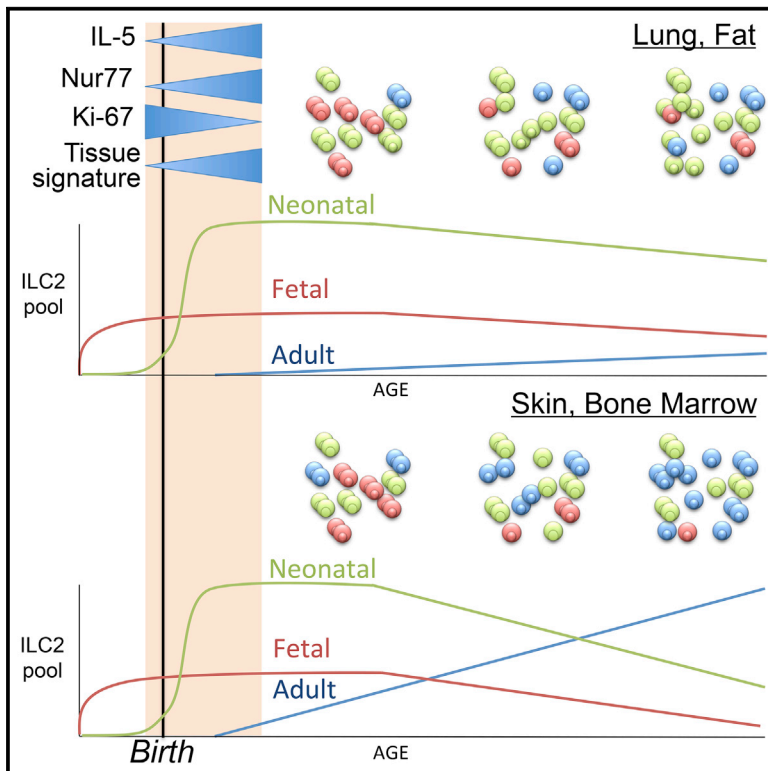


# Immunity

## Tissue-Resident Group 2 Innate Lymphoid Cells Differentiate by Layered Ontogeny and *In Situ* Perinatal Priming

### Graphical Abstract



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### In Brief

The perinatal period is critical for the distribution of innate tissue-resident immune cells within developing organs. Using temporally controlled fate mapping of group 2 innate lymphoid cells (ILC2s), Schneider and colleagues demonstrate that the neonatal period is accompanied by *de novo* ILC2 generation, systemic effector repertoire activation, and acquisition of tissue-specific signatures.

### Highlights

- Adult ILC2 pools are composed of cells of fetal, postnatal, and adult origin
- Adult ILC2 pool dilution by *de novo*-generated ILC2s is dynamic but varies by tissue
- Neonatal ILC2s activate effector repertoires and acquire tissue-specific signatures
- Worm infection drives expansion of preexisting ILC2 pools, but not *de novo* generation



# Tissue-Resident Group 2 Innate Lymphoid Cells Differentiate by Layered Ontogeny and *In Situ* Perinatal Priming

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## SUMMARY

The perinatal period is a critical window for distribution of innate tissue-resident immune cells within developing organs. Despite epidemiologic evidence implicating the early-life environment in the risk for allergy, temporally controlled lineage tracing of group 2 innate lymphoid cells (ILC2s) during this period remains unstudied. Using complementary fate-mapping approaches and reporters for ILC2 activation, we show that ILC2s appeared in multiple organs during late gestation like tissue macrophages, but, unlike the latter, a majority of peripheral ILC2 pools were generated *de novo* during the postnatal window. This period was accompanied by systemic ILC2 priming and acquisition of tissue-specific transcriptomes. Although perinatal ILC2s were variably replaced across tissues with age, the dramatic increases in tissue ILC2s following helminth infection were mediated through local expansion independent of *de novo* generation by bone marrow hematopoiesis. We provide comprehensive temporally controlled fate mapping of an innate lymphocyte subset with notable nuances as compared to tissue macrophage ontogeny.

## INTRODUCTION

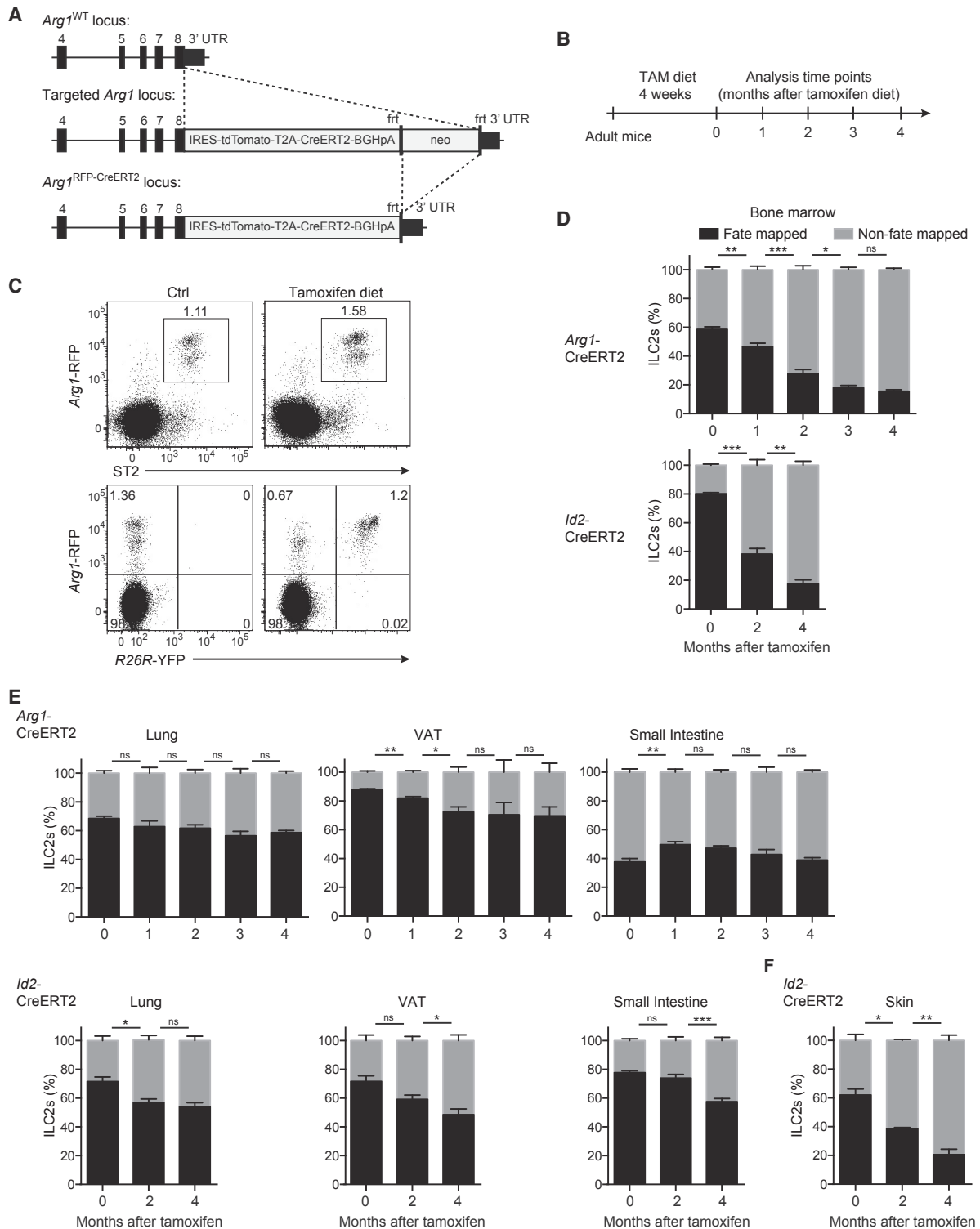
Asthma and allergic diseases represent a major health burden worldwide (Pawankar et al., 2011). Epidemiologic evidence has increasingly linked various environmental exposures during early years of life with the risk of developing allergies (Lambrecht and Hammad, 2017). The discovery of group 2 innate lymphoid cells (ILC2s; Moro et al., 2010; Neill et al., 2010; Price et al., 2010), which express type 2 cytokines in the absence of antigen receptors, offers an opportunity to look for functions of these cells independent of traditional host immune defense in efforts to reveal

the contextual roots of allergic immunity. Indeed, activation of these cells by a variety of epithelial cytokines, neuropeptides, and eicosanoids and involvement in metabolism, tissue repair, and remodeling in multiple organs, have begun to outline a previously unsuspected role for type 2 immunity integrated deeply into aspects of basal homeostasis (Ebbo et al., 2017; Klose and Artis, 2016; Kotas and Locksley, 2018). Further understanding could be gained by examining the development, activation, and turnover of these cells in tissues, particularly during early life when environmental inputs might be 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 (BM) 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 replaced the dogma that tissue-resident macrophages rely on constant replenishment from blood monocytes for homeostasis (van Furth and Cohn, 1968) with the discovery that recurrent waves of precursor cells—from early embryonic yolk sac macrophages, late embryonic fetal monocytes, and adult blood monocytes—layer together in differing proportions to comprise the tissue resident macrophage pool in different organs (McGrath et al., 2015; Perdiguero and Geissman, 2016). The ability to distinguish cells based on ontogeny has revealed unsuspected transcriptomic and epigenomic landscapes that differ among macrophages of different origins, whether from fetal hematopoiesis in the yolk sac and liver or from adult hematopoiesis in the BM (Gibbins et al., 2015; Lavin et al., 2014). ILC progenitors with the capacity to give rise to mature ILCs *in vitro* and *in vivo* after transfer into lymphopenic mice have been identified in fetal tissues (Bando et al., 2015; Ishizuka et al., 2016) and adult BM (Constantinides et al., 2014; Hoyler et al., 2012; Klose et al., 2014), but the relative contributions of these populations and their turnover among adult tissue ILC2 pools remain poorly studied, in part due to the lack of tools to reliably fate map and track these cells.

The development of ILC2s requires the transcriptional regulator Id2 (Boos et al., 2007; Moro et al., 2010; Yokota et al., 1999), and high Id2 expression is maintained in mature ILC2s





**Figure 1. Tissue ILC2s Are Replaced by De Novo Generated ILC2s at a Low Rate**

(A) Gene-targeting strategy for the *Arg1*<sup>RFP-CreERT2</sup> mouse.

(B–F) Adult *Arg1*<sup>RFP-CreERT2</sup>*R26R*<sup>YFP</sup> and *Id2*<sup>CreERT2</sup>*R26R*<sup>YFP</sup> mice were treated with tamoxifen chow according to schedule (B). (C) Expression of *Arg1*-RFP and ST2 (top) or R26R-YFP (bottom) by cells gated on CD45<sup>+</sup> cells isolated from the lung of *Arg1*<sup>RFP-CreERT2</sup>*R26R*<sup>YFP</sup> mice after 4 weeks of tamoxifen diet. (D–F) Percentages of fate-mapped ILC2s in BM (D), lung, visceral adipose tissue (VAT), small intestine lamina propria (E), and skin (F) analyzed at indicated time points.

