phenotype of unconventional T cells is exemplified by their ability to secret cytokines and chemokines without prior antigen exposure upon thymic egress. Similarly, γδ-T cells, which mature and exit the thymus prior to RAG recombination of the γδ TCR, are largely effector-like cells and express a semi-variant TCR. Whereas, MAIT and NKT cells each comprise ∼2% of the T cell population in the lung, γδ-T cells are estimated to represent up to 8–20% of all resident pulmonary lymphocytes (107, 108). Although there is a growing understanding of the role of MAIT, NKT, and γδ-T cells in host protection from pathogens, at present their role and relevance in lung disease is largely unknown.

Unconventional T cells respond to microbial pathogens within the lung. NKT cells become activated in response to microbial CD1d-restricted lipids and upon exposure to inflammatory cytokines such as IL-12 (109, 110). Similarly to NKT cells, which respond predominantly to bacterial challenges, MAIT cells are activated by bacteria and yeasts and produce TNF-α and IFN-γ to control infection (111). γδ-T cells sense cellular stress-induced signals through TCR-dependent and -independent pathways. Activation through the γδ-TCR can occur through non-classical MHC molecules including T10/T22 and CD1 family members as well as butyrophilin 3A1 and viral glycoproteins (112). γδ-T cell ligands are still largely undefined but γδ-T cells have been shown to respond to phospholipids, viral proteins, and stress-induced molecules. γδ-T cells also can respond to pathogen-associated molecular patterns through expression of numerous pattern recognition receptors on their surface as well as NK receptor ligands Rae1 and MICA/B (113). Lung inflammation drives γδ-T cells to secrete IFN-γ and TNF-α and they have also been shown to be a potent source of IL-17. There is an obvious overlap in positioning, ligand recognition, activation, and effector function between the unconventional T cells. Unconventional T cells all express cell surface receptors for the detection of microbial products, which unlike the ILC population within the lung, gives them specificity to directly respond to infectious agents entering the

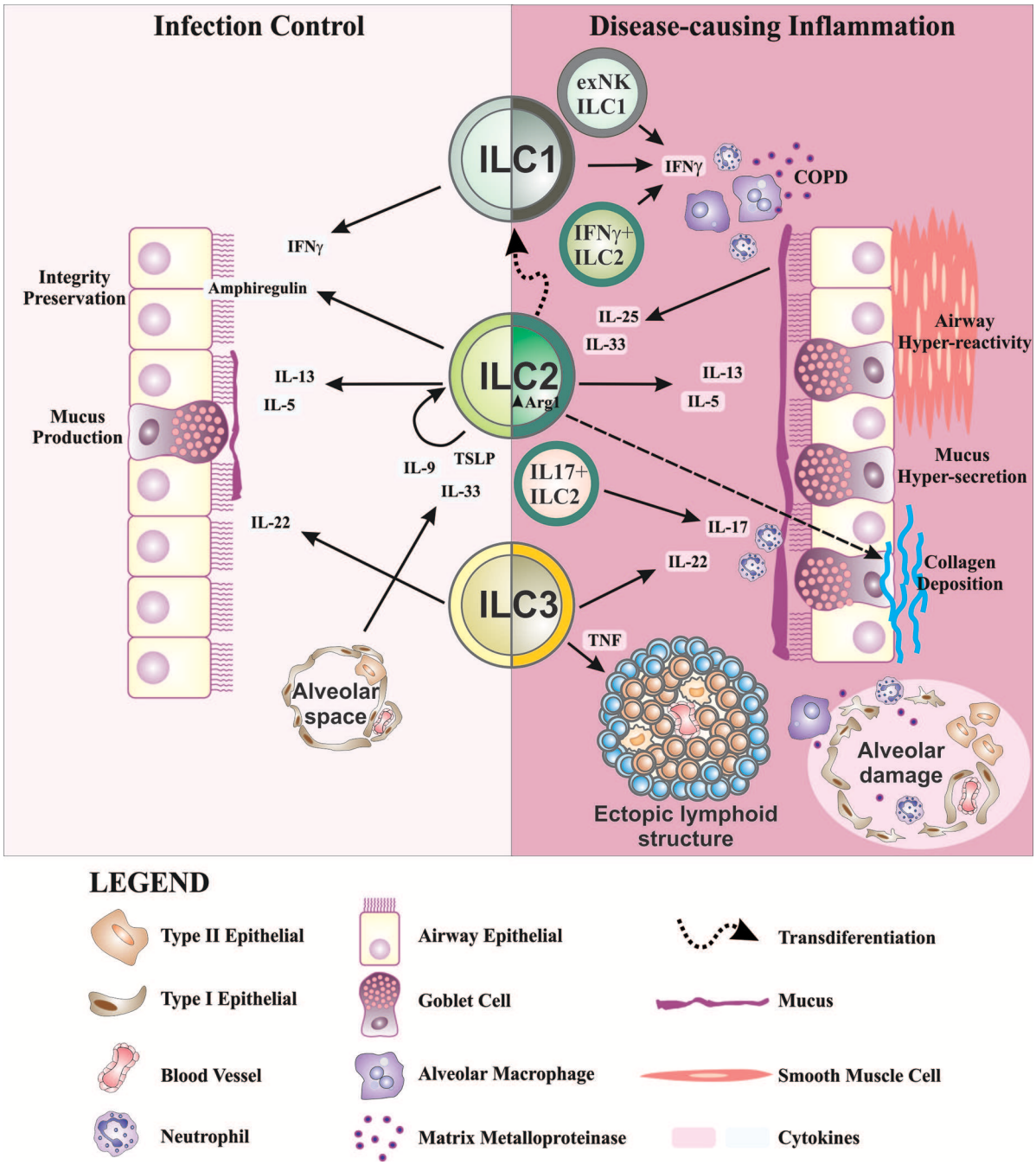

FIGURE 2 | Schematic representation of ILCs in lung health and disease. ILC2s are important for the preservation of airway barrier integrity during homeostasis. During inflammation in response to allergens, IL-33, and TSLP produced by alveolar type II epithelial cells are known to drive IL-9 production in ILC2s. Autocrine IL-9 promotes IL-5 and IL-13 production which drives mucus production from goblet cells in the airways. ILC2s also produce amphiregulin during infection to activate epithelial repair responses. IL-22, an important factor for the maintenance of epithelial barrier function, mucus production and tissue repair, is predominantly produced by ILC3s. In disease settings, IL-25 and IL-33 from airway epithelial cells induce IL-13 and IL-5 production from ILC2s which contribute to airway hyper-reactivity, mucus overproduction, and airway inflammation in asthma. Further dysregulation of ILC2s through increased expression of Arg1 promotes collagen synthesis and deposition causing lung fibrosis. In addition to the contribution of IFN-γ^+ from ILC1s in recruiting and activating alveolar macrophages in COPD, there is accumulation of IFN-γ^+ ILC2s. Similar alterations in ILC phenotype are seen in asthma with the production of IL-17, primarily produced by ILC3s, co-expressed with IL-13 by ILC2s causing neutrophilia in COPD and asthma. Airway remodeling, an important feature in asthma and COPD, may in part be supported by LTi-like ILC3s and their participation in the formation of ectopic lymphoid structures. The above findings demonstrate a highly complex interplay of ILCs, cytokines and other inflammatory mediators within the lung. The highly plastic nature of ILCs, which follow cues from the inflammatory milieu, causes them to become dysregulated during chronic lung disease due to an overt and persistent onslaught of inflammatory mediators.

lung rather than to a diverse inflammatory milieu. Given their cytolytic capacity and cytokine profiles, unconventional T cells appear to be a specialized innate-like group of cells that are poised to act in early host defense to a range of pathogens and their bi-products. It also suggests that unconventional T cells may play a specific and perhaps intersecting role during infection of the chronically inflamed lung, contributing to exacerbations.

NKT Cells

The most widely studied subset of NKT cells are invariant NKT (iNKT) cells, expressing a TCR with limited diversity that responds to microbial glycolipid antigens. Upon activation, iNKT cells rapidly produce IFN-γ and IL-4 among other cytokines, indicating that they act early in the immune response and can coordinate both the innate and adaptive arms.

The role of iNKT cells in asthma so far is inconclusive [reviewed in (114)]. Bronchial asthma is classically thought to be a Th2 cell-associated disease characterized by $CD4^+$ Th2 cells producing IL-4, −5, and −13. Yet, there are reports that a significant proportion of lymphocytes in the blood are CD1d-restricted $CD4^+$ iNKT cells that inversely correlate with atopic indexes in asthmatic patients (115, 116). However, the inverse has also been reported in the BAL where fewer than 2% of T cells were $CD4^+$ iNKT in patients with asthma, a number which did not differ significantly from the number found in healthy subjects (117). Nevertheless, using NKT cell deficient mice it has been shown that the absence $V_\alpha 14i$ NKT cells protects mice from developing allergen-induced airway hyper-reactivity (118). Thus, much more work is required to fully appreciate the contribution of iNKT cells to asthmatic airway disease.

In COPD, the role of iNKT cells is as confounding with $CD4^+$ iNKT numbers in the BAL and sputum unchanged, although a conflicting study reported that the frequency of iNKT cells in the peripheral blood was significantly reduced in subjects with COPD compared to healthy individuals and further reduced in those COPD patients with exacerbations (117, 119). The converse trends between the blood and BAL were also seen with respect to $CD4^+$ cells in a patient study of smokers with COPD. Smoking was found to increase the number of $CD8^+$ T cells and $CD8^+$ NKT-like cells in the BAL of COPD patients with a concurrent reduction of $CD4^+$ cells in the BAL and increase of $CD4^+$ in the blood as previously reported (120, 121), while another study found an increase in iNKT and NKT-like cells in BAL fluid from current smokers but not COPD patients (121). Indeed, the role of iNKT cells in COPD may more relate to exacerbations, which can be caused by infection with respiratory viruses and other pathogens. iNKT cells have been shown to contribute to COPD following viral challenge with Sendai virus and subsequent glycolipid presentation by dendritic cells, which leads to the secretion of IL-13 and alternative activation of alveolar macrophages (122). This identifies a possible role for iNKT cells in viral exacerbations of COPD, which may extend to other chronic lung diseases.

iNKT cells have been shown to induce airway hyper-responsiveness in the absence of adaptive immunity, which would suggest that iNKT cells and ILCs may interact within the lung. A study has addressed this using glycolipid antigens in a murine model of asthma. iNKT cells activated by glycolipid antigens stimulated the production of IL-33 from alveolar macrophages which in turn activated ILC2 to produce IL-13 and contribute to airway hyper-responsiveness (123). NKT cells themselves have been shown to be a source of IL-33 during influenza infection (19). Taken together, these results suggest that iNKT crosstalk in the lung is highly complex and involves other innate cells such as ILC2s.

MAIT Cells

In humans, MAIT cells are in abundance in the peripheral blood and comprise 10% of lung mucosal T cells, which in addition to the limited diversity of the MAIT-TCR means that early within the immune response, MAIT cell responses significantly outnumber those from conventional αβ-T cells. MAIT cells display an effector-memory phenotype without prior clonal expansion and can secrete a range of pro-inflammatory cytokines including TNF-α, IL-17, and IFN-γ and also IL-4 upon TCR ligation (124–126).

Circulating MAIT cell numbers have been found to be significantly reduced in patients with COPD, correlating with disease severity and inflammatory activity (127, 128). Exacerbations are major drivers of morbidity and mortality in COPD. Since exacerbations can be driven by bacterial infection, and MAIT cell activation is elicited by precursors and derivatives of the riboflavin biosynthetic pathway conserved in bacteria and yeast, there is potential for their involvement in COPD exacerbations. Macrophages stimulated with live non-typeable *Haemophilus influenzae* (NTHi), the most common airway-colonizing bacterium in COPD, promoted a potent IFN-γ response from MAIT cells, providing strong evidence that NTHi is a target of MAIT cell immunity. Interestingly, MAIT cell number and immune responses were significantly impaired by corticosteroid treatment, suggesting that failure of MAIT cell immunity may mediate COPD immunopathology during infection (127). MAIT cell frequencies were markedly reduced in the blood and lung tissues of severe asthma patients, which was also related to corticosteroid treatment (129).

MAIT cells as well as NK cells and ILCs have been shown to be involved in asthma. MAIT cells, activated NK cells ($CD69^+$), ILC1, ILC2, and ILC3 cells have all been shown to positively correlate with each other and with reduced airflow in asthmatic patients (130). This would suggest an underlying bacterial challenge could be involved in the increase in MAIT cell numbers, although an increase in all ILC populations and their mixed response seems contradictory. In response to viral challenge by influenza A virus and the bacteria *Staphylococcus aureus* and NTHi, all of which are COPD-associated triggers, ILC populations were altered. Each pathogen induced ILC reprogramming in the lung by inducing the loss of GATA-3 expression, while increasing IL-12RβII, IL-18Rα, and T-bet expression (43). Similarly, exposure to cigarette smoke induced a loss of GATA-3 expression and emergence of an ILC1 population. Interestingly, exposure to cigarette smoke combined with viral infection augmented the ILC phenotypic switch, suggesting the response was due to pathogenic or environmental

insults (43). Understanding how MAIT cells contribute to these exacerbations and support ILC plasticity may define their role in pulmonary immunity and the immunopathology of chronic respiratory diseases.

γδ-T Cells

γδ-T cells are tissue-resident cells primarily located at mucosal sites within the body; in the lung they have been characterized as CD4 and CD8 double negative cells selectively expressing Vγ6, Vγ1, and Vγ4 gene segments (131). The γδ-T cells residing in the lung are potent producers of IL-17 whereas γδ-T cells expressing different Vγ gene segments in the skin, gut, liver, spleen, uterus, and peripheral blood can produce IL-17 or IFN-γ (131). The abundance of γδ-T cells in the lung supports tissue homeostasis, although γδ-T cells have also been shown to play critical roles in bacterial clearance and the prevention of inflammation and lung fibrosis (132).

Little is known about the roles of γδ-T cells in chronic lung diseases such as COPD except that they are an important component of tissue injury and remodeling. The specific localization of γδ-T cells in epithelial and mucosal tissues and their role in protecting and maintaining airway function, suggests that they are likely participants in chronic airway diseases. Cigarette smoke exposure is the major cause of COPD and has been directly implicated in neutrophil variant asthma, asthma-COPD overlap syndrome, interstitial lung disease, rheumatic lung disease, and infectious exacerbations of these conditions (87, 133–135). Studies have shown elevated numbers of γδ-T cells in the bronchial glands, lung parenchyma, peripheral blood and BAL of smokers compared to never smokers (136–138). Early reports demonstrated that γδ-T cell percentages in the BAL and peripheral blood increased in COPD patients compared to healthy controls and were further amplified by smoking (139, 140). In contrast, a more recent study reported significantly lower relative and absolute numbers of γδ-T cells in the sputum and BAL of patients with COPD than those with asthma or healthy subjects, which negatively correlated with FEV1 and smoking pack years (141). This finding contradicted earlier publications but failed to stratify patient subgroups due to small sample size whereas the previous studies may have been confounded by inhaled steroid and bronchodilator therapies, and thus, much more work is required to fully appreciate the contribution of γδ-T cells in COPD.

In many cases, airway infections can cause COPD exacerbations, which are an acute worsening of respiratory symptoms. IL-17 producing cells, in particular Th17 cells, play distinct roles in host defenses against diverse pathogens (142). Pathogens that invoke an IL-17 response involve innate immune cells such as γδ-T cells, NKT cells, MAIT cells, and ILC3s, as well as adaptive Th17 cells. Persistence of some bacteria such as *Pseudomonas aeruginosa* within the lower airways is common in patients with cystic fibrosis, non-cystic fibrosis bronchiectasis, and COPD. In a murine model of chronic pulmonary infection with *Pseudomonas aeruginosa*, there was a significant expansion of IL-17$^+$ cells in lung homogenates and of these, 50% were CD3$^-$ IL-17$^+$ ILC3s, likely to be LTi cells and 50% were

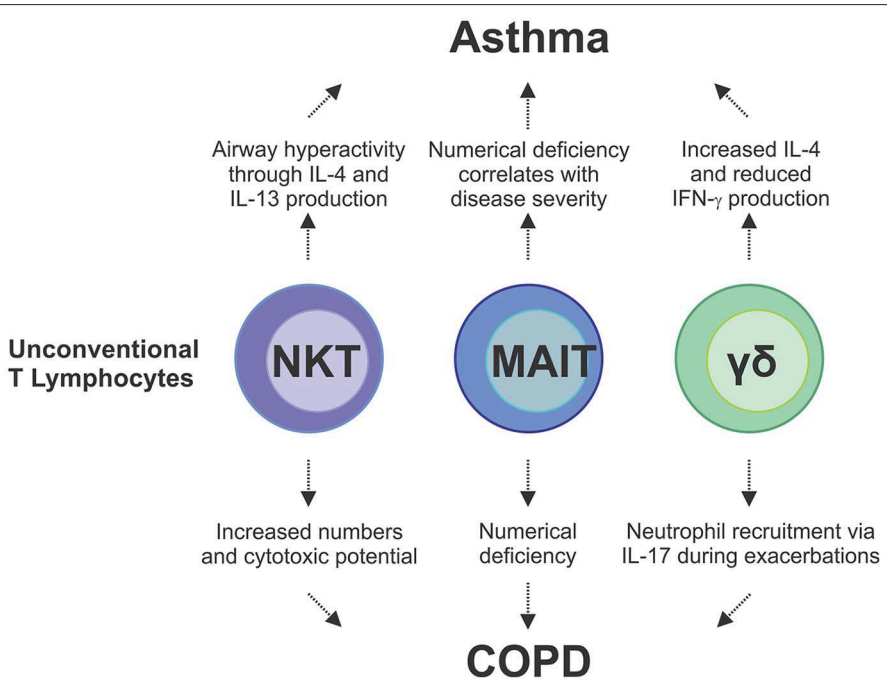

FIGURE 3 | Unconventional lymphocytes are implicated as pathogenic mediators of asthma and COPD and disease exacerbations. In asthma, IL-4 and IL-13 cytokines produced by NKT cells are directly involved in disease development which is compounded by additional IL-4 production from γδ-T cells. Conversely there is a reduction in IFN-γ producing γδ-T cells and MAIT cells, which correlates with disease severity in asthma and COPD. In COPD, there is an increase in NKT cell numbers and cytotoxicity in the lungs, and IL-17-mediated neutrophilia, which is driven by γδ-T cells, both of which contribute to lung damage. These studies demonstrate the complex interplay and multifaceted contributions from the different types of unconventional T cells to chronic lung disease.

$CD3^+$ T cells, split equally between γδ-T cells and Th17 cells, demonstrating a diverse range of cellular sources of IL-17 in chronic respiratory infection (143). Why a spectrum of IL-17-producing cells are generated during pulmonary infection and the roles of these different cell types, remains to be determined.

Just as cigarette smoke has been shown to increase the number of $IL\text{-}17A^+$ NKT cells in the lung, alternative cellular sources of IL-17A include NK cells and γδ-T cells which have been shown to become potent producers of IL-17A upon cigarette smoke exposure, with the frequency of $IL\text{-}17^+$ γδ-T cells significantly increasing in number (144). Clinical studies and experimental models of viral-induced COPD exacerbations provide strong evidence of ineffective anti-viral immunity in response to cigarette smoke. Although increased γδ-T cell numbers and production of IL-17A in response to cigarette smoke has been shown to be protective, pneumococcal challenge of mice chronically subjected to cigarette smoke led to defective production of IL-17 from γδ-T cells (145). Similarly, in the presence influenza A, mice exposed to cigarette smoke recovered poorly from an acute infection (146). During influenza infection, γδ-T cells acquire reciprocal production of IFN-γ and IL-17A, however, cigarette smoke exposure leads to repression of *IFN-γ* transcription (146). The contribution of unconventional T cells to chronic lung disease, including asthma and COPD, is only starting to be understood (**Figure 3**). As most chronic lung disease patients experience exacerbations during the course of their disease, an improved understanding of how γδ-T cells as well as MAIT and NKT cells contribute to the cytokine milieu during these insults to drive ILC plasticity and alter function is now required. This may offer important new insights into the way in which innate and innate-like cells contribute to COPD and asthma exacerbations, as well as other chronic pulmonary diseases.

CONCLUDING REMARKS

The development of innate and innate-like lymphocytes with overlapping phenotypes and functions has most likely evolved to provide robustness to the pulmonary immune system (147). ILCs and unconventional T cells may differ in cellular biology but they share common roles in tissue integrity preservation, lung homeostasis and immunity against infections. Compared with their conventional counterparts, it seems likely that ILCs and unconventional T cells have developed as specialized tissue-resident sensors to rapidly detect deviations in tissue integrity that arise from infection or injury. While it is clear that ILCs contribute to the maintenance of lung homeostasis, there is growing evidence to support the contribution of ILCs to a number of lung pathologies. ILCs have now been shown to become dysregulated in chronic lung disorders including asthma, COPD, chronic rhinosinusitis, and pulmonary fibrosis, and similarly, unconventional T cells are involved in the immunopathology of these diseases, particularly in the context of disease exacerbations. Recent advances have identified the highly heterogeneous and flexible nature of ILCs, which enable them to readily adapt to changing local tissue environmental conditions by altering their traits and functional attributes. This now introduces the question of how ILCs functionally integrate into the complex network of immune cells and stroma within the lung. In particular, the interplay and functional overlap between these innate cells and the other tissue-resident unconventional T cells requires further investigation. Whether or not these cell subsets co-regulate one another or function independently remains to be answered. We have discussed the dysregulation of ILC function during chronic lung disease, a feature that becomes more pronounced during exacerbations due to the sensitivity of ILCs to the changing lung tissue microenvironment but also in part owing to the complex interplay between ILCs and unconventional T cells within the lung. Further studies should now be directed at understanding these complex relationships, particularly in the setting of chronic lung disease, which may reveal potential treatment targets and lead to the formulation of interventions against these prevalent and debilitating diseases.

AUTHOR CONTRIBUTIONS

JB conceived and wrote the manuscript. MH and ML contributed to the writing of the manuscript and prepared the figures.

FUNDING

Publishing costs for this article were covered by the International Union of Immunological Societies (IUIS).

ACKNOWLEDGMENTS

This work was supported by the International Union of Immunological Societies (JB) and the Central Clinical School, Monash University (MH), and funded by grants from the National Health and Medical Research Council, Australia (APP1141208 and APP1147267 to MH).

