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Origin of Tissue-Resident Group 2 Innate Lymphoid Cells

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Origin of Tissue-Resident Group 2 Innate Lymphoid Cells

by

Jinwoo Lee

DISSERTATION

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DOCTOR OF PHILOSOPHY

in

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Dedicated to my wife, Stacey Moon,

And my parents, Haeseong Lee and Junhee Kim.

Acknowledgements

As the saying goes, it takes a village to raise a child, and I have found that this saying holds exceptionally true for the process of obtaining a PhD. First and foremost, I'd like to express my deep gratitude for my thesis advisor, Dr. Rich Locksley. I first came to know Rich when I interviewed at UCSF for the MD/PhD program, which despite the seven years that has elapsed since I applied, still feels like yesterday. In the thirty or so minutes that we spent together talking about science, I found myself wanting to come to UCSF to continue the conversation. A few months after the interview, I committed to UCSF, and a couple years later when I was ready to begin the graduate school portion of my program, I was lucky enough to have Rich accept me into his lab. It would be a huge disservice to Rich for me to try to fit almost five years of mentoring (as well as the entirety of the medical school and graduate school courses he teaches) into one sentence, but I think the heart of his teachings can be encapsulated in a single statement: "Ask the most interesting questions." Time and time again, whether in the classroom or during lab meeting, Rich has engaged me to think about the far-reaching implications (and limitations) of our knowledge and to design experiments that have the potential to answer some of the most interesting questions in biology and immunology.

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Contributions to the Presented Work

Chapter 2 of this dissertation is work that is being prepared for submission as a manuscript. Co-authors of this work include Christoph Schneider, Jesse Nussbaum, Roberto Ricardo-Gonzalez, Steven Van Dyken, Hong-Erh Liang, Lucas Smith, Saul Villeda, Dedeepya Vaka, and David Erle. Christoph Schneider and Hong-Erh Liang generated a novel fate-mapping mouse strain, and Christoph Schneider contributed to fate-mapping experiments using the novel mouse strain. Jesse Nussbaum, Lucas Smith, and Saul Villeda contributed to parabiosis experiments. Roberto Ricardo-Gonzalez and Steven Van Dyken contributed to cell sorting and Dedeepya Vaka contributed to single-cell RNA-sequencing analysis of the sorted cells. Richard Locksley supervised the entirety of this work.

Origin of Tissue-Resident Group 2 Innate Lymphoid Cells

Jinwoo Lee

Abstract

Group 2 innate lymphocytes (ILC2s) are developmentally programmed to generate type 2 cytokines, perhaps creating the opportunity for understanding the origins of dysregulated allergic immune responses accompanied by adaptive Th2 responses. We used a fate-mapping approach to more fully investigate the ontogeny of these cells in the mouse during prenatal, neonatal, and adult life. Using several approaches, we define the neonatal window as a period of vigorous *de novo* ILC2 generation accompanied by ILC2 activation and proliferation in all tissues of the mouse. ILC2s generated during the neonatal period remain the major constituents of adult ILC2s in the lung and visceral adipose tissue. In contrast, neonatal-labeled ILC2s are progressively replaced by adult-derived ILC2s in skin and small intestine with age, suggesting that different tissue microenvironments influence the survival and/or turnover of ILC2s over the course of adult life. Despite increased ILC2 numbers generated in response to helminthic infection, the proportion of fate-mapped cells does not significantly change, suggesting that proliferation of existing cells rather than generation of new cells is the main mechanism by which ILC2 numbers increase under inflammatory conditions. These data uncover 3 distinct phases of ILC2 ontogeny and reveal unsuspected tissue-specific effects driving the maintenance and turnover of these potentially long-lived tissue resident cells.

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Chapter 1: Introduction

"Ancient" and "Modern" Immunity

The immune system is an incredible network of cells within the body that have the ability to defend us from infectious diseases and help us fight cancer[1]. This network of cells can respond to information from a variety of sources, including microorganisms, epithelial barriers, and even the nervous system[2], and this is achieved through a diverse cast of actors, whose actions and roles are interdependent on one another. The immune ensemble can be divided into two groups from a developmental perspective: the myeloid lineage, which includes cells such as granulocytes and macrophages, and the lymphoid lineage, which includes cells such as T cells and B cells[3]. Of the two, myeloid cells are the more evolutionarily ancient; in the experiment that formed the basis for the theory of phagocytosis back in the 1880s, Elie Metchnikoff famously observed the response of phagocytes to an implanted rose thorn in the larva of a starfish[4], a member of a phylum that diverged with the last common ancestor of humans almost 900 million years ago[5]. The molecular mechanisms by which these cells respond to pathogens are also largely conserved among fruit flies (in which the response mechanism was initially discovered)[6], sea urchins[7], sea sponges[8,9], mice[10], and humans[11], representing an immune apparatus that has most likely existed in some shape or form for almost a billion years.

In contrast, lymphoid cells have only been described thus far in vertebrate animals, which though more "modern" than invertebrates, nevertheless encompasses an evolutionary history of over 500 million years[5]. A remarkable innovation that occurred in lymphocytes was the introduction of enzymes capable of rearranging somatic gene segments that when combined, encodes a receptor protein. The somatic mutations induced by these enzymes allowed lymphocytes to collectively be able to respond to a diverse range

of ligands with each individual lymphocyte having a unique (or in some cases, dual[12]) specificity, and allowed lymphocytes to pass down those specificities to their progeny as well. These features establish the fundamental mechanism by which adaptive immunity is defined, first theorized by David Talmage[13] and Macfarlane Burnet[14] in 1957, and later shown experimentally with the discovery of the recombinase gene *Rag* in 1989[15]. In a striking example of convergent evolution, the usage of such a strategy to generate adaptive immunity has occurred independently at least twice throughout the evolution of vertebrates: (i) the aforementioned RAG-mediated recombination of immunoglobulin family gene segments in jawed vertebrates to generate T-cell receptors (TCRs) and B-cell receptors (BCRs), and (ii) cytosine-deaminase-mediated gene conversion of leucin-rich repeat family gene segments in jawless vertebrates to generate variable lymphocyte receptors (VLRs)[16]. The entirely separate evolution of two independent mechanisms for adaptive immunity in lymphocytes has another remarkable implication—that before the advent of adaptive immunity, there would have existed a primordial lymphocyte in the common ancestor to jawed and jawless vertebrates that predated TCRs, BCRs, and VLRs. In other words, because lymphocytes must have existed in some manner before pathways for generating adaptive immunity arose, the ancestral lymphocyte to T cells, B cells, and the T-cell-like and B-cell-like VLR-expressing cells of the lamprey would have been an innate lymphocyte.

(As an aside, it is technically possible that the first lymphocyte already had the biochemical pathways necessary to generate both types of adaptive antigen receptors but lost one of the two pathways over time. However, the absence of the *Rag* gene in any of the jawless vertebrates studied thus far would make the possibility that the primeval

lymphocyte had a RAG-based mechanism of generating adaptive immunity unlikely. On the other hand, it is worth noting that genes with sequence homology to *Rag* have been identified in certain species of invertebrates, such as the purple sea urchin[17] and the lancelet[18], suggesting vertical transmission of the *Rag* gene from the common ancestor of vertebrates and invertebrates, with subsequent loss of the gene in jawless vertebrates and some invertebrates. An alternative explanation is that the transposon containing the *Rag* genes was introduced multiple times throughout deuterostome evolution.)

Innate Lymphoid Cells

The recent discovery of innate lymphoid cells (ILCs)—lymphocytes that do not express an antigen receptor but bear marked similarities to T cells with regard to morphology and gene expression[19,20]—gives us insight into what the primitive lymphocyte may have looked like. Though modern ILCs have probably diverged and specialized in the hundreds of millions years since the original lymphocyte first arose, the study of ILCs can still provide us with a conceptual framework that can be generalizable to the study of other lymphocytes, especially adaptive immune cells that have innate-like function and immune cells that are associated with tissue and barrier function[21]. Just as the knowledge gained from discoveries regarding lamprey lymphocyte biology has not been restricted in application to lamprey immunity, the knowledge gained from a deeper comprehension of core similarities among different members of the immune system with similar functions can help us refine our interpretations about important principles of immunology.

One of the salient features of ILCs is how they closely parallel different helper T cell (T_H) subsets both in terms of the effector cytokines they produce and in terms of the

molecular machinery that regulates their effector functions. Group 1 ILCs (ILC1s) mirror T_H1 cells in the production of the cytokine interferon-γ (IFN-γ) and the involvement of the transcription factor T-bet for their function[22,23]. Group 3 ILCs (ILC3s) mirror T_H17 cells in the production of interleukin-17 (IL-17) and IL-22 and their dependence on the transcription factor RORγt for their development[24–28]. Finally, the group 2 ILCs (ILC2s) mirror the T_H2 cell subset in the production of the type 2 cytokines IL-5, IL-13[29,30], and IL-9[31,32] and their dependence on GATA-3 for their development and function[33]. (As another aside, it is noteworthy that there are subtle differences in cytokine output between ILC2s and T_H2 cells, namely in that we[30] and others[29,34] have not observed significant secretion of the signature type 2 cytokine IL-4 from ILC2s under physiological conditions in vivo, whereas it is one of the quintessential effector cytokines for T_H2 cells. Subsequent reports have described IL-4 secretion from ILC2s after ex vivo stimulation[35] as well as in vivo expression of *Il4* transcript[36], showing that ILC2s have the capacity to produce IL-4, but further investigation is needed to understand why IL-4 protein expression from ILC2s is not detected in mice in vivo, in contrast to IL-5 expression.)

However, unlike T cells, ILC2s lack the defining feature of adaptive immunity, namely the antigen receptor (a TCR or BCR), as alluded to earlier. Instead, ILC2s can be stimulated to make their effector cytokines by other cytokines—a mechanism of "cytokine-induced cytokine production". ILC2s express high levels of receptors for the cytokines IL-25, IL-33, and TSLP, and in vivo, they have been identified as the main activators of ILC2s[29,30,37,38]. These cytokines are commonly expressed in (but not restricted to) the epithelium, and existing data support a model (Figure 1.1) wherein release of these cytokines from epithelial cells following allergic or parasitic stimuli increases both ILC2

numbers and the proportion of those ILC2s that produce IL-5 and IL-13[39]. The model allows direct and acute activation of ILC2s to environmental perturbations. Furthermore, ILC2s are widely distributed across tissues in the body[30,40], including barrier surfaces such as the lung[30,41], small intestine[29], and skin[42,43], and are in fact the principal source of IL-5 and IL-13 in early type 2 immune responses[29,30,44]. Accordingly, they have the potential to play strategic roles in the immune response of asthmatic lung inflammation[37], atopic dermatitis[45], and parasitic worm infection[46].

More interestingly, recent studies have identified roles for type 2 cytokines and ILC2s in a broader range of biological contexts, including metabolic homeostasis[47] and the response of immune cells to circadian rhythm[40], suggestive of contributions to other processes that are unappreciated. The presence of ILC2s at non-barrier sites, such as the mesenteric adipose tissue[34] and perigonadal adipose tissue[47], lends credence to a non-immune role for ILC2s. From the time when initial reports described epithelium-associated cytokines as activators of ILC2s[29,37,38,48–50], reports have since been published describing the ability of ILC2s to respond to a diverse array of signaling molecules, including autocrine IL-9[31,32], TL1A (a TNF family member)[51], lipid mediators of inflammation[35,52,53], and neuropeptides[40,54–56] highlighting roles for ILC2s not only during perturbed states, but under homeostatic conditions as well. As such, ILC2s may act as key integrators of endogenous signals, which underscore the homeostatic functions of type 2 cytokines. The expanding domain of processes linked to type 2 immunity draws attention to the question of why we have the type 2 response and what value the type 2 response has to the fitness of an organism, the answer to which may be connected to the steady-state role of ILC2s in tissues.

Figure 1.1

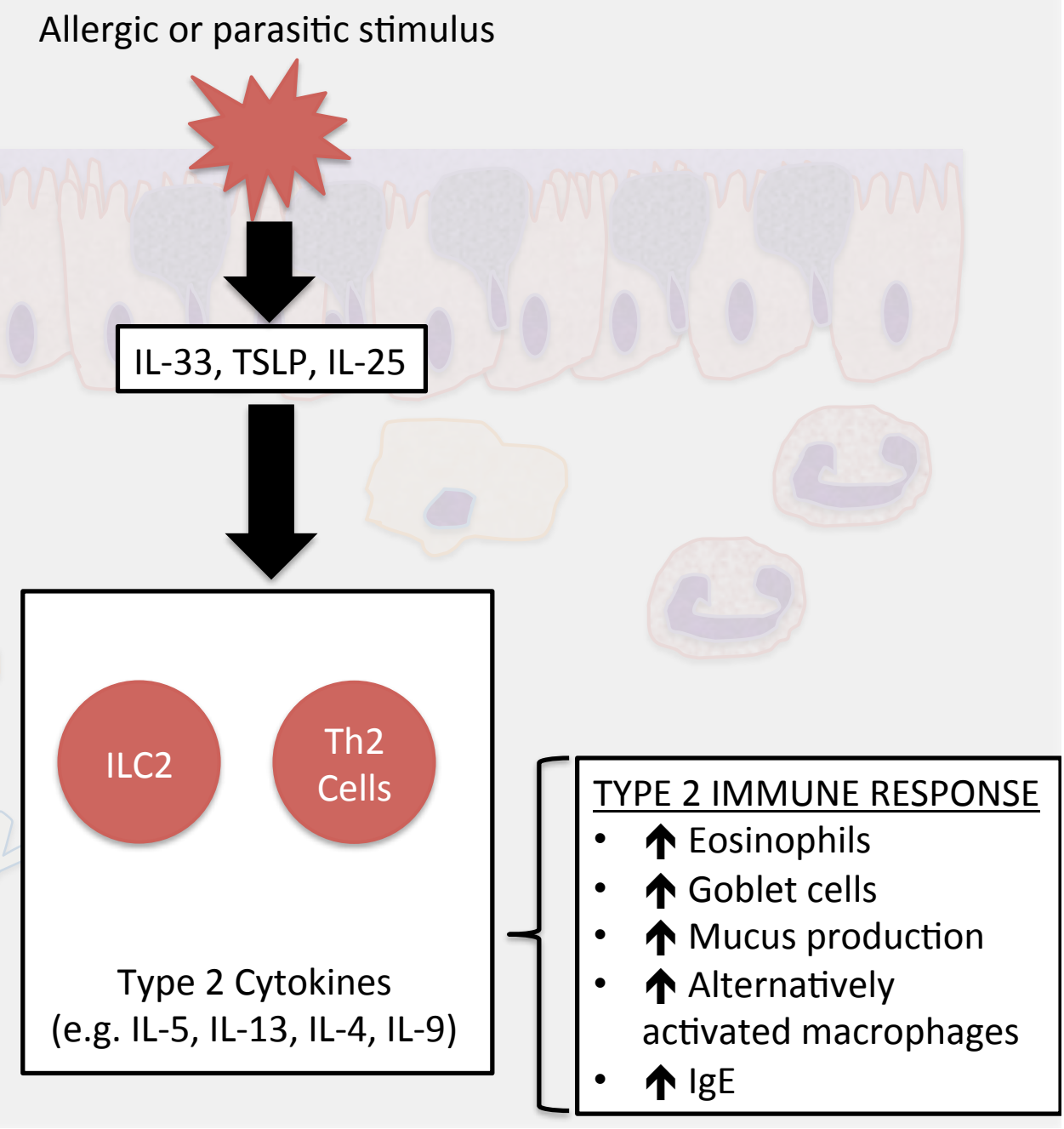
Allergic or parasitic stimulus
IL-33, TSLP, IL-25
ILC2
Th2 Cells
Type 2 Cytokines
(e.g. IL-5, IL-13, IL-4, IL-9)
TYPE 2 IMMUNE RESPONSE
↑ Eosinophils
↑ Goblet cells
↑ Mucus production
↑ Alternatively activated macrophages
↑ IgE

Figure 1.1: Activation of the type 2 immune response by epithelial cytokines
Allergic and parasitic stimuli can cause the release of epithelium-derived cytokines, such as IL-25, IL-33, and TSLP, which in turn can activate immune cells with receptors for these cytokines, such as ILC2s and fully differentiated T_H2 cells. ILC2s are major producers of the type 2 cytokines IL-5, IL-13, and IL-9 in vivo, and at later stages of the response, when adaptive immune cells begin to participate, T_H2 cells (and basophils) also produce type 2 cytokines, such as IL-4. Type 2 cytokines are responsible in large part for initiating and maintaining the increased production of mucus and immunoglobulin E (IgE) and the accumulation of eosinophils and alternatively activated macrophages (AAMs) that characterize what is called the type 2 immune response.