(legend continued on next page)

(Hoyler et al., 2012). Putative ILC precursors that express *Id2* are present in the BM (Constantinides et al., 2014; Klose et al., 2014), providing a potential source for these cells. ILC precursors that express the urea cycle enzyme *Arg1* have also been identified in the small intestine before birth (Bando et al., 2015), which suggests that ILCs can seed tissues prenatally and might arise from fetally generated precursors. Parabiosis experiments revealed minimal replacement of tissue ILC2s by circulating cells under steady-state conditions (Gasteiger et al., 2015; Moro et al., 2016), implying little contribution from bloodborne precursors and consistent with the designation of ILC2s as predominantly tissue-resident cells (Fan and Rudensky, 2016). Limitations and variability driven by the duration of parabiosis (Gasteiger et al., 2015; Huang et al., 2018) suggest that complementary approaches will be necessary to comprehensively assess contributions by cells that might be derived during different phases of development.

In this study, we have used *Arg1*-driven and *Id2*-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 lifespans of these cells in tissues and gain greater understanding of the role of ILC2s in local and systemic contributions to type 2 immunity. Our studies reveal three distinct waves of ILC2 development characterized by dispersal into tissues, expansion and activation of tissue-specific transcriptional programs, and maintenance, with evidence for niche-specific differences in local regulation of survival and turnover throughout life.

## RESULTS

### Tissue ILC2s Are Replaced by Newly Generated ILC2s at a Low Rate

We used two independent tamoxifen-inducible Cre mouse strains to lineage trace ILC2s and assess their temporal origin. First, we generated an *Arg1*<sup>RFP-CreERT2</sup> mouse strain, in which expression of red fluorescent protein (RFP) as well as tamoxifen-inducible Cre (CreERT2) is under the control of the endogenous arginase-1 (*Arg1*) promoter (Figure 1A). Consistent with previous reports (Bando et al., 2013, 2015; Monticelli et al., 2016), *Arg1*<sup>RFP-CreERT2</sup> mice accurately report *Arg1* expression in CD25<sup>+</sup>*Id2*<sup>GFP+</sup> ILC2s from the BM, lung, and adipose tissue (Figures S1A and S1B). In contrast, *Arg1* expression was absent in CD25<sup>-</sup>*Id2*<sup>GFP+</sup> BM ILC precursors (CHILPs), a fraction of ILC2s from the small intestine lamina propria (Figures S1A and S1B) (Schneider et al., 2018), and a majority of the ILC2s in the skin (Figures S1C–S1E), corroborating heterogeneity in *Arg1* expression among mature ILC2 subsets in different tissues (Ricardo-Gonzalez et al., 2018). In addition to ILC2s, the *Arg1* reporter was expressed by a subset of small intestinal ROR $\gamma$ <sup>+</sup> ILC3s (Bando et al., 2015; Robinette et al., 2015) and in F4/80<sup>hi</sup>MHCII<sup>low</sup> peritoneal macrophages (Figures S1A and S1F). Due to the absence of *Arg1* expression in ILC2s of certain tis-

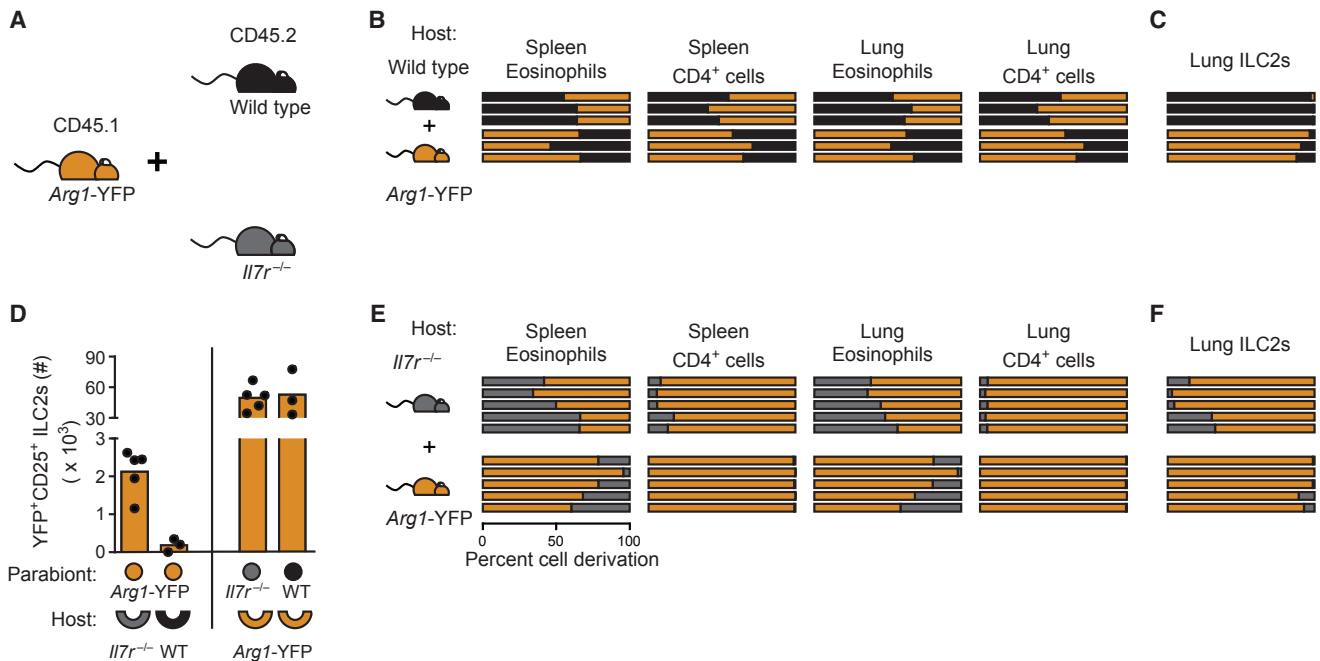
sues, and as a second and independent approach, we used a previously described *Id2*-CreERT<sup>2</sup> genetically targeted mouse (Rawlins et al., 2009) to compare and confirm results obtained using the *Arg1*<sup>RFP-CreERT2</sup> strain.

To assess the turnover of adult ILC2s, we crossed these two strains to R26R-EYFP or R26R-RFP reporter mice for fate mapping. Adult mice were fed tamoxifen diet for 4 weeks to enable activation of the floxed reporter allele and then analyzed monthly for 4 months after return to chow diet (Figures 1B and 1C). Notably, the BM ILC2 population was steadily diluted by newly generated unlabeled cells, as assessed by the decreasing proportion of fate-mapped cells in both strains of mice (Figure 1D). By 2–3 months after tamoxifen removal, fewer than half of BM ILC2s remained fate-mapped. Despite the dilution of ILC2s in the BM, there was minimal change in labeling frequency of tissue ILC2 populations over the course of 4 months following removal from tamoxifen (Figure 1E) with the exception of the skin (Figure 1F), suggesting a slow rate of tissue ILC2 replenishment or expansion through *de novo* generation from a non-fate-mapped population. To address whether changes in the size of ILC2 pools could contribute to the dilution of fate-mapping labeling in certain tissues, we determined the absolute numbers at distinct ages (2, 4, and 6 months). Notably, total ILC2 numbers gradually increased in the BM and skin, which showed the highest rate of fate-mapping label dilution (Figure S1G). In contrast, the numbers of ILC2s in the lung and VAT remained relatively stable over the same period of 4 months, consistent with little dilution of the percentages of fate-mapped cells in these organs. These results suggest that the differential decay in ILC2 fate-mapping frequencies might, at least in part, be caused by addition of *de-novo*-generated ILC2s to the pools of existing ILC2s rather than replacement of the latter. Taken together, these data are consistent with generally stable long-lived populations of ILC2s in most adult tissues but with more rapid turnover and continuous expansion of the ILC2 pools in BM and skin.

### Tissue ILC2s Do Not Recirculate or Repopulate Empty Niches at Steady State

To assess further 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 (Figure 2A). After 5 weeks, the lungs and spleens of WT recipients contained approximately equivalent numbers of CD4<sup>+</sup> T cells and eosinophils derived from each parabiont, indicating the free exchange of these circulating cells across the surgical anastomosis (Figures 2B, S2A, and S2B). However, consistent with previous reports over similar periods (Gasteiger et al., 2015; Huang et al., 2018; Moro et al., 2016), lung ILC2s showed <5% crossover between the paired mice, corroborating a relatively long tissue lifespan and limited replenishment from the circulation (Figures 2C and S2C). Because niche occupancy in the WT mice may have prevented circulating progenitors from engrafting between the

ILC2s gated as Lin<sup>-</sup> $\alpha$ 4 $\beta$ 7<sup>+</sup>IL-7R<sup>+</sup>Thy1<sup>+</sup>CD25<sup>+</sup> (BM), Lin<sup>-</sup>Thy1<sup>+</sup>ST2<sup>+</sup> (lung and VAT), Lin<sup>-</sup>IL17RB<sup>+</sup>KLRG1<sup>+</sup> (small intestine), or Lin<sup>-</sup>Thy1<sup>+</sup>ICOS<sup>+</sup> (skin). Data are from one experiment representative of at least three independent experiments (C) or pooled from multiple independent experiments and displayed as mean  $\pm$  SEM of 4–7 individual mice per group and time point (D–F). See also Figure S1.



**Figure 2. Low Progenitor Recirculation Frequency and Restricted Niche Availability Cause Minimal Replacement of Adult Tissue ILC2s**

(A) Schematic of CD45.1 *Arg1*-YFP reporter mice (yellow) parabiosis with either WT (black) or *Il7r*<sup>-/-</sup> (gray) mice.

(B and C) Percent derivation of (B) eosinophils and CD4<sup>+</sup> T cells from spleen and lung and (C) ILC2s from the lung; each bar represents a single mouse from the WT:*Arg1*-YFP pair.

(D) Numbers of lung *Arg1*<sup>+</sup>CD25<sup>+</sup>ILC2s; each dot represents one host mouse.

(E and F) Percent derivation of (E) eosinophils and CD4<sup>+</sup> T cells from the spleen and lung and (F) ILC2s from the lung; each bar represents a single mouse from the *Il7r*<sup>-/-</sup>:*Arg1*-YFP pair. Data are from one surgery cohort and representative of three independent surgery cohorts.