REFERENCES

1. Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity*. (2014) 41:354–65. doi: 10.1016/j.immuni.2014.09.005
2. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell*. (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
3. Yu YRA, O'Koren EG, Hotten DF, Kan MJ, Kopin D, Nelson ER, et al. A protocol for the comprehensive flow cytometric analysis of immune cells in normal and inflamed murine non-lymphoid tissues. *PLoS ONE*. (2016) 11:e0150606. doi: 10.1371/journal.pone.0150606
4. Dutton EE, Camelo A, Sleeman M, Herbst R, Carlesso G, Belz GT, et al. Characterisation of innate lymphoid cell populations at different sites in mice with defective T cell immunity. *Wellcome Open Res*. (2017) 2:117. doi: 10.12688/wellcomeopenres.13199.3

5. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol*. (2011) 12:1045–54. doi: 10.1038/ni.2131
6. Gasteiger, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science*. (2015) 350:981–5. doi: 10.1126/science.aac9593
7. Moro K, Kabata H, Tanabe M, Koga S, Takeno N, Mochizuki M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol*. (2016) 17:76–86. doi: 10.1038/ni.3309
8. Haas JD, Ravens S, Duber S, Sandrock I, Oberdorfer L, Kashani E, et al. Development of interleukin-17-producing γδ T cells is restricted to a functional embryonic wave. *Immunity*. (2012) 37:48–59. doi: 10.1016/j.immuni.2012.06.003
9. McKenzie DR, Kara EE, Bastow CR, Tyllis TS, Fenix KA, Gregor CE, et al. IL-17-producing γδ T cells switch migratory patterns between resting and activated states. *Nat Commun*. (2017) 8:15632. doi: 10.1038/ncomms15632
10. Huang Y, Guo L, Qiu J, Chen X, Hu-Li J, Siebenlist U, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol*. (2015) 16:161–9. doi: 10.1038/ni.3078
11. Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science*. (2018) 359:114–9. doi: 10.1126/science.aam5809
12. Mohapatra, Van Dyken SJ, Schneider C, Nussbaum JC, Liang HE, Locksley RM. Group 2 innate lymphoid cells utilize the IRF4-IL-9 module to coordinate epithelial cell maintenance of lung homeostasis. *Mucosal Immunol*. (2016) 9:275–86. doi: 10.1038/mi.2015.59
13. Martinez-Gonzalez, Steer CA, Takei F. Lung ILC2s link innate and adaptive responses in allergic inflammation. *Trends Immunol*. (2015) 36:189–95. doi: 10.1016/j.it.2015.01.005
14. De Grove KC, Provoost S, Verhamme FM, Bracke KR, Joos GF, Maes T, et al. Characterization and quantification of innate lymphoid cell subsets in human lung. *PLoS ONE*. (2016) 11:e0145961. doi: 10.1371/journal.pone.0145961
15. Hirose K, Ito T, Nakajima H. Roles of IL-22 in allergic airway inflammation in mice and humans. *Int Immunol*. (2018) 30:413–8. doi: 10.1093/intimm/dxy010
16. Prefontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, et al. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol*. (2010) 125:752–4. doi: 10.1016/j.jaci.2009.12.935
17. Byers DE, Alexander-Brett J, Patel AC, Agapov E, Dang-Vu G, Jin X, et al. Long-term IL-33-producing epithelial progenitor cells in chronic obstructive lung disease. *J Clin Invest*. (2013) 123:3967–82. doi: 10.1172/JCI65570
18. Lee JU, Chang HS, Lee HJ, Jung CA, Bae DJ, Song HJ, et al. Upregulation of interleukin-33 and thymic stromal lymphopoietin levels in the lungs of idiopathic pulmonary fibrosis. *BMC Pulm Med*. (2017) 17:39. doi: 10.1186/s12890-017-0380-z
19. Gorski SA, Hahn YS, Braciale TJ. Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. *PLoS Pathog*. (2013) 9:e1003615. doi: 10.1371/journal.ppat.1003615
20. Guo XJ, Dash P, Crawford JC, Allen EK, Zamora AE, Boyd DF, et al. Lung γδ T cells mediate protective responses during neonatal influenza infection that are associated with type 2 immunity. *Immunity*. (2018) 49:531–44.e6. doi: 10.1016/j.immuni.2018.07.011
21. Hong JY, Bentley JK, Chung Y, Lei J, Steenrod JM, Chen Q, et al. Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness through IL-25 and type 2 innate lymphoid cells. *J Allergy Clin Immunol*. (2014) 134:429–39. doi: 10.1016/j.jaci.2014.04.020
22. Han M, Rajput C, Hong JY, Lei J, Hinde JL, Wu Q, et al. The innate cytokines IL-25, IL-33, and TSLP cooperate in the induction of type 2 innate lymphoid cell expansion and mucous metaplasia in rhinovirus-infected immature mice. *J Immunol*. (2017) 199:1308–18. doi: 10.4049/jimmunol.1700216
23. Stier MT, Bloodworth MH, Toki S, Newcomb DC, Goleniewska K, Boyd KL, et al. Respiratory syncytial virus infection activates IL-13-producing group 2 innate lymphoid cells through thymic stromal lymphopoietin. *J Allergy Clin Immunol*. (2016) 138:814–24.e11. doi: 10.1016/j.jaci.2016.01.050
24. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med*. (2010) 363:1211–21. doi: 10.1056/NEJMoa0906312
25. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity*. (2012) 36:451–63. doi: 10.1016/j.immuni.2011.12.020
26. Nagata, Kamijuku H, Taniguchi M, Ziegler S, Seino K. Differential role of thymic stromal lymphopoietin in the induction of airway hyperreactivity and Th2 immune response in antigen-induced asthma with respect to natural killer T cell function. *Int Arch Allergy Immunol*. (2007) 144:305–14. doi: 10.1159/000106319
27. Denney L, Byrne AJ, Shea TJ, Buckley JS, Pease JE, Herledan GM, et al. Pulmonary epithelial cell-derived cytokine TGF-β1 is a critical cofactor for enhanced innate lymphoid cell function. *Immunity*. (2015) 43:945–58. doi: 10.1016/j.immuni.2015.10.012
28. McHedlidze T, Kindermann M, Neves AT, Voehringer D, Neurath MF, Wirtz S. IL-27 suppresses type 2 immune responses *in vivo* via direct effects on group 2 innate lymphoid cells. *Mucosal Immunol*. (2016) 9:1384–94. doi: 10.1038/mi.2016.20
29. Murphy KM, Stockinger B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol*. (2010) 11:674–80. doi: 10.1038/ni.1899
30. Pikovskaya O, Chaix J, Rothman NJ, Collins A, Chen YH, Scipioni AM, et al. Cutting Edge: eomesodermin is sufficient to direct type 1 innate lymphocyte development into the conventional NK lineage. *J Immunol*. (2016) 196:1449–54. doi: 10.4049/jimmunol.1502396
31. Cortez VS, Cervantes-Barragan L, Robinette ML, Bando JK, Wang Y, Geiger TL, et al. Transforming growth factor-β signaling guides the differentiation of innate lymphoid cells in salivary glands. *Immunity*. (2016) 44:1127–39. doi: 10.1016/j.immuni.2016.03.007
32. Cortez VS, Ulland TK, Cervantes-Barragan L, Bando JK, Robinette ML, Wang Q, et al. SMAD4 impedes the conversion of NK cells into ILC1-like cells by curtailing non-canonical TGF-β signaling. *Nat Immunol*. (2017) 18:995–1003. doi: 10.1038/ni.3809
33. Gao YF, Bald T, Ng SS, Young A, Ngiow SF, Rautela J, et al. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nat Immunol*. (2017) 18:1004–15. doi: 10.1038/ni.3800
34. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and−23 control plasticity of CD127(+) group 1 and group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity*. (2015) 43:146–60. doi: 10.1016/j.immuni.2015.06.019
35. Simoni Y, Fehlings M, Kloverpris HN, McGovern N, Koo SL, Loh CY, et al. Human innate lymphoid cell subsets possess tissue-type based heterogeneity in phenotype and frequency. *Immunity*. (2017) 46:148–61. doi: 10.1016/j.immuni.2016.11.005
36. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol*. (2016) 17:758–64. doi: 10.1038/ni.3482
37. Mindt BC, Fritz JH, Duerr CU. Group 2 innate lymphoid cells in pulmonary immunity and tissue homeostasis. *Front Immunol*. (2018) 9:840. doi: 10.3389/fimmu.2018.00840
38. Nussbaum JC, Van Dyken SJ, von Moltke J, Cheng LE, Mohapatra A, Molofsky AB, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature*. (2013) 502:245–8. doi: 10.1038/nature12526
39. Gury-BenAri M, Thaiss CA, Serafini N, Winter DR, Giladi A, Lara-Astiaso D, et al. The spectrum and regulatory landscape of intestinal innate lymphoid cells are shaped by the microbiome. *Cell*. (2016) 166:1231–46.e13. doi: 10.1016/j.cell.2016.07.043
40. Zhang K, Xu X, Pasha MA, Siebel CW, Costello A, Haczku A, et al. Cutting edge: notch signaling promotes the plasticity of group-2 innate lymphoid cells. *J Immunol*. (2017) 198:1798–803. doi: 10.4049/jimmunol.1601421
41. Bal SM, Bernink JH, Nagasawa M, Groot J, Shikhagaie MM, Golebski K, et al. IL-1β, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nat Immunol*. (2016) 17:636–45. doi: 10.1038/ni.3444

42. Ohne Y, Silver JS, Thompson-Snipes L, Collet MA, Blanck JP, Cantarel BL, et al. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat Immunol.* (2016) 17:646–55. doi: 10.1038/ni.3447
43. Silver JS, Kearley J, Copenhaver AM, Sanden C, Mori M, Yu L, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat Immunol.* (2016) 17:626–35. doi: 10.1038/ni.3443
44. Lim AI, Menegatti S, Bustamante J, Le Bourhis L, Allez M, Rogge L, et al. IL-12 drives functional plasticity of human group 2 innate lymphoid cells. *J Exp Med.* (2016) 213:569–83. doi: 10.1084/jem.20151750
45. Teunissen mBM, Munneke JM, Bernink JH, Spuls PI, Res PCM, Te Velde A, et al. Composition of innate lymphoid cell subsets in the human skin: enrichment of NCR(+) ILC3 in lesional skin and blood of psoriasis patients. *J Invest Dermatol.* (2014) 134:2351–60. doi: 10.1038/jid.2014.146
46. Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol.* (2011) 12:1055–62. doi: 10.1038/ni.2104
47. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol.* (2013) 14:221–9. doi: 10.1038/ni.2534
48. Hughes T, Becknell B, McClory S, Briercheck E, Freud AG, Zhang X, et al. Stage 3 immature human natural killer cells found in secondary lymphoid tissue constitutively and selectively express the TH 17 cytokine interleukin-22. *Blood.* (2009) 113:4008–10. doi: 10.1182/blood-2008-12-192443
49. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proc Natl Acad Sci USA.* (2010) 107:10961–6. doi: 10.1073/pnas.1005641107
50. Hughes T, Becknell B, Freud AG, McClory S, Briercheck E, Yu J, et al. Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. *Immunity.* (2010) 32:803–14. doi: 10.1016/j.immuni.2010.06.007
51. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature.* (2013) 494:261–5. doi: 10.1038/nature 11813
52. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, et al. Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity.* (2010) 33:736–51. doi: 10.1016/j.immuni.2010.10.017
53. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet.* (2008) 372:1107–19. doi: 10.1016/S0140-6736(08)61452-X
54. Starkey MR, McKenzie AN, Belz GT, Hansbro PM. Pulmonary group 2 innate lymphoid cells: surprises and challenges. *Mucosal Immunol.* (2019) 12:299–311. doi: 10.1038/s41385-018-0130-4
55. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity.* (2001) 15:985–95. doi: 10.1016/S1074-7613(01)00243-6
56. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol.* (2012) 42:1106–16. doi: 10.1002/eji.201142018
57. Kondo Y, Yoshimoto T, Yasuda K, Futatsugi-Yumikura S, Morimoto M, Hayashi N, et al. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int Immunol.* (2008) 20:791–800. doi: 10.1093/intimm/dxn037
58. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol.* (2012) 129:191–8 e1–4. doi: 10.1016/j.jaci.2011.09.041
59. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J Allergy Clin Immunol.* (2013) 132:933–41. doi: 10.1016/j.jaci.2013.05.012
60. Li Y, Chen S, Chi Y, Yang Y, Chen X, Wang H, et al. Kinetics of the accumulation of group 2 innate lymphoid cells in IL-33-induced and IL-25-induced murine models of asthma: a potential role for the chemokine CXCL16. *Cell Mol Immunol.* (2019) 16:75–86. doi: 10.1038/s41423-018-0182-0
61. Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, et al. Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol.* (2012) 10:e1001255. doi: 10.1371/journal.pbio.1001255
62. Walford HH, Lund SJ, Baum RE, White AA, Bergeron CM, Husseman J, et al. Increased ILC2s in the eosinophilic nasal polyp endotype are associated with corticosteroid responsiveness. *Clin Immunol.* (2014) 155:126–35. doi: 10.1016/j.clim.2014.09.007
63. Fan D, Wang X, Wang M, Wang Y, Zhang L, Li Y, et al. Allergen-dependent differences in ILC2s frequencies in patients with allergic rhinitis. *Allergy Asthma Immunol Res.* (2016) 8:216–22. doi: 10.4168/aair.2016.8.3.216
64. van Rijt L, von Richthofen H, van Ree R. Type 2 innate lymphoid cells: at the cross-roads in allergic asthma. *Semin Immunopathol.* (2016) 38:483–96. doi: 10.1007/s00281-016-0556-2
65. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol.* (2014) 134:671–8 e4. doi: 10.1016/j.jaci.2014.06.024
66. Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol.* (2016) 137:75–86 e8. doi: 10.1016/j.jaci.2015.05.037
67. Stadhouders R, Li BWS, de Bruijn MJW, Gomez A, Rao TN, Fehling HJ, et al. Epigenome analysis links gene regulatory elements in group 2 innate lymphocytes to asthma susceptibility. *J Allergy Clin Immunol.* (2018) 142:1793–807. doi: 10.1016/j.jaci.2017.12.1006
68. Kim HY, Lee HJ, Chang YJ, Pichavant M, Shore SA, Fitzgerald KA, et al. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med.* (2014) 20:54–61. doi: 10.1038/nm.3423
69. Hekking PP, Loza MJ, Pavlidis S, de Meulder B, Lefaudeux D, Baribaud F, et al. Pathway discovery using transcriptomic profiles in adult-onset severe asthma. *J Allergy Clin Immunol.* (2018) 141:1280–90. doi: 10.1016/j.jaci.2017.06.037
70. Schnyder B, Lima C, Schnyder-Candrian S. Interleukin-22 is a negative regulator of the allergic response. *Cytokine.* (2010) 50:220–7. doi: 10.1016/j.cyto.2010.02.003
71. Besnard AG, Sabat R, Dumoutier L, Renauld JC, Willart M, Lambrecht B, et al. Dual Role of IL-22 in allergic airway inflammation and its cross-talk with IL-17A. *Am J Respir Crit Care Med.* (2011) 183:1153–63. doi: 10.1164/rccm.201008-1383OC
72. Taube C, Tertilt C, Gyulveszi G, Dehzad N, Kreymborg K, Schneeweiss K, et al. IL-22 is produced by innate lymphoid cells and limits inflammation in allergic airway disease. *PLoS ONE.* (2011) 6:e21799. doi: 10.1371/journal.pone.0021799
73. Gray J, Oehrle K, Worthen G, Alenghat T, Whitsett J, Deshmukh H. Intestinal commensal bacteria mediate lung mucosal immunity and promote resistance of newborn mice to infection. *Sci Transl Med.* (2017) 9:eaaf9412. doi: 10.1126/scitranslmed.aaf9412
74. Liao B, Cao PP, Zeng M, Zhen Z, Wang H, Zhang YN, et al. Interaction of thymic stromal lymphopoietin, IL-33, and their receptors in epithelial cells in eosinophilic chronic rhinosinusitis with nasal polyps. *Allergy.* (2015) 70:1169–80. doi: 10.1111/all.12667
75. Shaw JL, Fakhri S, Citardi MJ, Porter PC, Corry DB, Kheradmand F, et al. IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps. *Am J Respir Crit Care Med.* (2013) 188:432–9. doi: 10.1164/rccm.201212-2227OC
76. Miljkovic D, Bassiouni A, Cooksley C, Ou J, Hauben E, Wormald PJ, et al. Association between group 2 innate lymphoid cells enrichment, nasal polyps and allergy in chronic rhinosinusitis. *Allergy.* (2014) 69:1154–61. doi: 10.1111/all.12440
77. Ho J, Bailey M, Zaunders J, Mrad N, Sacks R, Sewell W, et al. Group 2 innate lymphoid cells (ILC2s) are increased in chronic rhinosinusitis

with nasal polyps or eosinophilia. *Clin Exp Allergy*. (2015) 45:394–403. doi: 10.1111/cea.12462
78. Poposki JA, Klingler AI, Tan BK, Soroosh P, Banie H, Lewis G, et al. Group 2 innate lymphoid cells are elevated and activated in chronic rhinosinusitis with nasal polyps. *Immun Inflamm Dis*. (2017) 5:233–43. doi: 10.1002/iid3.161
79. Ebbo M, Crinier A, Vely F, Vivier E. Innate lymphoid cells: major players in inflammatory diseases. *Nat Rev Immunol*. (2017) 17:665–78. doi: 10.1038/nri.2017.86
80. Suzuki M, Sze MA, Campbell JD, Brothers JF 2nd, Lenburg ME, McDonough JE, et al. The cellular and molecular determinants of emphysematous destruction in COPD. *Sci Rep*. (2017) 7:9562. doi: 10.1038/s41598-017-10126-2
81. Donovan C, Starkey MR, Kim RY, Rana BMJ, Barlow JL, Jones B, et al. Roles for T/B lymphocytes and ILC2s in experimental chronic obstructive pulmonary disease. *J Leukoc Biol*. (2019) 105:143–50. doi: 10.1002/JLB.3AB0518-178R
82. Monticelli LA, Buck MD, Flamar AL, Saenz SA, Tait Wojno ED, Yudanin NA, et al. Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation. *Nat Immunol*. (2016) 17:656–65. doi: 10.1038/ni.3421
83. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Vicari C, Capelli A, et al. T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol*. (2009) 157:316–24. doi: 10.1111/j.1365-2249.2009.03965.x
84. Shikhagaie MM, Bjorklund AK, Mjosberg J, Erjefalt JS, Cornelissen AS, Ros XR, et al. Neuropilin-1 is expressed on lymphoid tissue residing LTi-like group 3 innate lymphoid cells and associated with ectopic lymphoid aggregates. *Cell Rep*. (2017) 18:1761–73. doi: 10.1016/j.celrep.2017.01.063
85. Prieto A, Reyes E, Bernstein ED, Martinez B, Monserrat J, Izquierdo JL, et al. Defective natural killer and phagocytic activities in chronic obstructive pulmonary disease are restored by glycophosphopeptical (inmunoferon). *Am J Respir Crit Care Med*. (2001) 163:1578–83. doi: 10.1164/ajrccm.163.7.2002015
86. Urbanowicz RA, Lamb JR, Todd I, Corne JM, Fairclough LC. Altered effector function of peripheral cytotoxic cells in COPD. *Respir Res*. (2009) 10:53. doi: 10.1186/1465-9921-10-53
87. Urbanowicz RA, Lamb JR, Todd I, Corne JM, Fairclough LC. Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients. *Respir Res*. (2010) 11:76. doi: 10.1186/1465-9921-11-76
88. Freeman CM, Stolberg VR, Crudgington S, Martinez FJ, Han MK, Chensue SW, et al. Human CD56+ cytotoxic lung lymphocytes kill autologous lung cells in chronic obstructive pulmonary disease. *PLoS ONE*. (2014) 9:e103840. doi: 10.1371/journal.pone.0103840
89. Wortham BW, Eppert BL, Motz GT, Flury JL, Orozco-Levi M, Hoebe K, et al. NKG2D mediates NK cell hyperresponsiveness and influenza-induced pathologies in a mouse model of chronic obstructive pulmonary disease. *J Immunol*. (2012) 188:4468–75. doi: 10.4049/jimmunol.1102643
90. Stolberg VR, Martin B, Mancuso P, Olszewski MA, Freeman CM, Curtis JL, et al. Role of CC chemokine receptor 4 in natural killer cell activation during acute cigarette smoke exposure. *Am J Pathol*. (2014) 184:454–63. doi: 10.1016/j.ajpath.2013.10.017
91. Motz GT, Eppert BL, Wortham BW, Amos-Kroohs RM, Flury JL, Wesselkamper SC, et al. Chronic cigarette smoke exposure primes NK cell activation in a mouse model of chronic obstructive pulmonary disease. *J Immunol*. (2010) 184:4460–9. doi: 10.4049/jimmunol.0903654
92. Kearley J, Silver JS, Sanden C, Liu Z, Berlin AA, White N, et al. Cigarette smoke silences innate lymphoid cell function and facilitates an exacerbated type I interleukin-33-dependent response to infection. *Immunity*. (2015) 42:566–79. doi: 10.1016/j.immuni.2015.02.011
93. Borchers MT, Wesselkamper SC, Curull V, Ramirez-Sarmiento A, Sanchez-Font A, Garcia-Aymerich J, et al. Sustained CTL activation by murine pulmonary epithelial cells promotes the development of COPD-like disease. *J Clin Invest*. (2009) 119:636–49. doi: 10.1172/JCI34462
94. Borchers MT, Harris NL, Wesselkamper SC, Vitucci M, Cosman D. NKG2D ligands are expressed on stressed human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. (2006) 291:L222–31. doi: 10.1152/ajplung.00327.2005
95. Jackson DJ, Makrinioti H, Rana BM, Shamji BW, Trujillo-Torralbo MB, Footitt J, et al. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations *in vivo*. *Am J Respir Crit Care Med*. (2014) 190:1373–82. doi: 10.1164/rccm.201406-1039OC
96. Cayrol C, Girard JP. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr Opin Immunol*. (2014) 31:31–7. doi: 10.1016/j.coi.2014.09.004
97. Chang YJ, Kim HY, Albacker LA, Baumgarth N, McKenzie AN, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol*. (2011) 12:631–8. doi: 10.1038/ni.2045
98. C.Bauer MT, Morissette MC, Stampfli MR. The influence of cigarette smoking on viral infections: translating bench science to impact COPD pathogenesis and acute exacerbations of COPD clinically. *Chest*. (2013) 143:196–206. doi: 10.1378/chest.12-0930
99. Vashist N, Trittel S, Ebensen T, Chambers BJ, Guzman CA, Riese P. Influenza-activated ILC1s contribute to antiviral immunity partially influenced by differential GITR expression. *Front Immunol*. (2018) 9:505. doi: 10.3389/fimmu.2018.00505
100. Wynn TA. Integrating mechanisms of pulmonary fibrosis. *J Exp Med*. (2011) 208:1339–50. doi: 10.1084/jem.20110551
101. Hams E, Armstrong ME, Barlow JL, Saunders SP, Schwartz C, Cooke G, et al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc Natl Acad Sci USA*. (2014) 111:367–72. doi: 10.1073/pnas.1315854111
102. Bonniaud P, Martin G, Margetts PJ, Ask K, Robertson J, Gauldie J, et al. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. *Am J Respir Cell Mol Biol*. (2004) 31:510–6. doi: 10.1165/rcmb.2004-0158OC
103. Marvie P, Lisbonne M, L'Helgoualc'h A, Rauch M, Turlin B, Preisser L, et al. Interleukin-33 overexpression is associated with liver fibrosis in mice and humans. *J Cell Mol Med*. (2010) 14:1726–39. doi: 10.1111/j.1582-4934.2009.00801.x
104. Jiang D, Liang J, Hodge J, Lu B, Zhu Z, Yu S, et al. Regulation of pulmonary fibrosis by chemokine receptor CXCR3. *J Clin Invest*. (2004) 114:291–9. doi: 10.1172/JCI16861
105. Khalil N, Parekh TV, O'Connor R, Antman N, Kepron W, Yehaulaeshet T, et al. Regulation of the effects of TGF-beta 1 by activation of latent TGF-beta 1 and differential expression of TGF-beta receptors (T beta R-I and T beta R-II) in idiopathic pulmonary fibrosis. *Thorax*. (2001) 56:907–15. doi: 10.1136/thorax.56.12.907
106. Aquino-Galvez A, Perez-Rodriguez M, Camarena A, Falfan-Valencia R, Ruiz V, Montano M, et al. MICA polymorphisms and decreased expression of the MICA receptor NKG2D contribute to idiopathic pulmonary fibrosis susceptibility. *Hum Genet*. (2009) 125:639–48. doi: 10.1007/s00439-009-0666-1
107. Trottein F, Paget C. Natural killer T cells and mucosal-associated invariant T cells in lung infections. *Front Immunol*. (2018) 9:1750. doi: 10.3389/fimmu.2018.01750
108. Born WK, Lahn M, Takeda K, Kanehiro A, O'Brien RL, Gelfand EW. Role of gammadelta T cells in protecting normal airway function. *Respir Res*. (2000) 1:151–8. doi: 10.1186/rr26
109. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature*. (2005) 434:520–5. doi: 10.1038/nature03407
110. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C 3rd, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. (2005) 434:525–9. doi: 10.1038/nature03408
111. Le Bourhis L, Martin E, Peguillet I, Guihot A, Froux N, Core M, et al. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol*. (2010) 11:701–8. doi: 10.1038/ni.1890
112. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol*. (2010) 10:467–78. doi: 10.1038/nri2781
113. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity*. (2009) 31:321–30. doi: 10.1016/j.immuni.2009.06.020