Type 2 Cytokines

Allergic disorders, such as seasonal allergic rhinitis and eczema, as well as parasitic infections, are all characterized by a particular type of immune response, involving the influx of eosinophils, production of immunoglobulin E, secretion of mucus, and activation of alternatively activated macrophages[57]. As mentioned earlier, this constellation of reactions is termed a type 2 immune response, and at the organismal level, the symptoms associated with a type 2 immune response can manifest themselves as experiences commonly encountered in modern life—runny noses, itchy eyes, sneezing. However, more dangerous forms of allergic dysfunction, such as severe food or drug allergies, as well as allergic asthma, can have much more dire consequences for the individual, including in the worst cases, morbidity and mortality.

The type 2 immune response is initiated and maintained in large part by the type 2 cytokines IL-4, IL-5, and IL-13[57]. These genes reside together in a ~200-kilobase (kb) region, which also includes the *Kif3a* and *Rad50* genes, on mouse chromosome 11 (chromosome 5 in humans). Two other related genes, encoding the cytokines IL-3 and GM-CSF, also reside on the same chromosome approximately 500 kb upstream of the *Il5/Il13/Il4* region. The arrangement of these genes is conserved between mice and humans, and the close relationship among these genes goes beyond spatial proximity in the genome. The IL-4Rα receptor subunit is shared between the receptors for IL-4 and IL-13, and similarly, the β_c receptor subunit (also called the common beta chain) is shared among the receptors for IL-5, GM-CSF, and IL-3. The similarities in receptor binding and signaling among these genes, as well as the conservation of the organization of these genes among species, make it highly likely that these cytokine genes were the product of gene

duplication events during vertebrate evolution despite their relatively low sequence identity (~23% identity between IL-4 and IL-13, ~25% identity between IL-5 and GM-CSF)[58]. Another related gene, encoding IL-9, resides approximately 3 megabases downstream of the *Il5/Il13/Il4* region in humans (but is located on a different chromosome in mice).

While all of these genes may be intimately related evolutionarily, the "core" drivers of the mucus accumulation, increased smooth muscle responsiveness, eosinophilia, and adaptive IgE responses seen in type 2 immune processes have been found to be IL-4, IL-5, and IL-13, with mounting evidence for involvement of IL-9[59–63]. Administration of neutralizing anti-IL-4 antibodies to mice infected with the larvae of *Nippostrongylus brasiliensis*, a parasitic worm that is a strong stimulator of the type 2 immune response, suppresses IgE production[64] , and administration of anti-IL-4 also ameliorates airway hyperreactivity in mice that had been sensitized then challenged with aerosolized antigen[65]. Similarly, mice that are genetically deficient for IL-4 have low IgE levels, as well as diminished T_H2 cytokine production[66,67], demonstrating the role of IL-4 in IgE and T_H2 responses.

Because IL-4 and IL-13 are closely related genes and share a receptor component, the initial thought at the time of discovery was that IL-13 would have functions similar and redundant to IL-4. However, selective neutralization of IL-13 using an IL-13R-Fc antibody revealed that IL-13 was necessary for allergic responses, independent of IL-4[68,69]. Administration of the IL-13 blocking antibody to mice in an experimental asthma model abrogated the hyperresponsiveness of airway smooth muscle, the recruitment of eosinophils, and the hyperplasia of mucus-producing goblet cells[68,69]. Furthermore,

administration of recombinant IL-13 (or IL-4) alone without antigen sensitization or challenge was sufficient to induce the asthma phenotype seen in the experimental asthma model[68,69], demonstrating the importance of IL-13 and IL-4 in the pathogenesis of asthma.

The major effect of IL-5 in allergic responses appears to be on eosinophils, as administration of anti-IL-5 suppresses eosinophilia (but not airway hyperreactivity) in experimental asthma[65], and mice genetically deficient for IL-5 do not develop blood or tissue eosinophilia upon parasitic worm infection[70], demonstrating the role of IL-5 in eosinophilic responses. Furthermore, a baseline level of eosinophil generation and eosinophil accumulation in tissues also occurs at steady state, and IL-5—from ILC2s—has been found to regulate homeostatic eosinophilopoiesis and eosinophil tissue accumulation[40].

The discovery of the roles for type 2 cytokines has allowed the development of antibody-based therapies for allergic diseases. In particular, dupilumab, a monoclonal antibody to human IL-4Rα that blocks both IL-4 and IL-13 signaling, has been shown to be effective in clinical trials for the treatment of persistent, moderate-to-severe eosinophilic asthma[71,72] and moderate-to-severe atopic dermatitis[73,74]. Additionally, monoclonal antibodies to human IL-5 (mepolizumab[75–79] and reslizumab[80,81]) and IL-5Rα (benralizumab[82–85]) have already been approved for use in severe eosinophilic asthma[86], attesting to the potential of basic science research to lead to novel and meaningful interventions that improve human health. Further research into the cells that secrete these cytokines may yield even more advances in our treatment strategies for severe allergic diseases.

Why Allergy?

Although some of the major molecular pathways in allergic diseases have been identified, there is continuing uncertainty as to why allergies are widespread and increasing in prevalence, most noticeably in developed countries[87,88]. One explanation that has been proposed is the so-called "hygiene hypothesis", which in its original formulation[89], conjectured that decreases in household sizes and increases in hygiene standards have reduced the opportunities for early childhood infections that can prevent allergic diseases. The reduction in childhood infections was postulated to cause the higher occurrence of hay fever and other allergic diseases. This idea gained more traction with the introduction and acceptance of the T_H1/T_H2 paradigm in immunology[90], whereby the effect of viral or bacterial infections would be to bias the child's immune system toward the T_H1 (or T_H17) pathways and away from the T_H2 pathway. In this model, as infections became less and less prevalent, the T-cell pool would become more and more imbalanced and skewed toward T_H2 differentiation, leading to the amplified type 2 responses seen in many allergic disorders.

The results of a study that would seem to support this notion were obtained after the 1990 German reunification, when it became possible to compare prevalence rates of asthma and atopy between populations of similar ethnicity that had been living under very different environmental conditions[91]. The study found a lower prevalence of allergic symptoms in East Germany than in West Germany, and though the frequency of childhood infections was not directly measured, East German children had had more exposure to daycare settings and significantly more siblings (and were thus presumed to be at more risk for cross-infection) than West German children. These findings were replicated in

subsequent studies comparing other populations that were of similar ethnic backgrounds and in different environments[92,93], but have also been disputed by the fact that US daycare attendance increased over the time period when asthma prevalence was growing most rapidly, as well as studies showing mixed or conflicting results regarding the risk effects of childhood exposure to other children[94,95], bacterial colonization[96], and markers of infection[97,98]. Later epidemiological studies looking directly at the incidence of early-life infections and allergic disorders found no relationship between childhood infections and the risk of developing atopic dermatitis, asthma, or allergic rhinitis[99–101]. Further clinical counterevidence against the original "hygiene hypothesis" can be found in the strong positive correlation between persistent asthma and neonatal respiratory viral infections[102], particularly with respiratory syncytial virus (RSV)[103], and in mouse models, microbial exposure can have either protective or exacerbating effects on allergic disease, depending on the exposure[104].

These contrary findings bring to light the fact that the complex processes underlying the development of allergy cannot be reduced to the generalization that allergy is caused by a lack of infections, and it has become clear that the hygiene hypothesis as originally articulated had oversimplified the myriad effects of microbial infection. Likewise, the argument of T_H1/T_H2 counterbalance must also be viewed with a degree of skepticism, as not only allergies, but also autoimmune diseases mediated by T_H1 and/or T_H17 processes are increasing in prevalence[105,106]. In addition, T_H2-inducing parasitic worm infections have been found to protect against, rather than aggravate, allergic symptoms in mouse models and human studies[107,108].

In light of these data, the concept of the hygiene hypothesis has pivoted by incorporating recent developments regarding the contribution of regulatory T (T_{reg}) cells to immune tolerance[109] and recognizing the importance of the commensal microbiota[110,111]. Since its original inception in 1989, the hygiene hypothesis in its current iteration[112] has evolved into a much more sophisticated argument, namely that aspects of a "Western" lifestyle, such as diet, antibiotics access, and indoor life, lead to altered and dysregulated immune responses through direct and indirect effects on the individual's immune system and commensal microbiome. (Aside: The current version of the hygiene hypothesis now seems to have little relation with the term "hygiene" in the colloquial sense. Indeed, studies have found no link between allergic disease incidence and personal hygiene or home cleanliness[113]. Commensal bacteria are far more likely to be disrupted by diet, antibiotics, and other facets of a Western lifestyle than it is by cleanliness behaviors.)

Despite numerous revisions to the original theory, one constant throughout the different versions of the hygiene hypothesis was the importance of the early postnatal developmental window in determining the magnitude of environmental and microbial influences. Several of the risk factors and protective factors identified for allergic diseases (such as the mode of delivery[114], breastfeeding[115], and number of infections within the first year of life[106]) affect the microbiome and host immune system during a vulnerable and impressionable period when organs are still developing, epithelial barriers are becoming established, and host defense mechanisms are being educated. The presence of ILC2s in tissues during this critical window, which is before the establishment of mature T-cell responses, as well as their known involvement in type 2 immune processes, makes ILC2s an enticing population to study during development.

But Why Allergy?

As described in the previous section, various environmental and lifestyle factors, particularly during early life, appear to influence the development of allergic diseases. But the question of why we have an allergic immune response in the first place still remains. What are the precise origins of type 2 immunity? The type 2 immune response certainly did not evolve to inflict hay fever on us, and the prevailing view is that allergic disorders reflect maladaptation of normal physiological responses. So what is the normal physiologic function of this arm of immunity and this family of cytokines?

The "textbook" explanation for type 2 immune cells and cytokines is that they mediate anti-helminthic immunity[3], and T_H2 immunity does seem to play a significant part in host resistance to infection with helminthes, such as hookworms or roundworms. Blocking IL-13 signaling in mice following infection with *N. brasiliensis*[116] prevents worm expulsion, whereas normally all worms are expelled from mice within a couple weeks of infection. However, it is important to note that while *N. brasiliensis* is used extensively in mice to model type 2 immune responses, *N. brasiliensis* is not a parasitic worm that is adapted to mice. The primary natural hosts for *N. brasiliensis* are in fact, rats, in which the worms can persist and continue laying eggs for prolonged periods, despite eliciting similar type 2 responses, such as eosinophilia[117], goblet cell hyperplasia[118], and induction of IgE[119]. Among feral animals or traditional human populations, the evidence for meaningful immune protection against parasites, which by definition have adapted to and co-evolved with their hosts, remains ambiguous, and the presumed anti-helminthic roles of eosinophils and IgE have also been called into question[120].

That being said, the health burden of helminthic infections is not inconsequential, particularly among young children in non-Westernized regions of the world, where helminthic infections can have profound detrimental effects on health and development[121]. More than an estimated 200 million people are infected with schistosomes (parasitic blood flukes) worldwide[122], causing up to 200,000 deaths per year[123,124]. Given the substantial morbidity caused by helminthes that may not have fully adapted to their hosts (as a fully adapted parasite would presumably not kill its host), it is possible that the T_H2 arm of adaptive immunity grew out of a need to control such errant parasites. Indeed, in addition to the requirement for IL-13, $CD4^+$ T cells are also essential for worm expulsion in mouse models of helminthic infection[116], lending support for this viewpoint.

However, CD4 T cells are not the only cell type capable of producing type 2 cytokines, and the more intriguing possibility is that adaptive T-cell-mediated immunity co-opted parts of an already existing homeostatic pathway to mediate the expulsion of harmful parasites and noxious stimuli. The part of the pathway we observe in allergic and parasitic contexts is the so-called "weep and sweep" response, but the type 2 cytokine network itself may be deeply rooted in the maintenance of organ physiology, including the processes related to epithelial differentiation and serous and mucous secretion that manifest themselves in allergic responses. The numerous T-independent roles of type 2 cytokines in glucose homeostasis[125], peripheral nutrient metabolism[126], and tissue regeneration[127–129] are suggestive of the true origins of type 2 immunity being seated not in the adaptive control of parasitic infections but in the innate regulation of responses to perturbations in homeostasis. Thus, the study of the steady-state role of innate type 2 immune cells, particularly the role of basally activated ILC2s, may provide a window into

the foundation of type 2 immunity. To go a step further, it would be of additional interest to study innate type 2 immunity during periods of growth and development when the principal programs governing organ physiology get engaged for the first time.

Chapter 2: Origin of Group 2 Innate Lymphoid Cells

Abstract

Group 2 innate lymphocytes (ILC2s) are developmentally programmed to generate type 2 cytokines, perhaps creating the opportunity for understanding the origins of dysregulated allergic immune responses accompanied by adaptive Th2 responses. We used complementary fate-mapping approaches to more fully investigate the ontogeny of these cells in the mouse during prenatal, neonatal, and adult life. Here, we define the neonatal window as a period of vigorous de novo ILC2 generation accompanied by ILC2 proliferation, activation, and acquisition of tissue-specific expression profiles. ILC2s generated during the neonatal period remain the major constituents of adult ILC2s in the lung and perigonadal adipose tissue, although neonatally labelled ILC2s are progressively replaced by adult-derived ILC2s in skin and small intestine with increasing age, suggesting that different tissue microenvironments influence the survival and/or turnover of ILC2s over the course of adult life. Despite increased ILC2 numbers generated in response to helminthic infection, the proportion of fate-mapped cells does not significantly change, suggesting that proliferation of existing cells rather than generation of new cells is the main mechanism by which ILC2 numbers increase under inflammatory conditions. These data uncover 3 distinct phases of ILC2 ontogeny and reveal unsuspected dynamic complexities and tissue-specific effects driving the identity, maintenance, and turnover of these important tissue-resident cells.

Introduction

Asthma and allergic diseases, such as hay fever, food allergy, and eczema, affect more than 60 million people in the United States across all age groups, and represent a

major health burden to patients, their families, health care systems, and governments worldwide[130]. Epidemiologic evidence has increasingly linked various environmental exposures during early years of life with the risk of developing allergies, as broadly delineated by the "hygiene hypothesis"[112]. The discovery of group 2 innate lymphoid cells[29,30,34], or ILC2s, which express type 2 cytokines in the absence of antigen receptors, offer an opportunity to look for functions of these cells independent of traditional host immune defense in hopes of revealing the contextual purpose of allergic immunity. Indeed, activation of these cells by a variety of epithelial cytokines[29,30,34,43], neuropeptides[40,54–56], and eicosanoids[35,53], and involvement in aspects of metabolism[47] and tissue repair and remodeling in multiple organs[41,131,132], have begun to outline an unsuspected role for type 2 immunity integrated deeply into aspects of basal homeostasis[133–135]. Further understanding might be gained by examining the development, activation, and turnover of these cells in tissues over the lifespan, and particularly during early life when epidemiologic evidence suggests that environmental inputs may be especially critical.

