Published in final edited form as:

Science. 2018 January 05; 359(6371): 114–119. doi:10.1126/science.aam5809.

S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense

Yuefeng Huang^{1,*†}, Kairui Mao^{2,*}, Xi Chen¹, Ming-an Sun³, Takeshi Kawabe¹, Weizhe Li², Nicholas Usher^{1,4}, Jinfang Zhu¹, Joseph F. Urban Jr.⁵, William E. Paul^{1,‡}, Ronald N. Germain^{1,2,†}

¹Laboratory of Immunology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Bethesda, MD 20892, USA.

²Laboratory of Systems Biology, NIAID, National Institutes of Health, Bethesda, MD 20892, USA.

³Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA.

⁴Department of Undergraduate Biology, Cornell University, Ithaca, NY 14853, USA.

⁵Diet, Genomics, and Immunology Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture (USDA), Beltsville, MD 20705, USA.

Abstract

Innate lymphoid cells (ILCs) are innate counterparts of adaptive T lymphocytes, contributing to host defense, tissue repair, metabolic homeostasis, and inflammatory diseases. ILCs have been considered to be tissue-resident cells, but whether ILCs move between tissue sites during infection has been unclear. We show here that interleukin-25- or helminth-induced inflammatory ILC2s are circulating cells that arise from resting ILC2s residing in intestinal lamina propria. They migrate to diverse tissues based on sphingosine 1-phosphate (S1P)-mediated chemotaxis that promotes lymphatic entry, blood circulation, and accumulation in peripheral sites, including the lung, where they contribute to anti-helminth defense and tissue repair. This ILC2 expansion and migration is a behavioral parallel to the antigen-driven proliferation and migration of adaptive lymphocytes to effector sites and indicates that ILCs complement adaptive immunity by providing both local and distant tissue protection during infection.

Innate lymphoid cells (ILCs) (1, 2) lack antigen-specific receptors but, upon suitable stimulation, produce effector cytokines that parallel those made by antigen-induced T helper subsets (3). Various ILC progenitors have been identified in fetal liver and bone marrow (4), whereas mature ILCs are abundant in mucosal tissues and provide immune protection against pathogens early in infection (5–8). ILCs are also found in nonmucosal sites, such as

[†]Corresponding author. yuefeng.huang@nih.gov (Y.H.); rgermain@niaid.nih.gov (R.N.G.).

^{*}These authors contributed equally to this work.

[‡]Deceased.

secondary lymphoid tissues, and can mediate the transition from innate to adaptive immune responses (9, 10) while also playing important roles in epithelial tissue repair (11), fat metabolism (12, 13), and tumor immune surveillance (14). One issue that remains incompletely explored involves the specific origin(s) of ILCs present in diverse tissue sites and whether mature ILCs move between sites when infection demands. This is of special importance in the case of ILC2s and helminthic infections, because the latter typically involve different tissues such as the intestinal tract and lungs. Here we show that inflammatory ILC2s (iILC2s) (15) induced by helminths or the cyto-kine interleukin-25 (IL-25) migrate between tissues in response to activating signals. We also show how this migration is mediated and demonstrate the important role of such interorgan trafficking in host defense.

To probe the origin of tissue-resident ILCs, we monitored their numbers in the lung and small intestine of mice from postnatal day 1 through adulthood. On day 1, a few hundred GATA-3^{hi} ILC2s and ROR γ t⁺ ILC3s each were detected in the lungs (fig. S1A) and small intestine (fig. S1B). ILC2s, but not ILC3s, dramatically increased in number in the lung in the first week after birth and became the dominant ILC population, expanding further over the subsequent 3 weeks (fig. S1A). The greatest increase in ILCs in the small intestine occurred 2 to 4 weeks after birth (fig. S1B), the same time period that gut microbiota diversity increases. The percentage of Ki-67⁺ ILCs was ~80% on day 1 after birth, then decreased to a stable 5 to 10% in adults. This represents a lower proliferation rate relative to CD4 T cells, of which 20 to 30% were Ki-67⁺ in adults (fig. S1, C and D).