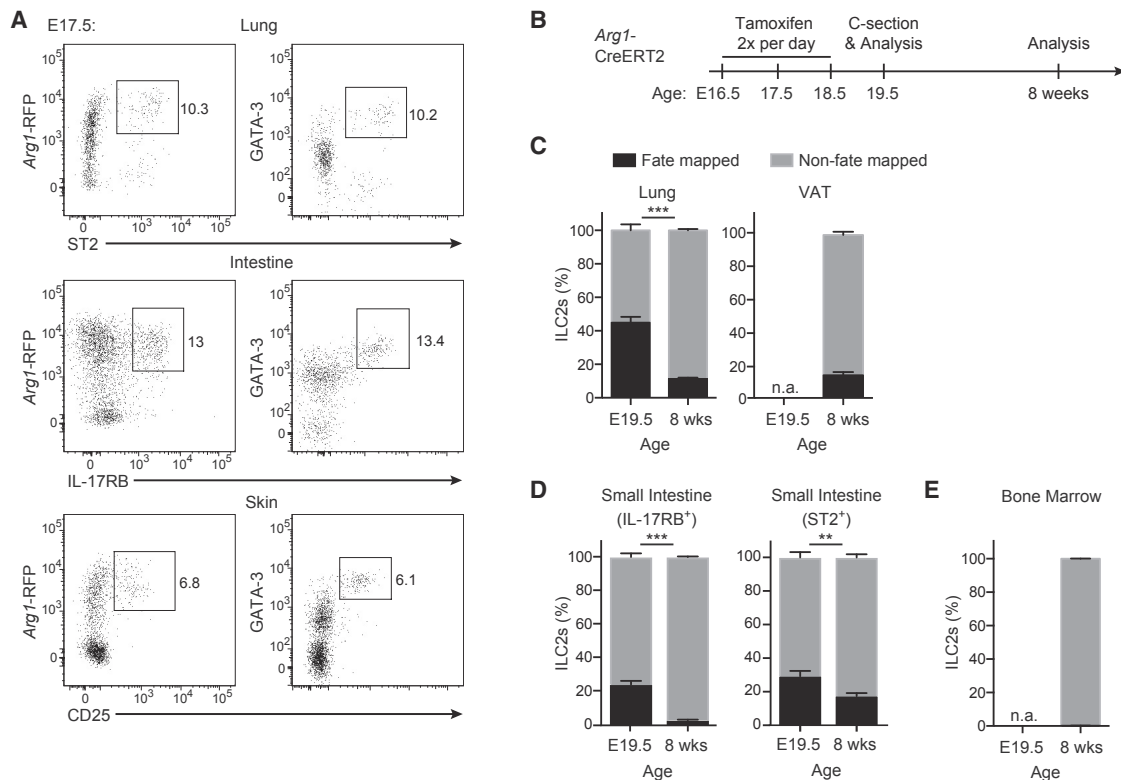
See also Figure S2.

parabionts, we also tested the ability of ILC2s to reconstitute IL-7 receptor  $\alpha$  (IL-7R $\alpha$ )-deficient recipients, which lack ILCs, B cells, and T cells. Although the percentage of parabiont-derived ILC2s was higher in the IL-7R $\alpha$ -deficient host (Figures 2D–2F), 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 those in *Arg1*-YFP (IL-7R $\alpha$ -sufficient) hosts (Figure 2D), confirming that ILC2s are predominantly long-lived tissue-resident cells, which maintain themselves during adulthood largely independent from replenishment by rare circulating precursors. We observed the same patterns of replacement when assessing ILC2s in the adipose tissue and in the small intestine lamina propria (Figures S2D and 2E), and thus extend prior observations using these types of approaches (Gasteiger et al., 2015; Huang et al., 2018; Moro et al., 2016). To assess the possibility that an inherently impaired niche in the lymphopenic IL-7R $\alpha$ -deficient parabionts may have prevented engraftment of circulating cells, we quantified the number of ILC2-supporting mesenchymal cells (Dahlgren et al., 2019; Koga et al., 2018) in *Il7r*<sup>-/-</sup> mice. The frequency and number of PDGFR $\alpha$ <sup>+</sup>gp38<sup>+</sup> stromal cells in the lung and their expression of important niche factors, interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP), were comparable between *Il7r*<sup>-/-</sup> and WT mice (Figures S2F and S2G), suggesting that the ILC2 niche is not obviously altered in these lymphopenic mice.

### 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 fate-mapping strains and two conditions of parabiosis suggested that tissue ILC2 pools are established prior to reaching adulthood. Many innate tissue-resident cells, including tissue macrophages, are seeded during fetal development (Ginhoux and Guillems, 2016), and previous work in our laboratory has characterized *Arg1*-YFP-expressing ILC precursors in the fetal small intestine (Bando et al., 2015). As assessed using flow cytometry, *Arg1*-RFP<sup>+</sup> cells with a surface marker expression profile of mature ILC2s (e.g., Lin<sup>-</sup>IL-7R $\alpha$ <sup>+</sup>Thy1<sup>+</sup>ST2<sup>+</sup>) appeared as early as embryonic day 17.5 (E17.5) in the fetal lung, small intestine, and skin (Figure 3A). These cells expressed GATA-3 (Figure 3A) and Ki-67 (Figure S3A), consistent with active cell proliferation.

To determine whether these fetal tissue-seeding *Arg1*<sup>+</sup> ILC2s contribute to adult ILC2 pools, we performed lineage-tracing experiments by administering tamoxifen prenatally during E16.5–E18.5 and analyzing the labeled tissue ILC2 populations at 2 months of age (Figure 3B). Labeling efficiency at E19.5 in lung and small intestine was robust (30%–45%); however, the absence of visceral adipose tissue (VAT) in the embryos and the absence of fetal BM ILC2s precluded analysis in these tissues (Figures 3C–3E). By 2 months after birth, however, only a fraction (5%–10%) of the ILC2s in lung, small intestine, and



**Figure 3. Prenatal ILC2Ps Seed Tissues during Fetal Development and Contribute to the Adult ILC2 Tissue Pool**

(A) Expression of *Arg1* (left) or GATA-3 (right), and ST2, IL-17RB, or CD25 by cells gated on Lin<sup>-</sup>CD45<sup>+</sup> cells isolated from the lung, small intestine, and skin of *Arg1*<sup>RFP-CreERT2</sup> reporter mice on embryonic day 17.5 (E17.5).

(B–E) Pregnant *Arg1*<sup>RFP-CreERT2</sup>*R26R*<sup>YFP</sup> mice treated with tamoxifen according to schedule in (B). (C–E) Percentages of fate-mapped ILC2s in lung and VAT (C), small intestine lamina propria (D), and BM (E) analyzed in E19.5 and 2-month-old mice. ILC2s gated as Lin<sup>-</sup>Thy1<sup>+</sup>ST2<sup>+</sup> (lung and VAT), Lin<sup>-</sup>IL-17RB<sup>+</sup>KLRG1<sup>+</sup>, or Lin<sup>-</sup>ST2<sup>+</sup>KLRG1<sup>+</sup> as indicated for the small intestine or Lin<sup>-</sup>IL-17RB<sup>+</sup>Thy1<sup>+</sup>CD25<sup>+</sup> (BM); n.a., not assessed. Data from one experiment representative of at least three independent experiments (A) or pooled from multiple independent experiments and displayed as mean ± SEM of 6–8 individual mice per group and time point (C–E).

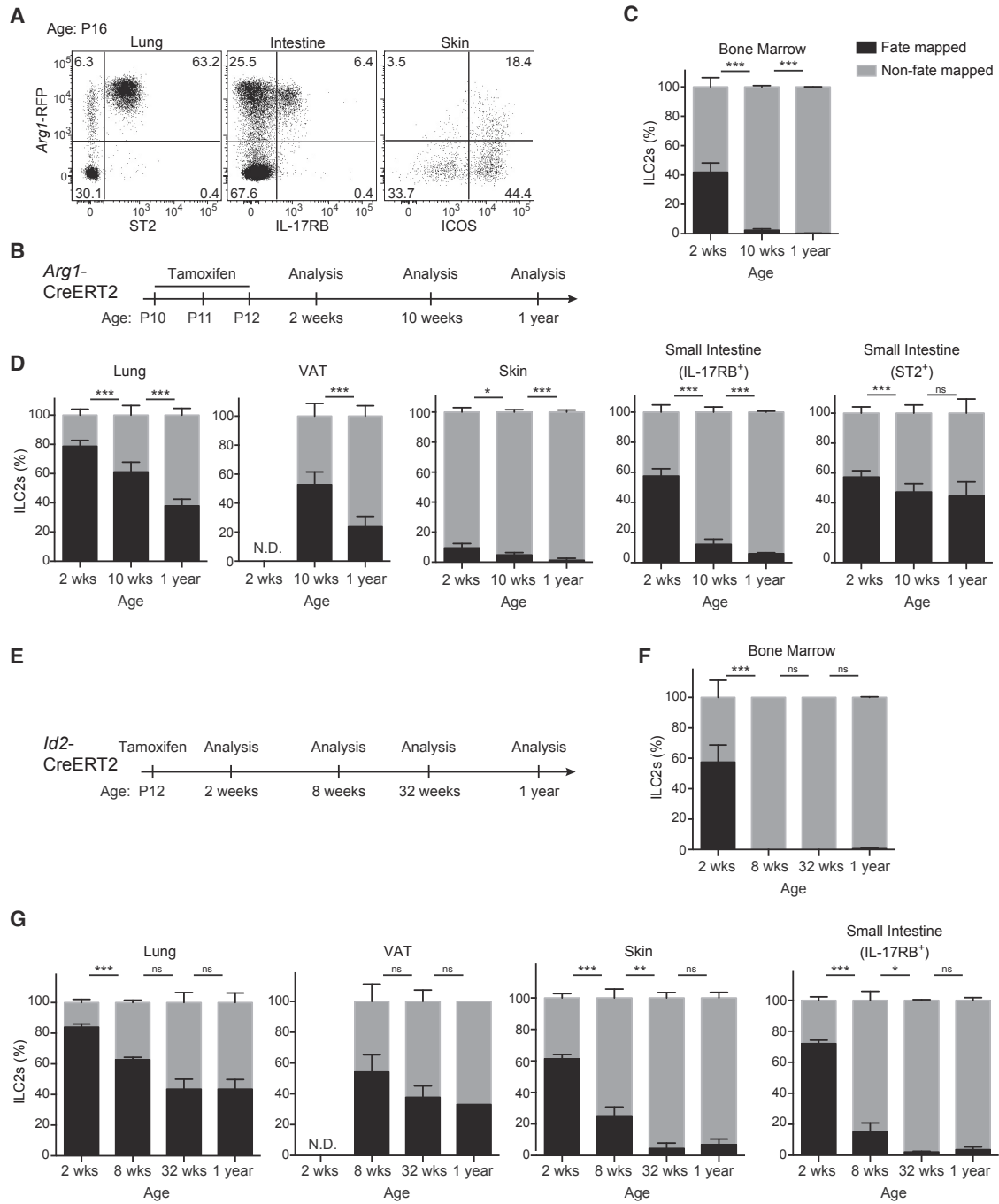
See also Figure S3.

VAT remained labeled and were thus embryonically derived (Figures 3C and 3D). Proportions of the latter were greater in lung and VAT than in small intestine. Although a majority of Gata-3<sup>+</sup> ILC2s in the fetal small intestine co-expressed the subunits of the IL-33 and IL-25 receptors, ST2 and IL-17RB, only a small fraction of their adult counterparts expressed ST2 (Figure S3B). However, the fraction of fetally labeled cells was higher among ST2<sup>+</sup> ILC2s than ST2<sup>-</sup> ILC2s, suggesting differential contribution of fetal-derived *Arg1*<sup>+</sup> cells to the heterogeneous ILC2 compartments in the adult small intestine (Figures 3D and S3C). Notably, none of the putative ILC2 precursors (ILC2Ps) in the BM remained labeled in the adult (Figure 3E), suggesting that post-birth BM *Arg1*<sup>+</sup> ILC2s have an origin temporally distinct from and with no contribution from prenatally derived ILC2s.