114. Umetsu DT, Dekruyff RH. Natural killer T cells are important in the pathogenesis of asthma: the many pathways to asthma. *J Allergy Clin Immunol*. (2010) 125:975–9. doi: 10.1016/j.jaci.2010.02.006
115. Akbari O, Faul JL, Hoyte EG, Berry GJ, Wahlstrom J, Kronenberg M, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med*. (2006) 354:1117–29. doi: 10.1056/NEJMoa053614
116. Koh YI, Shim JU, Wi JO, Han ER, Jin NC, Oh SH, et al. Inverse association of peripheral blood CD4(+) invariant natural killer T cells with atopy in human asthma. *Hum Immunol*. (2010) 71:186–91. doi: 10.1016/j.humimm.2009.10.011
117. Vijayanand P, Seumois G, Pickard C, Powell RM, Angco G, Sammut D, et al. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. *N Engl J Med*. (2007) 356:1410–22. doi: 10.1056/NEJMoa064691
118. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med*. (2003) 9:582–8. doi: 10.1038/nm851
119. Chi SY, Ban HJ, Kwon YS, Oh IJ, Kim KS, Kim YI, et al. Invariant natural killer T cells in chronic obstructive pulmonary disease. *Respirology*. (2012) 17:486–92. doi: 10.1111/j.1440-1843.2011.02104.x
120. Forsslund, Mikko M, Karimi R, Grunewald J, Wheelock AM, Wahlstrom J, and Skold CM, Distribution of T-cell subsets in BAL fluid of patients with mild to moderate COPD depends on current smoking status and not airway obstruction. Chest (2014) 145:711–22. doi: 10.1378/chest.13-0873
121. Eriksson Strom J, Pourazar J, Linder R, Blomberg A, Lindberg A, Bucht A, et al. Cytotoxic lymphocytes in COPD airways: increased NK cells associated with disease, iNKT and NKT-like cells with current smoking. *Respir Res*. (2018) 19:244. doi: 10.1186/s12931-018-0940-7
122. Kim EY, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med*. (2008) 14:633–40. doi: 10.1038/nm1770
123. Kim HY, Chang YJ, Subramanian S, Lee HH, Albacker LA, Matangkasombut P, et al. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J Allergy Clin Immunol*. (2012) 129:216–27 e1–6. doi: 10.1016/j.jaci.2011.10.036
124. Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood*. (2011) 117:1250–9. doi: 10.1182/blood-2010-08-303339
125. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol*. (2015) 16:1114–23. doi: 10.1038/ni.3298
126. Rahimpour A, Koay HF, Enders A, Clanchy R, Eckle SB, Meehan B, et al. Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J Exp Med*. (2015) 212:1095–108. doi: 10.1084/jem.20142110
127. Hinks TS, Wallington JC, Williams AP, Djukanovic R, Staples KJ, Wilkinson TM. Steroid-induced deficiency of mucosal-associated invariant T cells in the chronic obstructive pulmonary disease lung. Implications for nontypeable haemophilus influenzae infection. *Am J Respir Crit Care Med*. (2016) 194:1208–18. doi: 10.1164/rccm.201601-0002OC
128. Kwon YS, Jin HM, Cho YN, Kim MJ, Kang JH, Jung HJ, et al. Mucosal-Associated invariant T cell deficiency in chronic obstructive pulmonary disease. *COPD*. (2016) 13:196–202. doi: 10.3109/15412555.2015.1069806
129. Hinks TS, Zhou X, Staples KJ, Dimitrov BD, Manta A, Petrossian T, et al. Innate and adaptive T cells in asthmatic patients: Relationship to severity and disease mechanisms. *J Allergy Clin Immunol*. (2015) 136:323–33. doi: 10.1016/j.jaci.2015.01.014
130. Ishimori A, Harada N, Chiba A, Harada S, Matsuno K, Makino F, et al. Circulating activated innate lymphoid cells and mucosal-associated invariant T cells are associated with airflow limitation in patients with asthma. *Allergol Int*. (2017) 66:302–9. doi: 10.1016/j.alit.2016.07.005
131. Cheng M, Hu S. Lung-resident gammadelta T cells and their roles in lung diseases. *Immunology*. (2017) 151:375–84. doi: 10.1111/imm.12764
132. Simonian PL, Roark CL, Wehrmann F, Lanham AM, Born WK, O'Brien RL, et al. IL-17A-expressing T cells are essential for bacterial clearance in a murine model of hypersensitivity pneumonitis. *J Immunol*. (2009) 182:6540–9. doi: 10.4049/jimmunol.0900013
133. Hodge G, Mukaro V, Holmes M, Reynolds PN, Hodge S. Enhanced cytotoxic function of natural killer and natural killer T-like cells associated with decreased CD94 (Kp43) in the chronic obstructive pulmonary disease airway. *Respirology*. (2013) 18:369–76. doi: 10.1111/j.1440-1843.2012.02287.x
134. Pichavant M, Remy G, Bekaert S, Le Rouzic O, Kervoaze G, Vilain E, et al. Oxidative stress-mediated iNKT-cell activation is involved in COPD pathogenesis. *Mucosal Immunol*. (2014) 7:568–78. doi: 10.1038/mi.2013.75
135. Wang J, Urbanowicz RA, Tighe PJ, Todd I, Corne JM, Fairclough LC. Differential activation of killer cells in the circulation and the lung: a study of current smoking status and chronic obstructive pulmonary disease (COPD). *PLoS ONE*. (2013) 8:e58556. doi: 10.1371/journal.pone.0058556
136. Ekberg-Jansson A, Andersson B, Avra E, Nilsson O, Lofdahl CG. The expression of lymphocyte surface antigens in bronchial biopsies, bronchoalveolar lavage cells and blood cells in healthy smoking and never-smoking men, 60 years old. *Respir Med*. (2000) 94:264–72. doi: 10.1053/rmed.1999.0735
137. Majo J, Ghezzo H, Cosio MG. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J*. (2001) 17:946–53. doi: 10.1183/09031936.01.17509460
138. Richmond I, Pritchard GE, Ashcroft T, Corris PA, Walters EH. Distribution of gamma delta T-cells in the bronchial tree of smokers and non-smokers. *J Clin Pathol*. (1993) 46:926–30. doi: 10.1136/jcp.46.10.926
139. Roos-Engstrand E, Ekstrand-Hammarstrom B, Pourazar J, Behndig AF, Bucht A, Blomberg A. Influence of smoking cessation on airway T lymphocyte subsets in COPD. *COPD*. (2009) 6:112–20. doi: 10.1080/15412550902755358
140. Pons J, Sauleda J, Ferrer JM, Barcelo B, Fuster A, Regueiro V, et al. Blunted gamma delta T-lymphocyte response in chronic obstructive pulmonary disease. *Eur Respir J*. (2005) 25:441–6. doi: 10.1183/09031936.05.00069304
141. Urboniene D, Babusyte A, Lotvall J, Sakalauskas R, Sitkauskiene B. Distribution of gammadelta and other T-lymphocyte subsets in patients with chronic obstructive pulmonary disease and asthma. *Respir Med*. (2013) 107:413–23. doi: 10.1016/j.rmed.2012.11.012
142. Vanaudenaerde BM, Verleden SE, Vos R, De Vleeschauwer SI, Willems-Widyastuti A, Geenens R, et al. Innate and adaptive interleukin-17-producing lymphocytes in chronic inflammatory lung disorders. *Am J Respir Crit Care Med*. (2011) 183:977–86. doi: 10.1164/rccm.201007-1196PP
143. Bayes HK, Ritchie ND, Evans TJ. Interleukin-17 is required for control of chronic lung infection caused by pseudomonas aeruginosa. *Infect Immun*. (2016) 84:3507–16. doi: 10.1128/IAI.00717-16
144. Bozinovski S, Seow HJ, Chan SP, Anthony D, McQualter J, Hansen M, et al. Innate cellular sources of interleukin-17A regulate macrophage accumulation in cigarette- smoke-induced lung inflammation in mice. *Clin Sci (Lond)*. (2015) 129:785–96. doi: 10.1042/CS20140703
145. Pichavant M, Sharan R, Le Rouzic O, Olivier C, Hennegrave F, Remy G, et al. IL-22 defect during streptococcus pneumoniae infection triggers exacerbation of chronic obstructive pulmonary disease. *EBioMedicine*. (2015) 2:1686–96. doi: 10.1016/j.ebiom.2015.09.040
146. Hong MJ, Gu BH, Madison MC, Landers C, Tung HY, Kim M, et al. Protective role of gammadelta T cells in cigarette smoke and influenza infection. *Mucosal Immunol*. (2018) 11:894–908. doi: 10.1038/mi.2017.93
147. Vivier E, van de Pavert SA, Cooper MD, Belz GT. The evolution of innate lymphoid cells. *Nat Immunol*. (2016) 17:790–4. doi: 10.1038/ni.3459

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

ORIGINAL RESEARCH
published: 07 August 2019
doi: 10.3389/fimmu.2019.01849

Invariant NKT Cell-Mediated Modulation of ILC1s as a Tool for Mucosal Immune Intervention

Stephanie Trittel[1†], *Neha Vashist*[1,2†], *Thomas Ebensen*[1], *Benedict J. Chambers*[2], *Carlos A. Guzmán*[1‡] *and Peggy Riese*[1*‡]

[1] Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany, [2] Department of Medicine, Center for Infectious Medicine, Karolinska Institute, Karolinska University Hospital Huddinge, Stockholm, Sweden

Non-NK group 1 innate lymphoid cells (ILC1s), mainly investigated in the mucosal areas of the intestine, are well-known to contribute to anti-parasitic and anti-bacterial immune responses. Recently, our group revealed that lung ILC1s become activated during murine influenza infection, thereby contributing to viral clearance. In this context, worldwide seasonal influenza infections often result in severe disease outbreaks leading to high morbidity and mortality. Therefore, new immune interventions are urgently needed. In contrast to NK cells, the potential of non-NK ILC1s to become functionally tailored by immune modulators to contribute to the combat against mucosal-transmitted viral pathogens has not yet been addressed. The present study aimed at assessing the potential of ILC1s to become modulated by iNKT cells activated through the CD1d agonist αGalCerMPEG. Our results demonstrate an improved functional responsiveness of murine lung and splenic ILC1s following iNKT cell stimulation by the mucosal route, as demonstrated by enhanced surface expression of TNF-related apoptosis-inducing ligand (TRAIL), CD49a and CD28, and increased secretion of IFNγ. Interestingly, iNKT cell stimulation also induced the expression of the immune checkpoint molecules GITR and CTLA-4, which represent crucial points of action for immune regulation. An *in vivo* influenza infection model revealed that intranasal activation of ILC1s by αGalCerMPEG contributed to increased viral clearance as shown by reduced viral loads in the lungs. The findings that ILC1s can become modulated by mucosally activated iNKT cells in a beneficial manner emphasize their up to now underestimated potential and renders them to be considered as targets for novel immune interventions.

Keywords: ILC1, iNKT cell, αGalCerMPEG, intra nasal, influenza virus, modulation

OPEN ACCESS

Edited by:
Joanna Kirman,
University of Otago, New Zealand

Reviewed by:
Jochen Mattner,
University of Erlangen Nuremberg, Germany
Ian Hermans,
Victoria University of Wellington, New Zealand

***Correspondence:**
Peggy Riese
peggy.riese@helmholtz-hzi.de

[†]*Shared first authorship*

[‡]*Shared last authorship*

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

Received: *13 December 2018*
Accepted: *23 July 2019*
Published: *07 August 2019*

Citation:
Trittel S, Vashist N, Ebensen T, Chambers BJ, Guzmán CA and Riese P (2019) Invariant NKT Cell-Mediated Modulation of ILC1s as a Tool for Mucosal Immune Intervention. Front. Immunol. 10:1849. doi: 10.3389/fimmu.2019.01849

INTRODUCTION

The recent discovery of innate lymphoid cells (ILCs) expanded the pool of heterogenic innate immune cell populations. ILCs are classified into group 1, 2, and 3 ILCs according to the expression of the transcription factors T-bet, GATA-3, and RORγt, respectively. They can rapidly secrete diverse cytokines upon stimulation and were shown to represent the innate counterparts of T_H1, T_H2 and T_H17 cells (1–3).

Group 1 ILCs encompass conventional NK cells as well as non-NK cells, named ILC1s. In contrast to NK cells, ILC1s express the surface receptor CD127, but lack the expression of the transcription factor Eomes (4, 5). The cytotoxic activity of ILC1s is much less prominent and mainly mediated via the expression of the TNF-related apoptosis-inducing ligand (TRAIL), whereas the killing of viral or transformed cells via inherent granule-mediated cytotoxicity is a main functional feature of NK cells next to cytokine secretion. With regard to the cytokine profile secreted upon stimulation, ILC1s primarily produce IFNγ and TNFα, similar to NK cells. Thus, ILCs expressing CD127 and T-bet but not Eomes and displaying weak cytotoxicity are considered as ILC1s.

ILCs were mainly found to be enriched at mucosal sites, where they contribute to the first line of host defense as well as to tissue homeostasis (6–8). ILC1s are also present in all secondary lymphoid tissues, albeit at very low frequencies (9). With regard to protective immunity, ILC1s were already shown to contribute to combat against bacterial as well as parasitic infections, and they were described in association with cytokine-mediated inflammation (10–13). Their impact in viral infection is less well-addressed and currently under investigation. Here, ILC1s are considered to be involved in anti-viral immunity against HIV and chronic hepatitis B (14, 15). Murine studies also suggest a role of ILC1s in cytomegalovirus and adenovirus infections (16–18). The beneficial impact of ILC1s during mucosally transmitted influenza infection as well as the importance of their cross-talk with other immune cells was recently reported by our group. In this context, differential expression of the immune checkpoint molecule GITR was shown to influence ILC1 functionality. IFNγ-secreting ILC1s displayed a differential GITR expression profile. ILC1s over-expressing GITR rather showed diminished IFNγ production. These observations were confirmed in an influenza infection model in which GITR-blocking could reverse ILC1 functionality (19). In general, GITR is described to co-stimulate T cell functionality as well as NK cell cytotoxicity (20, 21). However, its overexpression leads to ILC1 regulation in the course of influenza infection. These findings highlight its complex and differential effects depending on the cell type and immunological setting. Besides GITR, CTLA-4 represents a prominent immune checkpoint molecule that might prove useful for ILC1 modulation. CTLA-4 was shown to harbor a regulatory impact on T cells and was also described to inhibit IFNγ secretion by NK cells in response to mature DCs (22, 23). These findings also suggest an implication in ILC1 functionality.

The described features render ILC1s interesting targets for mucosal immune interventions. The development of novel formulations that act directly at the port of pathogen entry, such as influenza, are moving into the focus of vaccinology. However, despite the advances made in the field of adjuvant research, there is still a strong need for the development of mucosal immune modulators suitable for implementation in vaccine formulations. In this regard, a pegylated derivative of α-galactosylceramide (αGalCerMPEG) was demonstrated to exhibited potent mucosal adjuvant properties (24, 25). The glycolipid antigens, αGalCer and αGalCerMPEG are presented by APCs via the non-classical MHC class I molecule CD1d and recognized by invariant NKT (iNKT) cells. Upon activation, iNKT cells secrete a variety of cytokines that initiate down-stream activation of other innate and adaptive immune cells (26–28). Its strong modulating impact on inherent NK cell features upon subcutaneous (s.c.) administration further points to a possible impact on ILC1 features. However, the capacity of ILC1s to become modulated and their emerging impact on anti-viral mucosal immunity is still elusive and was therefore the aim of the present study.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2b) female mice aged 8–10 weeks were purchased from Harlan Winkelmann GmbH (now Envigo, Borchen, Germany). Jα281$^{-/-}$ and RAG2$^{-/-}$ mice were bred at the animal facility of the Helmholtz Center for Infection Research (HZI), Braunschweig. Mice were treated according to local and European community guidelines. They were housed under pathogen-free conditions in individual ventilated cages with food and water *ad-libitum*. The performed animal experiments were approved by the local government in Braunschweig (Germany) under the animal permission codes AZ: 33.42502-04-13/1281 and AZ: 33.19-42502-04-16/2280.

Administration of αGalCerMPEG

The glycolipid αGalCer was pegylated at the HZI according to the published protocols (24). Briefly, αGalCer was mixed with methyl-PEG-COOH and the resulting αGalCerMPEG was purified by silica gel chromatography. Its purity was assessed by HPLC. Mice were administered with a single dose of αGalCerMPEG (5 μg (=1.7 nmol) diluted in PBS) via the intranasal (i.n., 20 μl) or the subcutaneous (s.c., 50 μl) route. For the i.n. administration, mice were briefly anesthetized by inhalation anesthesia.

Influenza Strains and Infection

The mouse-adapted influenza A/PR/8/34 (H1N1 PR8) strain was kindly provided by Dr. Paulina Blazejewska and Dr. Klaus Schughart (HZI). Mice were infected with a single dose containing $2*10^3$ foci forming units (ffu)/animal of H1N1 diluted in 20 μl PBS via the i.n. route. For this, mice were anesthetized by intra-peritoneal (i.p.) injection of ketamine/Xylazin (1 mg ketamine/0.1 mg Xylazin per 10 g body weight). The body weight was monitored on a daily basis.

Sample Collection

Mice were euthanized and lungs, spleens and draining lymph nodes (dLNs, cervical and mediastinal) were collected and separately mashed through a 100 μm nylon strainer. Spleen and dLN homogenates were subjected to erythrocyte lysis. Lung homogenates were subsequently incubated at 37°C in 5% FCS RPMI 1640 (Life technologies, UK) containing 0.2 mg/ml collagenase D (Roche, Germany) and 20 μg/ml DNase I (Roche, Germany). Lymphocyte suspensions were purified by density gradient centrifugation (Easycoll, Biochrome GmbH, Germany). Lung and spleen lymphocytes were incubated in medium containing brefeldin A (5 μg/ml) and monensin (6 μg/ml) for

3 h at 37°C. Blood samples were collected via the retro-orbital plexus. Serum was isolated upon centrifugation and stored at −80°C until further use. Bronchoalveolar lavage (BAL) samples were collected by two intratracheal washes with 1 ml 5% FCS PBS. Serum and BAL samples were subsequently analyzed by cytometric bead array according to the manufacturer's protocol (Affymetrix/eBioscience).

Foci Assay

The viral burden of infected mice was assessed using lung homogenates that were prepared on ice in PBS supplemented with 0.1% BSA using the Polytron 2100 homogenizer (4,000 rpm-−20 s/sample). The supernatant, cleared by centrifugation, was stored at −80°C until further use. The assay was performed as described previously (19). Briefly, serial dilutions of the lung homogenates were incubated with MDCK cells and the influenza nucleocapsid was detected by ELISA. Foci were counted under a microscope and viral titers were calculated as ffu/ml.

Flow Cytometry Analysis

The prepared single cell suspensions were subjected to flow cytometry. Upon incubation with Fc-block (CD16/CD32, 2.4G2, Fc block, BD Biosciences), cells were stained for surface markers and subsequently fixed and permeabilized for intracellular and intranuclear staining steps according to the manufacturer's protocol (BD Bioscience, USA/Foxp3 staining kit, eBioscience, USA). The samples were processed on a FACS LSR II and Fortessa (BD Bioscience, USA) and the subsequent analysis was performed using the FlowJo software (TreeStar Inc.). ILC1s were identified according to the gating strategy published recently [(19) and **Figure S1**]. Briefly, living single lineage negative NKp46$^+$ lymphocytes were gated on CD90$^+$CD127$^+$ cells and ILC1s were identified by the expression of T-bet and the lack of Eomes expression. The following antibodies were used: LIVE/DEAD Fixable Blue Dead cell stain kit (UV excitation, Invitrogen, USA), CD90.2 (53.2-1, BV785, Biolegend), CD127 (A7R34, PE-Cy5/biotin, Biolegend), NKp46 (29A1.4, A700, BD Bioscience/19A1.4, APC, eBioscience), CD3/CD19/Gr1/Ter-119 (17A2/6D5/RB6-8C5/TER-119, BV421, Biolegend), TRAIL (N2B2, biotin–streptavidin BV650, Biolegend), GITR (YGITR765, PE, Biolegend), CD28 (37.51, PerCP-Cy5.5, Biolegend), CTLA-4 (UC10-489, BV605, Biolegend; stained for surface and intracellular expression simultaneously), CD49a (Ha31/8, BV510, BD Bioscience), CD11c (N418, PE-Cy7, Biolegend), CD11b (M1/70, BV605, Biolegend), B220 (RA3-6B2, PE-Cy5, Biolegend), CD80 (16- 10A1, BV421, Biolegend), CD86 (GL-1, BV650, Biolegend), IFNγ (XMG1.2, BV711, Biolegend), T-bet (4B10, PE-Cy7, Biolegend), Eomes (Dan11mag, FITC, eBioscience), RORγt (AFKJS-9, APC, eBioscience). The t-distributed stochastic neighbor embedding (tSNE) analysis was performed using the FlowJo software (Version 10.5.3, TreeStar Inc.).

In vitro Models

For assessing the impact of αGalCerMPEG on ILC1 activation *in vitro*, αGalCerMPEG-loaded bone marrow derived dendritic cells (BMDCs), sorted splenic NKT cells (B220$^-$ CD11c$^-$ NK1.1$^+$ CD4$^+$ CD8$^+$ cells, sorted using a FACS Aria II cell sorter) and *in vitro*-generated ILC1s were co-cultured. ILC1s were generated from RAG2$^{-/-}$ bone marrow cells *in vitro* as described earlier (19). NKT cells were sorted on a FACS Aria II cell sorter using the following antibodies: CD4 (GK1.5, FITC, eBioscience), CD8 (53–6.7, FITC, BD), NK1.1 (PK136, PE-Cy7, eBioscience), B220 (RA3–6B2, Pacific Blue, BioLegend), CD11c (N418, PB, BioLegend). BMDCs were generated as previously described (19). Briefly, bone marrow cells were incubated in the presence of 100 ng/ml FLT-3 ligand (Peprotech, USA) for 7–8 days. For co-culture studies these BMDCs were primed overnight with 300 ng/ml αGalCerMPEG and subsequently co-cultured in complete media supplemented with 300 ng/ml of αGalCerMPEG overnight at a ratio of 6:6:1. To address the *in vitro* impact of αGalCerMPEG on ILC1s in the context of H1N1 infection, the co-culture was set up with H1N1-infected BMDCs. For this, BMDCs were infected for 1 h with the wild type mouse-adapted H1N1 PR8 strain at a multiplicity of infection (MOI) of 1. The BMDCs were subsequently cultured for 5 h at 37°C with 5% CO_2. After 6 h, the BMDCs were harvested, washed, counted, and used in the described co-culture experiments. For all co-culture settings, brefeldin A (5 μg/ml) and monensin (6 μg/ml) were added for the last 3 h of incubation. The phenotypic and functional analysis of the cells was performed by flow cytometry as described above.

Statistical Analysis

The data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, USA). Independent groups were statistically compared using Mann-Whitney test and for the comparison of multiple groups, the One-way ANOVA statistical analysis was applied. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Intranasal Administration of αGalCerMPEG Induces an ILC1 Activating Cytokine Milieu

The previous finding that ILC1s contribute to the clearance of H1N1 infection via cross-talk with other immune cells renders them an interesting target for mucosal immune modulation (19). Thus, the impact of the iNKT cell-activating CD1d agonist αGalCerMPEG on ILC1 functionality was investigated. Wild type mice received a single dose of αGalCerMPEG (5 μg/animal) by i.n. route to gain insight into its impact on the mucosal and systemic cytokine environment. At different time points, serum and BAL samples were analyzed with regards to changes in the concentration of various cytokines known to impact adaptive as well as innate immune cell populations. After 6 h an increased expression of IL-4 was detected in serum as well as BAL samples, peaking 12 h after administration. In serum and BAL samples, αGalCerMPEG administration induced increased levels of IL-12 as early as 12 and 6 h after treatment, respectively. After 12 and 24 h enhanced

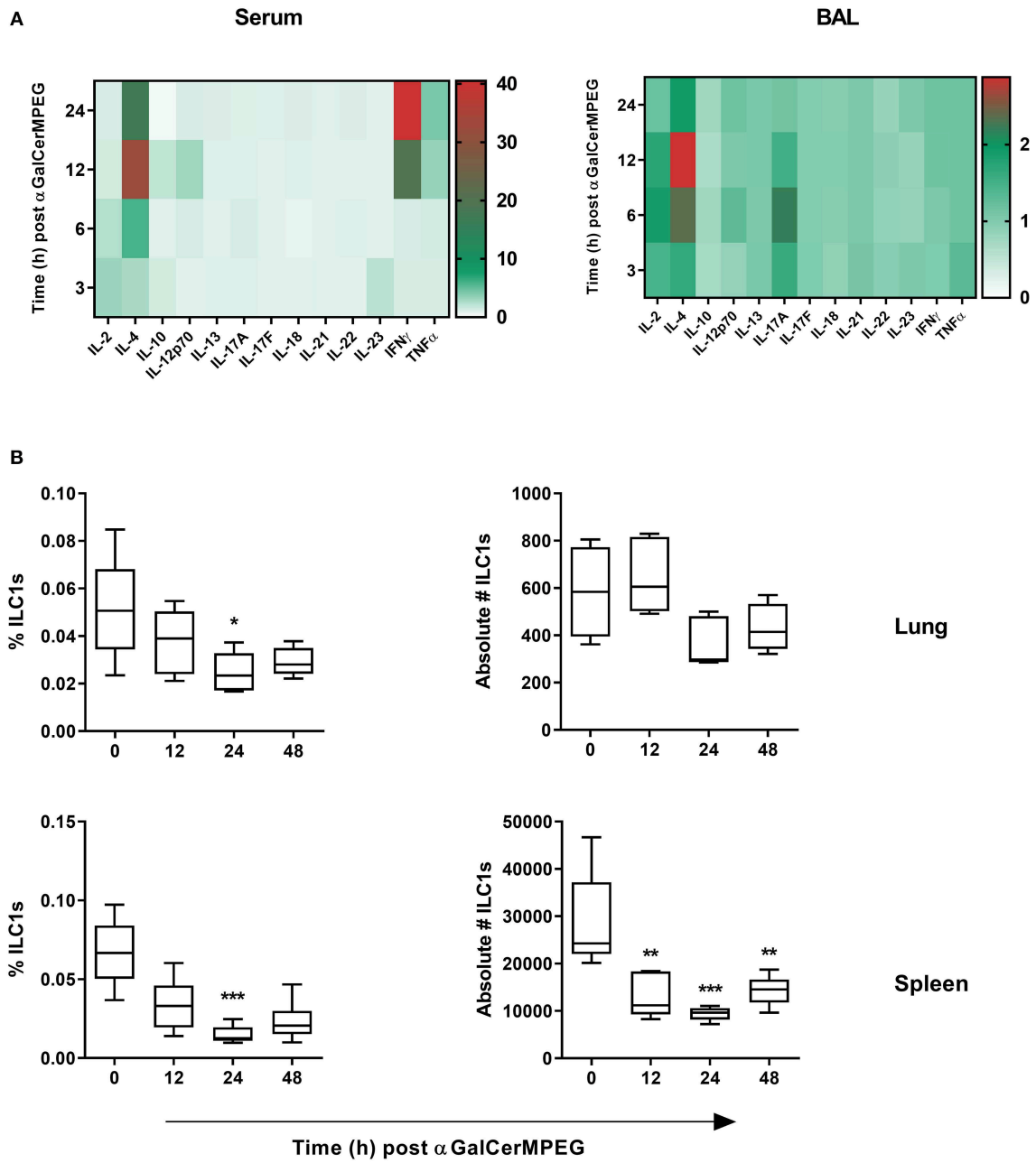

FIGURE 1 | αGalCerMPEG administration induces enhanced secretion of ILC1 stimulating cytokines and reduced frequencies and absolute numbers of ILC1s. Wild type mice were administered i.n. with a single dose of αGalCerMPEG (5 μg). Sera and BAL samples were collected at the indicated time points post-administration and evaluated for their cytokine profile by CBA. **(A)** Fold change of MFI of cytokines detected in sera and BAL as compared to untreated controls. The shown data are derived from one experiment ($n = 4$). Lung and splenic lymphocytes were stained for ILC1s ($CD3^-$, $CD19^-$, $Gr1^-$, $Ter119^-$, $CD90.2^+$, $CD127^+$, $NKp46^+$, T-bet^+, and $Eomes^-$) to perform flow cytometry analysis. **(B)** Frequencies (of total cells) and absolute numbers of lung and splenic ILC1s. Box plots represent the range in frequency and absolute number variation with the horizontal line indicating the mean. MFI and frequency data are representative from one out of two independent experiments (each with $n = 4$–5). Asterisks denote significant values calculated by One-way ANOVA as compared to untreated samples; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

IFNγ levels were observed in serum but not in BAL samples (**Figure 1A**).

To investigate whether αGalCerMPEG-induced changes in the cytokine milieu influence ILC1s, lung and spleen derived lymphocytes were analyzed regarding their frequencies and absolute numbers by flow cytometry (**Figure S1**). After 24 h significantly decreased frequencies of splenic and lung-derived ILC1s were observed (**Figure 1B**). The absolute cell numbers

of lung ILC1s were marginally decreased 24 and 48 h post-administration, whereas splenic ILC1 numbers were significantly decreased at all investigated time points after αGalCerMPEG administration. The obtained data show that the administration of αGalCerMPEG supports the generation of an ILC1-activating cytokine environment while resulting in reduced frequencies and absolute numbers of ILC1s at both local and systemic levels.

Intranasal Administration of αGalCerMPEG Results in Enhanced ILC1 Activation

The observed impact of αGalCerMPEG on ILC1 frequencies and numbers prompted the functional evaluation of lung and splenic ILC1s. Therefore, following i.n administration of αGalCerMPEG, surface markers known to be expressed following ILC1 activation were evaluated at different time points. A significantly enhanced expression of TRAIL on lung ILC1s was observed 24 and 48 h after administration, whereas splenic ILC1 showed a significantly elevated expression at all analyzed time points (**Figure 2A**). The administration of αGalCerMPEG resulted in elevated expression of CD49a and CD28 after 24 and 48 h in both organs as compared to untreated controls (**Figure 2A**). The analysis of IFNγ secretion revealed significantly increased expression densities (MFI) as well as frequencies in the lung after 12 and 24 h (**Figure 2B**). The frequency of splenic IFNγ-secreting ILC1s was enhanced 24 and 48 h after administration of αGalCerMPEG and significantly increased expression densities were detected at all analyzed time points. These results demonstrate that ILC1s at mucosal as well as lymphoid tissues can be phenotypically and functionally modulated by administration of the glycolipid αGalCerMPEG. The stimulation of iNKT cells via the s.c. route resulted in a similar activation of lung and splenic ILC1s as observed for the i.n. route (**Figure S2**).