ILCs are generally considered to be tissue-resident cells (16, 17). To further study ILC localization in the steady state and during infection, parabiotic mice were used. Given the substantial effects of commensal bacteria on the host immune system, antibiotics were administered for only 2 weeks after surgery. A 50:50 exchange rate in blood leukocytes was observed 1 week after surgery (fig. S2A), and lung and intestinal CD4 T cells showed exchange rates of 50:50 and 30:70 to 40:60, respectively, 2 months after surgery (fig. S2B). In contrast, lung ILC2s and intestinal ILC3s did not appreciably exchange between the two partners even 6 to 8 months after surgery (Fig. 1, A and B), suggesting that these ILC subsets are largely self-maintained and that progenitors in bone marrow contribute little to their numbers in the steady state. Although intestinal ILC2 exchange was barely detected at 2 months, a modest but significant increase to a ~10% exchange rate was observed at 6 to 8 months after surgery (Fig. 1B). ILC2s in mesenteric lymph nodes (MLNs) showed a 20% exchange rate, whereas ILC3s in the MLNs did not exchange (Fig. 1C). Thus, although other ILC2 or ILC3 subsets appear to be self-maintained, intestinal ILC2s are refreshed at a low rate in the steady state.

We recently reported the existence of a lineage-negative (Lin⁻), KLRG1^{hi} ILC2 population that is induced in the lung, liver, MLNs, and spleen after treatment with IL-25 or inoculation with infective third-stage *Nippostrongylus brasiliensis* larvae (L3) (15). These iILC2s are distinct from the ILC2s that naturally reside in the lung (nILC2s) (18). However, the source of iILC2s in various organs remained undetermined. Thus, we investigated ILC2 activation and migration in response to IL-25 treatment. Intranasal administration of IL-25 did not elicit iILC2s in the lung, whereas intraperitoneal (i.p.) injection of IL-25 induced the

appearance of KLRG1^{hi}ST2⁻iILC2s (Fig. 1D), suggesting the absence of iILC2 precursors in the lungs. In vivo antibody labeling was performed in CD45.1⁺ CD45.2⁺ mice treated with IL-25. nILC2s were not labeled acutely by injected antibodies, indicating that they reside in parenchymal lung tissue. In contrast, like CD4 T cells, a large number of iILC2s were labeled, suggesting that they exist in the vascular space and circulate in the blood stream (Fig. 1E).

A parabiotic mouse model was used to further address the issue of iILC2 recirculation. After IL-25 i.p. injection into the CD45.1⁺ mouse of a parabiotic pair, KLRG1^{hi} iILC2s were found in the lungs of both the CD45.1⁺ mouse and its CD45.2⁺ partner (Fig. 1F). Notably, the majority of iILC2s in the lung and liver, 50% of iILC2s in the spleen, and 25% of iILC2s in MLNs in the CD45.2⁺ mouse were derived from its CD45.1⁺ partner (Fig. 1F and fig. S3, A to C), indicating that they are circulating cells. In contrast, Thy1^{hi} nILC2s in the lung were endogenously derived (Fig. 1F), suggesting that they did not circulate upon IL-25 treatment. Very few CD45.2⁺ iILC2s were found in the lung and liver (Fig. 1F and fig. S3A), possibly owing to the short half-life of exogenous IL-25 circulating via the blood to the partner mouse. Although IL-33 treatment induced lung nILC2 proliferation in both animals (Fig. 1G), based on increased Ki-67 expression (fig. S1C), nILC2 transfer between the parabiotic animals did not occur, indicating that these cells do not enter the circulation. Consistent with the IL-25 treatment data, 5 days after the inoculation of both parabiotic partners with *N. brasiliensis* L3, 35% of iILC2s in the lung were derived from the partner mouse, whereas the majority of nILC2s were endogenous (Fig. 1H). Thus, IL-25- or *N. brasiliensis*-induced iILC2s are circulating cells, distinct from tissue-resident ILC subsets.