### Early Postnatal ILC2s Comprise the Majority of the ILC2 Pool in Adult Lung and VAT

We and others have noted the acute expansion and activation of ILC2s that occur in the lung shortly after birth (Huang et al., 2018; de Kleer et al., 2016; Nussbaum et al., 2013; Saluzzo et al., 2017; Steer et al., 2017). Given these observations, we used lineage tracing in *Arg1*-CreERT2 and *Id2*-CreERT2 mice to assess the

contribution of early postnatal ILC2s to adult tissue ILC2 pools. At 2 weeks of age, ILC2s in the lung and small intestine express *Arg1*, in contrast to the skin, where, similar to adult mice, ILC2s expressed little *Arg1* (Figure 4A). *Arg1*-CreERT2 and *Id2*-CreERT2 pups were given tamoxifen on postnatal days 10–12 and analyzed at various time points to assess the numbers of label-retaining ILC2s in multiple tissues (Figures 4B and 4E). Notably, ILC2s in the BM were almost completely diluted and/or replenished by unlabeled cells within 2 months, suggesting their continuous *de novo* generation during early BM hematopoiesis (Figures 4C and 4F). In contrast, and as compared to the dilution of fetally labeled ILC2s, a significantly higher fraction (40%–70%) of fate-mapped ILC2s were present in adult lung, adipose tissues, small intestine, and skin (using *Id2*-CreERT2 mice, since *Arg1* is poorly expressed), and this was comparable in both strains of fate-mapping mice (Figures 4C–4G). There was some continuing dilution of fate-mapped ILC2s during the period between 2 and 8 weeks of age, indicating that *de novo* ILC2 generation likely continues beyond postnatal day 12, but, by 8 weeks of age, the tissue ILC2 pool remained relatively stable in the lung and VAT throughout the life of the mouse (Figures 4D and 4G); this pool of fate-mapped cells persisted in mice that were over



**Figure 4. Neonatal ILC2s Contribute to the Majority of the ILC2 Pool in Adult Tissues**

(A) Expression of *Arg1*-RFP and ST2, IL-17RB, or ICOS by cells gated on Lin<sup>-</sup>CD45<sup>+</sup> cells isolated from lung, small intestine, and skin of *Arg1*<sup>RFP-CreERT2</sup> reporter mice on postnatal day 16 (P16).

(B–F) Neonatal *Arg1*<sup>RFP-CreERT2</sup>*R26R*<sup>YFP</sup> and *Id2*<sup>CreERT2</sup>*R26R*<sup>RFP</sup> mice treated with tamoxifen according to schedule in (B and E). Percentages of fate-mapped ILC2s in BM (C) and lung, VAT, skin, and small intestine lamina propria (D) of *Arg1*<sup>RFP-CreERT2</sup>*R26R*<sup>YFP</sup> mice. Percentages of fate-mapped ILC2s in the BM (F) and lung, VAT, skin, and small intestine lamina propria (G) of *Id2*<sup>CreERT2</sup>*R26R*<sup>RFP</sup> mice. ILC2s gated as in Figure 3. Data are from one experiment representative of at least three independent experiments (A) or pooled from multiple independent experiments and displayed as mean ± SEM of 5–9 individual mice per group and time point (C–G).

1 year old. In contrast, in the skin and small intestine, ILC2s were replaced more rapidly by newly generated, unlabeled ILC2s, such that by 1 year of age, only ~5% of ILC2s remained among

postnatally labeled cells (Figures 4D and 4G), although postnatally mapped cells clearly remain for many months, even in these tissues. Consistently, heterogeneity was apparent in comparing

the proportions of fate-mapped and non-fate-mapped cells among the different tissues. In the small intestine, a subpopulation of KLRG1<sup>+</sup>ST2<sup>+</sup> ILC2s remained stable throughout adulthood, consistent with a predominant early postnatal origin for these cells (Figure 4D).

### Tissues of the Newborn Mouse Are Characterized by Vigorous ILC2 Activation and Proliferation that Is Dependent on IL-7R $\alpha$ 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 (de Kleer et al., 2016; Nussbaum et al., 2013; Saluzzo et al., 2017; Steer et al., 2017). Here, we demonstrated that this neonatal wave of ILC2 expansion and activation occurred systemically, as assessed kinetically in lung, small intestine, skin, and BM (Figure 5A). The development of ILC2s is dependent on the cytokine IL-7 (Moro et al., 2010), and we confirmed that the early expansion of ILC2s was also dependent on IL-7R signaling: prenatal administration of a blocking antibody to IL-7R $\alpha$  resulted in significantly decreased ILC2 frequencies in the lungs, small intestine, and skin of neonatal mice (Figure 5B). Despite three doses of anti-IL-7R, however, ILC2 frequencies largely recovered by 9 weeks of age in the lungs, small intestine, skin, and VAT (Figure 5B), consistent with substantial restorative capacity.

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 (Liang et al., 2011; Nussbaum et al., 2013). We found that an increasing proportion of ILC2s turned reporter positive for IL-5 and IL-13, although much smaller in frequency for the latter, over these first weeks of life (Figures 5C and 5D). Systemic opening of the *Il5* locus was coincident with proliferation in peripheral tissues and was essentially absent among BM ILC2s (Figure 5C). ILC2 activation during this period was not dependent on the presence of microbiota, as neither *Il5* transcript expression nor *Il13* transcript expression was decreased in ILC2s sorted from the tissues of gnotobiotic neonatal mice (Figures 5E and 5F).

We previously noted high mRNA expression of the nuclear hormone receptor Nur77, or *Nr4a1*, in ILC2s isolated from peripheral tissues as compared to the BM (Figure 5G; Ricardo-Gonzalez et al., 2018). *Nr4a1* is an immediate early gene expressed in hematopoietic cells in association with growth factor, cytokine, inflammatory, and proliferative signals and important in mediating cell survival during stress (Maxwell and Muscat, 2006). Lymphocyte activation of adaptive T and B cells can be monitored *in situ* using cells from *Nr4a1*-GFP reporter mice, and “tuning” of *Nr4a1*-GFP corresponds with the degree of integrated signals accumulating within the cell (Zikherman et al., 2012). We speculated that Nur77 expression might report the activation status of ILC2s similar to adaptive lymphocytes. Indeed, when stimulated with recombinant interleukin (rIL)-33 *in vivo* ILC2s in the BM and lung of *Nr4a1*-GFP mice responded with increased Nur77 reporter expression (Figures S4A and S4B). We then used *Nr4a1*-GFP mice to assess whether ILC2s engage these pathways during the period of early postnatal expansion and activation. Indeed, ILC2s underwent a marked increase in *Nr4a1*-reporter expression in the early postnatal period in tissues

that is maintained into adulthood (Figure 5H); in contrast, *Nr4a1*-reporter expression remained low among ILC2s from fetal liver and adult BM. Notably, postnatal induction of IL-5 expression in peripheral ILC2s correlated with high expression of *Nr4a1*-GFP, although analysis revealed slightly different kinetics in ILC2 priming between the different organs, including the lung, small intestine, and skin (Figure 5I).

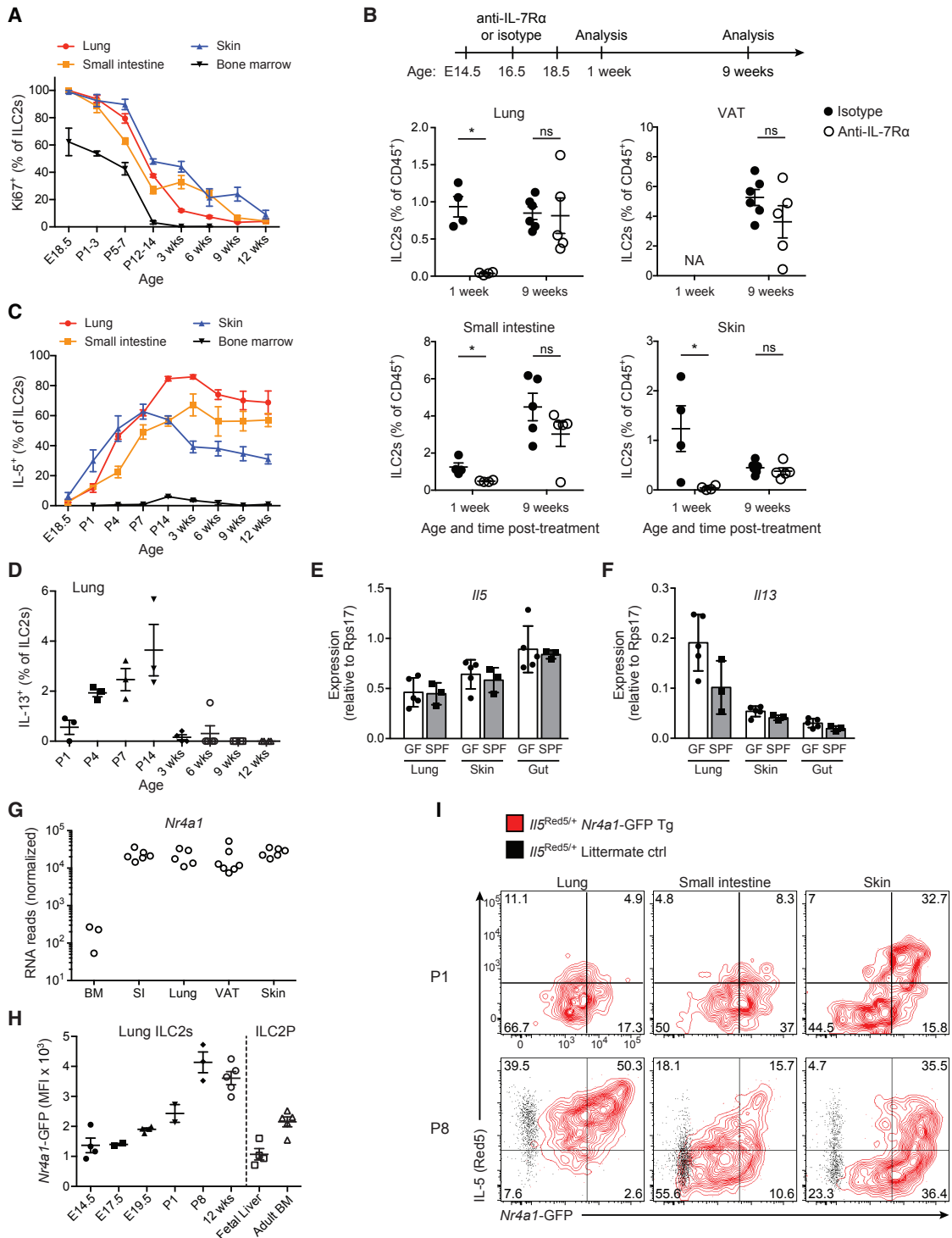
### Tissue-Specific Heterogeneity of ILC2s Is Established in the Early Postnatal Wave