Temporally restricted in vivo lineage tracing has accelerated our understanding of hematopoietic development from the conventional model that all leukocytes arise from bone marrow hematopoietic stem cells (HSCs) to the current model that progressive waves of immune cell generation begin in early development. In the macrophage field in particular, fate-mapping studies have overturned the dogma that tissue-resident macrophages rely on constant replenishment from blood monocytes for homeostasis[136], with the discovery that multiple waves of precursor cells—from early-embryonic yolk sac macrophages, from late-embryonic fetal monocytes, and from adult blood monocytes—layer together in differing proportions to comprise the tissue-resident macrophage pool in

different organs[137]. The ability to distinguish cells based on ontogeny has also allowed novel insights into how the transcriptomic and epigenomic landscapes differ among macrophages of different origins, whether from fetal hematopoiesis in the yolk sac and liver or from adult hematopoiesis in the bone marrow[138,139]. ILC progenitors with the capacity to give rise to mature ILCs in vitro and in vivo upon transfer into lymphopenic mice had been identified in fetal tissues[140–142] as well as in adult bone marrow[33,140,143], but the relative contributions of these populations and their turnover in adult tissue ILC2 pools remains poorly studied, in part due to the lack of tools to reliably fate-map and track these cells.

The development of innate lymphoid cells requires the transcriptional regulator Id2[34,144,145], and high levels of Id2 expression are maintained in mature innate lymphoid cells[33]. Putative ILC precursors that express Id2 are present in the bone marrow[140,143], providing a potential source for these cells. However, ILC precursors that express the urea cycle enzyme Arg1 are present in the small intestine before birth[141], which suggests that ILCs seed tissues prenatally, and might arise predominantly from embryonically generated precursors. Thus, bone marrow ILC2s may be another tissue-resident ILC2 population, and their true function in vivo as precursors has not been definitively addressed. Results from parabiosis experiments[146,147], in which pairs of congenically distinct mice are surgically joined for 4-8 weeks to allow sharing of circulating blood constituents, reveal minimal replacement of tissue ILC2s by circulating cells under steady-state conditions, implying little contribution from blood-borne precursors, and consistent with the designation of ILC2s as predominantly tissue-resident cells[148]. As a caveat, cells with limited blood dwell time and low blood frequency at steady state may not show an appreciable rate of

crossover between the two parabionts during the conjoined period, and a slow rate of replacement from putative bone marrow precursors cannot be conclusively ruled out. Indeed, one study noted a slight increase in ILC2 crossover when mice are parabiosed for 4-5 months rather than 4-6 weeks[146], and similar increases were observed in parabionts challenged with IL-25 or helminthic infection and anastomosed up to 8 months[149].

Whether tissue-resident ILC2s represent embryonically established ILC2s that are long-lived, and thus resemble long-lived tissue-resident cells such as microglia[150,151], alveolar macrophages[152–154], and Langerhans cells[155], or whether new cells continuously replace these cells, akin to the case for blood monocyte-derived intestinal macrophages[156], remains unclear. These two possibilities are not mutually exclusive; the tissue ILC2 population may reflect a heterogeneous pool of embryonically derived and adult-derived cells that have distinct behaviors. Indeed, recent studies using more refined reagents suggest that intestinal macrophages represent populations of both long-lived cells potentially seeded during fetal hematopoiesis and more frequently replaced cells populated from monocytes from adult bone marrow[157]. Even following allergic or parasitic challenge, when numbers of ILC2s increase by up to 8-fold in affected tissues, parabiosis experiments suggest that the increase in ILC2s is largely a result of proliferation in situ rather than recruitment from circulating progenitors[146].

We used Id2-driven and Arg1-driven fate-mapping strategies together with transcriptome profiling to assess the ontogeny and turnover of ILC2s in mouse tissues over the period from prenatal tissue seeding to adulthood in order to clarify the origins and lifespan of these cells in tissues, and to gain greater understanding of the role of ILC2s in local and systemic contributions to type 2 immunity.

Results

Tissue ILC2s are replaced by newly generated ILC2s at a low rate

To determine the temporal origin of ILC2s, we used two independent tamoxifen-inducible Cre mouse strains to lineage-trace ILC2s. First, we generated a novel *Arg1-RFP-CreER^{T2}* mouse strain, in which expression of red fluorescent protein (RFP) as well as tamoxifen-inducible Cre (CreER^{T2}) is under the control of the endogenous arginase-1 (*Arg1*) promoter (Fig. 2.1A). Consistent with previous reports[141,158], *Arg1-RFP-CreER^{T2}* mice accurately demonstrate *Arg1* expression in CD25^{+}Id2^{GFP+} ILC2s from the bone marrow, lung, and adipose tissue. In contrast, *Arg1* expression was absent in CD25^{-}Id2^{GFP+} bone marrow ILC precursors (CHILPs), skin-resident ILC2s, and in a fraction of ILC2s from the small intestine lamina propria[159], confirming heterogeneity in Arg1 expression among mature ILC2 subsets in different tissues (Ricardo-Gonzalez, Van Dyken et al, under review). As a second and independent approach, we used a previously described tamoxifen-inducible Cre strain, the *Id2-CreER^{T2}* knock-in mouse strain[160], to compare and confirm results obtained using the *Arg1-RFP-CreER^{T2}* strain.

Figure 2.1

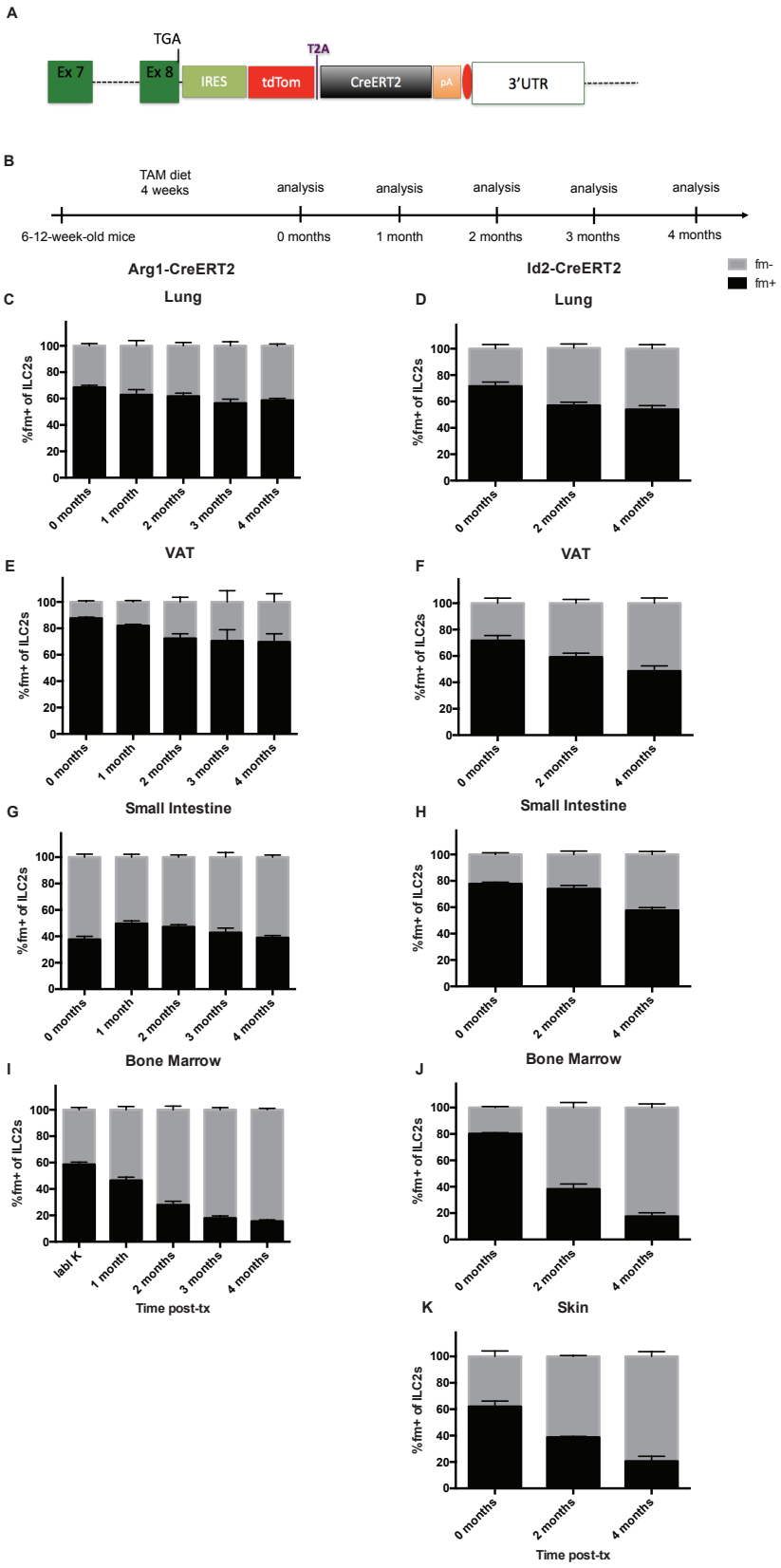
A
TGA
T2A
Ex 7
Ex 8
IRES
tdTom
CreERT2
pA
3'UTR
B
TAM diet
4 weeks
analysis
analysis
analysis
analysis
analysis
6-12-week-old mice
0 months
1 month
2 months
3 months
4 months
Arg1-CreERT2
Id2-CreERT2
fm-
fm+
C
Lung
D
Lung
E
VAT
F
VAT
G
Small Intestine
H
Small Intestine
I
Bone Marrow
J
Bone Marrow
K
Skin
%fm+ of ILC2s
Time post-tx

Figure 2.1. Arg1-RFP-CreER^{T2} and Id2-CreER^{T2} fate mapping shows a slow rate of replacement by newly generated cells.
(A) Schematic of the *Arg1* locus with the inserted IRES-tdTomato-CreERT2-pA construct. **(B)** Schedule of tamoxifen administration and analysis for adult fate mapping. **(C-I)** Fate mapped ILC2 percentages using the *Arg1-RFP-CreER^{T2}* and *Id2-CreER^{T2}* mouse strains in the (C,D) lung, (E,F) visceral adipose tissue, (G,H) small intestine lamina propria, and (I) back skin. ILC2s were gated as Lin$^-$Thy1$^+$ST2$^+$ (lung and VAT), Lin$^-$IL17RB$^+$KLRG1$^+$ (small intestine), or Lin$^-$Thy1$^+$ICOS$^+$ (skin). Data are displayed as mean ± SEM of 4-7 individual mice from at least 2 independent experiments per labeling and analysis time point.

To assess the turnover of adult ILC2s, we first crossed these strains to R26R reporter mice[161,162]. Adult mice (6-12 weeks of age) were placed on tamoxifen diet for 4 weeks, and then analyzed monthly for 4 months after return to chow diet (Fig. 2.1B). Notably, the bone marrow ILC2 population was steadily replaced by newly generated unlabeled cells, as assessed by the decreasing proportion of fate-mapped cells in both strains of mice (Fig. 2.1I,J). By 2-3 months after removal from tamoxifen diet, fewer than half of bone marrow ILC2s remained fate-mapped. Despite the replacement of ILC2s in the bone marrow, there was minimal replacement of tissue ILC2 populations by newly generated ILC2s over the course of 4 months following removal from tamoxifen diet (Fig. 2.1C-H), with the notable exception of the skin (Fig. 2.1K), suggesting a very slow rate of tissue ILC2 replenishment through de novo generation from a non-fate-mapped population. Taken together, these data are consistent with generally stable long-lived populations of ILC2s in most adult tissues, but with evidence for more rapid turnover in bone marrow and skin.

Tissue ILC2s do not recirculate or repopulate empty niches at steady state

To further assess the homeostatic turnover of tissue ILC2s, we performed parabiosis experiments in which CD45.1 mice carrying an Arg1 YFP reporter allele (*Yarg*) were surgically conjoined with CD45.2 wild-type (WT) mice (Fig. 2.2A). After 5 weeks, the lungs and spleens of WT recipients contained approximately equivalent numbers of $CD4^+$ T cells and eosinophils derived from each parabiont, indicating the free exchange of these circulating cells across the surgical anastomosis (Fig. 2.2B). However, consistent with previous reports over similar periods[146,147,149], lung ILC2s showed <5% crossover between the paired mice, suggesting a relatively long tissue lifespan and limited replenishment from

the circulation. Because niche occupancy in the WT mice may have prevented circulating progenitors from engrafting between the parabionts, we also tested the ability of ILC2s to reconstitute IL-7Rα-deficient recipients, which lack ILCs, B cells, and T cells. Although the percentage of parabiont-derived ILC2s was higher in the IL-7Rα-deficient host (Fig. 2.2D,E), perhaps due to increased niche availability and/or the presence of uncontested growth factors, including IL-7, the total number of ILC2s was still substantially below wild-type levels (Fig. 2.2D), confirming that ILC2s are predominantly long-lived tissue-resident cells, which maintain themselves during adulthood largely independent from replenishment by circulating precursors. We observed the same patterns of replacement when assessing ILC2s in the adipose tissue and in the small intestine lamina propria (Fig.2.3C,D), and thus extend prior observations using these types of approaches[146,147].

Figure 2.2

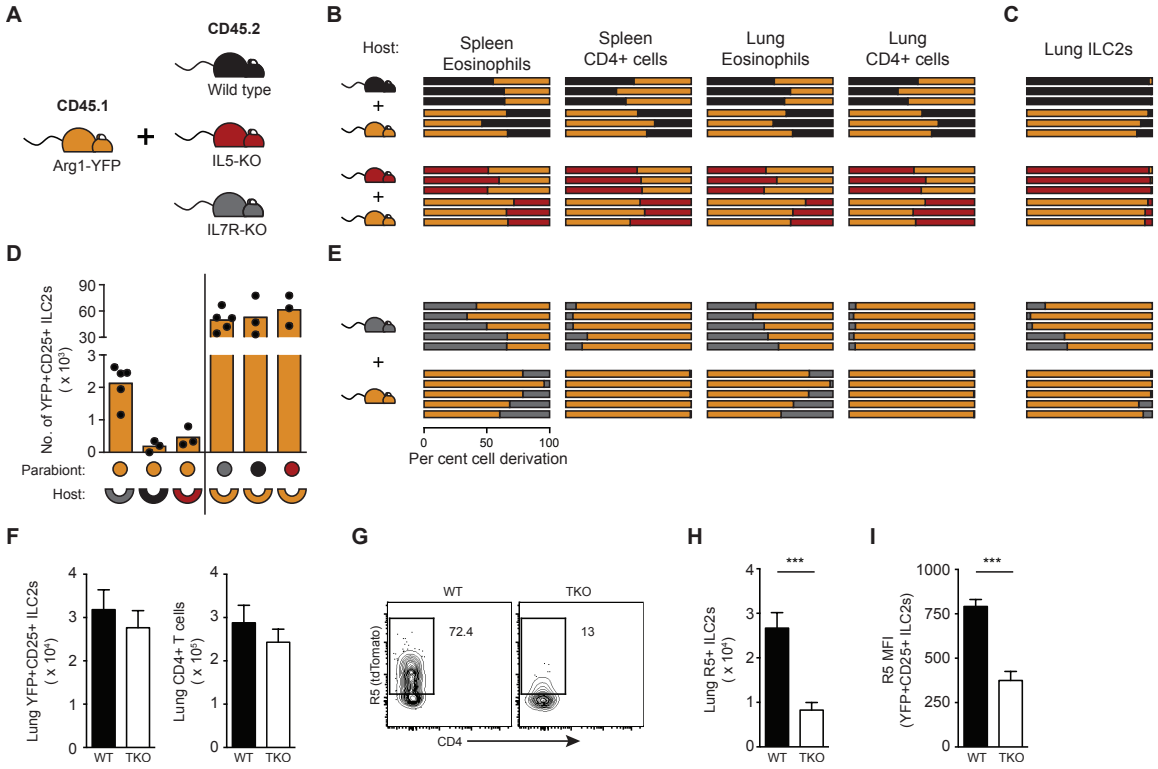

Figure 2.2. Parabiosis shows minimal replacement of tissue ILC2s at steady state.