To identify the source of circulating iILC2s, total leukocytes were isolated from different CD45.1⁺ *Rag1*^{-/-} mouse tissues and then transferred into CD45.2⁺ *Rag1*^{-/-} mice; this was followed by IL-25 treatment. Unexpectedly, cells from small intestine lamina propria (siLP) gave rise to high numbers of rapidly proliferating iILC2s in the lungs of the recipients, whereas transferred bone marrow cells gave rise to few iILC2s, and lung cells gave rise to none (Fig. 2A). This suggested that the intestine contains an enriched source of “pre-iILC2s.” siLP leukocytes were then divided into three groups, and similar cell transfer experiments were performed. Only KLRG1⁺ ILC2s from the siLP could give rise to iILC2s in the lung of recipients—neither Lin⁺ cells nor KLRG1⁻ ILCs could do so (Fig. 2B)—suggesting that IL-25-induced iILC2s in peripheral sites are derived from intestinal ILC2s. In addition, we purified lung nILC2s, bone marrow (BM) ILC2 progenitors, and siLP ILC2s and transferred equal numbers of these populations to recipients; we then administered IL-25 treatment. Intestinal ILC2s were much more efficient in giving rise to iILC2s than were BM ILC2 progenitors. Most iILC2s in the recipients were Ki-67⁺, indicating that they were proliferating rather than just phenotypically converted from donor cells (Fig. 2C). Although BM ILC2 progenitors may contribute to peripheral responses in chronic conditions, they are unlikely to be the major source of iILC2s during acute helminthic infections, owing to the lack of local IL-25 producers such as the tuft cells of the gastrointestinal tract (19–21).

Flow cytometry revealed that IL-25-induced iILC2s and siLP ILC2s possess similar surface-marker phenotypes (Fig. 2D). To further characterize ILC2 populations, we

examined the transcriptomes of BM ILC2 progenitors, lung nILC2s, IL-33-activated lung nILC2s, intestinal ILC2s, IL-25-induced lung iILC2s, and MLN iILC2s by RNA sequencing (RNA-seq). Despite the difference in location, lung and MLN iILC2s have very similar transcriptome profiles (Fig. 2E). Intestinal ILC2s showed the closest resemblance in gene expression pattern to iILC2s, providing additional evidence that the source of IL-25-induced iILC2s is intestinal ILC2s. Activated lung nILC2s and lung iILC2s showed highly distinct gene expression patterns, although they were in the same tissue (Fig. 2F). iILC2s produce more IL-13, whereas nILC2s produce more IL-9. We also found that iILC2s express high levels of CCR9 and some inhibitory receptors such as KLRG1 and TIGIT. In addition, we found that iILC2s produce IL-17A, which is consistent with our previous findings (15), whereas nILC2s express a higher level of the receptor (IL-18R1) and receptor-associated protein (IL-1RAcP) involved in responses to IL-18 and IL-1 (Fig. 2F), cytokines that are important for the conversion of lung ILC2s into ILC1s (22–24).

iILC2s appear in the lungs during the early stage of pulmonary *N. brasiliensis* larval migration and disappear after the expulsion of adult worms from the intestine (15). In parabiotic mouse experiments, KLRG1^{hi} iILC2s were no longer detected in the lungs 12 days after a 3-day IL-25 treatment. However, among lung nILC2s in the untreated CD45.2⁺ mouse partner, ~10% were CD45.1⁺ (fig. S4A). Among lung nILC2s, ~16% were derived from the parabiotic donor 20 days after inoculation of *N. brasiliensis* into both partners (fig. S4B), suggesting that iILC2s can contribute to the lung nILC2 pool late in infection. Notably, among siLP ILC2s of the CD45.2⁺ mouse, ~40% were CD45.1⁺ (fig. S4A). iILC2s were found to express high levels of integrin $\alpha 4\beta 7$ (25) and gut-homing receptor CCR9 (26) (fig. S4C), suggesting that iILC2s also have the potential to return to the gut after infection. CD62L expression was detected on iILC2s in the MLNs, but not in the lung. Thus, iILC2s present in peripheral sites are a transient cell population, converting into nILC2s in tissues such as the lung and/or migrating back to the gut.