Developing ILC2s position 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- to 14-week-old mice on the same day (to eliminate batch effects) and used single-cell RNA-sequencing analysis to assess cell and tissue heterogeneity. Early postnatal ILC2s clustered distinctly from adult ILC2s (Figures 6A and 6B) and were enriched for cells showing increased transcripts associated with cellular activation (*Il5*, *Il13*, *Nr4a1*, and *Mki67*) as compared to adult ILC2s (Figures 6C–6F and S5A–S5F). However, when analyzed in aggregate, early postnatal ILC2s from different tissues localized with adult ILC2s from the same tissues and consistent with a tissue-organizing expression pattern that has already developed during the early postnatal period during the period of marked ILC2 expansion (Figure 6G). In addition to transcripts known to be associated with ILC2 activation, certain chemokine genes (*Ccl1* and *Cxcl2*) were upregulated in the early postnatal ILC2s (Figures 6E, 6F, S6A, S6D, and S6G), suggesting that ILC2s could play a role in the early recruitment of other immune cells into tissues. A subset of tumor necrosis factor (TNF) superfamily gene transcripts, particularly those involved in lymphotoxin-beta receptor signaling (*Ltb* and *Tnfrsf14*), were present in both early postnatal and adult ILC2s (Figures S6B, S6C, S6E, and S6F), and although these genes were not differentially expressed in early postnatal ILC2s to a statistically significant degree, the expression of these genes further suggests a potential role for ILC2s in orchestrating processes associated with immune development. 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 gene-expression patterns.

### 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 (Figure 1), their dynamics during acute immune perturbations have been less carefully quantitated. We used *Nippostrongylus brasiliensis*, a migratory helminth that induces a vigorous systemic type 2 immune response with marked tissue effects in both the lungs and small intestine (Camberis et al., 2003), as underscored by a nearly 8-fold increase in ILC2 numbers (Figures 7A and 7B). Using mice with postnatally fate-mapped ILC2s, we found that even after infection with *N. brasiliensis*, the proportion of fate-mapped cells showed minimal decrease in the lung, spleen, and mesenteric lymph node (LN; Figures 7B–7D), despite the considerable





**Figure 5. Vigorous Perinatal ILC2 Activation and Proliferation Is Associated with Nur77 Expression**

(A) Percentages of Ki-67<sup>+</sup> ILC2s in the lung, small intestine lamina propria, skin, and BM analyzed at the indicated embryonic (E) and postnatal (P) time points. (B) Pregnant mice treated with anti-IL-7R $\alpha$  blocking antibody (A7R34) on E14.5, E16.5, and E18.5, and percentages of ILC2s among CD45<sup>+</sup> cells analyzed in lung, VAT, small intestine, and skin in 1- and 9-week-old mice. (C) Percentages of IL-5-reporter<sup>+</sup> ILC2s in lung, small intestine, skin, and BM of *I/5*<sup>Red5</sup> mice analyzed at the indicated time points. (D) Percentages of IL-13-reporter<sup>+</sup> ILC2s in lung of *I/13*<sup>Sm13</sup> mice. (E and F) Expression of *I/5* (E) and *I/13* (F) by ILC2s sorted from individual specific pathogen-free (SPF) or germ-free neonatal mice analyzed by qPCR. (legend continued on next page)

increase in ILC2 numbers. These data suggest a minor contribution of *de-novo*-generated ILC2s even in an inflammatory setting. Of note, essentially none of the putative BM ILC2Ps were fate mapped at the time of infection, ruling out any major contribution of ILC2Ps to the expansion of ILC2s that accompanies migratory helminth infection (Figure 7E). Furthermore, we found that the differential ILC2 fate-mapping frequencies between tissues such as the lung and small intestine-draining lymph nodes were maintained after infection (Figures 7B and 7D). Altogether, our studies suggest that the increases in ILC2s in the context of migratory helminth infection are predominantly mediated through local expansion of preexisting ILC2 pools at sites of inflammation.

## DISCUSSION

Using fate mapping in combination with markers for proliferation and activation, we make several observations relevant to the understanding of ILC2 biology. First, we identified three temporally distinct origins for tissue-resident ILC2s: fetally derived ILC2s that were distributed to tissues before birth, postnatally derived ILC2s that were generated in the period from birth through weaning, and adult-derived ILC2s that slowly diluted the preexisting ILC2s. Second, we showed that steady-state ILC2 replacement by *de novo* ILC2s generated in adult life was dynamic but tissue specific, with slow turnover in lung, fat, and small intestine but more rapid dilution in BM and skin. Third, using a combination of reporter mice and single-cell transcriptomics, we have shown that the perinatal seeding of peripheral tissues coincided with the period when ILC2s expand, activate their effector repertoire, and establish a tissue-specific transcriptomic signature that remains through life (Ricardo-Gonzalez et al., 2018). Lastly, using a mouse model of helminth infection, we found that the dominant mechanism by which ILC2 numbers increase in the lung after *N. brasiliensis* infection is the expansion of preexisting ILC2s rather than *de novo* generation of new ILC2s.

Our studies reveal that the adult tissue ILC2 pool comprises populations that are both prenatally and postnatally derived and furthermore that the postnatally derived population, in many tissues, arises largely within the first few weeks of life with minor contribution and replenishment by subsequent adult BM HSC-derived hematopoiesis. These findings reveal similarity between ILC2s and other innate-like lymphocytes (Carding and Egan, 2002; Montecino-Rodriguez et al., 2016), and even regulatory T (Treg) cells (Yang et al., 2015), and highlight the temporal window for niche seeding by tissue-resident immune cells in developing organs (Eberl et al., 2004; Gentek et al., 2018; Ginhoux and Guillems, 2016; Hoeffel and Ginhoux, 2015; Sawa et al., 2010; Yang et al., 2015). Whether the early postnatal ILC2 populations identi-

fied here originate from conventional HSC-dependent BM hematopoiesis or from an alternative source, such as multipotent progenitors in the spleen (Ghaedi et al., 2016) or progenitors already present within the tissue, requires further study.

Our fate-mapping studies also highlighted notable differences in the ontogeny of tissue macrophages and ILC2s, which both appeared in multiple organs during late gestation. Tissue macrophage populations were largely established during fetal development and derived predominantly from embryonic progenitors that originated in the yolk sac and fetal liver (Ginhoux and Guillems, 2016). Here, we demonstrated that a majority of the peripheral ILC2 pools were generated *de novo* during a postnatal window, which diluted the proportion of fetal ILC2s, and persisted into adulthood. Whether these populations derive from distinct progenitors, which might include fetal and adult HSCs (Beaudin and Forsberg, 2016), remains unknown. Notably, a transient wave of developmentally restricted HSCs with higher lymphoid cell production compared to fetal and adult HSCs and greater capacity to generate innate-like B and T lymphocytes has been described (Beaudin et al., 2016). In support of this, recent fate mapping of adult HSCs revealed their minimal contribution to B-1a cells and tissue macrophage compartments, but ILCs were not analyzed (Sawai et al., 2016). The observation that these stereotyped developmental trajectories early in life appear to be conserved even in humans highlight the need for a better understanding of the perinatal dynamics in immune development (Olin et al., 2018).

Our fate-mapping approach revealed dynamic complexity underlying ILC2 turnover and dilution, which manifests striking parallels with the varying kinetics observed for macrophage turnover in different tissues, with the differences in adult HSC contribution becoming increasingly apparent with age after fate mapping. Notably, subsets of tissue-resident macrophages in the intestine (Shaw et al., 2018) and skin (Tamoutounour et al., 2013) have higher rates of replacement than other tissues, including the lung (Ginhoux and Guillems, 2016), mirroring observations generated here for ILC2s. The intestine and skin are impacted by dietary and microbial stimuli and undergo dynamic phases of remodeling, including continuous small intestinal epithelial renewal in the bowel and hair follicle cycling in the skin, which potentially alter existing niches or create new ones that might enable colonization by *de novo* generated ILC2s. These processes might also contribute to yet-unappreciated differences in subset-specific stability within an organ, as suggested by the differential dilution kinetics between ST2<sup>+</sup> and ST2<sup>-</sup> small intestinal ILC2s. Notably, among multiple peripheral tissues, ILC2s in the small intestine and skin express the highest amounts of IL-13 (Ricardo-Gonzalez et al., 2018), a dynamic marker of ILC2 activation (Liang et al., 2011), suggesting

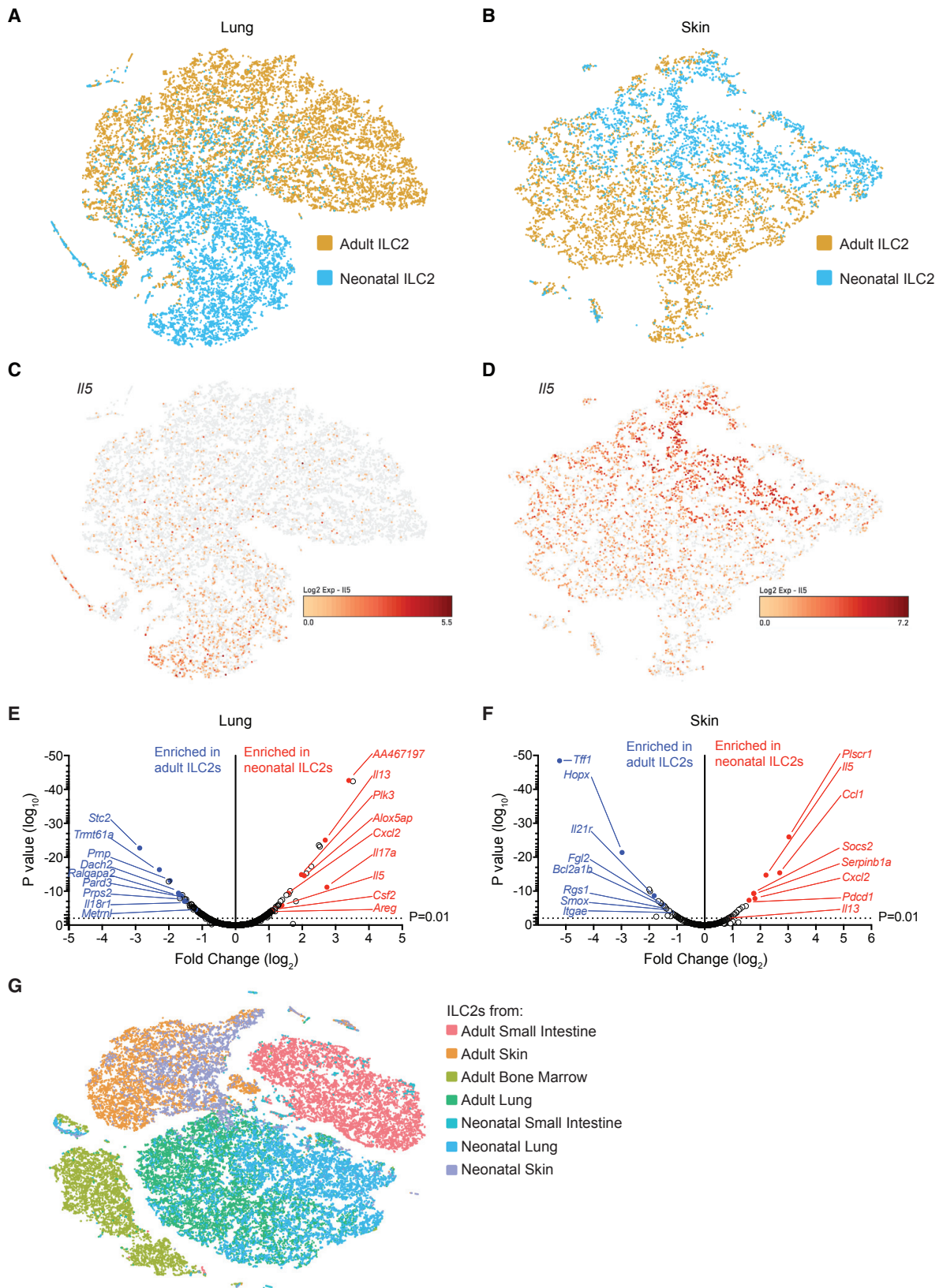
(G) RNA-sequencing (RNA-seq) analysis of Nur77 (*Nr4a1*) transcripts (fragments per kilobase of exon per million mapped reads [FPKM]; averaged) among sorted IL-5 reporter<sup>+</sup> ILC2s from indicated organs of adult mice (Ricardo-Gonzalez et al., 2018). BM, bone marrow.