The iNKT cell-dependent activation of different populations of the innate (e.g., NK cell and DCs) as well as the adaptive (e.g., $CD4^+$ and $CD8^+$ T cells) immune system by αGalCerMPEG was already shown Ebensen et al. (24). However, the importance of iNKT cells for the observed αGalCerMPEG-induced ILC1 activation is still elusive. Therefore, iNKT cell-deficient mice ($J\alpha281^{-/-}$) received αGalCerMPEG by i.n. route and the secretion of IFNγ by ILC1s was assessed. The lack of iNKT cells completely abolished αGalCerMPEG-induced enhanced IFNγ expression and the increased frequencies of $IFN\gamma^+$ ILC1s observed in the lungs and spleen of wild type mice (**Figure 2C** and **Figure S3**). These findings clearly show the iNKT cell dependency of the detected αGalCerMPEG-induced ILC1 activation after mucosal application.

αGalCerMPEG-Activated iNKT Cells Stimulate $GITR^+$ But Not $GITR^-$ ILC1s

We recently showed in a murine influenza infection model that the differential expression of GITR, a stimulatory immune checkpoint molecule, can influence the activation status of ILC1s. Here, we assessed whether the level of GITR expression by ILC1s and subsequently their functional responsiveness can be modulated by i.n. administered αGalCerMPEG. Significantly enhanced expression densities of GITR on lung ILC1s were observed 24 and 48 h after iNKT cell stimulation (**Figure 3A**). The increase in GITR expression was accompanied by elevated IFNγ secretion by $GITR^+$ but not $GITR^-$ ILC1s, which peaked 24 h after iNKT cell stimulation. For splenic ILC1s, increased expression levels of GITR were detected 12 and 24 h after i.n. αGalCerMPEG administration. Similar to lung ILC1s, splenic $GITR^+$ ILC1s showed significantly enhanced IFNγ secretion after 12 h whereas no changes were observed for $GITR^-$ ILC1s as compared to untreated controls (**Figure 3B**). The obtained finding reveals αGalCerMPEG-induced changes on the GITR expression level and enhanced cytokine secretion of $GITR^+$ ILC1 subsets.

ILC1 Stimulation by αGalCerMPEG Is Characterized by an Enhanced CTLA-4 Expression

Next to GITR, CTLA-4 represents an interesting stimulatory immune checkpoint molecule which is described to control T cell and NK cell activation. Thus, surface and intracellular CTLA-4 expression by lung and splenic ILC1s following i.n. administration of αGalCerMPEG was assessed. The stimulation of iNKT cells resulted in enhanced frequencies of lung $CTLA\text{-}4^+$ ILC1s as well as significantly increased expression of CTLA-4 after 48 and 84 h as well as 84 h, respectively (**Figure 4A**). In contrast, splenic ILC1s did not show meaningful changes in frequency or expression density with regard to CTLA-4. The αGalCerMPEG-induced up-regulation of CTLA-4 *in vivo* was further confirmed in an *in vitro* co-culture of BMDCs, sorted NKT cells and *in vitro*-generated ILC1s (**Figure S4A**).

Subsequently, $CLTA\text{-}4^+$ and $CTLA\text{-}4^-$ lung ILC1s were compared with regard to their surface expression of activation markers as well as the secretion of cytokines to assess the impact of CTLA-4 expression on ILC1 functionality. Strikingly, significantly enhanced expression densities of TRAIL and IFNγ were observed after 48, and 48 as well as 84 h, respectively, which correlate with an elevated expression of CTLA-4 (**Figure 4B**). Furthermore, enhanced GITR expression was observed in the $CTLA\text{-}4^+$ but not $CTLA\text{-}4^-$ ILC1 subsets 84 h after αGalCerMPEG administration. The expression of CD28 was not associated with CTLA-4 expression. These results demonstrate that αGalCerMPEG-mediated activation of ILC1s induces the expression of CTLA-4 on ILC1s. In addition, the observed correlation of CTLA-4 with enhanced expression of the activation markers TRAIL and GITR, and the secretion of IFNγ reveals that only activated lung ILC1s express CTLA-4. These findings were consistent with conducted *in vitro* co-culture experiments of BMDCs, sorted NKT cells and *in vitro* generated ILC1s, in which the αGalCerMPEG-induced secretion of IFNγ was restricted to the $CTLA\text{-}4^+$ ILC1 subset (**Figures S4A,B**).

Subsequently, it was investigated whether αGalCerMPEG induces changes in the expression patterns of CTLA-4 ligands on DCs. To this end, cells isolated from dLNs (mediastinal and cervical) of αGalCerMPEG-treated mice were analyzed with regard to the frequencies of different DC subsets and the expression of the CTLA-4 ligands CD80 and CD86. Significantly increased frequencies of pDCs were observed 48 h

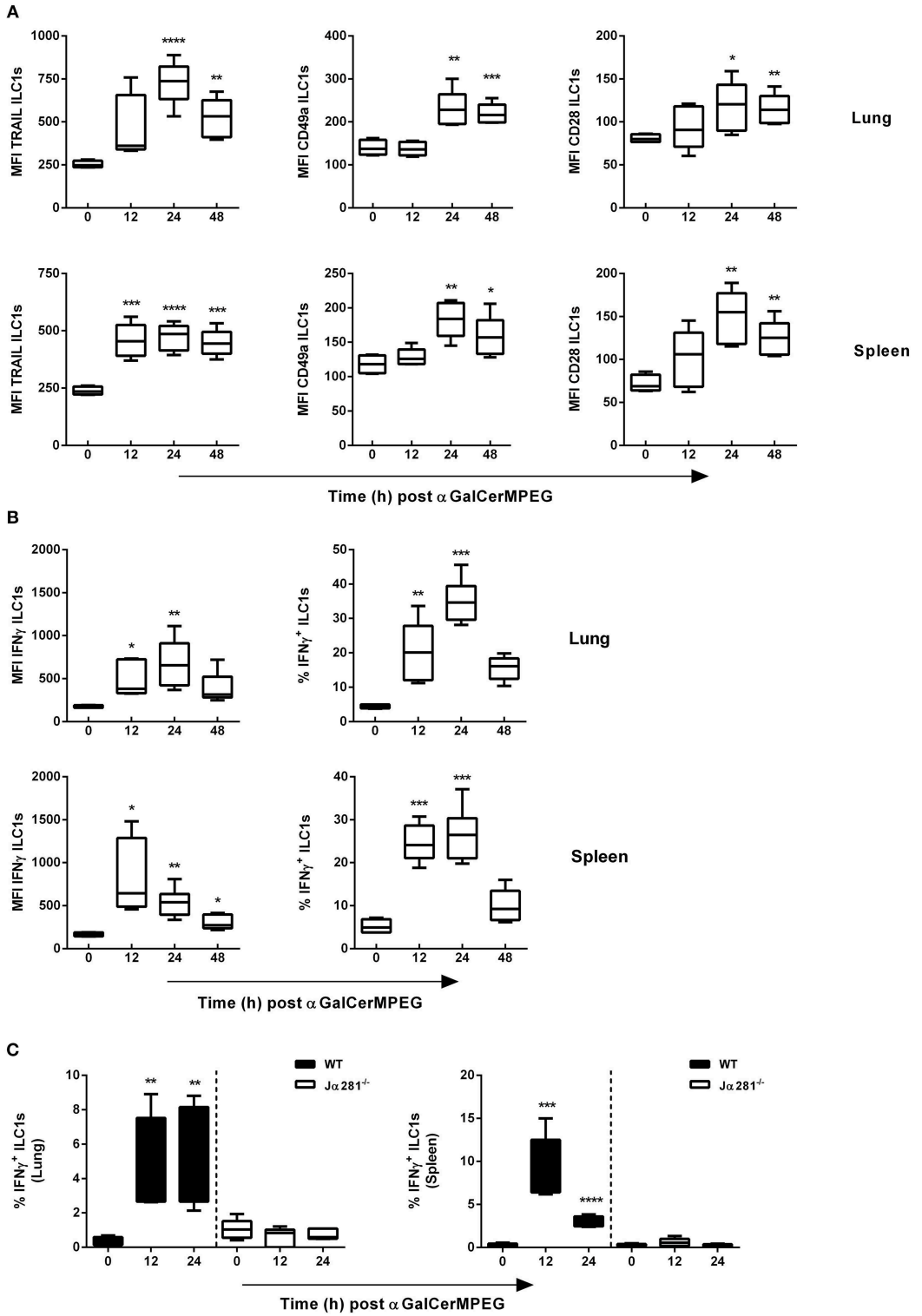

FIGURE 2 | αGalCerMPEG treatment results in activation of ILC1s. Wild type (WT) and Jα281$^{-/-}$ mice received a single dose of αGalCerMPEG (5 μg) by i.n. route. Subsequently, lung and splenic lymphocytes were stained for the surface activation markers TRAIL, CD49a and CD28 as well as IFNγ, and analyzed by flow cytometry upon 3 h incubation in the presence of monensin and brefeldin. **(A)** MFI of lung and splenic ILC1s expressing TRAIL, CD49a, and CD28. **(B)** MFI and frequency (of ILC1s) of IFNγ production by lung and splenic ILC1s. **(C)** Frequencies (of ILC1s) of lung and splenic IFNγ^{+} ILC1s derived from wild type and Jα281$^{-/-}$ mice. Box plots represent the range in MFI variation with the horizontal line indicating the mean. MFI data are representative from one out of two independent experiments (each with $n = 4$–6) for **(A)** and **(B)** and from one experiment ($n = 4$–6) for **(C)**. Asterisks denote significant values calculated by One-way ANOVA as compared with untreated samples; ****$p \leq 0.0001$; ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$.

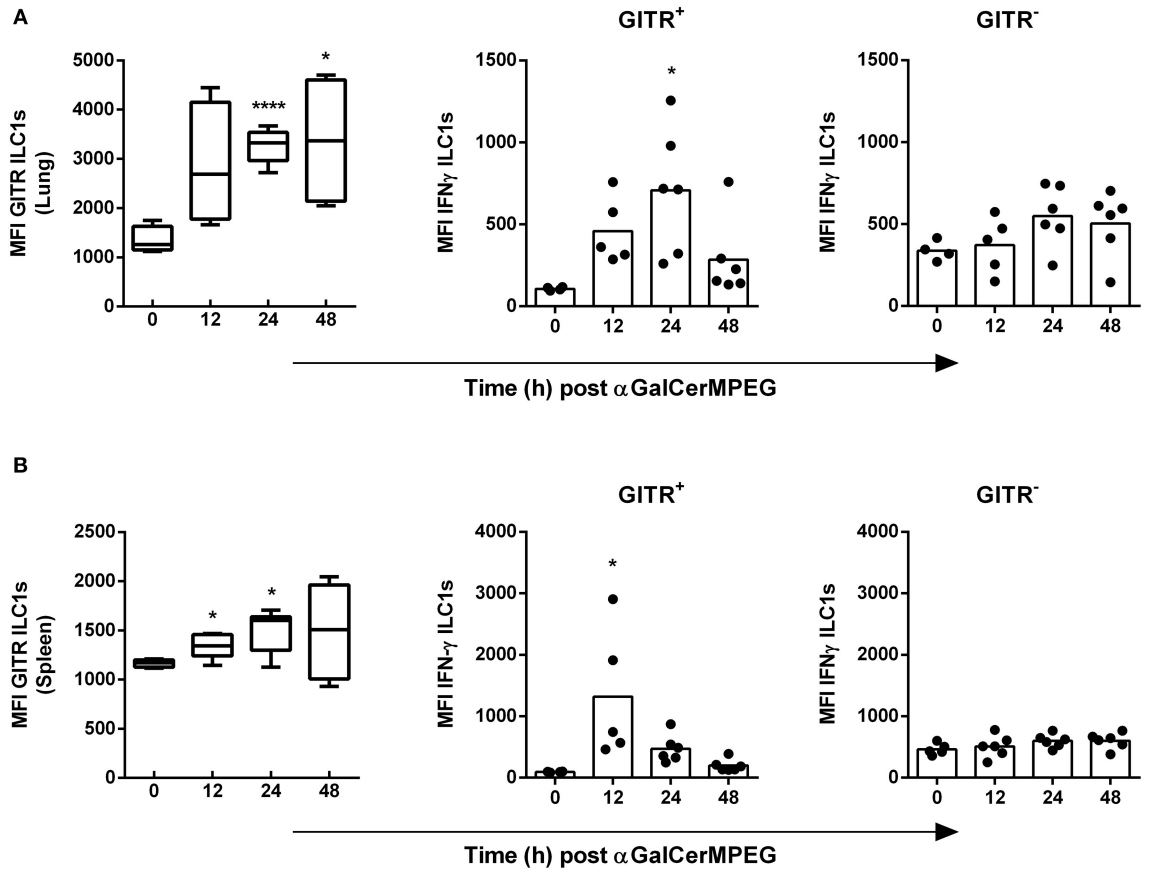

FIGURE 3 | Enhanced IFNγ secretion by GITR$^+$ ILC1s following i.n. treatment with αGalCerMPEG. Wild type mice were administered i.n. with a single dose of αGalCerMPEG (5 μg). Lung and splenic lymphocytes were stained for GITR and IFNγ expression by ILC1s and subjected to flow cytometry analysis. MFI of GITR on ILC1s and MFI of IFNγ by GITR$^+$ and GITR$^-$ ILC1s derived from **(A)** lung and **(B)** spleen. Box plots represent the range in MFI with the horizontal line indicating the mean. MFI data are representative from one out of two independent experiments (each with $n = 4$–6). Asterisks denote significant values calculated by One-way ANOVA and compared with untreated samples; $^{****}p \leq 0.0001$*; $p \leq 0.05$.

after treatment (**Figure 4C**). Furthermore, iNKT cell activation induced enhanced expression of the CTLA-4 ligand CD86 24 and 48 h later, whereas no impact on CD80 was observed. Interestingly, the frequency of cDCs was not affected by αGalCerMPEG, but the expression of CD86 by cDCs was significantly increased after 48 h. The assessment of pulmonary DCs (MHC cl. II$^+$CD11c$^+$) revealed significantly enhanced frequencies as well as marginally increased expression of CD80 and CD86 upon i.n. administration of αGalCerMPEG (**Figure S4C**). The obtained data suggest an interaction of ILC1s with different DC subsets via the ligation of CTLA-4 to their respective ligands upon αGalCerMPEG-stimulation of iNKT cells.

Stimulation of iNKT Cells Activates ILC1s in the Course of Influenza Infection

Our group showed that ILC1s contribute to the clearance of influenza infections. Therefore, the observed phenotypic and functional modulation of ILC1s by αGalCerMPEG-activated iNKT cells raised the question of whether this approach can be used to improve protective immunity against influenza infections. In order to address this point, mice which received αGalCerMPEG i.n. and were infected 12 h later with influenza by the natural i.n. route. Mice treated with αGalCerMPEG as well as untreated but infected mice showed a body weight reduction starting from 2 to 3 days post-infection, respectively (**Figure 5A**). The viral burden in the lungs assessed 3 days post-infection revealed that pre-activation of iNKT cells caused a significant reduction of lung viral titers as compared to untreated influenza-infected mice (**Figure 5B**). These findings demonstrate that αGalCerMPEG boosts protective immune responses toward influenza infection. In order to assess the impact of αGalCerMPEG on ILC1s in the course of influenza infection, changes in their phenotype and functional responsiveness were analyzed. Flow cytometry analysis of lung- and spleen-derived lymphocytes was carried out 3 days post-infection. Influenza infection alone resulted in reduced frequencies of lung ILC1s which was further strengthened by prior iNKT cell activation. The absolute number of lung ILC1s

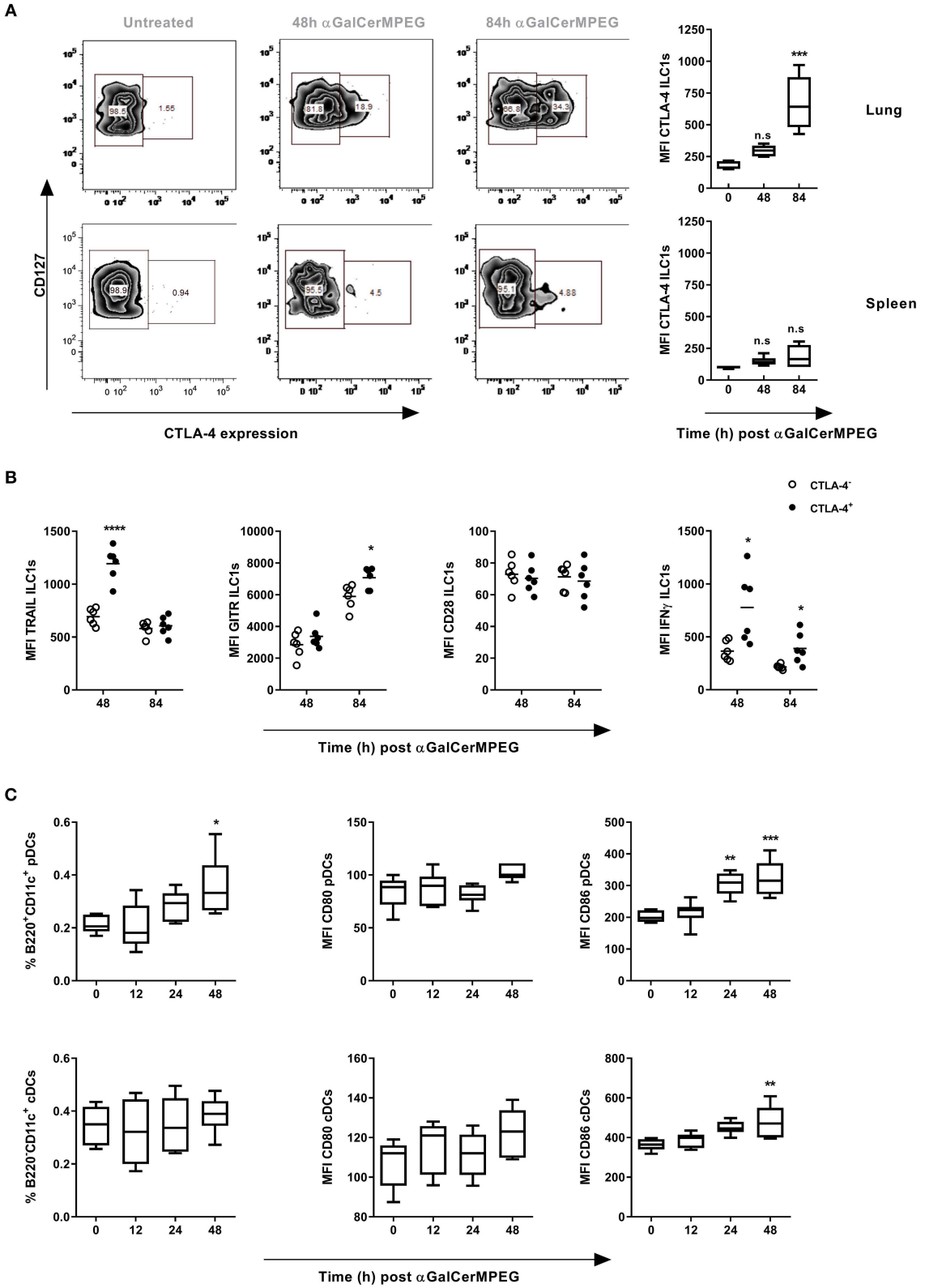

FIGURE 4 | αGalCerMPEG-induced enhanced functionality of lung ILC1s is associated with CTLA-4 expression. Wild type mice were treated i.n. with a single dose of αGalCerMPEG (5 μg). Lung and splenic lymphocytes were isolated 48 and 84 h after administration and stained for ILC1 expression of CTLA-4 and activation markers (TRAIL, GITR, CD28, IFNγ) for the flow cytometric analysis upon 3 h of incubation in the presence of monensin and brefeldin. **(A)** Representative dot plots

(Continued)

FIGURE 4 | show the expression of CTLA-4 on lung and splenic ILC1s. Diagrams show the expression density (MFI) of CTLA-4 by lung and splenic ILC1s. **(B)** MFI of TRAIL, GITR, CD28, and IFNγ by lung CTLA-4$^+$ and CTLA-4$^-$ ILC1s. DCs from dLNs (mediastinal and cervical) were processed for flow cytometry analysis and stained for markers related to pDC (B220$^+$CD11c$^+$) and cDC (B220$^-$CD11c$^+$) identification and maturation. **(C)** Frequencies (of living singlet cells) of DC subsets and MFI of CD80 and CD86. Box plots represent the range in MFI with the horizontal line indicating the mean. Scatter plots represent the range in MFI with the horizontal line indicating the mean. MFI and frequencies from one experiment are shown ($n = 4$–6). Asterisks denote significant values calculated by One-way ANOVA as compared to untreated samples **(A)** and **(C)** or by unpaired, two-tailed Student's *t*-test or comparing CTLA-4$^-$ and CTLA-4$^+$ **(B)**; $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

was not affected as compared to uninfected controls, but was significantly increased upon pre-treatment with αGalCerMPEG as compared to infected untreated mice (**Figure 5C**).

A significant drop in ILC1 frequencies and absolute numbers was detected in the spleen after infection as compared to uninfected controls (**Figure S5A**). Pre-treatment with αGalCerMPEG prior to infection could reverse this effect and resulted in significantly increased numbers of splenic ILC1s as compared to untreated but infected groups.

These results show that stimulated iNKT cells impact ILC1s and thus prompted the evaluation of a functional ILC1 modulation in the course of influenza infection. A significantly up-regulated expression of TRAIL and CD49a was observed on lung ILC1s derived from pre-treated influenza-infected mice as compared to the untreated infected and uninfected control groups (**Figure 5D**). For splenic ILC1s, increased expression densities of TRAIL and CD49a in the αGalCerMPEG-pre-treated infected group were observed as compared to the non-treated infected group (**Figure S5B**). Significantly enhanced GITR expression was also detected on splenic ILC1s upon influenza infection, whereas in the lung only minor changes were observed (**Figure 5D** and **Figure S5B**). The pre-treatment with αGalCerMPEG, however, resulted in an additionally increased GITR expression on ILC1s in both organs. With regard to CD28, an enhanced expression was observed on lung ILC1s derived from αGalCerMPEG pre-treated infected mice as compared to the uninfected as well as infected but untreated groups (**Figure 5D**). With regard to splenic ILC1s, influenza infection alone resulted in an up-regulated expression of CD28 that was not further boosted by the pre-activation of iNKT cells (**Figure S5B**).

These data show that αGalCerMPEG administration significantly alters the phenotype of ILC1s in the course of infection locally, at the site of administration and infection, as well as at systemic level.

In order to assess whether the phenotypic activation is accompanied by functional changes, lung and splenic ILC1s were subsequently assessed for their cytokine secretion capacity 3 days post-infection. The expression density of IFNγ by lung and splenic ILC1s was not affected by the infection alone as compared to uninfected controls. Pre-treatment with αGalCerMPEG significantly boosted the IFNγ expression density of lung and splenic ILC1s as compared to uninfected or untreated infected controls (**Figure 5E** and **Figure S5C**). The frequencies of IFNγ$^+$ lung ILC1s were significantly elevated upon infection, whereas splenic IFNγ$^+$ ILC1s were only marginally increased (**Figure 5E** and **Figure S5C**). The pre-activation of iNKT cells by αGalCerMPEG prior to the infection resulted in even higher frequencies of IFNγ$^+$ lung ILC1s, whereas only a minor additional effect was observed for spleen-derived ILC1s. The presented data prove that αGalCerMPEG can modify both local and systemic ILC1 responses during influenza infection. Furthermore, the data clearly demonstrate the capacity of αGalCerMPEG for boosting and fine-tuning ILC1 functionality in the course of an H1N1 influenza infection.

αGalCerMPEG-Activated ILC1s Expressing CTLA-4 Display Increased Functionality in Course of Influenza Infection

The observed impact of activated iNKT cells on the phenotype and functionality of lung and splenic ILC1s as well as the implication of CTLA-4 expression for ILC1 responsiveness also raised the question of whether CTLA-4 expression by ILC1s is affected in the course of influenza infection. To approach this assumption, the expression of CTLA-4 and IFNγ by ILC1s was assessed in an *in vitro* co-culture system comprising H1N1-infected BMDCs, NKT cells and *in vitro* generated ILC1s as previously described (19). A significantly enhanced expression density of CTLA-4 and IFNγ by ILC1s was observed when NKT cells and ILC1s were co-cultured with influenza-infected BMDCs in the presence of αGalCerMPEG as compared to co-cultures of H1N1-infected BMDCs in the absence of NKT cell stimulation or uninfected BMDCs and unstimulated NKT cells (**Figure 6A**). The functional analysis of ILC1s with regard to CTLA expression identified CTLA-4$^+$ ILC1s as the main producers of IFNγ in this *in vitro* setting.

In vivo, the i.n. administration of αGalCerMPEG resulted in an up-regulation of CTLA-4 on lung ILC1s. A beneficial role of memory T cell responses in the course of influenza infection was recently ascribed to the interaction of CTLA-4 with its corresponding ligands CD80/CD86 (29). Thus, to assess the impact of H1N1 infection on CTLA-4 expression, lung ILC1s were analyzed 3 days post-infection. Influenza infection alone did not impact the expression density or frequencies of CTLA-4$^+$ ILC1s as compared to uninfected controls, whereas prior iNKT cell stimulation by αGalCerMPEG induced significantly enhanced frequencies of CTLA-4$^+$ ILC1s as well as increased expression density levels (**Figures 6B,C**). To further assess the impact of CTLA-4 expression on ILC1 functionality, CTLA-4$^+$ and CTLA-4$^-$ ILC1s derived from αGalCerMPEG-treated H1N1-infected mice were analyzed 3 days post-infection. CTLA-4$^+$ lung ILC1s showed enhanced expression densities of TRAIL and GITR as compared to CTLA-4$^-$ ILC1s, whereas the expression of CD28 did not differ between both populations (**Figure 6D**). The production of IFNγ was marginally increased in the CTLA-4-expressing ILC1 subset.