To gain mechanistic insight into the effects of IL-25 on siLP pre-iILC2s and the control of their exit into the circulation, we used confocal imaging. The coexpression of GATA-3 and KLRG1 confirmed that KLRG1 is a reliable marker for intestinal ILC2s (fig. S5). In the steady state, CD3⁺ KLRG1⁺ ILC2s predominantly resided in the lamina propria, and only a small fraction were Ki-67⁺ (Fig. 3A, left). Thirty-six hours after IL-25 treatment, more than 50% of ILC2s were Ki-67⁺. Sixty hours after IL-25 treatment, ILC2 numbers dramatically increased, and the majority were Ki-67⁺ (Fig. 3, A and B). Intestinal ILC2s expressed substantial levels of cell-surface CD69, whereas levels in circulating iILC2s were lower (Fig. 3C). CD69 has an important role in the control of T cell migration between tissues. It is highly expressed on tissue-resident memory T cells, prolonging their residency, and on activated T cells in lymph nodes during the sequestration phase shortly after the initiation of an immune response. In contrast, its expression on trafficking T cells is low (27). Given that KLRG1^{hi} ILC2s were present in many peripheral sites 60 hours after IL-25 administration (fig. S3C), we hypothesized that activated intestinal ILC2s behave like activated T cells in lymph nodes, crossing the lymphatic endothelium, entering lymphatics, and entering the blood circulation. When Lyve-1 staining was used to delineate lymphatic vessels (28), KLRG1⁺ ILC2s were observed within these vessels in the villi of IL-25-treated mice but not of naïve mice (Fig. 3D). Three-dimensional image reconstruction confirmed the localization

of ILC2s with-in, rather than adjacent to, the lymphatic vessels (movie S1). iILC2s were also detected in peripheral blood 60 hours after IL-25 treatment (Fig. 3E). Thus, intestinal ILC2s rapidly proliferate after IL-25 stimulation and enter lymphatic vessels and then the blood, accumulating as iILC2s in many peripheral sites.

G protein-coupled sphingosine 1-phosphate (S1P) receptors are required for T lymphocyte egress from lymphoid organs across lymphatic endothelial barriers (29, 30). Thus, we examined the possible role of this chemotactic pathway in ILC migration. ILC2s or ILC3s in naïve mice did not express S1P receptors, but IL-25-induced iILC2s in the lung and MLNs, like CD4 T cells, expressed S1PR1 and S1PR4 (Fig. 4A). iILC2s in MLNs also expressed S1PR5 (Fig. 4A), which has been reported to be expressed on natural killer cells (31). FTY720, which antagonizes the S1P-signaling pathway, did not affect IL-25-induced intestinal ILC2 proliferation (Fig. 4, B and C) but blocked iILC2 accumulation in the lung, liver, and spleen and partially blocked iILC2 accumulation in MLNs (Fig. 4D). This is consistent with the tissue-specific differences in CD69 expression (Fig. 3C) (27, 32). Together, these results indicate that iILC2 cells use a similar molecular mechanism to that of conventional CD4⁺ and CD8⁺ T cells, which regulates their exit from tissues into the lymph as they move to a distant site of effector activity.

IL-25R⁺ ILC2 progenitors were recently identified in bone marrow (33). Thus, we addressed the relationship between BM ILC2 progenitors and iILC2s. IL-25R⁺ progenitors expressed ST2 but not KLRG1 in naïve mice (fig. S6, A and B). IL-25 treatment not only expanded the progenitor population, but also induced ST2⁺ KLRG1^{hi} iILC2s in the bone marrow (fig. S6B). FTY720 did not affect the expansion of ILC2 progenitors but abolished the presence of iILC2s in bone marrow (fig. S6, B and C). These results indicate that intestinally derived iILC2s can infiltrate the bone marrow and highlight the distinction between iILC2 and BM ILC2 progenitors.

Last, we investigated the physiological implications of ILC2 migratory responses during anti-helminth immunity. Inoculation of conventional *Rag1*^{-/-} mice with *N. brasiliensis* established a chronic adult worm infection in the intestine, which was cleared by the administration of IL-25 (Fig. 4E), suggesting that an increase in iILC2 numbers is sufficient to expel the worms even in the absence of adaptive lymphocytes. Parasitic *N. brasiliensis* L3 pass through the lungs early in the infection and cause inflammation and tissue damage. FTY720 treatment of *Rag1*^{-/-} mice inoculated with a dose of 500 L3 resulted in the death of 80% of the mice at early stages of infection (Fig. 4F). This mortality was largely prevented by the transfer of iILC2s into the circulating pool before FTY720 treatment, which compensated for the drug-induced blockade of endogenous iILC2s in the lung. Larvae were observed in the lungs (arrows) on day 5, and severe epithelial destruction was noted (asterisks) on day 8 postinoculation in FTY720-treated mice not given adoptive cell therapy (Fig. 4G). Prolonged worm residency and tissue damage could be prevented by iILC2 transfer. Furthermore, iILC2s were found to express higher levels of amphiregulin—a key contributor to epithelial tissue repair during infection—compared with nILC2s (Fig. 4H). Thus, intestinally derived iILC2s accumulate in the lung in a S1P-dependent manner and provide crucial protection at the early stage of infection, contributing to worm clearance, tissue repair, and host survival.