(A) Schematic of CD45.1 Arg1-YFP reporter mice (yellow) parabiosis with either WT (black) or IL7Rα-deficient (gray) mice. Percent derivation of **(B)** eosinophils and CD4 T cells from the spleen and lung and **(C)** ILC2s from the lung, where each bar represents a single mouse from the WT and Arg1-YFP pair. **(D)** Numbers of lung $Arg1^{YFP+}CD25^{+}$ ILC2s, where each dot represents one host mouse. Percent derivation of **(E)** eosinophils and CD4 T cells from the spleen and lung and **(F)** ILC2s from the lung, where each bar represents a single mouse from the IL7Rα-KO and Arg1-YFP pair.

Figure 2.3

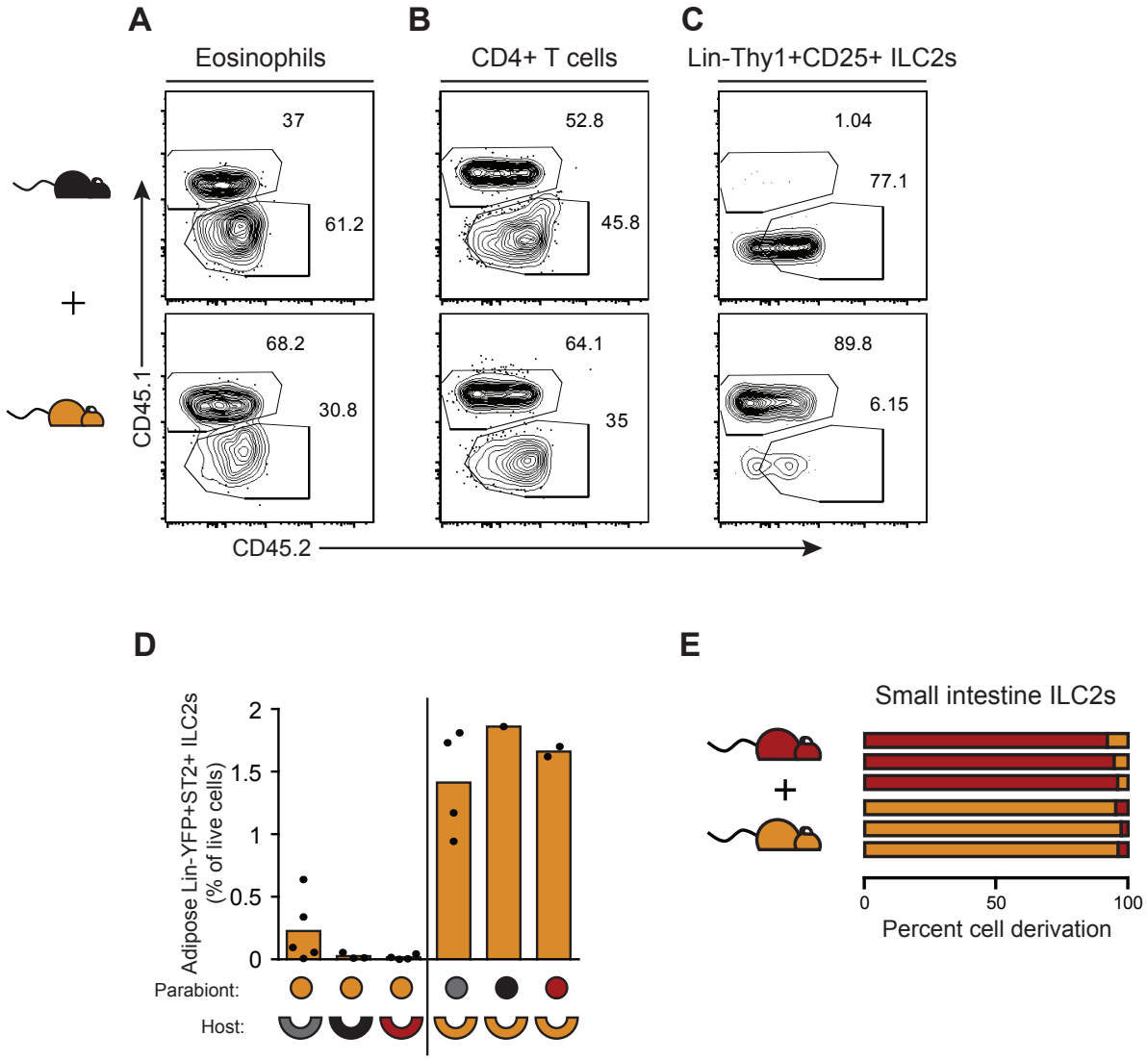

Figure 2.3. Representative flow cytometry plots for CD45.1 and CD45.2 gating and percentages of parabiont-derived ILC2s in adipose tissue and small intestine

Gating to differentiate CD45.1 and CD45.2 cells among **(A)** eosinophils, **(B)** $CD4^+$ T cells, and **(C)** ILC2s in the lungs of wild type CD45.2 (top) and Arg1-YFP CD45.1 (bottom) mice 5-6 weeks after parabiosis surgery. **(D)** $Arg1^{YFP+}CD25^+$ ILC2s in adipose tissue of host mice, enumerated as a percent of total live $CD45^+$ cells, where each dot represents one host mouse, with parabionts as labeled. **(E)** Percent derivation of lamina propria ILC2s in CD45.2 (red) and Arg1-YFP CD45.1 (yellow) pairs, where each bar represents a single mouse. Data are from one surgery cohort and representative of three independent surgery cohorts.

Prenatal ILC2 precursors seed tissues during fetal development and contribute to the adult ILC2 tissue pool

Our results using adult lineage tracing in two independent reporter strains and two conditions of parabiosis suggest that tissue ILC2 pools are established prior to reaching adulthood. Many innate tissue-resident cells, including tissue macrophages, are seeded during fetal development[163], and previous work in our laboratory has characterized Arg1-expressing ILC precursors in the fetal small intestine[141]. As assessed using flow cytometry, $Arg1^+$ cells with a surface marker expression profile of mature ILC2s (e.g. Lin^- $IL7R\alpha^+Thy1^+ST2^+$) appear as early as embryonic day 17.5 (E17.5) in the fetal lung, small intestine, and skin (Fig. 2.4A). These cells express GATA-3 and Ki-67, consistent with active cell proliferation (Fig. 2.5A,B).

Figure 2.4

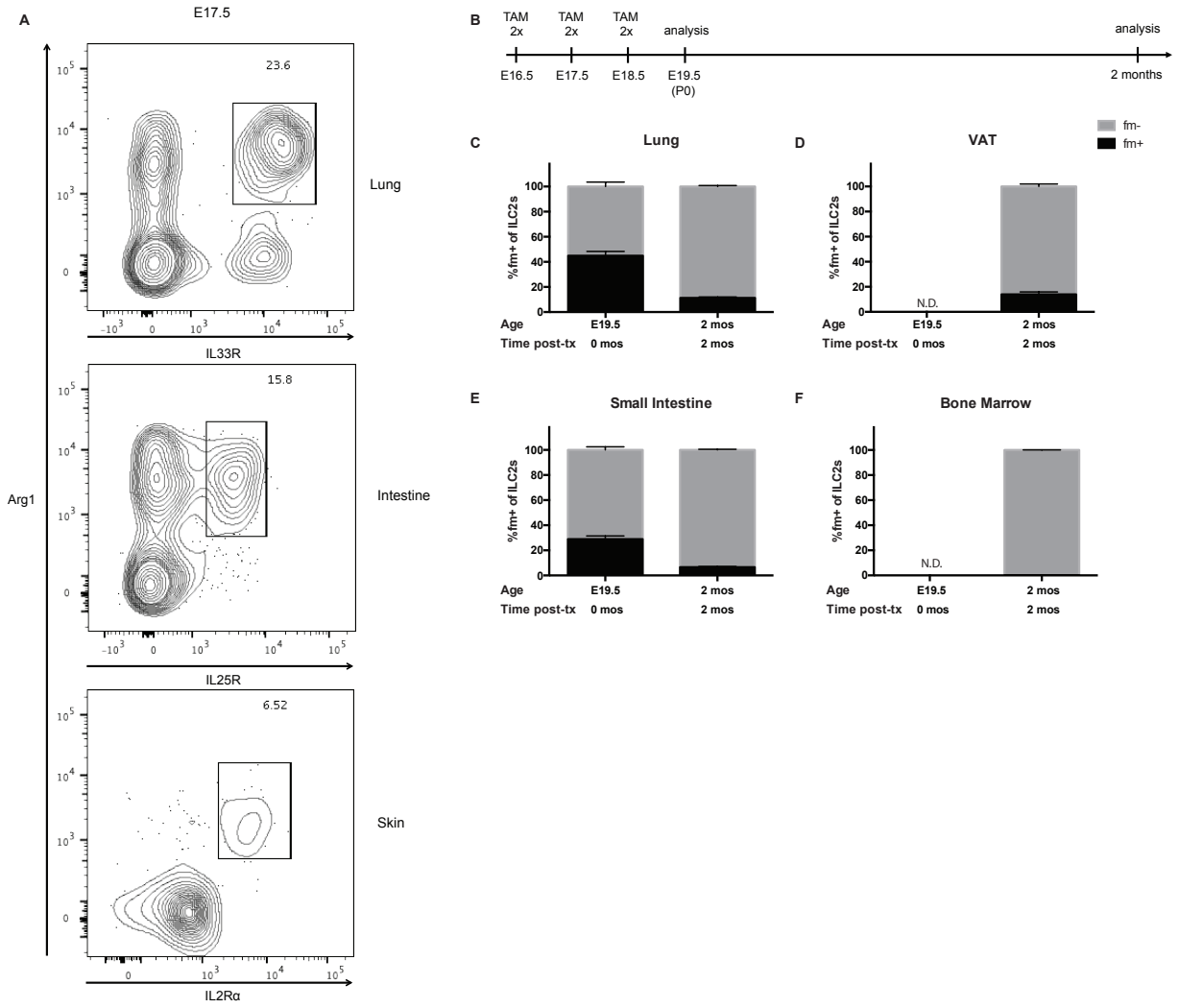

Figure 2.4. Prenatal fate mapping labels a subset of ILC2s in the adult.
(A) Flow cytometry plots pre-gated on live CD45$^+$Lin$^-$ cells, showing E17.5 lung, small intestine, and skin. **(B)** Schedule of tamoxifen administration for prenatal fate mapping. **(C-F)** Fate mapped ILC2 percentages using the *Arg1-RFP-CreER^{T2}* mouse strain in the (C) lung, (D) visceral adipose tissue, (E) small intestine lamina propria, and (F) bone marrow. ILC2s were gated as Lin$^-$Thy1$^+$ST2$^+$ (lung and VAT), Lin$^-$IL17RB$^+$KLRG1$^+$ (small intestine), or Lin$^-$IL7Rα$^+$Thy1$^+$CD25$^+$ (bone marrow). Data are displayed as mean ± SEM of 6-8 individual mice from at least 2 independent experiments per labeling and analysis time point.

Figure 2.5

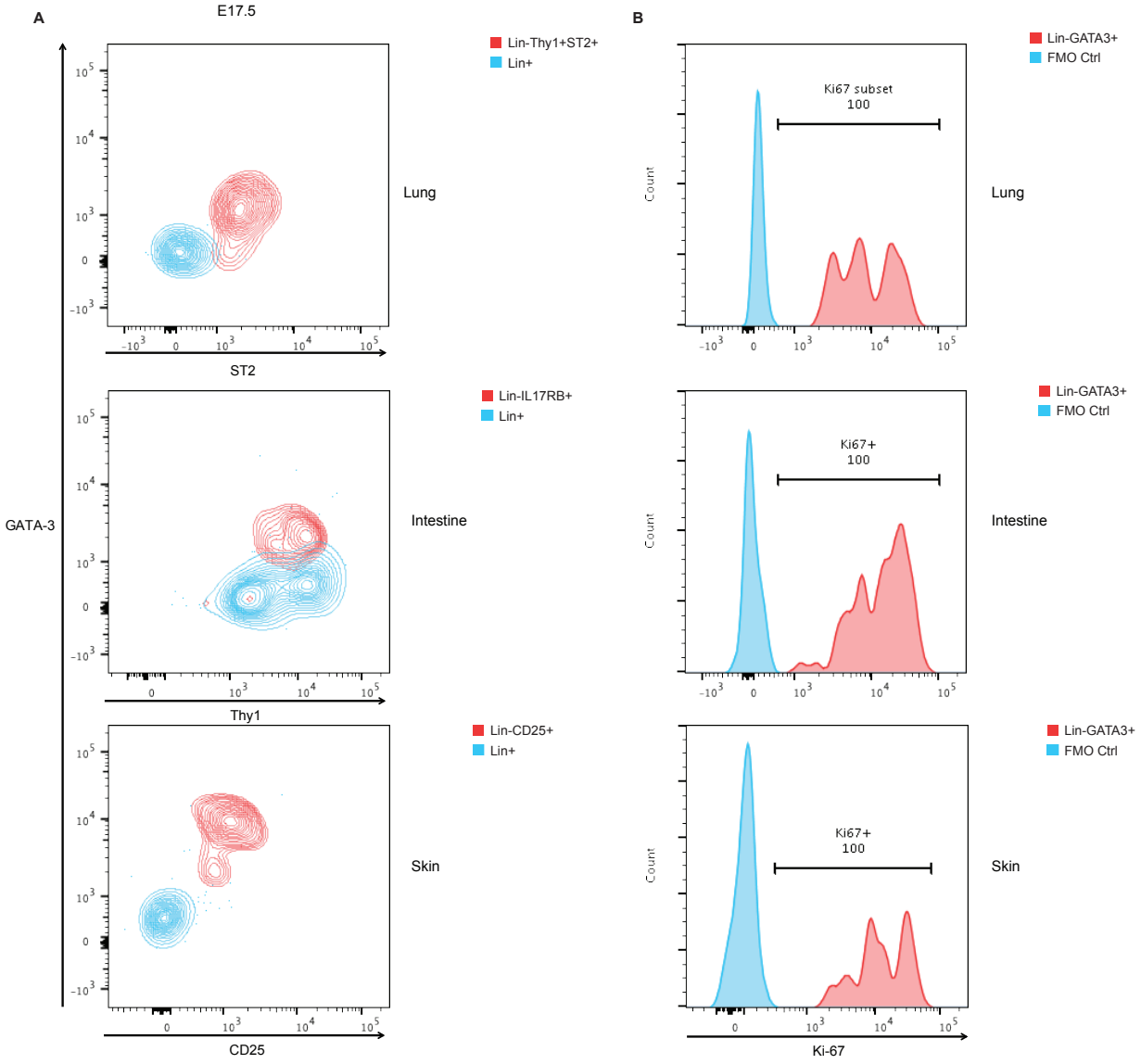

Figure 2.5. Representative flow cytometry plots of GATA-3 and Ki-67 staining on fetal ILC2s

(A) Flow cytometry plots of GATA-3 and surface marker expression, pre-gated on ILC2s (red) or Lin+ cells (blue), showing E17.5 lung, small intestine, and skin. **(B)** Flow cytometry histograms of Ki-67 expression, pre-gated on ILC2s shown in red, fluorescence minus one (FMO) control shown in blue, from E17.5 lung, small intestine, and skin. Data are representative of four independent experiments.