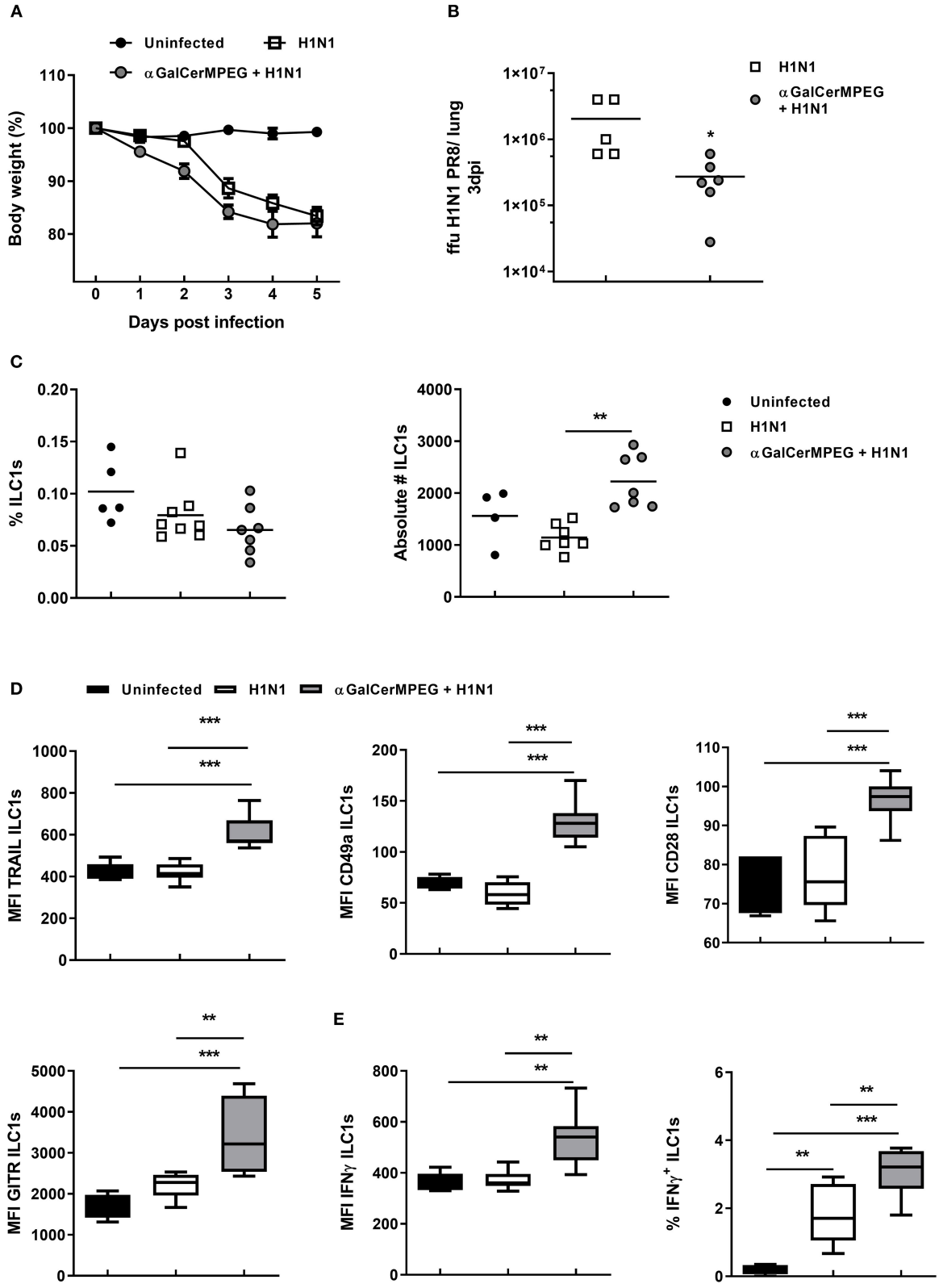

FIGURE 5 | H1N1 infection of αGalCerMPEG-treated mice induced ILC1 activation. Wild type mice were administered i.n. with a single dose of αGalCerMPEG (5 μg) 12 h prior to H1N1 infection ($2*10^3$ ffu). **(A)** Changes in body weight (%) post-infection. **(B)** Lung viral burden as determined 3 days post-infection by foci assay. Scatter plots represent viral loads as ffu per lung. Depicted data are representative from one out of two independent experiments (each with $n = 5–7$). Lung lymphocytes were

(Continued)

FIGURE 5 | isolated 3 days post-infection and analyzed for **(C)** frequencies and absolute numbers of ILC1s and **(D)** their expression density (MFI) of the activation markers TRAIL, CD49a, CD28, and GITR by flow cytometry analysis. Frequency and absolute cell number data are representative from one out of two independent experiments (each with $n = 5–7$). MFI data for the expression of the activation markers are shown from one experiment ($n = 5–7$). **(E)** Lung lymphocytes secreting IFNγ (MFI and frequencies) analyzed by flow cytometry following 3 h incubation in media with monensin and brefeldin. MFI and frequency data are representative from one out of two independent experiments (each with $n = 5–7$). Box plots represent the range in frequency variation as well as absolute cell number with the horizontal line indicating the mean. Asterisks denote significant values calculated by One-way ANOVA; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

These data confirm a link between the expression of CTLA-4 and the functionality of ILC1s upon mucosal stimulation with αGalCerMPEG in the course of influenza infection.

DISCUSSION

Our previously published studies demonstrated that ILC1s distinctly contribute to the clearance of influenza infection, partly by cross-talking with innate and adaptive immune cells (19). Here, an influenza-induced activation of ILC1s was shown by enhanced expression of different surface activation markers and secreted cytokines. These findings prompted the question of whether ILC1s can be modulated to promote anti-viral immunity. To address this issue, the impact of a pegylated derivative of the iNKT cell agonist αGalCer on ILC1 functionality was investigated. This compound was already shown to affect various innate and adaptive immune cells populations (24, 25, 30). In this context, αGalCerMPEG-activated iNKT cells were reported to modulate inherent NK cell features, thereby impacting anti-viral responses in a murine cytomegalovirus infection model (31). The close similarity of NK cells and ILC1s cells, together with the mucosal adjuvant properties of αGalCerMPEG (24, 32) suggest that it might also be a promising modulator of ILC1 functionality when administered by mucosal route.

The i.n. treatment of wild type mice with αGalCerMPEG resulted in enhanced levels of a variety of cytokines including the iNKT cell-characteristic cytokine IL-4 as well as IL-12 and IFNγ with differential patterns observed in serum and BAL samples. These findings indicate the promotion of T_H1 as well as T_H2 responses thus indicating a balanced adaptive immune response. Stimulation of iNKT cells with αGalCerMPEG resulted in the activation of ILC1s as shown by the increased expression of different activation markers as well as an enhanced cytokine production. Interestingly, this activation was not limited to the lung, but was also observed for splenic ILC1s. The observed increased expression of TRAIL correlates with an enhanced cytotoxic potential. In this regard, a previous report showed that stimulation of NKT cells with αGalCer induced an upregulated TRAIL expression by liver NK cells (33). Furthermore, TRAIL was reported to contribute to immunity against HIV, hepatitis, CMV and influenza amongst others (34, 35). The induction of TRAIL expression by ILC1s might thus directly contribute to anti-viral immunity. An enhanced activation status of ILC1s upon administration of αGalCerMPEG is further reflected by an elevated expression of CD49a. This is in line with earlier findings showing a correlation of enhanced CD49a expression and elevated functionality by T cells and NKT cells upon *in vivo* stimulation with concanavalin-A (36). CD49a was further described as a ligand implicated in retaining immune cells within tissues (37). Thus, besides its function as an activation marker, CD49a might promote the retention of activated ILC1s within the lung, thereby supporting local immune responses. CD28 expression was also found to be enhanced on ILC1s following αGalCerMPEG stimulation by the mucosal route. CD28 was reported to induce the proliferation of murine IL-2-activated NK cells *in vitro* and enhance their IFNγ-production via the interaction with its ligands CD80 and CD86 (38). Thus, the findings of increased expression of TRAIL, CD49a, and CD28 corroborates that αGalCerMPEG administered i.n. promotes the activation of ILC1s. Furthermore, the stimulation of iNKT cells resulted in increased IFNγ secretion by ILC1s. Interestingly, a previous study reported that CD49a expression on T cells drives IFNγ production, thereby promoting inflammation (36). An additional study showed that protection against *Toxoplasma gondii* infection could be mediated by $CD28^+$ NK cells displaying increased IFNγ production and cytotoxicity (39). A similar mechanism might be triggered in ILC1s following stimulation with αGalCerMPEG. The increased IFNγ secretion might be further elicited by the observed αGalCerMPEG-induced upregulation of IL-12 and IFNγ. These cytokines were described to play crucial roles in promoting IFNγ production by NK cells (40, 41). Treatment of $J\alpha281^{-/-}$ mice, which lack NKT cells, with αGalCerMPEG did not result in any changes with regard to the activation status and functionality of ILC1s as compared to untreated mice. This is consistent with recent studies from our group demonstrating that the αGalCerMPEG-mediated activation of NK cells is crucially dependent on the presence of iNKT cells as well as CD1d expression (24, 31). The s.c. administration of αGalCerMPEG showed the same stimulatory impact on lung and splenic ILC1s as the i.n. administration thus highlighting the potential of immune cell stimulation via the i.n. route.

Assessing the potential role of αGalCerMPEG-activated ILC1s in the course of H1N1 infection *in vivo* revealed reduced viral titers in mice pre-treated with αGalCerMPEG. The increase in the absolute numbers of lung ILC1s correlated with an increased expression of TRAIL, CD49a, and CD28 in mice treated with αGalCerMPEG prior to influenza infection. This suggests that pre-activated ILC1s exhibit a higher responsiveness, thereby contributing to viral clearance. The elevated CD28 levels on lung ILC1s indicate a potential role for the CD28-CD80/CD86 axis leading to improved ILC1s responsiveness during influenza infection. This is in line with a study describing a crucial impact of the CD28-CD80/CD86 axis for anti-viral $CD8^+$ T cell responses (42). Furthermore, an increased IFNγ secretion by lung ILC1s was observed in αGalCerMPEG-pre-treated and infected mice. Secretion of IFNγ

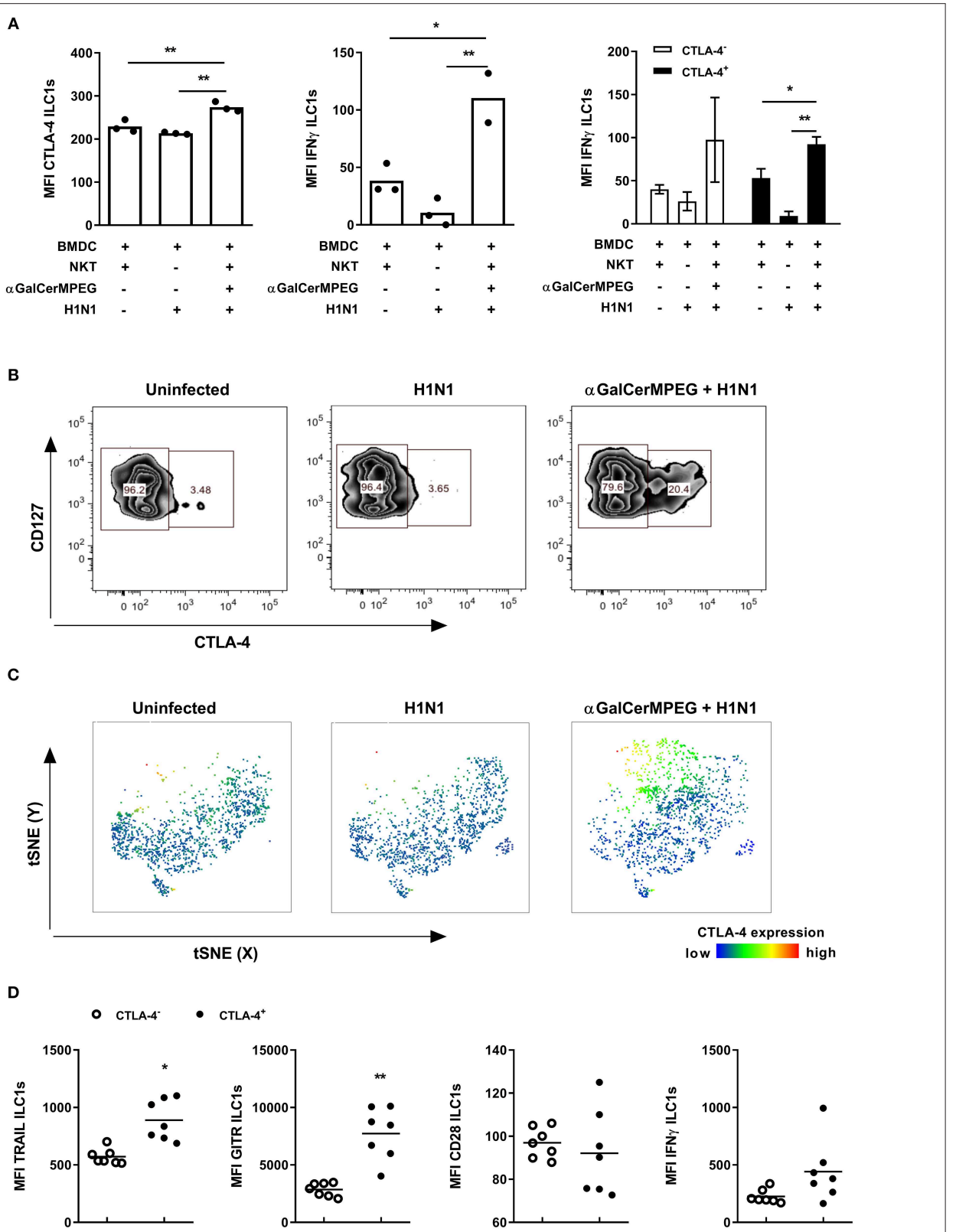

FIGURE 6 | αGalCerMPEG-induced CTLA-4 expression correlates with αGalCerMPEG-activated ILC1s. FLT-3L differentiated BMDCs were first treated overnight with αGalCerMPEG (300 ng/ml) and subsequently BMDCs were infected with H1N1 (MOI 1). BMDCs treated with αGalCerMPEG were co-cultured with *in vitro*-generated ILC1s and sorted splenic NKT cells overnight at a 6:6:1 ratio. ILC1s were assessed for the expression of CTLA-4 and IFNγ by flow cytometry following 3 h incubation

(Continued)

FIGURE 6 | in the presence of monensin and brefeldin. **(A)** MFI of ILC1s expressing CTLA-4 and IFNγ and MFI of IFNγ expression by CTLA-4$^+$ and CTLA-4$^-$ ILC1s. Bars represent mean ± SEM. MFI data are shown from one experiment (n = 3–4 technical replicates). Wild type mice were injected i.n. with a single dose of αGalCerMPEG (5 μg) 12 h prior to H1N1 infection (2*10^3 ffu). Lung lymphocytes were analyzed by flow cytometry 3 days post-infection with regard to CTLA-4 expression and markers related to ILC1 functionality following 3 h incubation in the presence of monensin and brefeldin. **(B)** Representative dot plots depicting CTLA-4 expression by lung ILC1s. **(C)** tSNE analysis of ILC1s indicating the expression density of CTLA-4. **(D)** Expression densities of TRAIL, GITR, CD28, and IFNγ by CTLA-4$^+$ and CTLA-4$^-$ lung ILC1s isolated from αGalCerMPEG-treated and influenza-infected mice. Box plots and scatter plots represent the range in MFI with the horizontal line indicating the mean. MFI data are displayed from one experiment (n = 5–8). Asterisks denote significant values as calculated by unpaired student's t-test or by One-way ANOVA; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

downstream of iNKT cell activation was previously shown to enhance the cytolytic activities of NK as well as virus-specific CD8$^+$ T cells, resulting in reduced influenza viral burden and enhanced survival (43). Similarly, stimulation of iNKT cells improved the disease outcome after influenza infection by boosting innate responses mediated by NK cells (44). Thus, the obtained results clearly indicate that stimulation of iNKT cells using αGalCerMPEG boosts the functionality of lung ILC1s, which might in turn contribute to early anti-viral immunity.

Next to the expression of activation markers and cytokine secretion, the impact of αGalCerMPEG-activated iNKT cells on the expression of the immune checkpoint molecules GITR and CTLA-4 by ILC1s was investigated. GITR signaling was already shown to inhibit regulatory T cells, thereby supporting the balance between effector and regulatory T cells and promoting CD4$^+$ and CD8$^+$ T cell responses (45–47). Recently, GITR was identified as a novel mechanism regulating ILC1 functionality *in vivo* in the course of influenza infection as well as *in vitro* upon cytokine stimulation (19). However, an impact of iNKT cell activation on GITR expression by ILC1s has not been reported so far. Interestingly, the activation of ILC1s by αGalCerMPEG-stimulated iNKT cells resulted in increased GITR expression with GITR$^+$ ILC1s displaying enhanced functionality. GITR up-regulation on memory CD8$^+$ T cells was previously demonstrated to result in an increased survival in the bone marrow dependent on IL-15 (48). Interestingly, elevated levels of the cytokine IL-15 were also observed in sera of αGalCerMPEG-treated mice (31). Thus, αGalCerMPEG-induced iNKT cell stimulation might boost the functional response of ILC1s by promoting their survival. The observation of increased GITR-L expression on DCs hints toward a potential interaction with GITR$^+$ ILC1s following iNKT cell stimulation (data not shown). In the influenza infection model, αGalCerMPEG-pre-treated mice showed an enhanced GITR expression on ILC1s as compared to infected untreated controls. This interaction might subsequently boost the potential of DCs to present antigens thus supporting enhanced anti-viral immunity. On the other hand, the elevated expression of GITR could also serve as a built-in safety feature to control immune activation. The ligation of GITR expressed on iNKT cells by an agonistic anti-GITR mAb was shown to negatively regulate their proliferation and cytokine secretion in response to αGalCer (49). Here, αGalCerMPEG-stimulated iNKT cells might initially trigger ILC1 activation but also promote GITR-upregulation thereby keeping the balance of immune activation and preventing overwhelming immune responses.

Besides the up-regulated expression of GITR on ILCs, iNKT cell stimulation by αGalCerMPEG induced an increased CTLA-4 expression on ILC1s over time. CTLA-4 has been extensively studied with regard to T cell biology and its role as an immune checkpoint molecule rendered it an attractive target for immune therapy (50–52). In this regard, the manipulation of CTLA-4 signaling (e.g., by using Ipilimumab) has been investigated in clinical trials and approved for treatment of advanced melanoma (53–55). The increased αGalCerMPEG-induced expression of the co-stimulatory receptor CD86 on dLN-derived DCs hints toward a potential interaction of CTLA-4$^+$ ILC1s with CD86$^+$ DCs. Interestingly, CTLA-4$^+$ ILC1s displayed a higher activation status at earlier time points. This observation concurs with T cell data showing high CTLA-4 expression on activated, proliferating T cells 24–72 h after stimulation (56). Enhanced CTLA-4 expression at later time points after iNKT cell stimulation was associated with a gradual decrease of responsiveness, as demonstrated by reduced IFNγ secretion and TRAIL expression and increased GITR expression. These findings suggest a regulatory function for CTLA-4 expressed by ILC1s. In this line, activated NK cells expressing CTLA-4 were described to display reduced IFNγ production in response to interaction with mature DCs (23). The mechanism of functional immune regulation might be explained by competition with the co-stimulatory receptor CD28 for their shared ligands CD80 and CD86 expressed by APCs. Compared to the constitutive expression of CD28, CTLA-4 is known to be expressed by activated immune cells at later time points exhibiting higher ligand affinity. Thus, CTLA-4 expression on ILC1s might compete with CD28, thereby regulating the functional responsiveness of iNKT cell-modulated ILC1s. However, further studies are required to confirm a direct regulatory impact of CTLA-4 on ILC1 functionality and ascertain the mode of action of CTLA-4-induced regulation of ILC1 responses. The observation of αGalCerMPEG-induced immune-modulation of ILC1s resulting in enhanced functionality suggest a beneficial effect on the anti-viral immune responses of ILC1s against influenza infection. However, immune stimulation requires to be regulated to prevent over-shooting responses that might lead to morbidity and mortality (57, 58). In this regard, the simultaneously enhanced ILC1-mediated anti-viral responses and subsequent upregulation of CTLA-4 and GITR expression on lung ILC1s highlight the potential of αGalCerMPEG as a novel immune-modulator which is not only capable of improving anti-viral immune responses, but also preventing over-stimulation of the immune system. In fact, immune regulation by CTLA-4 might represent a second built-in safety mechanism next to GITR crucial for preventing over-activation of the triggered

immune responses. This would be in consistence with our data showing that CTLA-4^+ ILC1s display enhanced GITR expression at later time points. In conclusion, the obtained data suggest ILC1 modulation as a valid approach for the establishment of immune interventions against viral infections affecting mucosal areas.

ETHICS STATEMENT

Mice were treated in consensus with local and European Community guidelines and were housed under specific pathogen free conditions in individual ventilated cages with food and water *ad libitum*. The performed animal experiments were approved by the local government in Braunschweig, Germany under the animal permission codes 33.42502-04-13/1281 and 33.19-42502-04-16/2280.

AUTHOR CONTRIBUTIONS

NV, PR, ST, TE, and BC designed the experiments. NV and ST performed experiments and acquired data. NV, PR, ST, and BC analyzed data. TE provided reagents. NV, ST, and PR wrote the manuscript draft. CG, ST, and PR discussed data and revised the final manuscript. All authors reviewed and edited the manuscript.

FUNDING

This research was funded by the European Research Training Group 1273 Strategies of human pathogens to establish acute and chronic infections (IRTG1273, DFG).

ACKNOWLEDGMENTS

We thank Elena Reinhard, Ulrike Bröder, and Hanna Shkarlet for their great technical assistance. $RAG2^{-/-}$ mice were kindly provided by Jochen Hühn.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01849/full#supplementary-material

REFERENCES

1. Eberl G, Di Santo JP, Vivier E. The brave new world of innate lymphoid cells. *Nat Immunol.* (2015) 16:1–5. doi: 10.1038/ni.3059
2. Padro Dietz C, Luong A. Innate lymphoid cells: the innate counterpart to T helper cells. *Adv Otorhinolaryngol.* (2016) 79:58–68. doi: 10.1159/000445130
3. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell.* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
4. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *J Exp Med.* (2014) 211:563–77. doi: 10.1084/jem.20131560
5. Zhang J, Marotel M, Fauteux-Daniel S, Mathieu AL, Viel S, Marcais A, et al. T-bet and Eomes govern differentiation and function of mouse and human NK cells and ILC1. *Eur J Immunol.* (2018) 48:738–50. doi: 10.1002/eji.201747299
6. Kim M, Kim CH. Colonization and effector functions of innate lymphoid cells in mucosal tissues. *Microbes Infect.* (2016) 18:604–14. doi: 10.1016/j.micinf.2016.06.005
7. Vivier E, van de Pavert SA, Cooper MD, Belz GT. The evolution of innate lymphoid cells. *Nat Immunol.* (2016) 17:790–4. doi: 10.1038/ni.3459
8. Neill DR, Flynn RJ. Origins and evolution of innate lymphoid cells: wardens of barrier immunity. *Parasite Immunol.* (2018) 40:e12436. doi: 10.1111/pim.12436
9. Kim CH, Hashimoto-Hill S, Kim M. Migration and tissue tropism of innate lymphoid cells. *Trends Immunol.* (2016) 37:68–79. doi: 10.1016/j.it.2015.11.003
10. Abt MC, Lewis BB, Caballero S, Xiong H, Carter RA, Susac B, et al. Innate immune defenses mediated by two ILC subsets are critical for protection against acute *Clostridium difficile* infection. *Cell Host Microbe.* (2015) 18:27–37. doi: 10.1016/j.chom.2015.06.011
11. Cording S, Medvedovic J, Aychek T, Eberl G. Innate lymphoid cells in defense, immunopathology and immunotherapy. *Nat Immunol.* (2016) 17:755–7. doi: 10.1038/ni.3448
12. Fuchs A. ILC1s in tissue inflammation and infection. *Front Immunol.* (2016) 7:104. doi: 10.3389/fimmu.2016.00104
13. Neill DR, Fallon PG. Innate lymphoid cells and parasites: ancient foes with shared history. *Parasite Immunol.* (2018) 40:e12513. doi: 10.1111/pim.12513
14. Yang Z, Tang T, Wei X, Yang S, Tian Z. Type 1 innate lymphoid cells contribute to the pathogenesis of chronic hepatitis B. *Innate Immun.* (2015) 21:665–73. doi: 10.1177/1753425915586074
15. Kloverpris HN, Kazer SW, Mjosberg J, Mabuka JM, Wellmann A, Ndhlovu Z, et al. Innate lymphoid cells are depleted irreversibly during acute HIV-1 infection in the absence of viral suppression. *Immunity.* (2016) 44:391–405. doi: 10.1016/j.immuni.2016.01.006
16. Carroll VA, Lundgren A, Wei H, Sainz S, Tung KS, Brown MG. Natural killer cells regulate murine cytomegalovirus-induced sialadenitis and salivary gland disease. *J Virol.* (2012) 86:2132–42. doi: 10.1128/JVI.06898-11
17. Schuster IS, Wikstrom ME, Brizard G, Coudert JD, Estcourt MJ, Manzur M, et al. TRAIL+ NK cells control CD4+ T cell responses during chronic viral infection to limit autoimmunity. *Immunity.* (2014) 41:646–56. doi: 10.1016/j.immuni.2014.09.013
18. Krueger PD, Narayanan S, Surette FA, Brown MG, Sung SJ, Hahn YS. Murine liver-resident group 1 innate lymphoid cells regulate optimal priming of anti-viral CD8+ T cells. *J Leukoc Biol.* (2017) 101:329–38. doi: 10.1189/jlb.3A0516-225R
19. Vashist N, Trittel S, Ebensen T, Chambers BJ, Guzman CA, Riese P. Influenza-activated ILC1s contribute to antiviral immunity partially influenced by differential GITR expression. *Front Immunol.* (2018) 9:505. doi: 10.3389/fimmu.2018.00505
20. Tone M, Tone Y, Adams E, Yates SF, Frewin MR, Cobbold SP, et al. Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. *Proc Natl Acad Sci USA.* (2003) 100:15059–64. doi: 10.1073/pnas.2334901100
21. Hanabuchi S, Watanabe N, Wang YH, Wang YH, Ito T, Shaw J, et al. Human plasmacytoid predendritic cells activate NK cells through glucocorticoid-induced tumor necrosis factor receptor-ligand (GITRL). *Blood.* (2006) 107:3617–23. doi: 10.1182/blood-2005-08-3419
22. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol.* (2001) 1:220–8. doi: 10.1038/35105024
23. Stojanovic A, Fiegler N, Brunner-Weinzierl M, Cerwenka A. CTLA-4 is expressed by activated mouse NK cells and inhibits NK Cell IFN-gamma production in response to mature dendritic cells. *J Immunol.* (2014) 192:4184–91. doi: 10.4049/jimmunol.1302091
24. Ebensen T, Link C, Riese P, Schulze K, Morr M, Guzman CA. A pegylated derivative of alpha-galactosylceramide exhibits