Here we have characterized in detail a distinct cell population that resides in the gut but can migrate to the lung and other distal sites and make substantial contributions to host defense. Tissue-resident ILC2s undergo interorgan migration, a property essential to their protective role in infection. This study also shows that epithelium-resident ILC2s move into lymphatics in a S1P-dependent manner by the same mechanism previously described for adaptive lymphocytes egressing from secondary lymphoid tissues. We hypothesize that S1P-dependent dissemination of activated effectors from one tissue through the lymphatics and blood to a distant site of infection evolved as a mechanism within the innate lymphoid system, which was later grafted onto the emerging adaptive system, rather than being a late development of the T cell adaptive immune system. Last, the ability of FTY720 to block ILC2 dissemination suggests that the immunosuppressive effects of this drug that have been attributed solely to the blockade of adaptive T cell migration from lymph nodes and the spleen need to be reassessed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank K. Weng, T. Moyer, and C. Henry for cell sorting; A. Moseman for technical assistance with parabiosis surgery; M. Wong for assistance with library preparation; J. M. Ward for analyzing histological sections; and the staff of the NIAID animal facility for the postoperative care of parabiotic mice. We thank E. Shevach and members of the Laboratories of Immunology and Systems Biology, NIAID, for discussions. This work was supported by the Intramural Research Program of NIAID, NIH, and by the USDA (8040-51000-058-00D). Y. Huang was also supported by a NIAID K99 award (1K99AI123350-01A1). RNA-seq data are available in the Gene Expression Omnibus database (accession number GSE104708). Y.F. designed, performed, and interpreted the majority of the experiments and drafted the manuscript. K.M. designed, performed, and interpreted confocal imaging experiments. X.C., T.K., and N.U. assisted with experiments. M.S. performed RNA-seq data analysis. W.L. assisted with image data analysis. J.Z. helped to design and interpret the experiments. J.F.U. provided *N. brasiliensis* and helped to design and interpret worm experiments. W.E.P. designed the experiments. R.N.G. designed the experiments, interpreted the data, and finalized the manuscript. All authors (with exception of W.E.P.) contributed to the discussion of experimental findings and preparation of the manuscript.

REFERENCES AND NOTES

1. Artis D, Spits H, Nature 517, 293–301 (2015). [PubMed: 25592534]
2. Klose CS, Artis D, Nat. Immunol. 17, 765–774 (2016). [PubMed: 27328006]
3. Eberl G, Colonna M, Di Santo JP, McKenzie AN, Science 348, aaa6566 (2015).
4. Zook EC, Kee BL, Nat. Immunol. 17, 775–782 (2016). [PubMed: 27328007]
5. Moro K et al., Nature 463, 540–544 (2010). [PubMed: 20023630]
6. Neill DR et al., Nature 464, 1367–1370 (2010). [PubMed: 20200518]
7. Price AE et al., Proc. Natl. Acad. Sci. U.S.A. 107, 11489–11494 (2010). [PubMed: 20534524]
8. Sonnenberg GF, Artis D, Nat. Med. 21, 698–708 (2015). [PubMed: 26121198]
9. Oliphant CJ et al., Immunity 41, 283–295 (2014). [PubMed: 25088770]
10. Mirchandani AS et al., J. Immunol. 192, 2442–2448 (2014). [PubMed: 24470502]
11. Monticelli LA et al., Nat. Immunol. 12, 1045–1054 (2011). [PubMed: 21946417]
12. Lee MW et al., Cell 160, 74–87 (2015). [PubMed: 25543153]
13. Brestoff JR et al., Nature 519, 242–246 (2015). [PubMed: 25533952]
14. Dadi S et al., Cell 164, 365–377 (2016). [PubMed: 26806130]
15. Huang Y et al., Nat. Immunol. 16, 161–169 (2015). [PubMed: 25531830]

16. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY, Science 350, 981–985 (2015). [PubMed: 26472762]
17. Moro K et al., Nat. Immunol. 17, 76–86 (2016). [PubMed: 26595888]
18. Huang Y, Paul WE, Int. Immunol. 28, 23–28 (2016). [PubMed: 26232596]
19. von Moltke J, Ji M, Liang HE, Locksley RM, Nature 529, 221–225 (2016). [PubMed: 26675736]
20. Howitt MR et al., Science 351, 1329–1333 (2016). [PubMed: 26847546]
21. Gerbe F et al., Nature 529, 226–230 (2016). [PubMed: 26762460]
22. Silver JS et al., Nat. Immunol. 17, 626–635 (2016). [PubMed: 27111143]
23. Ohne Y et al., Nat. Immunol. 17, 646–655 (2016). [PubMed: 27111142]
24. Bal SM et al., Nat. Immunol. 17, 636–645 (2016). [PubMed: 27111145]
25. Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC, J. Immunol. 152, 3282–3293 (1994). [PubMed: 7511642]
26. Svensson M et al., J. Clin. Invest. 110, 1113–1121 (2002). [PubMed: 12393847]
27. Park CO, Kupper TS, Nat. Med. 21, 688–697 (2015). [PubMed: 26121195]
28. Banerji S et al., J. Cell Biol. 144, 789–801 (1999). [PubMed: 10037799]
29. Allende ML, Dreier JL, Mandala S, Proia RL, J. Biol. Chem. 279, 15396–15401 (2004). [PubMed: 14732704]
30. Matloubian M et al., Nature 427, 355–360 (2004). [PubMed: 14737169]
31. Walzer T et al., Nat. Immunol. 8, 1337–1344 (2007). [PubMed: 17965716]
32. Shiow LR et al., Nature 440, 540–544 (2006). [PubMed: 16525420]
33. Yu Y et al., Nature 539, 102–106 (2016). [PubMed: 27749818]

Fig. 1. IL-25- or helminthic infection-induced inflammatory ILC2s are circulating cells that differ from tissue-resident ILCs.

(A to C) CD45.1⁺ and CD45.2⁺ mice were surgically connected to generate parabiotic partners. Percentages of host-derived cells were analyzed among ILC2s and ILC3s 2 to 3 weeks, 2 months, and 6 to 8 months after surgery. ILC2s were gated as lineage-negative (Lin⁻) CD127⁺ GATA-3^{hi}, and ILC3s were gated as Lin⁻ CD127⁺ RORγt⁺. MLNs, mesenteric lymph nodes. (D) B6 mice were treated with IL-25 intranasally or intraperitoneally (i.p.) daily for 3 days. Lung leukocytes were then analyzed by flow cytometry. Cells were gated on CD45.2⁺ Lin⁻ CD127⁺. (E) CD45.1⁺ CD45.2⁺ mice were treated with IL-25 i.p. daily for 3 days. Mice were intravenously injected with phycoerythrin-labeled antibodies against CD45.1 and euthanized 5 minutes later for analysis of CD45.2 on Lin⁻ CD127⁺ ST2⁺ KLRG1^{int} naturally lung-residing ILC2s (nILC2s), Lin⁻ CD127⁺ ST2⁺ KLRG1^{hi} inflammatory ILC2s (iILC2s), and CD4⁺ T cells in the lungs without perfusion. (F and G) Two months after surgery, the CD45.1⁺ partner of each parabiotic pair was treated i.p. with IL-25 (F) or IL-33 (G) daily for 3 days. Cells in the lungs were then analyzed. nILC2s were gated as Lin⁻ CD127⁺ Thy1^{hi} KLRG1^{int}, and iILC2s were gated as Lin⁻ CD127⁺ Thy1^{lo} KLRG1^{hi}. (H) Two months after surgery, both mice in each parabiotic pair were inoculated with ~200 infective *N. brasiliensis* larvae (L3). Cells in the lungs were analyzed on day 5 postinoculation. Means ± SEM from six to eight mice at each time point in (A) to (C) and from three mice in (D). ****P 0.0001; NS, not significant; unpaired two-tailed *t* test. Results are representative of at least two independent experiments.

Fig. 2. Intestinal ILC2s are the immediate source of iILC2s.