To determine whether these fetal tissue-seeding Arg1$^+$ ILC2s contribute to adult ILC2 pools, we performed lineage-tracing experiments by administering tamoxifen prenatally during E16.5-18.5, birthing pups on foster mothers, and analyzing the labeled tissue ILC2 populations at 2 months of age (Fig. 2.4B). Labeling efficiency on postnatal day 1 (P1) in lung and small intestine was robust (30-45%), and more efficient than in bone marrow (<10%); the absence of visceral adipose tissue (VAT) in the embryos and the absence of ILC2s in fetal bone marrow precluded analysis. By two months post-birth, however, only a fraction (5-10%; one-fifth to one-third of the initially labeled fraction) of the ILC2s, in the lung, small intestine, and visceral adipose tissue (VAT) remain labeled and were thus embryonically derived (Fig. 2.4D-F); proportions of the latter were greater in lung and VAT than in small intestine. Notably, none of the putative ILC2 precursors (ILC2Ps) in the bone marrow remain labeled in the adult (Fig. 2.4G), suggesting that post-birth bone marrow Arg1$^+$ ILC2s have a distinct temporal origin from prenatally derived ILC2s.

Neonatal ILC2s contribute to the majority of the ILC2 pool in adult tissues

We, as well as others, have defined the acute expansion and activation of ILC2s that occur in the lung shortly after birth[40,149,164–166]. Given these observations, we used lineage tracing in *Arg1-RFP-CreER^{T2}* and *Id2-CreER^{T2}* mice to assess the contribution of neonatal ILC2s to adult tissue ILC2 pools. Neonatal mice were given tamoxifen on P10-P12 and analyzed 2 weeks, 2 months, and 1 year later to assess numbers of label-retaining ILC2s in various tissues. As compared to the efficiency of prenatal labeling, a significantly higher fraction (40-70%) of fate-mapped ILC2s were present in adult lung, adipose tissues, small intestine, and skin, and this was comparable between both strains of fate-mapping mice

(Fig. 2.6A-L). There is some continuing dilution of fate-mapped ILC2s during the period between 2 and 8 weeks of age, suggesting that de novo ILC2 generation may continue beyond P12, but after 8 weeks of age, the tissue ILC2 pool remains relatively stable in the lung and VAT throughout the life of the mouse; unexpectedly, this pool of fate-mapped cells persists in mice that are over 1 year old. In contrast, in the skin and small intestine, ILC2s are replaced more rapidly by newly generated, unlabeled ILC2s, such that, by 1 year of age, only ~5% of ILC2s remain neonatally derived (Fig. 2.6D,E,J,L), although neonatal cells clearly remain for many months even in these tissues. Consistently, heterogeneity is apparent in comparing the proportions of fate-mapped and non-fate-mapped cells among the different tissues. Interestingly, in the small intestine, a subpopulation of $KLRG1^{+}ST2^{+}$ ILC2s remains stable throughout adulthood, consistent with a predominant neonatal origin of these cells (Fig. 2.6G).

Figure 2.6

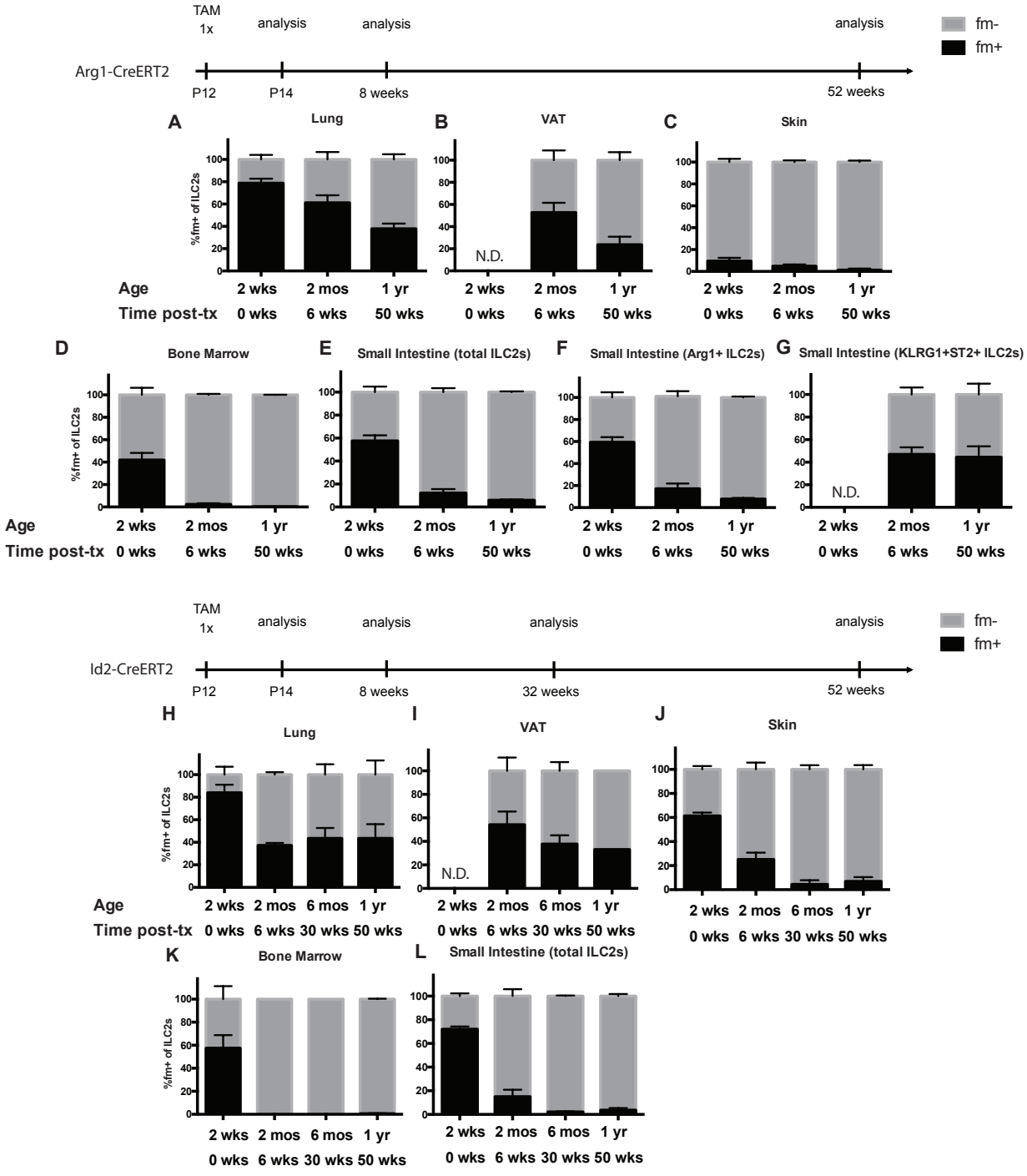

Figure 2.6. Neonatal fate mapping labels a majority of ILC2s in the adult lung and visceral adipose tissue, but not in the skin and small intestine.
(A-G) Fate mapped ILC2 percentages using the *Arg1-RFP-CreER^{T2}* mouse strain in the (A) lung, (B) visceral adipose tissue, (C) back skin, (D) bone marrow, and (E-G) small intestine lamina propria. **(H-L)** Fate mapped ILC2 percentages using the *Id2-CreER^{T2}* mouse strain in

the (H) lung, (I) visceral adipose tissue, (J) back skin, (K) bone marrow, and (L) small intestine lamina propria. ILC2s were gated as $Lin^{-}Thy1^{+}ST2^{+}$ (lung and VAT), $Lin^{-}Thy1^{+}ICOS^{+}$ (skin), or $Lin^{-}IL7R\alpha^{+}Thy1^{+}CD25^{+}$ (bone marrow). Total ILC2s in the small intestine were gated as $Lin\text{-}IL17RB^{+}KLRG1^{+}$, except for panels F and G. Data are displayed as mean ± SEM of 5-9 individual mice from at least 2 independent experiments per labeling and analysis time point.

Tissues of the newborn mouse are characterized by vigorous ILC2 activation and proliferation that is dependent on IL-7Rα signaling

Prior studies, including our own, have noted extensive proliferation and activation of ILC2s in lung that occurs around the time of birth and extends through weaning[40,164–166]. Here, we demonstrate that this neonatal wave of ILC2 expansion occurs systemically, as assessed kinetically in the lung, small intestine, skin, and bone marrow (Fig. 2.7A-D). The development of ILC2s is dependent on the cytokine IL-7[34], and we confirm that the early expansion of ILC2s is also dependent on IL-7R signaling, as prenatal administration of a blocking antibody to IL-7Rα results in significantly decreased ILC2 frequencies in the lungs, small intestine, and skin of neonatal mice (Fig. 2.7E-G). Despite three doses of anti-IL-7Rα, however, ILC2 frequencies in the small intestine, skin, and visceral adipose tissue substantially recovered by 9 weeks of age (Fig. 2.7F-H) consistent with restorative capacity. In contrast, the reduction in lung ILC2s persists, and ILC2 frequencies remain low in the lungs of mice that were 9 weeks old when compared to their isotype-treated littermates, suggesting that a temporal window may restrict ILC2 development in lung tissue (Fig. 2.7E).

Figure 2.7

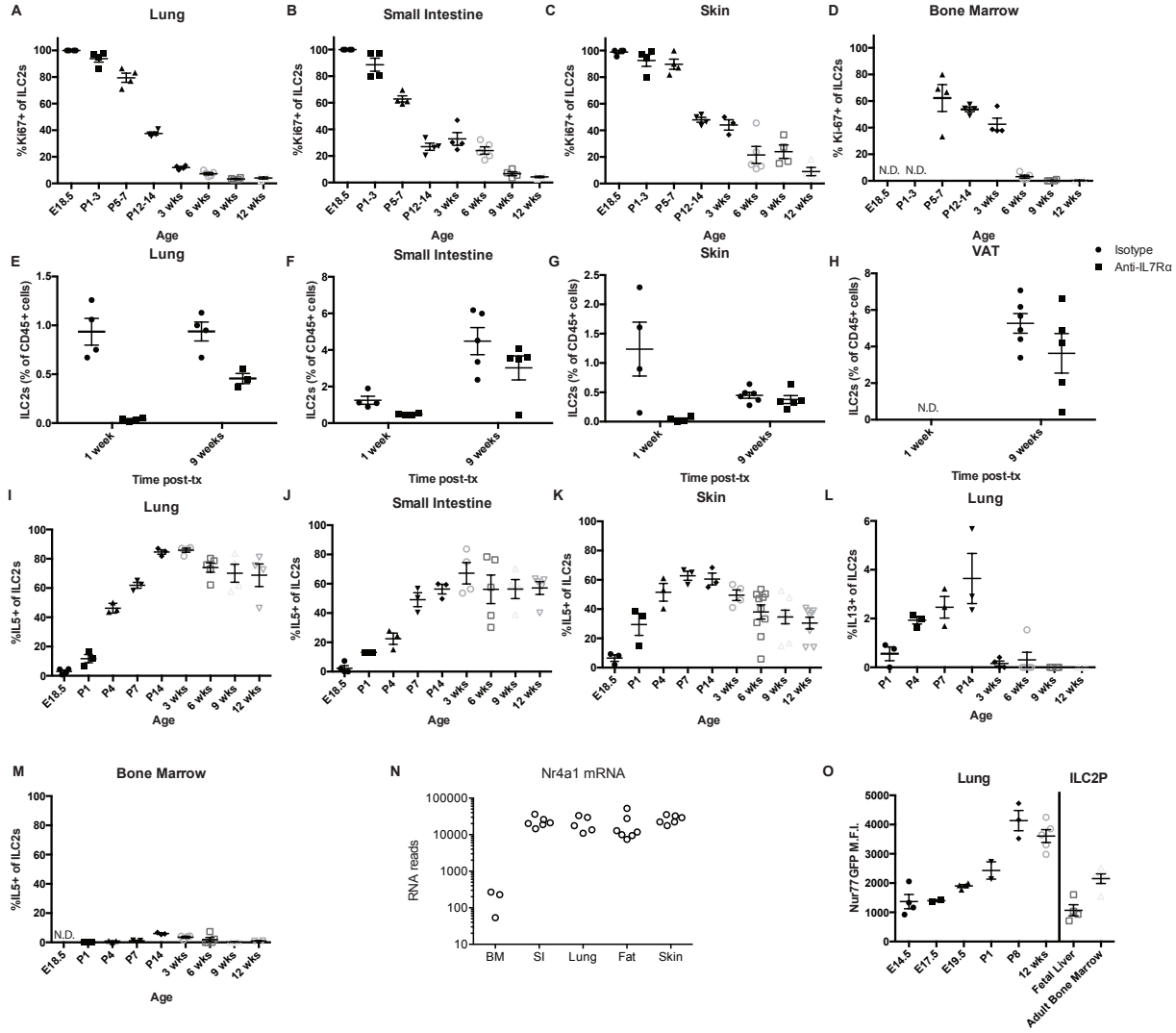

Figure 2.7. The neonatal window is a period of IL7R-dependent tissue ILC2 proliferation and activation.

(A-D) Percent of ILC2s that are positive for the proliferation marker Ki-67 in the (A) lung, (B) small intestine lamina propria, (C) back skin, and (D) bone marrow. **(E-H)** ILC2 frequencies in mice treated with 3 doses of anti-IL7Rα blocking antibody (A7R34) on E14.5, E16.5, and E18.5. Data are representative of two independent experiments. **(I-K)** Percent of ILC2s that are reporter positive for IL-5 in the (I) lung, (J) small intestine lamina propria, (K) back skin. **(L)** Percent of lung ILC2s that are reporter positive for IL-13. **(M)** Percent of bone marrow ILC2s that are reporter positive for IL-5. **(N)** Numbers of reads for *Nr4a1* mRNA transcript assessed by RNA sequencing of ILC2s from bone marrow, small intestine, lung, perigonadal fat, and skin. Each circle represents one mouse. **(O)** Median fluorescence intensity of Nur77-GFP in Nur77$^+$ ILC2s. Each dot represents one mouse. ILC2s were gated as Lin$^-$Thy1$^+$ST2$^+$ (lung and VAT), Lin$^-$IL17RB$^+$KLRG1$^+$ (small intestine),

$Lin^-Thy1^+ICOS^+$ (skin), $Lin^-IL7R\alpha^+Thy1^+CD25^+$ (bone marrow), or $Lin^-IL7R\alpha^+Arg1^{YFP+}$ (fetal liver). Each dot represents one mouse.

Given this developmental time window for ILC2 proliferation, we assessed expression of the defining type 2 cytokines for ILC2s, IL-5 and IL-13, using previously described and validated reporter mice[40,167]. We found that an increasing proportion of ILC2s turned reporter-positive for IL-5 over these first weeks of life (Fig. 2.7I-K), as well as for IL-13, although smaller in frequency (Fig. 2.7L). Activation of the reporters was coincident with proliferation in peripheral tissues, but essentially absent in bone marrow (Fig. 2.7M). We previously noted high mRNA expression of the nuclear hormone receptor Nur77, or Nr4a1, in ILC2s isolated from different peripheral tissues compared to the bone marrow (Fig. 2.7N). Nur77 is an immediate early gene expressed in hematopoietic cells in association with the response to growth factors, cytokines, and proliferative or inflammatory signals, and important in mediating cell survival during stress[168]. Lymphocyte activation of adaptive T and B cells can be analyzed in situ using Nur77-GFP reporter mice, and "tuning" of Nur77-GFP corresponds with the degree of integrated signals accumulating within the cell[169]. We used Nur77-GFP mice to assess whether ILC2s engage these survival pathways during the period of perinatal expansion and activation. Indeed, tissue ILC2s undergo a marked increase in Nur77 expression levels after birth that is maintained into adulthood (Fig. 2.7O); in contrast, Nur77 expression is low in fetal liver and adult bone marrow.