improved biological properties. *J Immunol.* (2007) 179:2065–73. doi: 10.4049/jimmunol.179.4.2065
25. Zygmunt BM, Weissmann SF, Guzman CA. NKT cell stimulation with alpha-galactosylceramide results in a block of Th17 differentiation after intranasal immunization in mice. *PLoS ONE.* (2012) 7:e30382. doi: 10.1371/journal.pone.0030382
26. Burdin N, Brossay L, Kronenberg M. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur J Immunol.* (1999) 29:2014–25. doi: 10.1002/(SICI)1521-4141(199906)29:06<2014::AID-IMMU2014>3.0.CO;2-G
27. Swann J, Crowe NY, Hayakawa Y, Godfrey DI, Smyth MJ. Regulation of antitumour immunity by CD1d-restricted NKT cells. *Immunol Cell Biol.* (2004) 82:323–31. doi: 10.1111/j.0818-9641.2004.01254.x
28. Au-Yeung BB, Fowell DJ. A key role for Itk in both IFN gamma and IL-4 production by NKT cells. *J Immunol.* (2007) 179:111–9. doi: 10.4049/jimmunol.179.1.111
29. Teijaro JR, Njau MN, Verhoeven D, Chandran S, Nadler SG, Hasday J, et al. Costimulation modulation uncouples protection from immunopathology in memory T cell responses to influenza virus. *J Immunol.* (2009) 182:6834–43. doi: 10.4049/jimmunol.0803860
30. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res.* (1995) 7:529–34.
31. Riese P, Trittel S, May T, Cicin-Sain L, Chambers BJ, Guzman CA. Activated NKT cells imprint NK-cell differentiation, functionality and education. *Eur J Immunol.* (2015) 45:1794–807. doi: 10.1002/eji.201445209
32. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol.* (2016) 17:758–64. doi: 10.1038/ni.3482
33. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med.* (2001) 193:661–70. doi: 10.1084/jem.193.6.661
34. Cummins N, Badley A. The TRAIL to viral pathogenesis: the good, the bad and the ugly. *Curr Mol Med.* (2009) 9:495–505. doi: 10.2174/156652409788167078
35. Stegmann KA, Bjorkstrom NK, Veber H, Ciesek S, Riese P, Wiegand J, et al. Interferon-alpha-induced TRAIL on natural killer cells is associated with control of hepatitis C virus infection. *Gastroenterology.* (2010) 138:1885–97. doi: 10.1053/j.gastro.2010.01.051
36. Chen Y, Peng H, Chen Y, Wei H, Sun R, Tian Z. CD49a promotes T-cell-mediated hepatitis by driving T helper 1 cytokine and interleukin-17 production. *Immunology.* (2014) 141:388–400. doi: 10.1111/imm.12201
37. Chapman TJ, Topham DJ. Identification of a unique population of tissue-memory CD4+ T cells in the airways after influenza infection that is dependent on the integrin VLA-1. *J Immunol.* (2010) 184:3841–9. doi: 10.4049/jimmunol.0902281
38. Nandi D, Gross JA, Allison JP. CD28-mediated costimulation is necessary for optimal proliferation of murine NK cells. *J Immunol.* (1994) 152:3361–9.
39. Hunter CA, Ellis-Neyer L, Gabriel KE, Kennedy MK, Grabstein KH, Linsley PS, et al. The role of the CD28/B7 interaction in the regulation of NK cell responses during infection with Toxoplasma gondii. *J Immunol.* (1997) 158:2285–93.
40. Liu B, Mori I, Hossain MJ, Dong L, Takeda K, Kimura Y. Interleukin-18 improves the early defence system against influenza virus infection by augmenting natural killer cell-mediated cytotoxicity. *J Gen Virol.* (2004) 85(Pt 2):423–8. doi: 10.1099/vir.0.19596-0
41. Hwang I, Scott JM, Kakarla T, Duriancik DM, Choi S, Cho C, et al. Activation mechanisms of natural killer cells during influenza virus infection. *PLoS ONE.* (2012) 7:e51858. doi: 10.1371/journal.pone.0051858
42. Fuse S, Obar JJ, Bellfy S, Leung EK, Zhang W, Usherwood EJ. CD80 and CD86 control antiviral CD8+ T-cell function and immune surveillance of murine gammaherpesvirus 68. *J Virol.* (2006) 80:9159–70. doi: 10.1128/JVI.00422-06
43. Ishikawa H, Tanaka K, Kutsukake E, Fukui T, Sasaki H, Hata A, et al. IFN-gamma production downstream of NKT cell activation in mice infected with influenza virus enhances the cytolytic activities of both NK cells and viral antigen-specific CD8+ T cells. *Virology.* (2010) 407:325–32. doi: 10.1016/j.virol.2010.08.030
44. Ho LP, Denney L, Luhn K, Teoh D, Clelland C, McMichael AJ. Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection. *Eur J Immunol.* (2008) 38:1913–22. doi: 10.1002/eji.200738017
45. Nocentini G, Riccardi C. GITR: a modulator of immune response and inflammation. *Adv Exp Med Biol.* (2009) 647:156–73. doi: 10.1007/978-0-387-89520-8_11
46. van Olffen RW, Koning N, van Gisbergen KP, Wensveen FM, Hoek RM, Boon L, et al. GITR triggering induces expansion of both effector and regulatory CD4+ T cells *in vivo*. *J Immunol.* (2009) 182:7490–500. doi: 10.4049/jimmunol.0802751
47. Snell LM, McPherson AJ, Lin GH, Sakaguchi S, Pandolfi PP, Riccardi C, et al. CD8 T cell-intrinsic GITR is required for T cell clonal expansion and mouse survival following severe influenza infection. *J Immunol.* (2010) 185:7223–34. doi: 10.4049/jimmunol.1001912
48. Snell LM, Lin GH, Watts TH. IL-15-dependent upregulation of GITR on CD8 memory phenotype T cells in the bone marrow relative to spleen and lymph node suggests the bone marrow as a site of superior bioavailability of IL-15. *J Immunol.* (2012) 188:5915–23. doi: 10.4049/jimmunol.1103270
49. Chen S, Ndhlovu LC, Takahashi T, Takeda K, Ikarashi Y, Kikuchi T, et al. Co-inhibitory roles for glucocorticoid-induced TNF receptor in CD1d-dependent natural killer T cells. *Eur J Immunol.* (2008) 38:2229–40. doi: 10.1002/eji.200838167
50. Walker LS, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. *Trends Immunol.* (2015) 36:63–70. doi: 10.1016/j.it.2014.12.001
51. Seidel JA, Otsuka A, Kabashima K. Anti-PD-1 and anti-CTLA-4 therapies in cancer: mechanisms of action, efficacy, and limitations. *Front Oncol.* (2018) 8:86. doi: 10.3389/fonc.2018.00086
52. Zhao Y, Yang W, Huang Y, Cui R, Li X, Li B. Evolving roles for targeting CTLA-4 in cancer immunotherapy. *Cell Physiol Biochem.* (2018) 47:721–34. doi: 10.1159/000490025
53. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med.* (2013) 369:122–33. doi: 10.1056/NEJMoa1302369
54. Callahan MK, Kluger H, Postow MA, Segal NH, Lesokhin A, Atkins MB, et al. Nivolumab plus ipilimumab in patients with advanced melanoma: updated survival, response, and safety data in a phase I dose-escalation study. *J Clin Oncol.* (2018) 36:391–8. doi: 10.1200/JCO.2017.72.2850
55. Turajlic S, Gore M, Larkin J. First report of overall survival for ipilimumab plus nivolumab from the phase III Checkmate 067 study in advanced melanoma. *Ann Oncol.* (2018) 29:542–3. doi: 10.1093/annonc/mdy020
56. Sansom DM. CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology.* (2000) 101:169–77. doi: 10.1046/j.1365-2567.2000.00121.x
57. Peiris JS, Hui KP, Yen HL. Host response to influenza virus: protection versus immunopathology. *Curr Opin Immunol.* (2010) 22:475–81. doi: 10.1016/j.coi.2010.06.003
58. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the eye of the cytokine storm. *Microbiol Mol Biol Rev.* (2012) 76:16–32. doi: 10.1128/MMBR.05015-11

Conflict of Interest Statement: CG and TE are named as inventors in a patent application covering the use of αGalCerMPEG as adjuvant (EP 05 022 771.9). This does not alter our adherence to the Frontier Immunology policies on sharing data.

The remaining 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: 06 September 2019
doi: 10.3389/fimmu.2019.02121

OPEN ACCESS

Edited by:
Jessica Borger,
Monash University, Australia

Reviewed by:
Ennio Carbone,
Università degli studi Magna Græcia di Catanzaro, Italy
Anja Fuchs,
Washington University in St. Louis, United States

***Correspondence:**
Camilla Jandus
camilla.jandus@chuv.ch

†Present address:
Romain Loyon,
Univ. Bourgogne Franche-Comté, INSERM, EFS BFC, UMR1098, Interactions Hôte-Greffon-Tumeur/Ingénierie Cellulaire et Génique, Besançon, France; Department of Medical Oncology, University Hospital of Besançon, Besançon, France
Bérengère Salomé,
Department of Oncological Sciences, Precision Immunology Institute, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, United States

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

Received: *14 December 2018*
Accepted: *23 August 2019*
Published: *06 September 2019*

Citation:
Loyon R, Jary M, Salomé B, Gomez-Cadena A, Galaine J, Kroemer M, Romero P, Trabanelli S, Adotévi O, Borg C and Jandus C (2019) Peripheral Innate Lymphoid Cells Are Increased in First Line Metastatic Colorectal Carcinoma Patients: A Negative Correlation With Th1 Immune Responses. Front. Immunol. 10:2121. doi: 10.3389/fimmu.2019.02121

Peripheral Innate Lymphoid Cells Are Increased in First Line Metastatic Colorectal Carcinoma Patients: A Negative Correlation With Th1 Immune Responses

***Romain Loyon*[1†], *Marine Jary*[2,3,4], *Bérengère Salomé*[1†], *Alejandra Gomez-Cadena*[1], *Jeanne Galaine*[2], *Marie Kroemer*[2,5], *Pedro Romero*[1], *Sara Trabanelli*[1], *Olivier Adotévi*[2,3], *Christophe Borg*[2,3] *and Camilla Jandus*[1*]**

[1] *Department of Oncology UNIL CHUV, Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, Lausanne, Switzerland,* [2] *Univ. Bourgogne Franche-Comté, INSERM, EFS BFC, UMR1098, Interactions Hôte-Greffon-Tumeur/Ingénierie Cellulaire et Génique, Besançon, France,* [3] *Department of Medical Oncology, University Hospital of Besançon, Besançon, France,* [4] *INSERM CIC-1431, Clinical Investigation Center in Biotherapy, University Hospital of Besançon, Besançon, France,* [5] *Department of Pharmacy, University Hospital of Besançon, Besançon, France*

Several distinct innate lymphoid cell (ILC) populations have been recently identified and shown to play a critical role in the immediate immune defense. In the context of tumors, there is evidence to support a dual role for ILCs with pro- or antitumor effects, depending on the ILC subset and the type of cancer. This ambivalent role has been particularly well-described in colorectal cancer models (CRC), but the presence and the evolution of ILCs in the peripheral blood of metastatic CRC (mCRC) patients have not yet been explored. Here, we investigated the distribution of ILC subsets in 96 mCRC patients who were prospectively included in the "Epitopes-CRC02" trial. Peripheral blood mononuclear cells (PBMCs) were analyzed by flow cytometry at metastatic diagnosis and after 3-months of treatment. The treatments consisted of Oxaliplatin-based chemotherapies for 76% of the patients or Folfiri (5FU, Irinotecan) chemotherapies for 14% of patients. Compared to healthy donors, the frequency of total ILCs was dramatically increased at metastatic diagnosis. $CD56^+$ ILC1-like cells were expanded, whereas ILC2, NCR^- ILCP and NCR^+ ILCP subsets were decreased. Combined analysis with the systemic anti-telomerase hTERT Th1 CD4 response revealed that patients with low anti-TERT Th1 CD4 responses had the highest frequencies of total ILCs at diagnosis. Of those, 91% had synchronous metastases, and their median progression-free survival was 7.43 months (vs. 9.17 months for the other patients). In these patients, ILC1 and ILC2 were significantly decreased, whereas $CD56^+$ ILC1-like cells were significantly increased compared to patients with low frequency of total ILCs and high anti-TERT responses. After treatment, the NCR^+ ILCP were further decreased irrespective of the chemotherapy regimen, whereas the balance between ILC1 and $CD56^+$ ILC1-like cells was modulated mainly by the Folfiri regimen in favor of ILC1. Altogether our results describe the effects of

different chemotherapies on ILCs in mCRC patients. We also establish for the first time a link between frequency of ILCs and anti-tumor CD4 T cell responses in cancer patients. Thus, our study supports an interest in monitoring ILCs during cancer therapy to possibly identify predictive biomarkers in mCRC.

Keywords: ILC, metastatic colorectal cancer, Th1, chemotherapy, immunomonitoring

INTRODUCTION

Colorectal cancer (CRC) has been used as a model to demonstrate the role of the immune system in cancer, notably to establish the prognostic role of memory T cell infiltration and Th17 predominance (1). Fifteen percent of localized CRC patients present microsatellite instability (MSI), with a higher mutation load conferring a higher response to immunotherapy (2). The immune response is also involved in CRC microsatellite stability (MSS), for example in consensus molecular subtype (CMS) four CRC patients (3). Research at the metastatic stage is ongoing in order to better define the CRC-stage-linked immune responses. It was previously described that cytotoxic innate immune cells, so called conventional natural killer (NK) cells, were almost absent in human colorectal tumors, despite efficient T cell infiltration (4). In contrast, a recent study on 13 localized human CRC and 13 lung tumors showed that helper innate immune cells, the recently identified family of innate lymphoid cells (ILCs) are present in these tumors, at different levels (5).

Distinct helper ILC populations have been reported to play a critical role on the maintenance of tissue homeostasis and immediate immune host defense (6). ILCs exhibit transcription factor and cytokine profiles that phenocopy the three major T helper (Th) cell subsets: Th1, Th2, and Th17/22. Thus, ILCs are classified into three subgroups, ILC1, ILC2, and ILC3 (7–9). The ILC3 subgroup can be further subdivided into NCR^+ ILC3 that express the natural cytotoxicity receptor (NCR) NKp46, and NCR^- ILC3, which include lymphoid tissue inducer (LTi) cells. In addition, an extensive analysis of ILC from human peripheral blood and tissue showed recently that ILCs with c-Kit^+ phenotype, previously proposed to represent human ILC3, are enriched in multi-potent and uni-potent ILC precursors (ILCP) in the peripheral blood that can give rise to all know ILC subsets (10).

ILCs produce high amounts of different cytokines very early after infection and tissue damage, suggesting potential pro- and anti-oncogenic roles of these cells during the early phases of carcinogenesis. ILC1s represent a very early source of IFN-γ that is mainly associated with a protective role against tumors through the upregulation of MHC molecules (11–13), the induction of Th1 polarization, and the activation of macrophages (14). Recently, we identified $CD56^+$ ILC1-like cells, an ILC population within the ILC1 compartment, which constitutively expresses CD56 and CD127, but lack c-Kit; similar to Stage 4b NK cells they lack KIR and CD16, but they express NKG2A. $CD56^+$ ILC1-like cells possess cytotoxic potential and have anti-leukemic effects (Salome et al., in revision). Similarly, $CD103^+$ cytotoxic intraepithelial ILC1-like cells were previously reported in localized colorectal tumors (5) and were shown to directly kill tumor cells in a perforin-dependent manner (15, 16). Both pro- and antitumor roles of ILC2 in cancer biology have been suggested. Indeed, we and others reported on the expansion of ILC2, which contributed to the suppression of antitumor responses via an IL-13/monocytic-myeloid derived suppressor cell (M-MDSC) axis (17–19). In CRC, high levels of the ILC2-activating alarmin IL-33 and the prototypic ILC2 cytokine IL-4 are associated with poor prognosis (20, 21). In addition to this protumorigenic role, ILC2 also produce IL-5, which induces the selective expansion of eosinophils (22) whose degranulation has been shown to improve prognosis in various types of cancer, including CRC (23). Furthermore, secretion of IL-13 by ILC2 may promote the migration of activated DCs to tumor-draining lymph nodes resulting in cytotoxic T cell activation (24). ILC3 have also been described to play a protective role in carcinogenesis by limiting tissue damage through the secretion of IL-22 in a model of chronic colitis (25). However, IL-22 producing $CCR6^+$ ILC3 may increase the tumorigenic potential of colon cancer (26–28). NCR^- ILC3-derived IL-22 can act on colon epithelial cells to sustain tumor progression. Moreover, IL-17 was shown to inhibit tumor progression by acting on T cells (29) and to contribute to reduced tumor growth and metastasis in mice inoculated with a colon cancer cell line (26). Nevertheless, an IL-23-Th17 gene signature in resected CRC was associated with a worse prognosis and found to predict rapid progression to metastatic disease, which demonstrates the potential pro-tumorigenic role of ILC3 (30).

These observations suggest the involvement of ILCs in natural tumor immunity. Furthermore, while a putative role for ILCs in mediating the effect of chemo-immunotherapy was demonstrated in a mouse model of melanoma (31), there is limited data available on the impact of anti-cancer treatments on ILC frequency and phenotype in humans. A recent study on human ovarian cancer reported that an ILC3-like population that expresses IFN-γ and IL-22 suppressed the activation and proliferation of tumor-infiltrating lymphocytes (TIL) *ex vivo*, suggesting a potential benefit of depleting these cells before TIL-based immunotherapy (32). In addition, a link between the efficacy of immune checkpoint blockades and ILCs is currently under investigation because ILC2 cells have recently been shown to express PD-1 (33, 34). Moreover, growing evidence suggests that chemotherapy may also modulate ILC homeostasis, in particular by altering the composition of commensal microbiota (35).

In this study, we assessed the modulation of ILC subsets in parallel with antigen-specific CD4 T cell responses in a large cohort of metastatic CRC (mCRC) patients, at baseline and 3

months postchemotherapy. Since human telomerase (hTERT) is expressed in many cancer types and plays a crucial role in oncogenesis by providing proliferation, survival and anti-apoptotic signals necessary for tumor progression (36–38), we used hTERT as a hallmark of cancer and as a universal tumor antigen prototype (39–41). We monitored the systemic anti-hTERT Th1 CD4 response and explored the relationship between anti-hTERT responses and the ILC compartment. We report here that ILCs are increased in favor of ILC1 in the peripheral blood of chemotherapy-naïve mCRC patients, and that the frequency of ILCs is negatively correlated with the cancer-specific Th1 immune response. Moreover, we observed that chemotherapy regimens, especially Folfiri, act on specific ILC subsets without affecting the total ILC population in the peripheral blood.

MATERIALS AND METHODS

Patients and Healthy Donors

Metastatic colorectal cancer patients were enrolled from March 2013 to August 2016 in the "Epitopes-CRC02" trial (NCT02817178), a French multicentric prospective study assessing the impact of treatment on the CD4 T cell response. All patients were enrolled after signing an informed consent, in accordance with French regulation, and after approval by local and national ethics committees. All data were anonymized. Overall population characteristics are summarized in **Table S1**. For each patient, blood samples were collected before any metastatic cancer specific treatment (baseline) and after 3 months of a chemotherapy-based regimen. The materials used and analysis plan are illustrated in **Figure 1**. Briefly, we monitored ILCs by flow cytometry in 86 patients at baseline and 76 matched patients after chemotherapy. From the samples collected after treatment, we retained data for patients receiving the following chemotherapy-based regimens: (A) 5FU and Irinotecan (Folfiri) ($n = 19$), (B) 5FU and Oxaliplatin (Folfox) ($n = 40$), and (C) 5FU, Oxaliplatin and Irinotecan (Folfoxiri) ($n = 17$). Patients treated with 5FU only, patients without treatment data available or patients without available data at baseline were removed from the analysis. Of note, we chose to include five patients who received Oxaliplatin and Raltitrexed, a folic acid analog that inhibits thymidylate synthetase similar to 5FU, in the Folfox branch. A total of 86% of patients received a bevacizumab regimen as well, regardless of the chemotherapy protocol. For healthy donor controls, blood cells were collected from anonymous donors at the *Etablissement Français du Sang* (Besançon, France) and the *Transfusion Interregionale* CRS (Lausanne, Switzerland) using an apheresis kit preparation, after obtaining signed informed consent.

Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of patients and healthy donors by density centrifugation using a Ficoll gradient (GE healthcare). Cells were maintained in RPMI (Gibco) supplemented with 8% human serum, 100 U/ml penicillin-streptomycin (Invitrogen) and nonessential amino-acids.

Antibodies

The following anti-human antibodies were used for the lineage the cocktail: CD3-FITC, CD4-FITC, CD8a-FITC, CD14-FITC, CD15-FITC, CD16-FITC, CD19-FITC, CD20-FITC, CD33-FITC, CD34-FITC, FcεRI-FITC, and CD203c-FITC (all from Biolegend). In addition, these anti-human antibodies were used: CD335 (NKp46)-PE/Cy7, CD294 (CRTH2)-PE, CD127-BV421 from Biolegend, CD56-APCeF780 from eBioscience, and CD117 (ckit)-APC from BD Bioscience. For intracellular transcription factor staining the following anti-human antibodies were used: Tbet-PE/CF594, GATA3-PE/Cy7, and RORγt-PE from BD Bioscience. Corresponding isotype control antibodies were used as controls.

Staining and Flow Cytometry

To prepare for immunostaining, PBMCs were counted and suspended in FACS buffer (1X PBS, 50 µM EDTA, 0.2% BSA) prior to labeling with appropriate antibodies for 30 min in the dark at 4°C. The cells were then washed with 1X PBS. For the second step, cells were stained with the fixable viability dye, eFluor 506 (eBioscience), for 30 min at 4°C. The cells were then washed again with 1X PBS. Samples were evaluated on a FACS Canto II (BD Biosciences).

For transcription factor staining, after extracellular staining, cells were fixed and permeabilised using the InvitrogenTM eBioscience Foxp3/Transcription factor staining buffer Set according to manufacturer's recommendations. Intracellular antibodies were prepared in Permeabilisation buffer 1X and the intracellular staining was perform for 1 h at room temperature. After incubation, cells were washed with Permeabilisation buffer 1x twice and finally resuspended in FACs buffer. Samples were acquired using a Gallios II from Beckman Coulter.

Analysis were realized with FlowJo Software v 9.9.4. The gating strategy was set based on the isotype controls and applied in a standard format across all samples and all conditions.