(H and I) *Nr4a1* expression measured by flow cytometry in ILC2s of *Nr4a1*-GFP reporter mice on indicated time points.

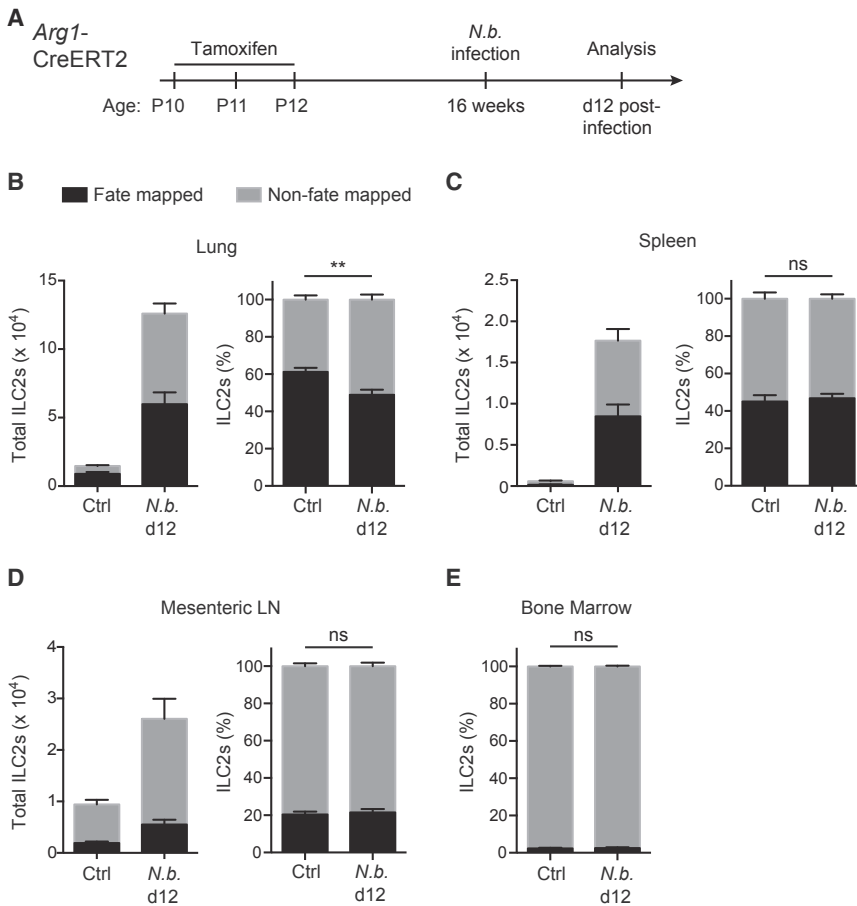
(H) Mean fluorescence intensity (MFI) of *Nr4a1*-GFP in ILC2s and ILC2Ps from lung, fetal liver and BM is displayed.

(I) Expression of *Nr4a1*-GFP and IL-5-reporter by ILC2s from lung, small intestine, and skin of *Il5<sup>Reds/+</sup>Nr4a1*-GFP mice on postnatal (P) days 1 and 8. ILC2s gated as Lin<sup>-</sup>Thy1<sup>+</sup>ST2<sup>+</sup> (lung and VAT), Lin<sup>-</sup>IL-17RB<sup>+</sup>KLRG1<sup>+</sup> (adult small intestine), Lin<sup>-</sup>IL-17RB<sup>+</sup> (neonatal small intestine), Lin<sup>-</sup>Thy1<sup>+</sup>CD25<sup>+</sup>ICOS<sup>+</sup> (skin), or Lin<sup>-</sup>IL-7R $\alpha$ <sup>+</sup>Thy1<sup>+</sup>CD25<sup>+</sup> (BM).

Data pooled from multiple independent experiments (A, C, D, G, and H) or from one experiment representative of at least two independent experiments (B and I) are shown (A and C; mean and SEM, n = 3–10). See also Figure S4.



(legend on next page)



### Figure 7. De Novo-Generated ILC2s Do Not Contribute to ILC2 Expansion Accompanying Migratory Helminth Infection

(A–E) *Arg1<sup>RFP-CreERT2</sup>R26<sup>YFP</sup>* mice tamoxifen-treated on P10–P12 and infected with *N. brasiliensis* (*N.b.*) 14 weeks later. Percentages of fate-mapped cells analyzed on day 12 post-infection and compared to uninfected littermates (A). (B–D) Numbers and percentages of fate-mapped and non-fate-mapped ILC2s in lung (B), spleen (C), and mesenteric LN (D). (E) Percentages of fate-mapped ILC2s in BM. ILC2s gated as Lin<sup>−</sup>IL-7R $\alpha$ <sup>+</sup>Thy1<sup>+</sup>CD25<sup>+</sup> (BM), Lin<sup>−</sup>Thy1<sup>+</sup>ST2<sup>+</sup> (lung and spleen), or Lin<sup>−</sup>IL-17RB<sup>+</sup>KLRG1<sup>+</sup> (mesenteric LN). Data are pooled from multiple independent experiments and displayed as mean  $\pm$  SEM of 8–9 mice.

with the period when ILC2s expand, begin to activate their cytokine repertoire, and establish a tissue-specific transcriptional signature. Although previously recognized as an important time window for pulmonary ILC2 activation associated with onset of breathing (Saluzzo et al., 2017), our studies demonstrated that ILC2 expansion and activation occurs systemically, suggesting that common niche factors might be involved in shaping a core effector repertoire, including IL-5 expression. While postnatal acquisition of this primed state appeared to be conserved across all tissues analyzed,

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 effects of increased migratory behavior, as recently described for ILC2s in the small intestine following helminth infection (Huang et al., 2018). In our studies, by comparing non-fate-mapped and fate-mapped ILC2s in tissues, we found that preexisting ILC2 pools expand in the lung after infection with *N. brasiliensis*, with minimal dilution of the preexisting fate-mapped population by cells generated *de novo* from unlabeled local or BM precursors. However, further studies are needed to assess if ILC2s generated during different developmental waves display functional heterogeneity as described for CD8<sup>+</sup> T cells (Smith et al., 2018).

Using markers for proliferation and activation, and corroborated by single-cell transcriptomics, we have shown that the perinatal establishment of peripheral ILC2 pools corresponded

the local environment also imprinted a tissue-defining transcriptional signature that resembled that of adult steady-state tissue ILC2s (Ricardo-Gonzalez et al., 2018) through mechanisms that remain to be identified. Therefore, ILC2s closely resemble fetal-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 in each tissue (Mass et al., 2016). Notably, despite clustering with their adult counterparts, our single cell RNA sequencing (scRNA-seq) analysis of postnatal ILC2s revealed differences in the expression of genes associated with activation, cell growth, chemokine production, and immune crosstalk.

Although the local signals underlying the developmental priming in the tissue remain to be determined, it is intriguing to speculate that the incipient crosstalk between ILC2s and other immune and non-immune cells during this period of organ

### Figure 6. Tissue-Specific Heterogeneity of ILC2s Is Established in the Early Postnatal Wave

(A–G) IL-5-reporter<sup>+</sup> ILC2s sorted from lung, skin, and small intestine of neonatal (P14) *Il5<sup>Red5/+</sup>* mice and analyzed by scRNA-seq together with ILC2s from adult tissues processed on the same day (Ricardo-Gonzalez et al., 2018).

(A and B) t-Distributed Stochastic Neighbor Embedding (t-SNE) visualization of adult and neonatal scRNA-seq datasets from lung (A) and skin (B).

(C and D) Relative single-cell *Il5* expression in lung (C) and skin (D) ILC2s.

(E and F) Volcano plots of differential gene expression between neonatal and adult ILC2s. Genes with an average expression of less than 1 transcript per 10 cells were excluded from analysis.

(G) t-SNE visualization of adult (BM, lung, adipose, skin, small intestine) and neonatal (lung, skin, small intestine) scRNA-seq datasets of sorted ILC2s.

See also Figures S5 and S6.

growth and remodeling might both guide and be shaped by the gene expression programs in 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 (Yang et al., 2015), 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 (Gollwitzer and Marsland, 2015; Kotas and Locksley, 2018).

In summary, we have demonstrated a previously unappreciated tripartite origin for tissue-resident ILC2s. Our studies reinforce the importance of the early postnatal window for the expansion and activation of resident immune cells and show that ILC2 replacement is dynamic and tissue dependent, revealing evidence for local regulation that remains unexplored. 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. Our findings bring ILC2s into the larger ongoing conversation regarding the influence of ontogeny and local signals on long-lived tissue-resident immune cells, which 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.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- CONTACT FOR REAGENT AND RESOURCE SHARING
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## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.immuni.2019.04.019>.