Tissue-specific heterogeneity of ILC2s is established in the neonatal wave

Developing neonatal ILC2s position themselves across tissues as they expand and activate, but how they compare to long-lived adult tissue ILC2s in their transcriptional profiles may elucidate previously unknown periods of tissue-driven differentiation. To assess this, we sorted ILC2s from 2-week-old mice and 8-14-week-old mice in a single

experiment on the same day (to eliminate batch effects), and used single-cell RNA-sequencing (scRNA-seq) analysis to assess cell and tissue heterogeneity using a graph-based clustering algorithm and visualized using *t*-distributed stochastic neighborhood embedding (*t*-SNE)[170]. As expected, perinatal ILC2s were enriched for cells showing elevated levels of transcripts associated with cellular activation and proliferation (*Il5, Il13, Nr4a1, Mki67*), compared to adult ILC2s (Fig. 2.8C,D & Fig. 2.9), and thus clustered distinctly from adult ILC2s (Fig. 2.8A,B). However, when analyzed in aggregate, neonatal ILC2s from different tissues localized in clusters with adult ILC2s from the same tissues, and consistent with a tissue-organizing expression pattern that has already developed during the perinatal window (Fig. 2.8E). Taken together, these data demonstrate that ILC2s, although first appearing during fetal hematopoiesis, establish their presence in tissues primarily during early postnatal development during a period of rapid expansion, priming, and acquisition of tissue-defining expression patterns, and remain in tissues as locally maintained resident cells.

Figure 2.8

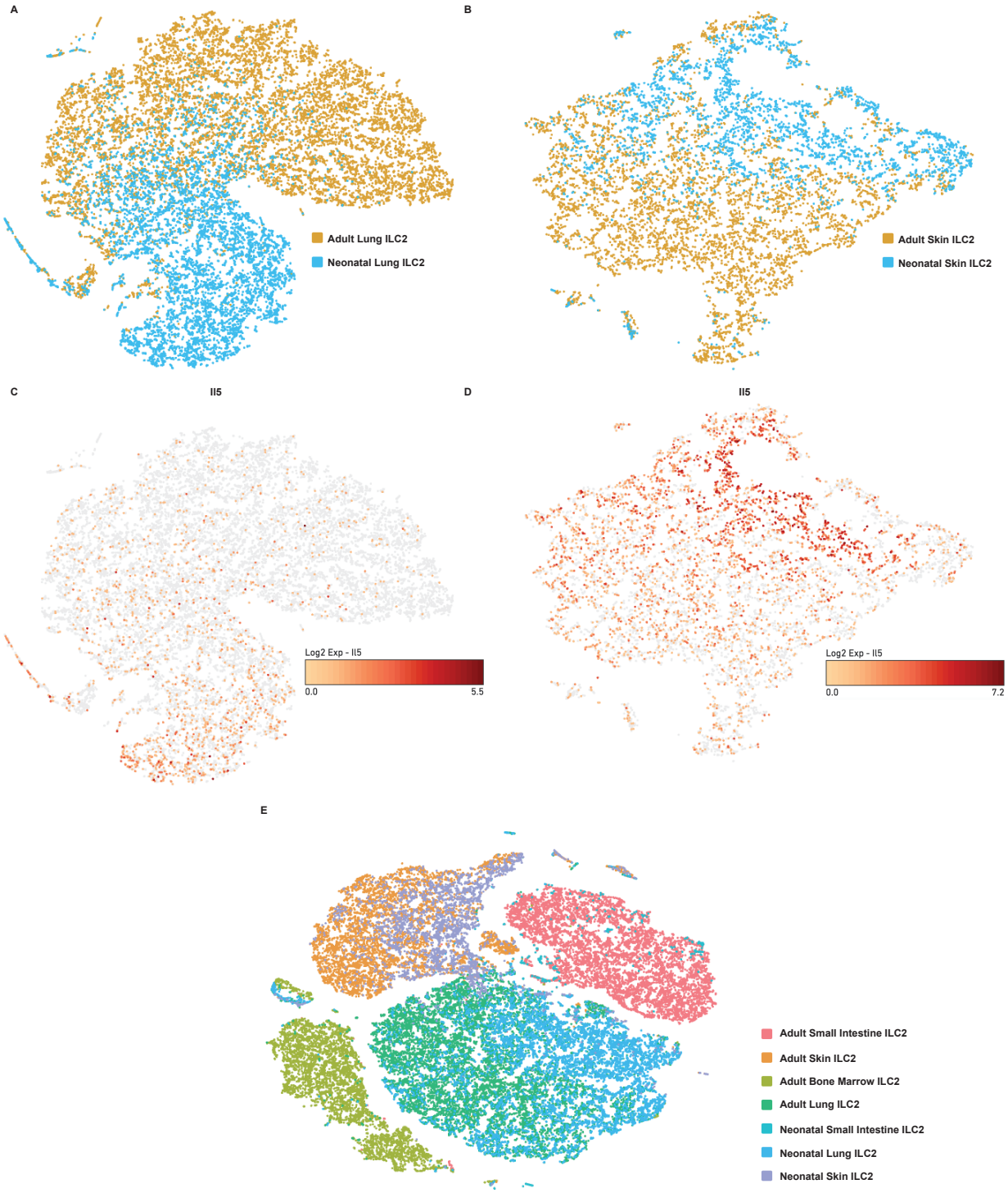

Figure 2.8. Single-cell RNA-seq analysis shows that neonatal ILC2s have a transcriptional profile consistent with increased activity.
(A,B) *t*-SNE visualization of adult and neonatal scRNA-seq datasets of sort-purified ILC2s from the (A) lung and (B) skin. **(C,D)** Relative single-cell *Il5* expression levels in (C) lung

and (D) skin ILC2s. **(E)** *t*-SNE visualization of adult and neonatal scRNA-seq datasets of sort-purified ILC2s from the lung, skin, small intestine, and bone marrow. ILC2s were sorted as $Lin^{-}IL5^{RFP+}$ cells from six 2-week-old (neonatal) and eight 8-14-week-old (adult) male mice on the same day.

Figure 2.9

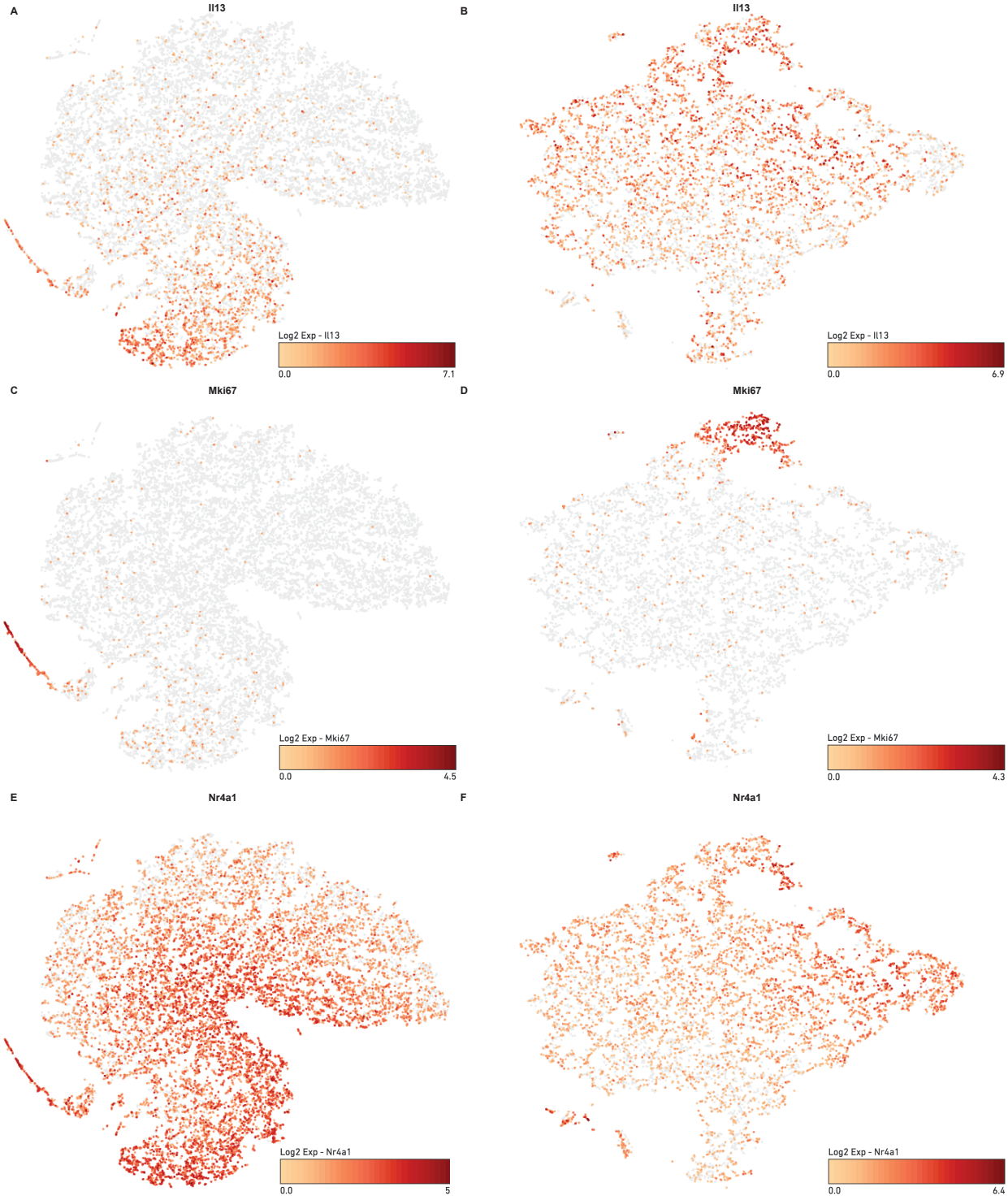

Figure 2.9. Expression of selected transcripts in scRNA-seq analysis of ILC2s from adult and neonatal lung and skin

(A,B) *Il13* expression visualized on t-SNE plot of scRNA-seq data (cf. Fig. 2.8A,B for age of tissue of origin) **(C,D)** *Mki67* expression visualized on t-SNE plot of scRNA-seq data (cf. Fig. 2.8A,B) **(E,F)** *Nr4a1* expression visualized on t-SNE plot of scRNA-seq data (cf. Fig. 2.8A,B)

Newly generated ILC2s do not contribute to ILC2 expansion accompanying migratory helminth infection

Although adult tissue ILC2s are dominated by a tissue-resident and infrequently replaced population in most tissues (Fig. 2.1), their dynamics during acute immune perturbations have been less carefully quantitated. We used *Nippostrongylus brasiliensis* (*N. brasiliensis*), a migratory helminth that induces a vigorous systemic type 2 immune response with marked tissue effects in both the lungs and small intestine[171], as underscored by a nearly 8-fold increase in ILC2 numbers (Fig. 2.10A). Using mice with neonatally fate-mapped ILC2s, we found that even after infection with *N. brasiliensis*, the proportion of fate-mapped cells showed a slight decrease (Fig. 2.10B,C), despite the considerable increase in ILC2 numbers. These data suggest a minor contribution of de novo ILC2s even in an inflammatory setting. Of note, essentially none of the putative bone marrow ILC2 precursors are fate-mapped at the time of infection, ruling out a major contribution of ILC2Ps to the expansion of ILC2s during helminthic infection (Fig. 2.10D). In addition, at steady state, ILC2s are not present in any significant numbers in the blood (Fig. 2.11A). However, after *N. brasiliensis* infection, a substantial number of ILC2s are found in the blood (Fig. 2.11B), but because the proportion that are fate-mapped resembles that of tissue ILC2s (Fig. 2.10E), this suggests that the blood ILC2s that arise during helminthic infection are tissue-derived, rather than bone marrow-derived, and consistent with a prior report[149].

Figure 2.10

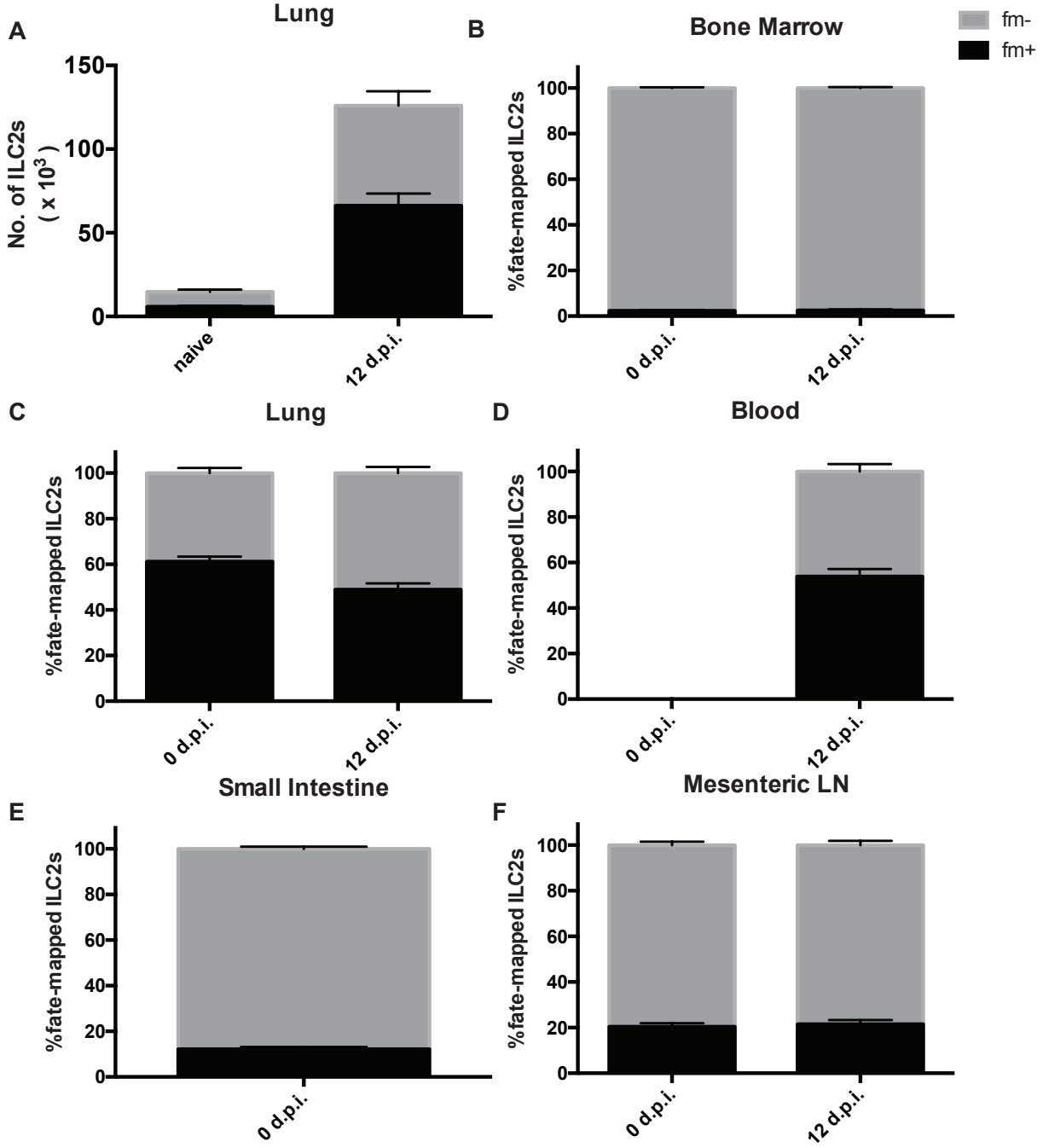

Figure 2.10. De novo generation is not the predominant mechanism by which ILC2s expand during helminth infection.

(A) Numbers of lung ILC2s before and after infection with *N. brasiliensis*. **(B-F)** Percentage of neonatally fate-mapped ILC2s using the *Arg1-RFP-CreER^{T2}* mouse in the (B) bone marrow, (C) lung, (D) blood, (E) small intestine lamina propria, and (F) mesenteric lymph nodes before and after infection. ILC2s were gated as Lin$^-$IL7Rα$^+$Thy1$^+$CD25$^+$ (bone marrow), Lin$^-$Thy1$^+$ST2$^+$ (lung), or Lin$^-$IL17RB$^+$KLRG1$^+$ (small intestine and mesenteric

LN). Data are displayed as mean ± SEM of 8-9 individual mice from at least 2 independent experiments per labeling and analysis time point.

Figure 2.11

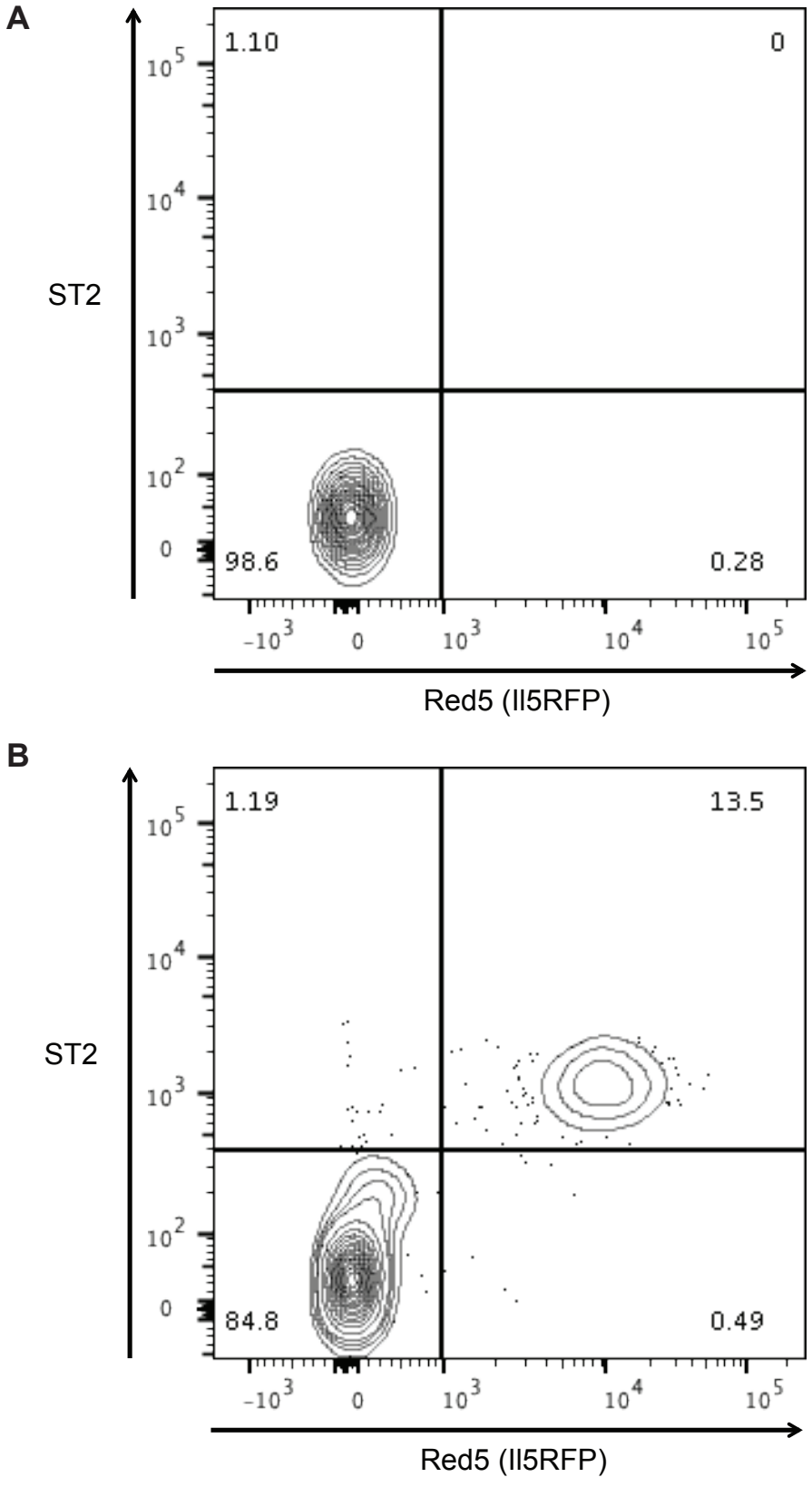
A
1.10
0
98.6
0.28
ST2
Red5 (Il5RFP)
B
1.19
13.5
84.8
0.49
ST2
Red5 (Il5RFP)

Figure 2.11. Representative flow cytometry plots of blood ILC2s before and after helminth infection

Flow cytometry plots of *Il5* reporter and ST2 expression of blood cells, pre-gated on live $CD45^{+}Lin^{-}$ cells from either **(A)** an uninfected mouse or **(B)** a mouse 10 days post-infection with *N*. *brasiliensis*. Data are representative of two independent experiments.

Discussion

Using fate-mapping in combination with proliferation and activation markers and transcriptional signatures, we make several observations relevant to the understanding of ILC2 biology. First, we have identified three temporally distinct origins for tissue-resident ILC2s—fetally derived ILC2s that are distributed to tissues before birth, neonatally derived ILC2s that are generated in the period from birth through weaning, and adult-derived ILC2s that slowly replenish and replace the pre-existing ILC2s. Second, we show that the steady-state rate of ILC2 replacement by new ILC2s generated in adult life is dynamic but tissue-specific, with slow turnover in lung, fat, and small intestine but relatively rapid replacement in the bone marrow and skin. Third, using markers for proliferation, cytokine reporter mice, Nur77-GFP reporter mice, and single-cell transcriptomics, we show that the neonatal wave corresponds with the period when ILC2s expand, begin to activate their effector cytokine repertoire, and establish a tissue-specific transcriptomic signature that remains through life. Lastly, using different methods, we corroborate the finding that the expansion of pre-existing ILC2s, likely tissue ILC2s, is the dominant mechanism by which ILC2 numbers increase in the lung after infection with *N. brasiliensis*. Taken together, our findings present strategic opportunities for the development of age- and tissue-specific interventions that might affect ILC2 numbers and function in positive or negative ways.

Our studies reveal that the tissue ILC2 pool comprises populations that are both prenatally and postnatally derived, and furthermore, that the postnatally derived population arises largely within the first few weeks of life with minor contribution and replenishment by subsequent bone marrow HSC-derived hematopoiesis. These findings reveal the striking similarity between ILC2s and other innate-like lymphocytes[172,173] and

even regulatory T (T_{reg}) cells[174], and highlight the temporal window for niche seeding by tissue-resident immune cells in developing organs[163,174–178]. Whether the early postnatal populations identified here originate from conventional HSC-dependent bone marrow hematopoiesis or from an alternative source, such as multipotent progenitors in the spleen[179] or from progenitors already present within the tissue, remains an open question. If the neonatal wave comes from a local progenitor pool that has yet to express *Id2* or *Arg1*, then the existence of a progenitor population that differentiates and expands in situ is an intriguing possibility. Further studies would be necessary to distinguish between these possibilities.

Our fate-mapping approach revealed unexpected dynamic complexity underlying ILC2 turnover in tissues. Whereas close to half of fate-mapped ILC2s from the skin are replaced by non-fate-mapped cells over the period of 4 months, lung and adipose tissue ILC2s take up to 1 year (half the typical lifespan of a laboratory mouse) for fate-mapped cells to be diluted to similar levels. The dynamic heterogeneity observed in ILC2s of different tissues manifests striking parallels with the varying kinetics observed for macrophage turnover in different tissues. Just as the rate of ILC2 replacement varies across tissues, the rate of adult HSC contribution to tissue macrophages also varies, with the differences becoming increasingly apparent with age after fate mapping. Notably, subsets of tissue-resident macrophages of the intestine[156,157] and dermis[180] have higher rates of replacement compared to alveolar macrophages[163], mirroring our observations for ILC2s. The intestine and skin are impacted by dietary and microbial stimuli, and even in barrier-housed mice, are markedly affected by weaning. These tissues also undergo dynamic phases of remodeling, including continuous epithelial renewal in the intestine and hair

follicle cycling in the skin. Notably, among multiple peripheral tissues, ILC2s in the small intestine and skin express the highest amounts of IL-13 (Ricardo-Gonzalez, Van Dyken et al, under review), a dynamic marker of ILC2 activation[167], suggesting functions devoted to homeostatic activities in these organs. Further studies will be required to determine the effect of recurring activation on ILC2 replenishment by circulating or local precursors, as well as the potential effect of increased migratory behavior as recently described for ILC2s in the small intestine following helminthic infection[149].

Using markers for proliferation and activation, and corroborated by single-cell transcriptomics, we show that the perinatal establishment of peripheral ILC2 pools corresponds with the period when ILC2s expand, begin to activate their cytokine repertoire, and establish a tissue-specific transcriptomic signature that persists throughout life. Increasingly recognized as an important time window for ILC2 activation, we provide a comprehensive characterization of those cells during neonatal development. Perinatal waves contributed to the postnatal expansion of ILC2 pools across all tissues and correlated with the acquisition of a primed and tissue-defining transcriptomic signature that resembled the signature of adult steady-state tissue ILC2s. Therefore, and perhaps not surprisingly, ILC2s closely resemble fetally derived tissue macrophages, not only with regard to the kinetics of their appearance, but also in their capacity to integrate into tissues and acquire organ-specific gene expression programs that are likely related to distinct functions[181]. Notably, despite clustering with their adult counterparts, our scRNA-seq analysis of neonatal ILC2s revealed significant differences in the expression of genes associated with activation, cell growth, and chemokine production. Although the local signals underlying the developmental priming in the tissue remain to be determined, it is

intriguing to speculate that incipient crosstalk between ILC2s and other immune and non-immune cells during this important period of organ growth and remodeling might both guide and be shaped by the gene expression programs of ILC2s. We might also speculate that similar interactions might be recapitulated later during life as part of homeostatic ILC2 activation and during tissue perturbation. Whether modulation of this early pool of ILC2s can have long-lasting consequences for the immune tone of the tissue, as has been described for neonatal regulatory T cells[174], will be an important area for further investigation, given the increasing recognition that perturbations during the neonatal period of life can have long-term effects with regard to immune function and disease susceptibility[182].

By comparing non-fate-mapped and fate-mapped ILC2s in tissue, we could demonstrate that expansion of pre-existing ILC2s is the dominant mechanism by which ILC2 numbers are increased in the lung after infection with *N. brasiliensis*, with minimal dilution of the pre-existing fate-mapped population. Further, there is no preferential expansion of the neonatally fate-mapped ILC2 pool after helminth infection, suggesting that ILC2s derived from early and later life respond equivalently to the tissue perturbations associated with *N. brasiliensis* infection. Further studies are needed to determine the impact of acute and chronic tissue perturbation on the turnover and positioning of ILC2s, and will enable a better understanding of inter-organ ILC2 crosstalk, including a recently described gut-lung axis[149], and in light of reports on circulating ILC precursors and ILC2s in humans[183,184].

In summary, we demonstrate a previously unappreciated tripartite origin for tissue-resident ILC2s, and in particular, our studies reinforce the importance of the early

postnatal window for the establishment of immune cells and reveal that ILC2 replacement is tissue-dependent. In addition, we show that in the setting of helminthic infection, the relative contributions of ILC2s from different temporal origins remain mostly unchanged, suggesting that homeostatic factors are dominant over inflammatory factors in determining the proportions of ILC2s of different origins. Looking forward, our discoveries delineate the interventional windows of ILC2 and type 2 cytokine modulation and can inform the prognostic value of interventions in early life or even in utero, broaching the possibility of therapeutically targeting specific ILC2 subsets based on ontogeny and the tissue of residency. Importantly, our findings bring ILC2s into the larger ongoing conversation regarding the influence of ontogeny and local signals on long-lived tissue-resident immune cells that are established early during development, and highlight the need for further studies addressing their roles during this important time window that has been associated with risk for human allergic diseases.

Experimental Procedures

Mice

Arg1-YFP (*Yarg*)[185], Il5-RFP (*Red5*)[40], and Il13-huCD4 (*Smart13*)[167] mice have been previously described. Id2-GFP, Id2-CreER^{T2}, R26R-eYFP, and *Il7ra*$^{-/-}$ mice were purchased from the Jackson Laboratory. R26R-RFP mice[162] were provided by E. Robey (Berkeley). Nur77-GFP mice were provided by J. Ziherman (UCSF)[169]. All mice were backcrossed to the C57BL/6 background for at least 10 generations. All experiments were conducted according to protocols approved by the UCSF Institutional Animal Care and Use Committee.

Arg1-RFP-CreER^{T2} reporter mice were generated by homologous gene targeting in C57BL/6 embryonic stem cells. The previously published plasmid expresses tdTomato and CreER^{T2}, such that the cassette contains (from 5' to 3') an internal ribosomal entry site (IRES), the gene encoding tdTomato, the Thoseaasigna virus 2A (T2A) sequence, the gene encoding CreER^{T2}, and bovine growth hormone poly(A).

Tissue dissociation

Mouse lungs were perfused through the right cardiac ventricle with cold PBS and underwent an initial mechanical dissociation using the m_lung_01_01 program on the gentleMACS dissociator (Miltenyi Biotec). Lung tissues were then digested with LiberaseTM (Roche) and Dnase I (Roche) in pre-warmed RPMI-1640 for 30mins at 37°C. Further dissociation was performed using the m_lung_01_02 program on the gentleMACS dissociator. Single-cell suspensions were obtained after passing the homogenized samples through 70μ cell strainers.

Mouse intestines were flushed with PBS and Peyer patches were removed when visible in older mice. Intestines were filleted, rinsed, and were further washed with DTT (1x20mins) and EDTA (3x15mins) in Ca/Mg-free HBSS. After a final wash with HBSS (1x10mins), tissues were cut into small pieces approximately 5mm wide with scissors and then digested with Liberase TM (Roche) and Dnase I (Roche) in pre-warmed HBSS for 30mins at 37°C. Afterwards, the tissues were further dissociated using the m_intestine_01_01 program on the gentleMACS dissociator. Samples were then centrifuged on a 40/90 Percoll (GE Healthcare Biosciences) gradient to remove debris. Single-cell suspensions were obtained after passing the homogenized samples through 100μ cell strainers.

Mouse adipose tissue was cut into small pieces approximately 5mm wide with scissors and then digested with Liberase TM (Roche) and Dnase I (Roche) for 40mins at 37°C with vigorous shaking. Fatty debris was removed after centrifugation and single-cell suspensions were obtained after passing through a 70μ cell strainer.

Mouse skin was cut into small pieces approximately 5mm wide with scissors and then digested with Collagenase XI (Sigma), Hyaluronidase (Sigma), and Dnase I (Roche) in pre-warmed RPMI-1640 for 90mins at 37°C. Afterwards, the tissues were further dissociated using the C program on the gentleMACS dissociator. Single-cell suspensions were obtained after passing the homogenized samples through 70μ cell strainers.