Assessment of Spontaneous hTERT-Specific CD4 T Cell Responses in Cancer Patients

An IFN-γ ELISpot was conducted for 74 patients of the cohort at baseline, as previously described by Godet et al. (42). Briefly, spontaneous responses were assessed by IFN-γ ELISpot after an *in vitro* stimulation of PBMCs with a mixture of eight peptides derived from hTERT ($TERT_{44-58}$, $TERT_{578-592}$, $TERT_{921-935}$, $TERT_{1055-1069}$, $TERT_{541-555}$, $TERT_{573-587}$, $TERT_{613-627}$, and $TERT_{911-925}$) at 5 µg/ml for 10 days. Another peptide mixture, referred to as CEF (PANATecs), derived from influenza virus (Flu), Epstein Barr virus (EBV), and cytomegalovirus (CMV) was used to evaluate antiviral recall responses in the same patients. CEF IFN-γ ELISpot data were available for 69 of the 74 patients. After the 10-day incubation with the peptide mixtures, the cells (10^5 per well) were cultured in triplicate in the ELISpot plate with restricted peptides mixture or individual peptides at 5 µg/mL in X-vivo 15 medium (Ozyme, BE04–418) for 15 h. The IFN-γ spots were developed following the

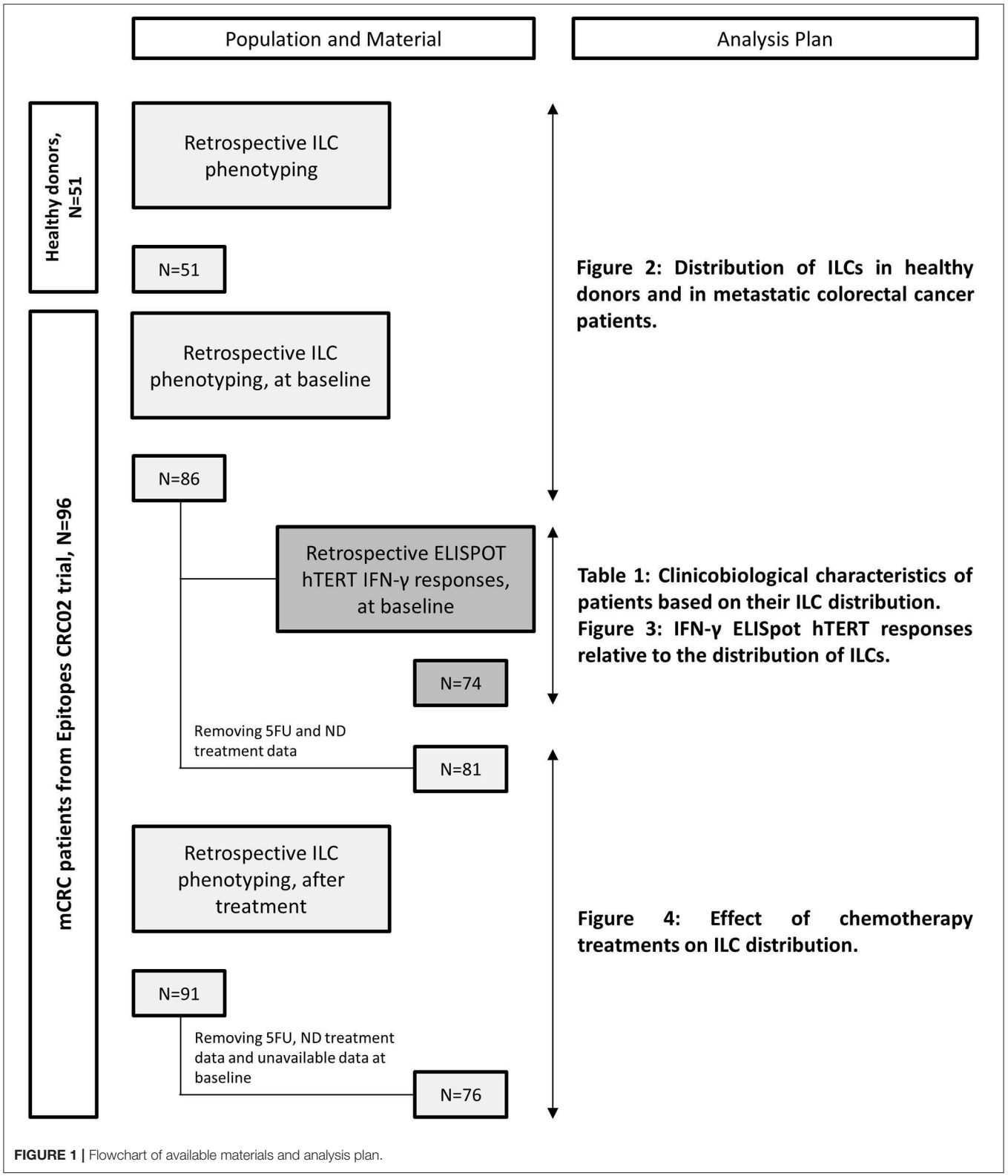

FIGURE 1 | Flowchart of available materials and analysis plan.

manufacturer's instructions (Diaclone, 856 051 020P). Spot-forming cells were counted using the C.T.L. Immunospot system (Cellular Technology Ltd.). The number of specific T cells expressed as the number of spot-forming cells per 10^5 cells was calculated after subtracting the negative control values (background). Responses were considered positive when the

number of IFN-γ spots was >10 and greater than twice the background. Responses with a background >100 spots were excluded from the analysis.

Statistical Analysis

Numerical data are expressed as the mean ± SEM (Standard Error Mean). Student's *t*-tests, ANOVAs and Chi-square tests were used for the evaluation of statistical significance, calculated with GraphPad Prism 8.0 or R Studio. *P*-values lower than 0.05 were considered significant with $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Progression free survival (PFS) was calculated from the date of study enrolment to the date of tumor progression. Kaplan-Meier survival curves were generated using R Studio® software (median with 95% confidence interval).

RESULTS

Chemotherapy-Naïve Metastatic Colorectal Cancer Patients Have Increased Frequencies of ILCs and Their Subsets' Distribution Is Skewed Toward ILC1

To evaluate the impact of mCRC on ILCs, we investigated ILC frequency and subset distribution in PBMCs of 86 chemotherapy naïve (baseline) mCRC patients included in the "Epitope-CRC02" study (**Table S1**). Fifty-one healthy donors (HD) were used as a control cohort. The percentage of total ILCs (ILCtot) (Lin^- $CD127^+$) was assessed by flow cytometry, as well as the five ILC subsets, defined as ILC1 (Lin^- $CD127^+$ $cKit^-$ $CRTH2^-$ $CD56^-$), $CD56^+$ ILC1-like (Lin^- $CD127^+$ $cKit^-$ $CRTH2^-$ $CD56^+$), ILC2 (Lin^- $CD127^+$ $CRTH2^+$), NCR^- ILCP (Lin^- $CD127^+$ $cKit^+$ $NKp46^-$), and NCR^+ ILCP (Lin^- $CD127^+$ $cKit^+$ $NKp46^+$) (**Figure 2A** and **Figure S1A**). In addition, we validated our gating strategy by assessing master transcription factor expression of each population by flow cytometry (**Figure S1B**). For ILC2, functionally distinct c-Kit^+ and c-Kit^- ILC2 have recently been described in healthy donors (43). Interestingly, in mCRC patients ILC2 were almost exclusively c-Kit^- (**Figure S2**). Thus, in subsequent analysis, ILC2 were analysis only based on the expression of CRTH2. We observed that the frequency of ILCtot are significantly increased in the PBMCs of mCRC patients at baseline compared to HD (**Figure 2B**). Moreover, the distribution of the ILC subsets was also distinct in chemotherapy-naïve mCRC patients (**Figures 2C,D**). The ILC2, NCR^+ ILCP and NCR^- ILCP subsets were significantly decreased in patients at baseline compared to HD, whereas the ILC1 and $CD56^+$ ILC1-like subsets were increased. We next stratified the patients according to their total ILC tercile distribution: ILCtot low [0.09–0.54%], $n = 29$; ILCtot medium [0.54–0.96%], $n = 29$; and ILCtot high [0.99–4.11], $n = 28$. For each group, the ILC subset distribution was analyzed, and the results showed that the ILC subset proportions are linearly modulated (**Figures 2E,F**). Deeper analysis revealed that the ILC1 population proportion was significantly decreased in ILCtot high patients, whereas $CD56^+$ ILC1-like cell proportion was increased. Of note, ILCtot low patients had the highest proportion of NCR^- ILCP and NCR^+ ILCP cells. Overall, these results show that ILCs are drastically increased in the peripheral blood, with a skewing toward ILC1s at CRC metastatic diagnosis. Moreover, patients with high proportions of ILCtot present the lowest rates of ILC1 and NCR^- ILCP cells in favor of $CD56^+$ ILC1-like cells.

Patient Clinicobiological Characteristics and ILC Distribution

To evaluate the link between ILCs and clinicobiological data we focused our analysis on 74 patients for which ILC frequencies and tumor-specific Th1 immunity (focusing on hTERT as a universal tumor-antigen) were available. The clinicobiological characteristics available for these patients are summarized in **Table 1**. Because we observed that ILCtot high patients presented at least 2-fold more ILCs than HD, for clinical correlations we decided to perform our analyses with merged ILCtot medium and ILCtot low patients (referred to hereafter as ILCtot low) (**Table 1**). ILCtot high patients did not differ from the rest of the cohort with regards to the age, gender, tumor location, time of metastases or metastatic location. Regarding biological and molecular parameters, no difference was observed between the two groups. Of note, ILC counts were not influenced by microsatellite status, RAS and BRAF mutations, carcinoembryonic antigen (CEA) value or lymphocyte count. Interestingly, only 13% of MSI tumors had an ILCtot high phenotype and the BRAF mutation prognostic biomarker was not enriched in the ILCtot high patients. Tumor response rate was also not different based on the profile of ILC distribution.

Next, some hypotheses were formulated regarding the different subsets of ILCs and were subsequently tested with our data. (i) We first hypothesized that MSI CRC patients harbor a higher mutational load and an interferon gamma (IFN-γ) signature. However, we observed that ILC subsets were distributed equally in the nine MSI tumors (data not shown), with the ILC1 subset count higher in only two patients. (ii) Next, inflammatory contextures, such as mucosal immunity or hepatic metastatic localization, were specifically assessed for ILC subsets. In the 68 synchronic patients with a primary colon tumor in place, the ILC subset proportions were largely the same. The ILC subset proportions were almost equally distributed among each different subset. (iii) We then hypothesized that cytokine availability following lymphopenia could enhance the number of ILCs, as has been described for NK cells after transplantation (44, 45). However, here we did not observe such an expansion, regardless of which ILC subset was considered. (iv) BRAF mutations are present in 5–8% of mCRC patients and are often associated with MSI tumors, however, less is known about BRAF mutations in MSS tumors. Of the nine BRAF mutated patients in our cohort, three had an MSS phenotype, however, none of these patients presented any specific enrichment for one ILC subset.

ILCs Are Negatively Correlated With the Th1 Anti-hTERT Immune Response

To explore the relationship between the adaptive immune system and ILCs, we took advantage of promiscuous hTERT-derived MHC class II peptides that allow for monitoring of anti-telomerase Th1 CD4 immunity in most HLA contexts

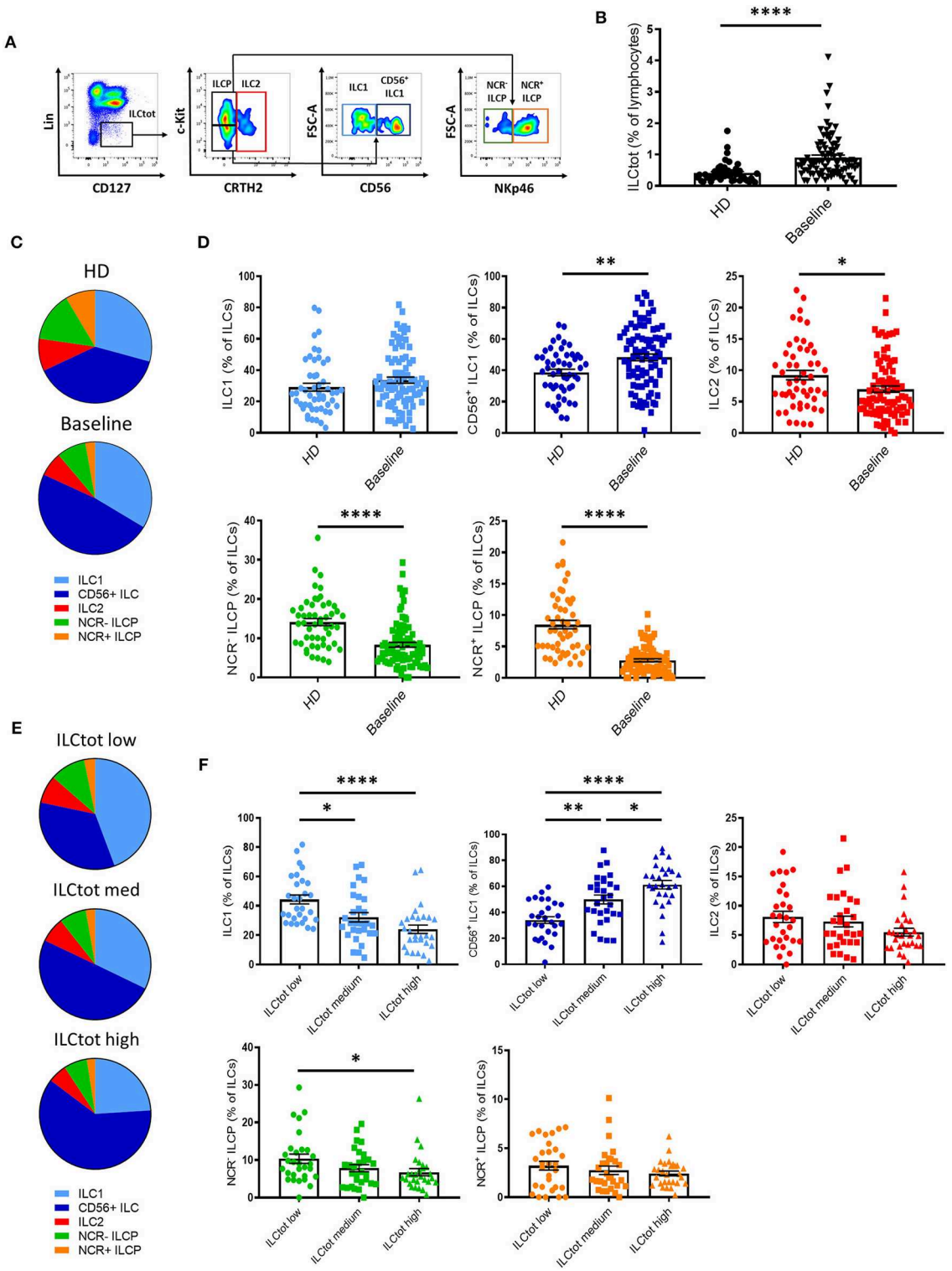

FIGURE 2 | Distribution of ILCs in healthy donors and in metastatic colorectal cancer patients. **(A)** Flow cytometry gating strategy for ILC subset identifications in PBMCs. **(B)** Frequencies of ILCtot. ILC subset distributions **(C)** and frequencies **(D)** were analyzed by flow cytometry in PBMCs of 51 healthy donors (HD) and 86 chemotherapy-naïve metastatic colorectal cancer patients (baseline). Distribution **(E)** and frequencies **(F)** of ILC subsets based on the frequency of ILCtot distributed in the low ($n = 29$), medium ($n = 29$), and high ($n = 28$) terciles. Columns, the means of ILC frequency for each patient; bars, SEM. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{****}p < 0.0001$, as determined by Student's *t*-test or ANOVA.

TABLE 1 | Clinicobiological characteristics of patients based on their ILC distribution.

	IFN-γ ELISpots hTERT patients at baseline	ILC low	ILC high	*p*
	n = 74 (%)	*n* = 50 (%)	*n* = 24(%)	
AGE-YEARS				
<65	*n* = 36 (49%)	22 (44%)	14 (61%)	
≥65	*n* = 37 (51%)	28 (56%)	9 (49%)	0.28
GENDER				
F	*n* = 27 (36%)	16 (32%)	11 (46%)	
M	*n* = 47 (64%)	34 (68%)	13 (54%)	0.37
TUMOR LOCATION				
Rectum	*n* = 19 (27%)	14 (30%)	5 (21%)	
Colon	*n* = 52 (73%)	33 (70%)	19 (79%)	0.60
MICROSATELLITES				
MSI	*n* = 8 (21%)	7 (28%)	1 (7%)	
MSS	*n* = 31 (79%)	18 (72%)	13 (93%)	0.26
RAS STATUS				
M	*n* = 33 (50%)	20 (45%)	13 (59%)	
WT	*n* = 33 (50%)	24 (55%)	9 (41%)	0.43
BRAF STATUS				
M	*n* = 8 (14%)	5 (11%)	3 (14%)	
WT	*n* = 58 (86%)	39 (89%)	19 (86%)	0.99
TIME OF METASTASIS				
Metachronous	*n* = 14 (20%)	12 (24%)	2 (8%)	
Synchronous	*n* =57 (80%)	35 (76%)	22 (92%)	0.16
METASTATIC LOCALIZATION				
Extra hepatic	*n* = 16 (22%)	*n* = 14 (29%)	*n* = 2 (8%)	
Hepatic and other	*n* = 19 (26%)	*n* = 12 (25%)	*n* = 7 (29%)	
Hepatic only	*n* = 37 (52%)	*n* = 22 (46%)	*n* = 15 (63%)	0.13
METASTATIC LOCALIZATION				
Extra hepatic/hepatic and other	*n* = 35 (49%)	*n* = 26 (54%)	*n* = 9 (37.5%)	
Hepatic only	*n* = 37 (51%)	*n* = 22 (46%)	*n* = 15 (62.5%)	0.28
LYMPHOCYTE COUNT				
<1,000	*n* = 9 (13%)	5 (11%)	4 (17%)	
≥1,000	*n* = 61 (87%)	41 (89%)	20 (83%)	0.75
ACE				
<20	*n* = 26 (43%)	19 (46%)	7 (35%)	
≥20	*n* = 35 (57%)	22 (54%)	13 (65%)	0.57
TUMOR RESPONSE				
Progression disease	*n* = 4 (7%)	2 (5%)	2 (10%)	
Stable disease	*n* = 21 (36%)	13 (35%)	8 (40%)	
Partial response	*n* = 29 (50%)	21 (55%)	8 (40%)	
Complete response	*n* = 4 (7%)	2 (5%)	2 (10%)	0.66

The ILCtot frequencies of patients at baseline were distributed into terciles. The results shown for the low (n = 25) and medium (n = 25) terciles were pooled and referred to as ILC low (n = 25 + 25 = 50), and the high tercile was referred to as ILC high (n = 24). ELISpot hTERT response data was available for all 74 patients, however, data for some clinicobiological characteristics are missing for some patients.

(42, 46, 47). For this purpose, IFN-γ production by PBMCs derived from 74 chemotherapy naïve mCRC patients exposed to hTERT promiscuous peptides was measured by ELISpot in short-term stimulation assays. hTERT-derived peptides were recognized by PBMCs from 18 of the patients analyzed (24.3%) (**Figure 3A**). A trend of lower frequency of ILCs was observed in these patients in comparison to the mCRC patients who had no Th1 responses after hTERT-peptide stimulation. For each ILCtot group, the number of IFN-γ spots after MHC class II restricted hTERT peptide stimulation were analyzed (**Figure 3B**). Interestingly, ILCtot low patients had a higher anti-telomerase Th1 response compared to patients with high ILC levels. As a comparison to the cancer antigen hTERT, we concurrently measured T cell reactivity against a mixture of viral peptides in the same patient group (**Figure 3C**). Anti-viral T cell responses were detected in most patients (76.8%). Unlike the systemic anti-hTERT Th1 response, the frequency of patients exhibiting antiviral T cell responses was equally distributed among the patient subgroups. These results suggest that the presence of circulating anti-hTERT Th1 responses is related to the ILCtot proportion. Interestingly, regardless of the ILCtot percentage, patients with the highest proportion of ILCP had a better Th1 response compared to patients with the lowest $NCR^{+/-}$ ILCP proportion (**Figure 3D**).

To explore the prognostic value of ILCtot high patients having lower anti-hTERT Th1 responses, we estimated the PFS of the ILCtot high and ILCtot low patients (**Figure S3**). In our cohort, the median of PFS was 7.43 months (95% CI: 6.44–14) for the ILCtot high patients, and 9.17 months (95% CI: 7.62–11.8) for the ILCtot low patients ($p = 0.3$). A better prognosis tendency was observed for the ILCtot low patients in our cohort, especially after 15 months.

Our results show that the frequency of ILCtot in the peripheral blood of mCRC patients at baseline is negatively correlated with anti-hTERT Th1 immune responses.

Chemotherapy Treatment of mCRC Modulates ILC Subsets' Distribution

Most conventional chemotherapies present immunogenic properties that at least partially contribute to their clinical efficacy (48). To evaluate the impact of chemotherapies on ILCs, we performed a comparative analysis of total and ILC subset frequency in peripheral blood of chemotherapy naïve mCRC patients at baseline and on the same patients after 3 months of treatment (TT in **Figure 4**) with the classical chemotherapy regimens, Folfiri (5FU and Irinotecan; $n = 19$), Folfox (5FU and Oxaliplatin; $n = 40$), and Folfoxiri (5FU, Oxaliplatin and Irinotecan; $n = 17$). The results showed that chemotherapies did not affect the frequency of total ILCs, which remain high after treatment (**Figure 4**). However, chemotherapies modulated the distribution of ILC subsets. The Folfiri regimen significantly reversed the balance between ILC1 and $CD56^+$ ILC1-like cells, while ILC2 were not affected by any of the treatments. All of the regimens induced a decrease in NCR^+ ILCP that reached significance in the Folfox group, whereas NCR^- ILCP were significantly increased in the Folfoxiri group. Collectively, these data highlight the ILC modulation potential of chemotherapy treatments.

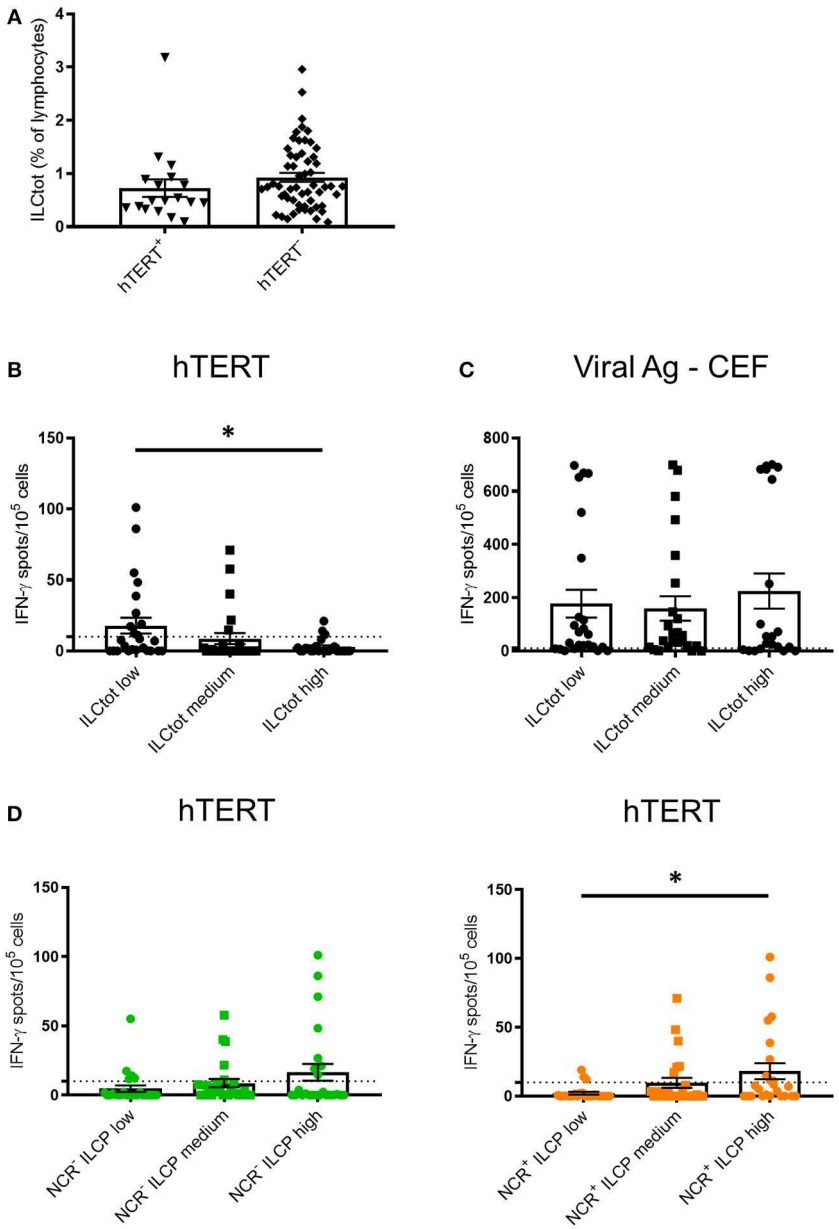

FIGURE 3 | IFN-γ ELISpot hTERT responses relative to the distribution of ILCs. PBMCs from 74 chemotherapy-naïve metastatic colorectal cancer patients were cultured with a mixture of 8 hTERT peptides ($TERT_{44-58}$, $TERT_{578-592}$, $TERT_{921-935}$, $TERT_{1055-1069}$, $TERT_{541-555}$, $TERT_{573-587}$, $TERT_{613-627}$, and $TERT_{911-925}$) at 5 μg/mL. The T cell reactivity against the hTERT peptides was detected by IFN-γ ELISpot assays, as described in the Material and Methods. Stimulation with Viral Ag-CEF peptides was used as control for T cell reactivity. **(A)** The frequency of ILCtot in the PBMCs of each patient was analyzed based on the positive ($n = 18$) or negative ($n = 56$) response to hTERT. **(B,C)** Results are shown as the means of IFN-γ spot numbers (magnitude) and the ILC frequencies (ILCtot distributed into low ($n = 25$), medium ($n = 25$) and high ($n = 24$) terciles) for hTERT responses **(B)** and Viral Ag-CEF responses **(C)**. **(D)** Results are shown as the means of IFN-γ spot numbers (magnitude) and frequencies of NCR^- ILCP (left) and NCR^+ ILCP (right) distributed among the low ($n = 25$), medium ($n = 25$) and high ($n = 24$) terciles. Columns, the means of spots from triplicate wells; bars, SEM. *$p < 0.05$, as determined by Student's *t*-test.