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## AUTHOR CONTRIBUTIONS

C.S. and J.L. conceived the study, designed and performed experiments, analyzed data, and wrote the manuscript with R.M.L. H.-E.L. designed the *Arg1<sup>RFP-CreERT2</sup>* reporter cassette and performed the embryonic stem cell work. H.-E.L., S.K., R.R.R.-G., and J.C.N. assisted with additional experiments. L.K.S. and S.A.V. assisted with parabiosis experiments. R.M.L. directed the study and wrote the paper with C.S. and J.L.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-mouse Siglec-F AF647 (clone E50-2440)	BD Biosciences	Cat# 562680
Anti-mouse CD45 BUV395 (clone 30-F11)	BD Biosciences	Cat# 565967
Anti-mouse CD3 AF488 (clone 17A2)	BioLegend	Cat# 100210
Anti-mouse CD3 PB (clone 17A2)	BioLegend	Cat# 100214
Anti-mouse CD3 PE/Cy7 (clone 17A2)	BioLegend	Cat# 100220
Anti-mouse CD4 BV711 (clone RM4-5)	BioLegend	Cat# 100550
Anti-mouse CD5 BV421 (clone 53-7.3)	BioLegend	Cat# 100629
Anti-mouse CD8 $\alpha$ PB (clone 53-6.7)	BioLegend	Cat# 100725
Anti-mouse/human CD11b PB (clone M1/70)	BioLegend	Cat# 101224
Anti-mouse CD25 BV605 (clone PC61)	BioLegend	Cat# 102036
Anti-mouse Thy1.2 BV785 (clone 30-H12)	BioLegend	Cat# 105331
Anti-mouse Gr-1 PB(clone RB6-8C5)	BioLegend	Cat# 108430
Anti-mouse NK-1.1 PB (clone PK136)	BioLegend	Cat# 108722
Anti-mouse CD49b PB (clone DX5)	BioLegend	Cat# 108918
Anti-mouse CD19 PB (clone 6D5)	BioLegend	Cat# 115523
Anti-mouse TER-119 PB (clone TER-119)	BioLegend	Cat# 116232
Anti-mouse CD11c PB (clone N418)	BioLegend	Cat# 117322
Anti-mouse TCR $\gamma/\delta$ BV421 (clone GL3)	BioLegend	Cat# 118120
Anti-mouse CD103 APC/Cy7 (clone 2E7)	BioLegend	Cat# 121431
Anti-mouse F4/80 PB (clone BM8)	BioLegend	Cat# 123124
Anti-mouse Fc $\epsilon$ RI $\alpha$ PB (clone MAR-1)	BioLegend	Cat# 134314
Anti-mouse NKp46 BV421 (clone 29A1.4)	BioLegend	Cat# 137612
Anti-mouse IL-17RB APC (clone 9B10)	BioLegend	Cat# 146308
Anti-mouse/rat/human ICOS APC (clone C398.4A)	BioLegend	Cat# 313510
Streptavidin APC	BioLegend	Cat# 405207
Anti-mouse EpCAM PerCP-Cy5.5	BioLegend	Cat# 118220
Anti-mouse PDGFR $\alpha$ APC	BioLegend	Cat# 135908
Anti-mouse Sca-1 BV711	BioLegend	Cat# 108131
Anti-mouse CD31 PE	BioLegend	Cat# 102407
Anti-mouse Podoplanin APC/Cy7	BioLegend	Cat# 127417
Anti-mouse Ki-67 FITC (clone SolA15)	eBioscience	Cat# 11-5698-82
Rat IgG2a kappa Isotype Control (eBR2a) FITC	eBioscience	Cat# 11-4321-80
Anti-human CD4 PE (clone RPA-T4)	eBioscience	Cat# 12-0049
Anti-mouse Gata-3 PE (clone TWAJ)	eBioscience	Cat# 12-9966-42
Anti-human CD4 APC (clone RPA-T4)	eBioscience	Cat# 17-0049
Anti-mouse KLRG1 PerCP-eF710 (clone 2F1)	eBioscience	Cat# 17-5893
Anti-mouse CD127 APC/eFluor780 (clone A7R34)	eBioscience	Cat# 47-1271-82
Anti-mouse ST2 Biotin (clone DJ8)	MD Biosciences	Cat# 101001B
Anti-mouse ST2 PE (clone DJ8)	MD Biosciences	Cat# 101001PE
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI)	Roche	Cat# 10236276001
Tamoxifen	Sigma	Cat# T5648
rIL-33	Biolegend	Cat# 580504

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Experimental Models: Organisms/Strains</b>		
Mouse: wild-type: C57BL/6	The Jackson Laboratory	Stock# 000664
Mouse: <i>Arg1</i> -RFP-CreER <sup>T2</sup>	Figure 1; this report	N/A
Mouse: R26R-EYFP: B6.129X1-Gt(ROSA)26Sor <sup>tm1EYFP</sup> Cos/J	The Jackson Laboratory	Stock# 006148
Mouse: Yarg: B6.129S4- <i>Arg1</i> <sup>tm1Lky</sup> /J	The Jackson Laboratory	Stock# 015857
Mouse: Id2-CreER <sup>T2</sup> : B6.129S(Cg)- <i>Id2</i> <sup>tm1.1(cre/ERT2)Blh</sup> /J	The Jackson Laboratory	Stock# 016222
Mouse: R26R-RFP	E. Robey, H. Luche, and H. Fehling (Luche et al., 2007)	N/A
Mouse: <i>Il7r</i> <sup>-/-</sup> : B6.129S7- <i>Il7r</i> <sup>tm1Imx</sup> /J	The Jackson Laboratory	Stock# 002295
Mouse: Red5: B6(C)- <i>Il5</i> <sup>tm1.1(cre)Lky</sup> /J	The Jackson Laboratory	Stock# 030926
Mouse: Smart13: B6.129S4(C)- <i>Il13</i> <sup>tm2.1Lky</sup> /J	The Jackson Laboratory	Stock# 031367
Mouse: Nur77-GFP	J. Zikherman (Zikherman et al., 2012)	N/A
Mouse: Id2-eGFP: B6.129S(Cg)- <i>Id2</i> <sup>tm2.1Blh</sup> /ZhuJ	The Jackson Laboratory	Stock# 016224
Mouse: B6 Cd45.1: B6.SJL- <i>Ptprc</i> <sup>a</sup> <i>Pepc</i> <sup>b</sup> /BoyJ	The Jackson Laboratory	Stock# 002014
<i>Nippostrongylus brasiliensis</i>	UCSF (R. Locksley)	N/A
<b>Oligonucleotides</b>		
<i>Rps17</i> (fwd) 5'-CGCCATTATCCCCAGCAAG-3'	Elim Biopharmaceuticals	N/A
<i>Rps17</i> (rev) 5'-TGTCGGGATCCACCTCAATG-3'	Elim Biopharmaceuticals	N/A
<i>Il33</i> (fwd) 5'-AAGACCAGGTGCTACTACGC-3'	Elim Biopharmaceuticals	N/A
<i>Il33</i> (rev) 5'-CTTCTCCCATCCACACCGT-3'	Elim Biopharmaceuticals	N/A
<i>Tsfp</i> (fwd) 5'-CCCTTCACTCCCCGACAAAA-3'	Elim Biopharmaceuticals	N/A
<i>Tsfp</i> (rev) 5'-CCTGAGTACCGTCATTTCTCTCA-3'	Elim Biopharmaceuticals	N/A
<i>Il5</i> (fwd) 5'-CTCTGTTGACAAGCAATGAGACG-3'	Elim Biopharmaceuticals	N/A
<i>Il5</i> (rev) 5'-TCTTCAGTATGTCTAGCCCCTG-3'	Elim Biopharmaceuticals	N/A
<i>Il13</i> (fwd) 5'-CCTGGCTCTTGCTTGCCCTT-3'	Elim Biopharmaceuticals	N/A
<i>Il13</i> (rev) 5'-GGTCTTGTGTGATGTTGCTCA-3'	Elim Biopharmaceuticals	N/A
<b>Software and Algorithms</b>		
FlowJo v.10.5	FlowJo	<a href="https://www.flowjo.com">https://www.flowjo.com</a>
Prism v.7.0	GraphPad	<a href="https://www.graphpad.com">https://www.graphpad.com</a>
Cell Ranger	10X Genomics	<a href="https://www.10xgenomics.com">https://www.10xgenomics.com</a>
Loupe Cell Browser	10X Genomics	<a href="https://www.10xgenomics.com">https://www.10xgenomics.com</a>

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Richard M. Locksley ([richard.locksley@ucsf.edu](mailto:richard.locksley@ucsf.edu)).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS****Mice**

*Arg1*<sup>Yarg</sup> (*Arg1*-YFP; B6.129S4-*Arg1*<sup>tm1Lky</sup>/J; 015857) (Reese et al., 2007), *Il5*<sup>Red5</sup> (Red5; B6(C)-*Il5*<sup>tm1.1(cre)Lky</sup>/J; 030926) (Nussbaum et al., 2013), and *Il13*<sup>Smart</sup> (Smart13; B6.129S4(C)-*Il13*<sup>tm2.1Lky</sup>/J; 031367) (Liang et al., 2011) mice have been previously described. Id2-GFP (B6.129S(Cg)-*Id2*<sup>tm2.1Blh</sup>/ZhuJ; 016224), *Id2*<sup>CreERT2</sup> (B6.129S(Cg)-*Id2*<sup>tm1.1(cre/ERT2)Blh</sup>/ZhuJ; 016222), *R26R*<sup>YFP</sup> (B6.129X1-Gt(ROSA)26Sor<sup>tm1(EYFP)Cos</sup>/J; 006148), *Rorc*( $\gamma$ )<sup>GFP</sup> (B6.129P2(Cg)-*Rorc*<sup>tm2Litt</sup>/J; 007572) and *Il7r*<sup>-/-</sup> (B6.129S7-*Il7r*<sup>tm1Imx</sup>/J; 002295) and wild-type (C57BL/6J; 000664) mice were purchased from the Jackson Laboratory. *R26R*<sup>RFP</sup> mice were provided by E. Robey (Berkeley). Nur77-GFP BAC transgene mice were provided by J. Zikherman (UCSF) (Zikherman et al., 2012).

*Arg1*<sup>RFP-CreERT2</sup> mice (*Arg1*-RFP) were generated by homologous gene targeting in C57BL/6 embryonic stem cells. In brief, the previously published plasmid pK0915-DT (Lexicon) containing the *Arg1*-YFP reporter (Reese et al., 2007), was modified to replace the eYFP and neo cassette with a tdTomato-T2A-CreERT2-frt-neo-frt sequence, such that the cassette now contained (in order from

5' to 3') encephalomyocarditis virus IRES, tandem dimer (td) Tomato, T2A self-cleaving peptide from *Thoesa asigna* virus, Cre recombinase fused to the estrogen receptor ligand binding domain (ER), bovine growth hormone poly(A), and a frt-flanked neomycin resistance cassette (suppl. Figure 1A). The final construct was linearized with NotI and transfected by electroporation into C57BL/6 embryonic stem cells. Cells were grown on irradiated feeders with the aminoglycoside G418 in the media, and neomycin-resistant clones were screened for 5' and 3' homologous recombination by PCR. Two clones were selected for injection into albino C57BL/6 blastocysts to generate chimeras, and the male pups with highest ratios of black-to-white coat color from a single clone were selected to breed with homozygous *Gt(ROSA26)<sup>FLP1/FLP1</sup>* females (Jackson Laboratories; 009086) to excise the neomycin resistance cassette. Deletion of neomycin was confirmed by PCR. Mice were then crossed to C57BL/6 mice to remove the FLP1 allele and subsequently crossed to B6.129X1-*Gt(ROSA)26Sor<sup>tm1(EYFP)Cos/J</sup>* (R26R-EYFP; Jackson Laboratories; 006148) for fate-mapping experiments.