Flow cytometry

Fc block (anti-mouse CD16/32, 2.4G2) was purchased from BioXCell. Rat anti-mouse CD4 (RM4-5), rat anti-mouse CD11b (M1/70), rat anti-mouse CD19 (ID3), rat anti-mouse B220 (RB6-8C5), and rat anti-mouse CXCR5 (2G8) were purchased from BD Pharmingen; rat anti-mouse c-kit (2B8), rat anti-mouse CD3 (17A2), rat anti-mouse CD5 (53-7.3), rat anti-mouse CD127 (A7R34), rat anti-mouse NKp46 (29A1.4), rat anti-mouse LPAM (DATK32), rat anti-human/mouse GATA-3 (TWAJ), and mouse anti-mouse NK1.1 (PK136) antibodies were purchased from eBioscience; Armenian hamster anti-mouse CD11c (N418), rat anti-mouse CD45 (30-F11), rat anti-mouse Ter119 (TER-119), Armenian hamster anti-human/mouse/rat ICOS (C398.4A), rat anti-mouse CD25 (PC61), and rat anti-mouse Flt3 (A2F10) antibodies were purchased from Biolegend; and rat anti-mouse ST2 (DJ8) antibodies were purchased from MD Bioproducts. Lin- cells were defined as lacking CD3, CD4, CD5, CD11b, CD11c, CD19, NK1.1, Gr-1, FcεRIα, and Ter119. DAPI and Live/Dead (Invitrogen) was used for dead-cell exclusion for live and fixed samples, respectively.

Samples were analyzed on a LSRFortessa X20 (BD Biosciences) with five lasers (355nm, 405nm, 488nm, 561nm, and 640nm). Samples were gated by FSC-A/SSC-A to exclude debris, FSC-H/FSC-W for single cells, and gated to exclude dead cells by DAPI (unfixed samples) or Live/Dead (fixed samples). Data analysis was performed using FlowJo (Treestar).

Parabiosis

Parabiosis surgery was performed following previously described procedures, which resulted in 40-60% mixing of circulating blood cells after 2 weeks.[73] Briefly, pairs of mice were joined by suturing mirror-image peritoneal openings, in addition to the elbow and knee joints, followed by stapling of the skin (9-mm autoclip, Clay Adams). Each mouse was then injected intraperitoneally (i.p.) with Baytril antibiotic and Buprenex analgesic, and over the first week of recovery after the surgery, each mouse received daily i.p. injections of 250cc normal saline. Several parameters, including pair weight and grooming behavior, were analyzed weekly after surgery to monitor overall health and recovery. We observed >70% survival at 5 weeks. Between 5 and 6 weeks after surgery, the pairs were euthanized and organs collected for processing and flow cytometric analysis.

In vivo treatments

IL-7Rα blockade was performed by injecting 3 doses of 200μg InVivoMAb anti-mouse IL-7Rα (A7R34) intravenously (i.v.) into timed pregnant females on E14.5, E16.5, and E18.5.

Cell sorting and scRNA-seq

ILC2s were sorted from homogenized mouse lung, skin, and small intestine as live ($DAPI^-$) $CD45^+$Lin(CD3,CD4,CD5,CD8α,CD11b,CD11c,CD19,NK1.1, NKp46,Gr-1,F4/80,Ter-

119,DX5)$^{-}$Il5^{RFP+} cells. Bone marrow cells were sorted as live CD45^{+}Lin^{-}Thy1^{+}Arg1^{YFP+} cells. Cells were sorted using a MoFlo XDP (Beckman Coulter) into ice-cold 0.5% BSA in PBS and processed on the same day through the Chromium Single Cell 3' v2 Library Kit (10X Genomics) per the manufacturer's protocol. Each channel was loaded with 5000-25000 cells from each tissue, yielding 400-3,000 single cells for analysis from each tissue. The cells were then partitioned into Gel Beads in Emulsion in the instrument, where cell lysis and barcoded reverse transcription of RNA occurred, followed by amplification, shearing, and 5' adaptor and sample index sequence ligation. Libraries were sequenced on an Illumina HiSeq 4000. Single Cell 3' libraries use standard Illumina sequencing primers for both sequencing and index reads. They were run using paired-end sequencing with single indexing where Read 1 is 26 cycles and Read 2 is 98 cycles.

Analysis of scRNA-seq data

FASTQ files were generated utilizing the Cell Ranger FASTQ ("cellranger mkfastq"; 10X Genomics) pipeline, through which resulting bcl files from sequencing are de-multiplexed using bcl2fastq (v. 2.17). The resultant paired-end FASTQ files were aligned utilizing the Cell Ranger Count ("cellranger count"; 10X Genomics) pipeline, though which FASTQ files are aligned to the mm10 reference transcriptome (Ensembl GRCm38 primary assembly) using STAR, an open source splicing-aware RNA-seq aligner, and then single-cell gene counts are generated. Outputs from the Cell Ranger Count pipeline were then run through the Cell Ranger Aggregator ("cellranger aggr") pipeline to combine data from neonatal and adult tissue ILC2 samples. Dimensionality reduction was also performed through the Cell Ranger pipeline, using principal components analysis (PCA) via a Python implementation of a modified version of the augmented implicitly restarted Lanczos

bidiagonalization algorithm (IRLBA)[186]. Data visualization of the PCA-reduced data was also performed through the Cell Ranger pipeline, using a modified version of the C++ reference implementation of *t*-SNE by Maaten, L.J.P. van der (2014)[187].

Infection with *N. brasiliensis*

Mice were infected subcutaneously with 500 L3-stage *N. brasiliensis* larvae and were analyzed at the indicated timepoints post-infection.

Statistical analysis

Statistical data analysis was performed using Prism 6 (GraphPad) using the two-tailed unpaired Student's t-test when indicated.

Chapter 3: Concluding Remarks

Model

Following from the results described in Chapter 2, I propose a developmental model in which tissue-resident ILC2s originate from specific waves during prenatal and postnatal windows (Fig. 3.1). The first wave arises prenatally in the late embryo, potentially signifying an anticipatory influx of ILC2s in preparation for birth and/or seeding of tissues by ILC2s that is on cue with the processes that govern tissue growth during gestation. The second wave arises soon after birth, which seems to trigger both the activation and proliferation of ILC2s; the majority of the tissue ILC2 populations are established during this wave in the first few weeks of life in the mouse. The extent of replacement of the pre-established ILC2s by the third contributor, which comprises newly derived ILC2s in the fully developed adult, differs among tissues. ILC2s from the skin and small intestine are replaced at a more rapid rate by adult-derived cells, compared to ILC2s from the lung and visceral adipose tissue, possibly reflecting differences in exposure to microbial or other environmental stimuli or differences in homeostatic maintenance and tissue turnover.

Several questions still remain in the proposed model, particularly questions regarding the functional consequences of having ILC2s of different origins in different tissues. Further studies will be necessary to determine the degree to which ontogeny contributes to the functional identity of different tissue ILC2s and whether these differences can be exploited for the benefit of the host.

Figure 3.1

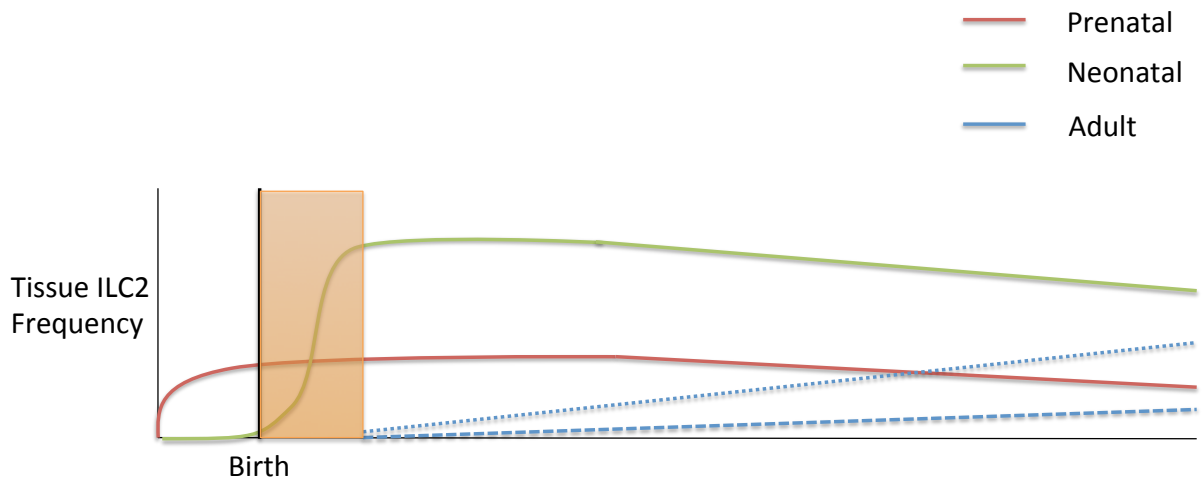

Figure 3.1. A model of ILC2 development
Three contributors exist for the tissue ILC2 pool throughout the mouse lifespan, represented on the x-axis, in the proposed model. The first wave (shown as a red line) seeds tissues in the late embryo before birth (indicated by the black vertical line). The second wave (shown as a green line) arises directly after birth during a window of activation and proliferation (indicated by the shaded orange region) and gives rise to a substantial proportion of the tissue ILC2 pool. The magnitude of the third wave, or "trickle", depends on the tissue (shown by the dashed and dotted blue lines of differing slopes). The rate of replacement of early-life-derived ILC2s by adult-derived ILC2s is higher in tissues such as the skin and intestine, compared to the lung.

Implications of Group 2 Innate Lymphoid Cell Heterogeneity

We have shown that the kinetics of ILC2 replacement is tissue-dependent, suggesting that the heterogeneity observed in ILC2s across different tissues may partially be due to differences in origin. Further studies will be needed to determine the influence and the relative contributions of ontogeny, developmental signals, tissue-specific signals, and environmental signals in defining the functional identity of different tissue ILC2s.

Tissue-specific factors may be a main cause of the differences seen in turnover and kinetics among different tissue ILC2s. In support of this, we have observed differential cytokine receptor expression on ILC2s from different tissues that seems to correspond with the availability of those cytokines in the particular tissues (Ricardo-Gonzalez, Van Dyken et al, under review). Variances in the nature of homeostatic signals required for ILC2 maintenance in different tissues are likely to influence ILC2 turnover. Additionally, many of the known activators of ILC2s are cytokines that are expressed by epithelial cells, and while adult lung epithelial cells are normally quiescent, gut and skin epithelial cells are renewed at a high rate, which could in turn affect the renewal of ILC2s that may depend on epithelium-derived maintenance factors.

In the case of the intestine, it is noteworthy that a recent study found that intestinal macrophages actually comprise multiple subsets, one of which is long-lived and not replaced by adult monocytes and one of which has a high rate of replacement from blood monocytes[157]. Similar subsets appear to exist within the gut ILC2 pool, with a $ST2^+$ subset that is more stably labeled after fate mapping and an $IL17RB^+KLRG1^+$ subset that is more rapidly replaced. Further studies will be necessary to determine the significance of this subset of gut ILC2s subset in relation to their activity, and also whether the features of

differential intestinal macrophage and ILC2 turnover are related to common tissue-specific signals.

Interrelated with tissue-specific factors, there may also be niche-specific factors that influence ILC2 turnover. For example, the spatial permissiveness of the lung ILC2 niches, described to be collagen-rich regions[40], may be much lower than that of the small intestine lamina propria. Moreover, just as ILC2s display heterogeneity within tissues, the niches themselves are likely to be heterogeneous, and thus the characteristics of the niche-providing cells, such as the recently described podoplanin-expressing mesenchymal cells in the mesentery[188], may also influence ILC2 turnover. For instance, pericryptal IL-33-expressing fibroblasts have been described in the intestine[189], and one might speculate that these stromal cells may serve as a more static niche for the intestinal ILC2s that express ST2 (IL-33R) in the intestine, compared to the more dynamic and rapidly replaced IL-25-expressing epithelial tuft cells[190], which would presumably be more important for sustaining the intestinal ILC2s that express IL17RB (IL-25R). Further studies will be required to determine the causes of tissue-specific differences in ILC2 replacement.

Single-cell RNA-sequencing analysis of neonatal ILC2s with adult ILC2s from corresponding tissues showed that neonatal ILC2s clustered distinctly from adult ILC2s, though a fair amount of overlap between the two populations was also observed. When analyzed in aggregate with neonatal and adult ILC2s from all tissues, neonatal ILC2s clustered with their adult tissue counterparts, suggesting that tissue-specific signatures (and thus, tissue-specific ILC2 heterogeneity) are adopted in early life. This is in agreement with our observations that differential expression of tissue-biased cytokine receptors on ILC2s occurs in early life.

Additionally, the activation of ILC2s post-birth resembles how ILC2s respond during states of inflammation. An interesting possibility is that the manner by which ILC2s respond to inflammatory stimulation is a reflection of how they became activated as they were becoming established in the neonatal window. Thus, neonatal ILC2 activation may represent a "priming" event that potentiates ILC2s for later function, and the ILC2 response during states of inflammation may simply be a revisitation of the steps that the ILC2 had taken during development. Further investigation would be needed to determine the significance of such a priming event during the neonatal period, and whether different developmental programs exist for different ILC2s and how much this affects their effector function.

During immune perturbation after infection with *N. brasiliensis*, we observe a blood ILC2 population that by cell surface marker expression and fate-mapped percentage most closely resembles lung and adipose tissue ILC2s, rather than bone marrow ILC2s, where the fate-mapped percentage is close to zero. Recently, a migratory IL-25R^{+}KLRG1^{+} ILC2 population that peaks 5-8 days after infection with *N. brasiliensis* has been described[149]. These cells purportedly originate in the small intestine and migrate to the lung. However, the fate-mapped percentage of the blood ILC2s we observe is much higher than the intestinal ILC2s, making it unlikely that these cells are of intestinal origin. More likely, the blood ILC2s that we observe represent a second wave of circulating ILC2s that are distinct from the migratory IL-25R^{+} ILC2 population described by Huang et al., particularly given the differences in the time points of analysis (5-8 days post-infection versus 10-12 days post-infection). On the other hand, we cannot rule out that the fate-mapped ILC2s in the blood represent a preferential expansion and emigration of the few remaining fate-mapped

intestinal ILC2s. Further studies will be needed to definitively prove the origin of blood ILC2s that arise in the aftermath of *N. brasiliensis* infection.

Summary

Despite the growing evidence for the importance of type 2 immunity and ILC2s in deep-seated physiological processes, little had been known about how these cells are laid down developmentally in tissues and how these cells are primed for their functions. To gain a finer comprehension of ILC2 ontogeny, we used two fate-mapping tools to elucidate three distinct waves of ILC2 development in fetal, neonatal, and adult life. This study has determined that the establishment of tissue ILC2s occurs in early life, which may be clinically valuable knowledge in the face of any potential therapy targeting these cells. We have also revealed that the origins of ILC2s in different tissues are not static, and rather that ILC2s show dynamic heterogeneity with regard to ontogeny that is tissue-specific. Furthermore, this study has identified and characterized an important window of activation for these cells that occurs shortly after birth. Lastly, we show that immune perturbations that occur in a model of helminth-induced inflammation do not drastically alter the proportions of cells of differing origins, signifying that de novo generation of ILC2s is not responsible for the ILC2 expansion during allergic inflammation.

Additional investigation will be required to determine whether modulation of ILC2s during the perinatal window can result in long-lasting changes to developing tissues and the immune "tone" of the immune cells that populate these tissues. Given the significance of early life events in the development of biased immune responses, it is tempting to speculate that these lasting effects would be mediated by a long-lived tissue-resident immune cells. Further work will also be necessary to determine the generalizability of our findings and how closely the innate lymphoid population parallels tissue-resident myeloid populations in terms of functional ontogeny.

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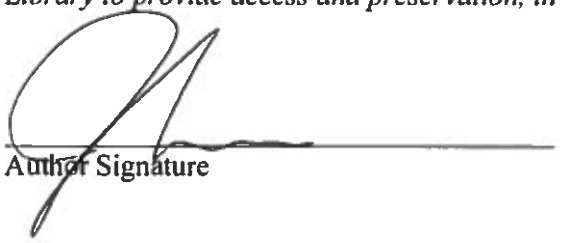

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