(A) Total leukocytes from lung, bone marrow, or siLP of CD45.1⁺ *Rag1*^{-/-} mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into CD45.2⁺ *Rag1*^{-/-} mice; this was followed by a 3-day treatment with IL-25 injected i.p. Cells in the lungs were analyzed for CD45.1⁺ donor cells. Blue line, Lin⁻ KLRG1^{hi} iILC2s; gray shaded area, CD45.1⁺ cells labeled with CFSE before transfer. (B) siLP leukocytes were divided into the three indicated groups by cell sorting and subjected to a similar transfer experiment to that described in (A). Left, cells were gated on CD45.1⁺ Lin⁻; right, CD45.1⁺ iILC2 numbers in the lungs. (C) Equal numbers of lung nILC2, siLP ILC2, and bone marrow (BM) ILC2 progenitors were sorted and subjected to a similar transfer experiment to that described in (A). (D) Comparison of surface markers on lung iILC2s in IL-25-treated mice and lung nILC2s and intestinal ILC2s in naïve mice by flow cytometry. (E) Principal component analysis of the transcriptomes of different ILC2 populations identified by RNA-seq. The analysis is based on log₁₀[transcripts per million (TPM) + 1] of 53 immune-related genes. (F) Scatter plot comparing the gene expression patterns from RNA-seq between activated lung nILC2s and lung iILC2s. The x and y axes show the log₁₀(TPM + 1) values for each sample. Only genes with at least a twofold difference were plotted. Means ± SEM from three mice in (A) to (C). Data are representative of two independent experiments in (A) to (D).

Fig. 3. Intestinal ILC2s proliferate and enter lymphatics in response to IL-25.

(A) Immunofluorescence staining of ilea from B6 mice 0, 36, and 60 hours (h) after treatment with IL-25. The lower panels are magnified views of the boxed areas in the upper panels. (B) Quantification of Ki-67⁺ ILC2s in the small intestine. (C) Comparison of CD69 expression on siLP ILC2s, lung iILC2s, and MLN iILC2s by flow cytometry. (D) Immunofluorescence staining of ilea from mice 0 and 60 hours after treatment with IL-25. Arrows, KLRG1⁺ ILC2s within a lymphatic vessel. (E) iILC2s in peripheral blood or lungs were analyzed from B6 mice 0, 36, and 60 hours after treatment with IL-25. Images are representative of three gut sections from two mice in each group. Data are representative of two independent experiments in (C) and (E). Means $\pm$ SEM; $n = 6$ to 9 and 3 for each group in (B) and (E), respectively. *** $P < 0.001$; unpaired two-tailed t test.

Fig. 4. Intestinally derived iILC2s migrate and provide protection to the lung in a S1P-dependent manner.

(A) S1P receptor expression by the indicated cell populations was assessed by real-time polymerase chain reaction. Intestinal ILC2s and ILC3s and MLN CD4 T cells were obtained from naïve mice, whereas iILC2s in the lung or MLNs were from IL-25-treated mice. (B) Immunofluorescence staining of ilea from mice that were untreated, treated with IL-25, or treated with IL-25 plus FTY720 for 3 days. (C) Quantification of Ki-67⁺ iILC2s in (B). (D) B6 mice were treated with IL-25 or with IL-25 plus FTY720 i.p. for 3 days, and the iILC2s in indicated tissues were analyzed by flow cytometry. (E) Worm burden on day 14 in the small intestine of the mice infected with ~300 *N. brasiliensis* L3. IL-25 treatment was given every other day in the third group. UD, undetected. (F) *Rag1*^{-/-} mice were inoculated with ~500 *N. brasiliensis* L3 (Nb), and FTY720 treatment started on day 3 and continued daily

until day 10, in two groups. In the transfer group, $\sim 3 \times 10^6$ iILC2s sorted from IL-25-treated mice were transferred intravenously into each animal on the same day as worm inoculation. Death and survival were monitored until day 13, and the difference between two groups was determined by the log-rank (Mantel-Cox) test. (G) Histology of lung sections from *Rag1*^{-/-} mice inoculated with ~ 300 *N. brasiliensis* L3. Arrows, worm larvae; asterisk, tissue destruction. (H) Amphiregulin mRNA levels in nILC2s and iILC2s, which were sorted from lungs on day 5 postinoculation with *N. brasiliensis*. Means $\pm$ SEM; $n=3$ in (A), (D), (E), and (H) and 4 to 6 in (C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; unpaired two-tailed t test. Data in (A), (D), and (E) are representative of three experiments; data in (F) are the summary of two experiments ($n = 10$). Images are representative of three sections from two mice of each group in (B) and (G).