All mice were generated on or backcrossed to the C57BL/6 background. Mice were maintained in the University of California San Francisco specific pathogen-free animal facility or UCSF Gnotobiotic Core Facility in accordance with the guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center. SPF animals were housed in individually ventilated cage units that are changed every three weeks for maintenance cages and every other week for breeding cages; cage bottoms are covered with autoclaved bedding and nesting material for enrichment. Mice were fed irradiated food (PicoLab Mouse Diet 20, 5058M) and drink from autoclaved bottles or automatic watering system. All animals were manipulated using standard procedures including filtered air exchange stations, chlorine-based disinfection of gloves and work surfaces within manipulations with animals; personnel protection equipment (PPE) (disposable gowns, gloves, head caps, and shoe covers) is required to enter the facility. Experiments were carried out using age and gender matched groups.

## METHOD DETAILS

### 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 50  $\mu$ g/ml Liberase TM (Roche) and 25  $\mu$ g/ml DNase I (Roche) in pre-warmed RPMI-1640 for 30 mins 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 70  $\mu$ m cell strainers.

Mouse intestines were flushed with PBS and Peyer patches were removed when visible in older mice. Intestines were opened, thoroughly cleaned with PBS, and incubated for 20 min in 20 mL HBSS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) supplemented with 2% fetal calf serum (FCS), 10 mM HEPES (UCSF Cell Culture Facility) and 5 mM DTT. Supernatants were discarded and intestines were incubated for 15 min in 10 mL HBSS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) supplemented with 2% fetal calf serum (FCS), 10 mM HEPES (UCSF Cell Culture Facility) and 5 mM EDTA solution. This step was repeated twice using fresh solution. Next, intestines were incubated for 10 min in 20 mL HBSS (with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) supplemented with 3% FCS and 10 mM HEPES. After incubation, intestines were gently vortexed, cut into small pieces and incubated for 30 min in 5 mL HBSS (with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) supplemented with 3% FCS, 10 mM HEPES, 100  $\mu$ g/ml Liberase TM (Roche) and 30  $\mu$ g/ml DNase I (Roche). All the incubations were performed with gentle rocking at 37°C. After digest, intestines were mechanically dissociated in GentleMACS C tubes (Miltenyi Biotec) using program m\_intestine\_01, passed through a 100  $\mu$ m filter, and washed. The resulting cell pellet was resuspended in 5 mL 40% Percoll (Sigma-Aldrich), underlaid with 5 mL 90% Percoll and centrifuged at 2000 rpm for 20 min at 20°C. The 40/90 interphase of the Percoll gradient was harvested, washed, and stained for flow cytometry.

Perigonadal visceral adipose tissue (VAT) was harvested and finely minced with scissors, followed by incubation for 40 min at 37°C in RPMI-1640 containing 100  $\mu$ g/ml Liberase TM (Roche) and 50  $\mu$ g/ml DNase I (Roche). The tissue was passed through 100  $\mu$ m nylon filters, washed, and subjected to red blood cell lysis (PharmLyse; BD) before final suspension in FACS buffer.

Back skin was cut into small pieces and digested with 2 mg/ml Collagenase XI (Sigma C9407), 0.5 mg/ml Hyaluronidase (Sigma H3506), and 100  $\mu$ g/ml DNase I (Roche) in pre-warmed RPMI-1640 for 90 min at 37°C with vigorous shaking. Afterward, the tissues were further dissociated using the C program on the gentleMACS dissociator. Single-cell suspensions were obtained after passing the homogenized samples through 70 $\mu$ m 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  $\alpha$ 4 $\beta$ 7 (DATK32), rat anti-human/mouse GATA-3 (TWAJ), rat anti-mouse MHC class II (M5/114.15.2), and mouse anti-mouse NK1.1 (PK136) antibodies were purchased from eBioscience; Armenian hamster anti-mouse CD11c (N418), Syrian hamster anti-mouse KLRG1 (2F1), 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), rat anti-mouse Flt3 (A2F10), rat anti-mouse IL-17RB (9B10), rat anti-mouse CD90.2 (30-H12), rat anti-mouse F4/80 (BM8), rat anti-mouse CD115 (AFS98), PDGFR $\alpha$  (APA5), Sca-1 (D7), CD31 (390), EpCAM (G8.8), and Podoplanin (8.1.1) 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, CD8 $\alpha$ , CD11b, CD11c, CD19, NK1.1, F4/80,

Gr-1, Fc $\epsilon$ R1 $\alpha$ , and Ter119. DAPI and Live/Dead (Invitrogen) was used for dead-cell exclusion for live and fixed samples, respectively. For intracellular staining of transcription factors, the FoxP3 Transcription Factor Staining Buffer Set (eBiosciences) was used according to manufacturer's instructions.

Samples were analyzed on a LSRFortessa X20 (BD Biosciences) with five lasers (355nm, 405nm, 488nm, 561nm, and 640nm). Samples were gated by FSC-A and SSC-A to exclude debris, FSC-H and 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 (Villeda et al., 2014). 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.

### Mouse treatments and infections

Mice were treated with tamoxifen dissolved at 20 mg/ml in corn oil (Sigma) for *Id2*<sup>CreERT2</sup>, or at 40 mg/ml in EtOH supplemented with 1 volume of Cremophor EL (Sigma C5135) and 2 volumes of PBS for a final concentration of 10 mg/ml for *Arg1*<sup>RFP-CreERT2</sup> mice. Pregnant females were treated by oral gavage with 1 mg tamoxifen and 0.5 mg progesterone (Sigma P0130) twice daily on E16, 17 and 18. Day of embryonic development was estimated by taking the day of vaginal plug observation as E0.5. Because tamoxifen treatment during pregnancy interferes with normal delivery, pups were delivered by caesarean sections on E19.5 and neonates were fostered by lactating females. Neonatal pups were injected i.p. with 1.5 mg on P10 for *Id2*<sup>CreERT2</sup>, or 400  $\mu$ g on P10,11 and 12 for *Arg1*<sup>RFP-CreERT2</sup> mice. Adult mice were fed a tamoxifen chow (Envigo TD.130858) for 4 weeks. IL-7R $\alpha$  blockade was performed by injecting 3 doses of 200  $\mu$ g InVivoMAb anti-mouse IL-7R $\alpha$  (A7R34, BioXCell) i.v. into timed pregnant females on E14.5, E16.5, and E18.5. For Nur77 stimulation, mice were injected i.p. with 1  $\mu$ g rIL-33 (Biolegend) 16h prior to analysis. For infections with *N. brasiliensis*, mice were injected subcutaneously with 500 L3-stage larvae and analyzed at the indicated time points post-infection.

### Cell sorting and scRNA-seq

ILC2s were sorted from homogenized mouse lung, skin, and small intestine as live (DAPI<sup>-</sup>) CD45<sup>+</sup>Lin<sup>-</sup>(CD3,CD4,CD5,CD8 $\alpha$ , CD11b,CD11c,CD19,NK1.1, NKp46,Gr-1,F4/80,Ter-119,DX5)I/I5<sup>RFP+</sup> cells. BM cells were sorted as live CD45<sup>+</sup>Lin<sup>-</sup>Thy1<sup>+</sup>Arg1<sup>YFP+</sup> 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 single cells (neonatal small intestine), 3030 single cells (neonatal skin), 6348 single cells (neonatal lung), 7547 single cells (adult small intestine), 6115 single cells (adult skin), 11615 single cells (adult lung), and 4990 single cells (adult BM) for analysis. 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, through 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) (Baglama and Reichel, 2005). 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 van der Maaten (2014).

### Accession Codes

scRNA-seq data generated in this study are deposited in Gene Expression Omnibus (GEO) under accession code GSE1126924 and GSE117568.

### Quantitative RT-PCR

Single-cell epithelial suspensions were isolated, stained as described earlier and sorted using a MoFlo XDP (Beckman Coulter). RNA was isolated using the Micro Plus RNeasy kit (QIAGEN) and reverse transcribed using the SuperScript Vilo Master Mix (Life Technologies). The resulting cDNA was used as template for quantitative PCR (qPCR) with the Power SYBR Green reagent on a StepOnePlus cycler (Applied Biosystems). Transcripts were normalized to *Rps17* (40S ribosomal protein S17) expression and relative expression shown as  $2^{-\Delta Ct}$ . Primer sequences (see also Key Resources Table): *Rps17*, 5'-CGCCATTATCCCCAGCAAG-3', 5'-TGTCGGGATC CACCTCAATG-3'; *I133*, 5'-AAGACCAGGTGCTACTACGC-3', 5'-CTTCTCCCATCCACACCGT-3'; *Tslp*, 5'-CCCTTCACTCCCCGA CAAAA-3', 5'-CCTGAGTACCGTCATTTCTCTCA-3'; *I15*, 5'-CTCTGTTGACAAGCAATGAGACG-3', 5'-TCTTCAGTATGTCTAGCCC CTG-3'; *I113*, 5'-CCTGGCTCTTGCTTGCCTT-3', 5'-GGTCTGTGTGATGTTGCTCA-3'.

### ILC2 culture and chemokine detection

$\text{Lin}^{-}\text{CD45}^{+}\text{Thy1}^{+}\text{Red5}^{+}$  ILC2s from lung and skin were FACS sorted and 2500 (lung) or 1000 (skin) cells per well were cultured in RPMI-1640 (Sigma) containing 10% FBS, 10 mM HEPES (Sigma), 100  $\mu\text{M}$  non-essential amino acids (Sigma), 1 mM sodium pyruvate (GIBCO), 100 U/mL penicillin (GIBCO), 100  $\mu\text{g}/\text{mL}$  streptomycin (GIBCO), 50  $\mu\text{M}$  2-mercaptoethanol (GIBCO), 10 ng/ml rIL-7 (R&D Systems), 10 ng/ml rIL-2 (R&D Systems), and 10 ng/ml rIL-33 (BioLegend) in 96-well round bottom plates at 37°C under 5%  $\text{CO}_2$ . After 3 days, supernatants were collected and the concentration of CXCL2 was measured by ELISA (ThermoFisher, EMCXCL2).

### QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were performed using randomly assigned mice without investigator blinding. All data points and n values reflect biological replicates. No data were excluded. Where noted in the figures, statistical significance was calculated using two-tailed non-parametric Mann-Whitney *U* test or unpaired, two-tailed *t* tests for the comparison between two experimental groups, or one-way ANOVA for multiple comparisons. Experimental groups included a minimum of three biological replicates. Intragroup variation was not assessed. All statistical analysis was performed using Prism 6 (GraphPad Software). Figures display means  $\pm$  s.e.m. as indicated. No statistical methods were used to predetermine sample size.