DISCUSSION

ILC dysregulation in terms of frequency and subset composition has already been described in hematological malignancies and some solid tumors (18, 19, 49–51). Here we analyzed ILC populations in mCRC patients pre- and postchemotherapy. We showed that ILCtot are drastically increased in the peripheral blood, suggesting cytokine stimulation in the ILC compartment. IL-7, largely described as a key stimulator of ILC proliferation and survival (52), could play such a role. Indeed, IL-7 was recently described as particularly elevated in the serum of CRC patients, especially in patients with metastases (53). Deeper

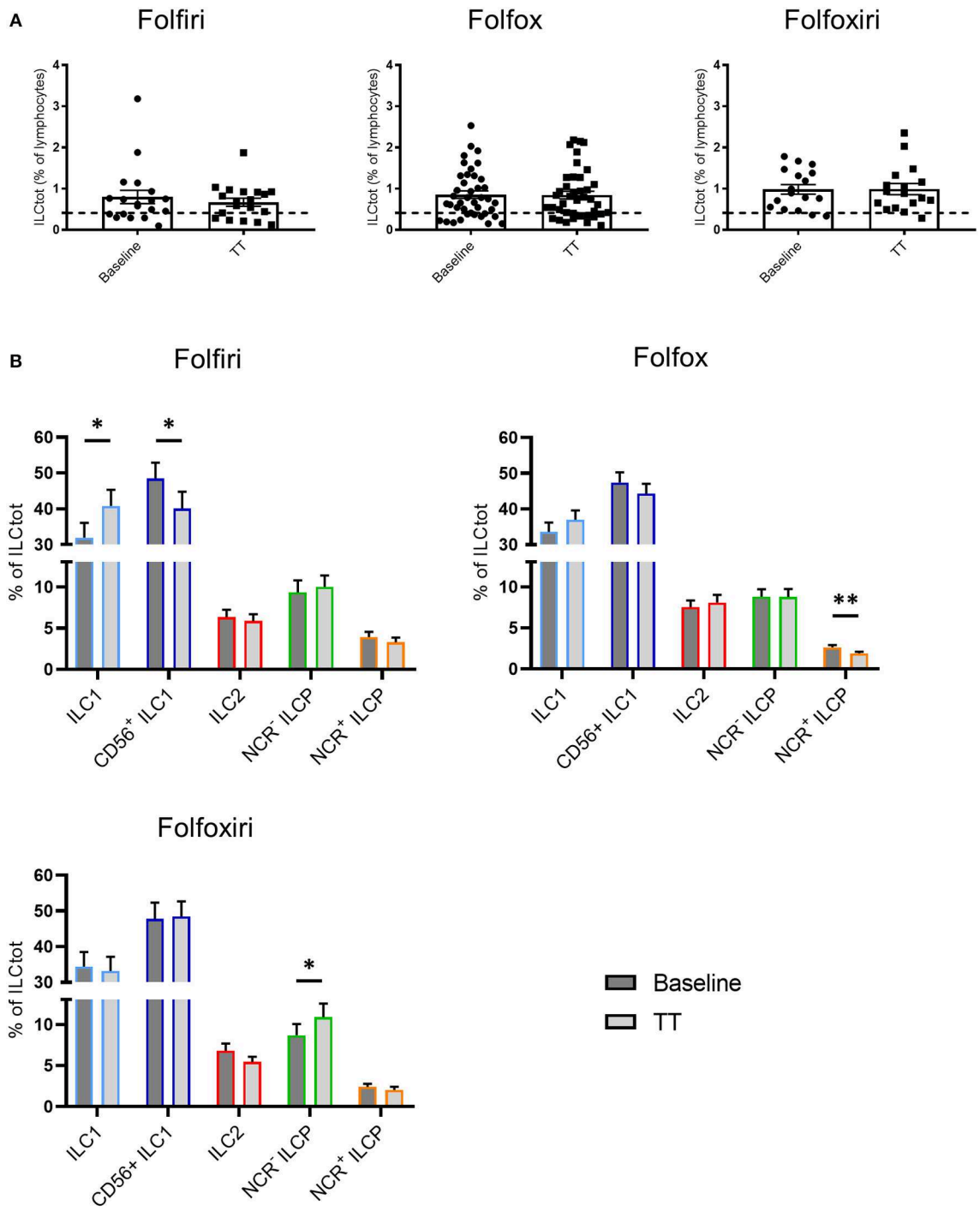

FIGURE 4 | Effect of chemotherapy treatments on ILC distribution. Frequencies of ILCtot **(A)** and ILC subsets **(B)** were analyzed by flow cytometry in PBMCs from chemotherapy-naïve mCRC patients (baseline) and after 3 months of treatment (TT). The mean frequency of ILCtot for HD is represented by a dashed line. Each patient received either the 5FU/Irinotecan regimen (Folfiri) (baseline/TT matched patients, $n = 19$), the 5FU/Oxaliplatin regimen (Folfox) (baseline/TT matched patients, $n = 40$), or the 5FU/Oxaliplatin/Irinotecan regimen (Folfoxiri) (baseline/TT matched patients $n = 17$). $^{*}p < 0.05$, $^{**}p < 0.005$, as determined by Student's *t*-test.

analysis revealed that the distribution of ILC subsets was altered in CRC patients. Indeed, only ILC1 and CD56^{+} ILC1-like cells were increased in mCRC patients at baseline compared to HD, whereas ILC2 and especially NCR^{-} ILCP and NCR^{+} ILCP were decreased. Recently, increased TGF-β in the tumor microenvironment was described as a key factor driving immune

evasion and metastases in CRC (54). Interestingly, the conversion of NK cells into noncytotoxic ILC1 has been reported as a novel mechanism of tumor immune escape, being dependent on TGF-β (55, 56). Indeed, the plasticity of ILCs could impact their role in cancer development. Collectively, these data suggest that ILC1 and ILC1-like cells could be increased due to TGF-β in mCRC patients. Further investigations are necessary to understand if the plasticity of ILCs is involved in this conversion or if the proliferation of $CD56^+$ ILC1-like cells is specifically favored.

Immunity against cancer is mainly mediated by Th1 cells (57, 58). Th1 immune infiltration is correlated to a better prognosis for patients, notably in CRC. The molecular analysis of cytotoxic and helper T cell populations in 125 colorectal tumor specimens by Galon et al. (30) showed that patients with high Th1 cluster expression have prolonged disease-free survival. More recently, data suggested that circulating tumor-specific T cells might reflect *in situ* events in the tumor (59, 60), since cancer immunity is a dynamic process that involves cell trafficking through the peripheral blood. Our results showed that higher systemic IFN-γ Th1 responses correlated with a lower frequency of total ILCs, but higher proportions of ILCP subsets. Thus, IFN-γ produced by ILC1 could drive the adaptive response toward a cancer specific Th1 profile. ILCP cells seem to also be associated with the Th1 immune response because the highest proportions of ILCP cells were found in patients with the strongest Th1-derived IFN-γ responses. It has been recently reported that NCR^+ ILCP cells are increased in human non-small cell lung cancer (NSCLC) tissue and might contribute to the formation of protective tumor-associated tertiary lymphoid structures (61). These observations might explain the specific increase in anti-tumor CD4 T cell responses.

Even if no significant PFS differences could be observed when comparing ILCtot low vs. high patients, there was a tendency for higher PFS in ILCtot low patients with high Th1 hTERT-related responses. These observations are in line with previous studies where spontaneous universal cancer peptide (UCP)-specific T-cell immune responses correlated with increased overall survival of patients responding to chemotherapy in NSCLC and anal squamous cell carcinoma (42, 62). Interestingly, no significant differences were observed between each group regarding the clinicobiological characteristics. However, it is interesting to note that the ILCtot high group was composed of only 13% MSI tumors, even if these observations are based on a few patients and considering that MSI tumors are rare in metastatic setting. In addition, MSI is known to increase the T cell repertoire involved in tumor immunity conferring a higher response to immunotherapy (2).

The immune contexture determined at diagnosis influences the prognosis of cancer patients, including patients with CRC (63). Moreover, it turned out that widely used conventional chemotherapies modulated the composition and functionality of tumor immune infiltrates and this affected disease outcome (48, 64). In addition, even if chemotherapy causes massive lymphodepletion, this could ultimately reset the immune system by favoring a rebound replenishment of various immune cell subsets and/or allow the emergence of a specific effector cell type with anticancer activity (65, 66). In this study, we explored the effects of chemotherapy regimens on the ILC compartment and we showed that the balance between NCR^+ ILCP and NCR^- ILCP was modulated. Indeed, NCR^+ ILC3 were decreased with all regimens, and especially with Folfox, whereas NCR^- ILCP were increased especially with Folfoxiri. Both regimens include Oxaliplatin, an agent already described to be an activator of tumor-targeting immune responses, mainly through the induction of immunogenic cell death (67). Folfiri does not include Oxaliplatin and is less commonly described as an immunologic modulator. However, Folfiri regimens induced a decrease in $CD56^+$ ILC1-like cells in favor of ILC1 cells. Interestingly, Folfiri has been described to support the expansion of circulating myeloid-derived suppressor cells (MDSCs), while this is not the case for Folfox (68). Thus, it would be interesting to explore the relationship between ILC1/ILC1-like cells and MDSCs in the context of mCRC.

To conclude, this study showed that ILCs are increased in the peripheral blood of chemotherapy-naïve mCRC patients and that their subset distribution is modulated. Moreover, the frequency of ILCs was found to be negatively correlated with the cancer-specific Th1 immune response. Finally, we showed that chemotherapy regimens act on the ILC compartment, especially Folfiri treatment, which modulated the balance between ILC1 and ILC1-like cells. Altogether, these results highlight the importance of considering the ILC compartment in the monitoring of immune responses in cancer to better define immune scores and eventually to identify useful biomarkers.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ANSM (*Agence Nationale de Sécurité du Médicament et des produits de santé*; no. 2012-A01377-36) with written informed consent obtained from all subjects in accordance with the Declaration of Helsinki. The protocol was approved by the independent Est-II French Committee for the Protection of Persons no. 12/672.

AUTHOR CONTRIBUTIONS

RL, BS, AG-C, and ST conducted the experiment. RL and MJ carried out data analysis. RL, MJ, CB, and CJ designed the research study. RL, MJ, BS, JG, MK, PR, ST, OA, CB, and CJ discussed the results, and wrote and/or reviewed the manuscript.

FUNDING

This work was supported by the Swiss National Science Foundation (SNSF), the Swiss Cancer League, the *Fondation pour la recherche Nuovo-Soldati*, *La Ligue contre le cancer,* and *the Interregional Programme Hospitalier pour la Recherche Clinique*.

ACKNOWLEDGMENTS

We are grateful to the patients for their dedicated collaboration and to the healthy donors for their blood and

tissue donation. We especially thank the Biomonitoring Platform of CHU Besançon for their management of patient samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02121/full#supplementary-material

Figure S1 | Flow cytometry gating strategy and expression of transcription factors for human ILC subsets. **(A)** Flow cytometry gating strategy for ILC subset identifications in PBMCs of a representative HD and a representative mCRC patient. **(B)** Transcription factors T-bet, GATA3 and RORγt were assessed by intracellular staining respectively on ILC1 and CD56$^+$ ILC1, ILC2, and ILCP. Representative example of the staining on PBMCs of a healthy donor. Gray histograms correspond to the FMO.

Figure S2 | Distribution of ILC2 c-Kit$^-$ and c-Kit$^+$ in healthy donors and metastatic colorectal cancer patients. Percentage of ILC2 c-Kit$^-$ and c-Kit$^+$ for HD and metastatic colorectal cancer patients at baseline and after treatment (TT). Columns, the means of ILC2 for each patient; bars, SEM. ****$p < 0.0001$, NS (non-significant) as determined by Student's *t*-test.

Figure S3 | Progression-Free Survival Kaplan-Meier curves of chemotherapy-naïve metastatic colorectal cancer patients. Patients ($n = 73$) stratified by the frequency of total ILCs distributed into low + medium ($n = 50$) vs. high ($n = 23$) terciles.

Table S1 | Clinicobiological characteristics of CRC patients. The table summarizes the clinicobiological characteristics of CRC patients enrolled in the "Epitopes-CRC02" trial (NCT02817178) before (baseline) and after chemotherapy treatment ($n = 96$).

REFERENCES

1. Amicarella F, Muraro MG, Hirt C, Cremonesi E, Padovan E, Mele V, et al. Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. *Gut.* (2017) 66:692–704. doi: 10.1136/gutjnl-2015-310016
2. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol.* (2010) 28:3167–75. doi: 10.1200/JCO.2009.26.7609
3. Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med.* (2015) 21:1350–6. doi: 10.1038/nm.3967
4. Halama N, Braun M, Kahlert C, Spille A, Quack C, Rahbari N, et al. Natural killer cells are scarce in colorectal carcinoma tissue despite high levels of chemokines and cytokines. *Clin Cancer Res.* (2011) 17:678–89. doi: 10.1158/1078-0432.CCR-10-2173
5. Simoni Y, Fehlings M, Kløverpris HN, McGovern N, Koo SL, Loh CY, et al. Human innate lymphoid cell subsets possess tissue-type based heterogeneity in phenotype and frequency. *Immunity.* (2017) 46:148–61. doi: 10.1016/j.immuni.2016.11.005
6. Klose CSN, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol.* (2016) 17:765–74. doi: 10.1038/ni.3489
7. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
8. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell.* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
9. Salomé B, Jandus C. Innate lymphoid cells in antitumor immunity. *J Leukoc Biol.* (2018) 103:479–83. doi: 10.1189/JLB.5MR0617-266R
10. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell.* (2017) 168:1086–100.e10. doi: 10.1016/j.cell.2017.02.021
11. Seliger B, Ruiz-Cabello F, Garrido F. IFN inducibility of major histocompatibility antigens in tumors. *Adv Cancer Res.* (2008) 101:249–76. doi: 10.1016/S0065-230X(08)00407-7
12. Kunz M, Toksoy A, Goebeler M, Engelhardt E, Bröcker E, Gillitzer R. Strong expression of the lymphoattractant C-X-C chemokine Mig is associated with heavy infiltration of T cells in human malignant melanoma. *J Pathol.* (1999) 189:552–8. doi: 10.1002/(SICI)1096-9896(199912)189:4<552::AID-PATH469>3.0.CO;2-I
13. Mukai S, Kjaergaard J, Shu S, Plautz GE. Infiltration of tumors by systemically transferred tumor-reactive T lymphocytes is required for antitumor efficacy. *Cancer Res.* (1999) 59:5245–9.
14. Hu X, Chakravarty SD, Ivashkiv LB. Regulation of interferon and toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunol Rev.* (2008) 226:41–56. doi: 10.1111/j.1600-065X.2008.00707.x
15. Dadi S, Chhangawala S, Whitlock BM, Franklin RA, Luo CT, Oh SA, et al. Cancer immunosurveillance by tissue-resident innate lymphoid cells and innate-like T cells. *Cell.* (2016) 164:365–77. doi: 10.1016/j.cell.2016.01.002
16. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol.* (2016) 17:758–64. doi: 10.1038/ni.3482
17. Bie Q, Zhang P, Su Z, Zheng D, Ying X, Wu Y, et al. Polarization of ILC2s in peripheral blood might contribute to immunosuppressive microenvironment in patients with gastric cancer. *J Immunol Res.* (2014) 2014:923135. doi: 10.1155/2014/923135
18. Chevalier MF, Trabanelli S, Racle J, Salomé B, Cesson V, Gharbi D, et al. ILC2-modulated T cell-to-MDSC balance is associated with bladder cancer recurrence. *J Clin Invest.* (2017) 127:2916–29. doi: 10.1172/JCI89717
19. Trabanelli S, Chevalier MF, Martinez-Usatorre A, Gomez-Cadena A, Salomé B, Lecciso M, et al. Tumour-derived PGD2 and NKp30-B7H6 engagement drives an immunosuppressive ILC2-MDSC axis. *Nat Commun.* (2017) 8:593. doi: 10.1038/s41467-017-00678-2
20. Di Stefano AB, Iovino F, Lombardo Y, Eterno V, Höger T, Dieli F, et al. Survivin is regulated by interleukin-4 in colon cancer stem cells. *J Cell Physiol.* (2010) 225:555–61. doi: 10.1002/jcp.22238
21. Zhou R, Qian S, Gu X, Chen Z, Xiang J. Interleukin-13 and its receptors in colorectal cancer (Review). *Biomed Rep.* (2013) 1:687–90. doi: 10.3892/br.2013.132
22. Sanderson CJ, Warren DJ, Strath M. Identification of a lymphokine that stimulates eosinophil differentiation *in vitro*. Its relationship to interleukin 3, and functional properties of eosinophils produced in cultures. *J Exp Med.* (1985) 162:60–74. doi: 10.1084/jem.162.1.60
23. Pretlow TP, Keith EF, Cryar AK, Bartolucci AA, Pitts AM, Pretlow TG, et al. Eosinophil infiltration of human colonic carcinomas as a prognostic indicator. *Cancer Res.* (1983) 43:2997–3000.
24. Saranchova I, Han J, Zaman R, Arora H, Huang H, Fenninger F, et al. Type 2 innate lymphocytes actuate immunity against tumours and limit cancer metastasis. *Sci Rep.* (2018) 8:2924. doi: 10.1038/s41598-018-20608-6
25. Huber S, Gagliani N, Zenewicz LA, Huber FJ, Bosurgi L, Hu B, et al. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature.* (2012) 491:259–63. doi: 10.1038/nature11535
26. Kryczek I, Lin Y, Nagarsheth N, Peng D, Zhao L, Zhao E, et al. IL-22(+)CD4(+) T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase DOT1L. *Immunity.* (2014) 40:772–84. doi: 10.1016/j.immuni.2014.03.010
27. Ji Y, Yang X, Li J, Lu Z, Li X, Yu J, et al. IL-22 promotes the migration and invasion of gastric cancer cells via IL-22R1/AKT/MMP-9 signaling. *Int J Clin Exp Pathol.* (2014) 7:3694–703.
28. Kirchberger S, Royston DJ, Boulard O, Thornton E, Franchini F, Szabady RL, et al. Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J Exp Med.* (2013) 210:917–31. doi: 10.1084/jem.20122308

29. Benchetrit F, Ciree A, Vives V, Warnier G, Gey A, Sautès-Fridman C, et al. Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. *Blood.* (2002) 99:2114–21. doi: 10.1182/blood.V99.6.2114
30. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res.* (2011) 71:1263–71. doi: 10.1158/0008-5472.CAN-10-2907
31. Moskalenko M, Pan M, Fu Y, de Moll EH, Hashimoto D, Mortha A, et al. Requirement for innate immunity and $CD90^+$ $NK1.1^-$ lymphocytes to treat established melanoma with chemo-immunotherapy. *Cancer Immunol Res.* (2015) 3:296–304. doi: 10.1158/2326-6066.CIR-14-0120
32. Crome SQ, Nguyen LT, Lopez-Verges S, Yang SYC, Martin B, Yam JY, et al. A distinct innate lymphoid cell population regulates tumor-associated T cells. *Nat Med.* (2017) 23:368–75. doi: 10.1038/nm.4278
33. Taylor S, Huang Y, Mallett G, Stathopoulou C, Felizardo TC, Sun MA, et al. PD-1 regulates $KLRG1^+$ group 2 innate lymphoid cells. *J Exp Med.* (2017) 214:1663–78. doi: 10.1084/jem.20161653
34. Yu Y, Tsang JCH, Wang C, Clare S, Wang J, Chen X, et al. Single-cell RNA-seq identifies a PD-1hi ILC progenitor and defines its development pathway. *Nature.* (2016) 539:102–6. doi: 10.1038/nature20105
35. Roy S, Trinchieri G. Microbiota: a key orchestrator of cancer therapy. *Nat Rev Cancer.* (2017) 17:271–85. doi: 10.1038/nrc.2017.13
36. Shay JW. Role of telomeres and telomerase in aging and cancer. *Cancer Discov.* (2016) 6:584–93. doi: 10.1158/2159-8290.CD-16-0062
37. Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat Rev Cancer.* (2011) 11:161–76. doi: 10.1038/nrc3025
38. Low KC, Tergaonkar V. Telomerase: central regulator of all of the hallmarks of cancer. *Trends Biochem Sci.* (2013) 38:426–34. doi: 10.1016/j.tibs.2013.07.001
39. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res.* (2009) 15:5323–37. doi: 10.1158/1078-0432.CCR-09-0737
40. Zanetti M. A second chance for telomerase reverse transcriptase in anticancer immunotherapy. *Nat Rev Clin Oncol.* (2017) 14:115–28. doi: 10.1038/nrclinonc.2016.67
41. Beatty GL, Vonderheide RH. Telomerase as a universal tumor antigen for cancer vaccines. *Expert Rev Vaccines.* (2008) 7:881–7. doi: 10.1586/14760584.7.7.881
42. Godet Y, Fabre E, Dosset M, Lamuraglia M, Levionnois E, Ravel P, et al. Analysis of spontaneous tumor-specific CD4 T-cell immunity in lung cancer using promiscuous HLA-DR telomerase-derived epitopes: potential synergistic effect with chemotherapy response. *Clin Cancer Res.* (2012) 18:2943–53. doi: 10.1158/1078-0432.CCR-11-3185
43. Hochdörfer T, Winkler C, Pardali K, Mjösberg J. Expression of c-Kit discriminates between two functionally distinct subsets of human type 2 innate lymphoid cells. *Eur J Immunol.* (2019) 49:884–93. doi: 10.1002/eji.201848006
44. Nguyen S, Dhedin N, Vernant JP, Kuentz M, Al Jijakli A, Rouas-Freiss N, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood.* (2005) 105:4135–42. doi: 10.1182/blood-2004-10-4113
45. Rey J, Fauriat C, Kochbati E, Orlanducci F, Charbonnier A, D'Incan E, et al. Kinetics of cytotoxic lymphocytes reconstitution after induction chemotherapy in elderly AML patients reveals progressive recovery of normal phenotypic and functional features in NK cells. *Front Immunol.* (2017) 8:64. doi: 10.3389/fimmu.2017.00064
46. Dosset M, Vauchy C, Beziaud L, Adotevi O, Godet Y. Universal tumor-reactive helper peptides from telomerase as new tools for anticancer vaccination. *Oncoimmunology.* (2013) 2:e23430. doi: 10.4161/onci.23430
47. Adotévi O, Dosset M, Galaine J, Beziaud L, Godet Y, Borg C. Targeting antitumor CD4 helper T cells with universal tumor-reactive helper peptides derived from telomerase for cancer vaccine. *Hum Vaccines Immunother.* (2013) 9:1073–7. doi: 10.4161/hv.23587
48. Galluzzi L, Buqué A, Kepp O, Zitvogel L, Kroemer G. Immunological effects of conventional chemotherapy and targeted anticancer agents. *Cancer Cell.* (2015) 28:690–714. doi: 10.1016/j.ccell.2015.10.012
49. Trabanelli S, Curti A, Lecciso M, Salomé B, Riether C, Ochsenbein A, et al. CD127+ innate lymphoid cells are dysregulated in treatment naïve acute myeloid leukemia patients at diagnosis. *Haematologica.* (2015) 100:e257–60. doi: 10.3324/haematol.2014.119602
50. de Weerdt I, van Hoeven V, Munneke JM, Endstra S, Hofland T, Hazenberg MD, et al. Innate lymphoid cells are expanded and functionally altered in chronic lymphocytic leukemia. *Haematologica.* (2016) 101:e461–4. doi: 10.3324/haematol.2016.144725
51. Salimi M, Wang R, Yao X, Li X, Wang X, Hu Y, et al. Activated innate lymphoid cell populations accumulate in human tumour tissues. *BMC Cancer.* (2018) 18:341. doi: 10.1186/s12885-018-4262-4
52. Yang J, Cornelissen F, Papazian N, Reijmers RM, Llorian M, Cupedo T, et al. IL-7-dependent maintenance of ILC3s is required for normal entry of lymphocytes into lymph nodes. *J Exp Med.* (2018) 215:1069–77. doi: 10.1084/jem.20170518
53. Krzystek-Korpacka M, Zawadzki M, Neubauer K, Bednarz-Misa I, Górska S, Wiśniewski J, et al. Elevated systemic interleukin-7 in patients with colorectal cancer and individuals at high risk of cancer: association with lymph node involvement and tumor location in the right colon. *Cancer Immunol Immunother.* (2017) 66:171–9. doi: 10.1007/s00262-016-1933-3
54. Tauriello DVF, Palomo-Ponce S, Stork D, Berenguer-Llergo A, Badia-Ramentol J, Iglesias M, et al. TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature.* (2018) 554:538–43. doi: 10.1038/nature25492
55. Gao Y, Souza-Fonseca-Guimaraes F, Bald T, Ng SS, Young A, Ngiow SF, et al. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nat Immunol.* (2017) 18:1004–15. doi: 10.1038/ni.3800
56. Cortez VS, Ulland TK, Cervantes-Barragan L, Bando JK, Robinette ML, Wang Q, et al. SMAD4 impedes the conversion of NK cells into ILC1-like cells by curtailing non-canonical TGF-β signaling. *Nat Immunol.* (2017) 18:995–1003. doi: 10.1038/ni.3809
57. Braumüller H, Wieder T, Brenner E, Aßmann S, Hahn M, Alkhaled M, et al. T-helper-1-cell cytokines drive cancer into senescence. *Nature.* (2013) 494:361–5. doi: 10.1038/nature11824
58. Kim HJ, Cantor H. CD4 T-cell subsets and tumor immunity: the helpful and the not-so-helpful. *Cancer Immunol Res.* (2014) 2:91–8. doi: 10.1158/2326-6066.CIR-13-0216
59. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature.* (2017) 541:321–30. doi: 10.1038/nature21349
60. Huang AC, Postow MA, Orlowski RJ, Mick R, Bengsch B, Manne S, et al. T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature.* (2017) 545:60–5. doi: 10.1038/nature22079
61. Carrega P, Loiacono F, Di Carlo E, Scaramuccia A, Mora M, Conte R, et al. NCR(+)ILC3 concentrate in human lung cancer and associate with intratumoral lymphoid structures. *Nat Commun.* (2015) 6:8280. doi: 10.1038/ncomms9280
62. Kim S, François E, André T, Samalin E, Jary M, El Hajbi F, et al. Docetaxel, cisplatin, and fluorouracil chemotherapy for metastatic or unresectable locally recurrent anal squamous cell carcinoma (Epitopes-HPV02): a multicentre, single-arm, phase 2 study. *Lancet Oncol.* (2018) 19:1094–106. doi: 10.1016/S1470-2045(18)30321-8
63. Anitei MG, Zeitoun G, Mlecnik B, Marliot F, Haicheur N, Todosi AM, et al. Prognostic and predictive values of the immunoscore in patients with rectal cancer. *Clin Cancer Res.* (2014) 20:1891–9. doi: 10.1158/1078-0432.CCR-13-2830
64. Senovilla L, Vacchelli E, Galon J, Adjemian S, Eggermont A, Fridman WH, et al. Trial watch: prognostic and predictive value of the immune infiltrate in cancer. *Oncoimmunology.* (2012) 1:1323–43. doi: 10.4161/onci.22009
65. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ.* (2014) 21:15–25. doi: 10.1038/cdd.2013.67
66. Romero AI, Chaput N, Poirier-Colame V, Rusakiewicz S, Jacquelot N, Chaba K, et al. Regulation of CD4(+)NKG2D(+) Th1 cells

in patients with metastatic melanoma treated with sorafenib: role of IL-15Rα and NKG2D triggering. *Cancer Res.* (2014) 74:68–80. doi: 10.1158/0008-5472.CAN-13-1186

67. Shalapour S, Font-Burgada J, Di Caro G, Zhong Z, Sanchez-Lopez E, Dhar D, et al. Immunosuppressive plasma cells impede T-cell-dependent immunogenic chemotherapy. *Nature.* (2015) 521:94–8. doi: 10.1038/nature14395
68. Kanterman J, Sade-Feldman M, Biton M, Ish-Shalom E, Lasry A, Goldshtein A, et al. Adverse immunoregulatory effects of 5FU and CPT11 chemotherapy on myeloid-derived suppressor cells and colorectal cancer outcomes. *Cancer Res.* (2014) 74:6022–35. doi: 10.1158/0008-5472.CAN-14-0657

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership