IL-25-responsive, lineage-negative KLRG1\textsuperscript{hi} cells are multipotential ‘inflammatory’ type 2 innate lymphoid cells

Yuefeng Huang\textsuperscript{1}, Liying Guo\textsuperscript{1}, Jin Qiu\textsuperscript{2}, Xi Chen\textsuperscript{1}, Jane Hu-Li\textsuperscript{1}, Ulrich Siebenlist\textsuperscript{3}, Peter R Williamson\textsuperscript{2}, Joseph F Urban Jr\textsuperscript{4} & William E Paul\textsuperscript{1}

Innate lymphoid cells (ILCs) are lymphocyte-like cells that lack T cell or B cell antigen receptors and mediate protective and repair functions through cytokine secretion. Among these, type 2 ILCs (ILC2 cells) are able to produce type 2 cytokines. We report the existence of an inflammatory ILC2 (iILC2) population responsive to interleukin 25 (IL-25) that complemented IL-33-responsive natural ILC2 (nILC2) cells. iILC2 cells developed into nILC2-like cells \textit{in vitro} and \textit{in vivo} and contributed to the expulsion of \textit{Nippostrongylus brasiliensis}. They also acquired IL-17-producing ability and provided partial protection against \textit{Candida albicans}. We propose that iILC2 cells are transient progenitors of ILCs mobilized by inflammation and infection that develop into nILC2-like cells or ILC3-like cells and contribute to immunity to both helminths and fungi.

Innate lymphoid cells (ILCs) are lymphocyte-like cells that lack specific antigen receptors yet produce effector cytokines that match those of CD4\textsuperscript{+} helper T cell subsets. ILCs are classified into three major groups on the basis of their cytokine-producing potential: interferon-\(\gamma\) (IFN-\(\gamma\))-producing type 1 ILCs (ILC1 cells); interleukin 13 (IL-13)- and IL-5-producing type 2 ILCs (ILC2 cells); and IL-17- or IL-22-producing type 3 ILCs (ILC3 cells)\textsuperscript{1–3}. ILCs provide immunity against infectious agents, participate in inflammatory responses and mediate lymphoid organogenesis and tissue repair, particularly at mucosal barriers. They provide early control of pathogen invasion in a non–antigen-specific manner and are often a critical first line of immune defense.

ILC2 cells have an important role in type 2 immunity. They were independently described by several groups and were designated ‘natural helper cells,’ ‘nuocytes’ or ‘innate helper 2 cells’\textsuperscript{4–6}. Although there are some differences between these cells in their tissue localization and surface marker patterns, they are considered by many to represent a single cell type, now designated ‘ILC2’ on the basis of their production of type 2 cytokines and associated functions. A separate IL-25-responsive innate cell population has been described, called ‘multipotent progenitors’ (MPP\textsuperscript{type2} cells)\textsuperscript{7}. MPP\textsuperscript{type2} cells contribute to helminth immunity but, in contrast to ILC2 cells, can develop into monocytoids and macrophages or mast cells and are of the myeloid lineage rather than the lymphoid lineage\textsuperscript{8}.

ILC2 cells have a critical role in immune responses to helminths, such as \textit{Nippostrongylus brasiliensis}. Dysfunction of ILC2 cells causes a substantial delay in worm expulsion\textsuperscript{9}. ILC2 cells also participate in allergic inflammation. They mediate influenza virus–induced airway hyper-reactivity and protease allergen–induced eosinophil lung inflammation\textsuperscript{10,11} and have been reported to promote allergic atop dermatitis in humans\textsuperscript{12}. ILC2 cells also function in tissue remodeling. They contribute to lung tissue repair during viral infection and mediate hepatic fibrosis\textsuperscript{13,14}.

ILC2 cells act through diverse effector pathways. Through the production of IL-13, they induce epithelial goblet cells to secrete mucus and contribute to tissue repair by producing amphiregulin\textsuperscript{10,13}. By secreting IL-5 and IL-9, ILC2 cells control eosinophil homeostasis and mast cell activation\textsuperscript{15,16}. It has been reported that ILC2 cells promote the B cell production of antibody and activation of CD4\textsuperscript{+} T cells\textsuperscript{17–19}.

ILC2 development depends on the action of the transcription factor Id2 and expression of the IL-2 receptor \(\gamma\)-chain (the common \(\gamma\)-chain (\(\gamma_c\)), but not on the RAG recombinase\textsuperscript{1,18}. The transcription factor GATA-3 is indispensable for the development and survival of ILC2 cells\textsuperscript{19,20}. The transcription factors ROR\(\gamma\)t and TCF-1 are also required for the generation of ILC2 cells\textsuperscript{21,22}. Additionally, the transcriptional determinant Gfi1 has been reported to promote the development of ILC2 cells and to regulate their cytokine-producing phenotype\textsuperscript{23}.

Two epithelial cell–derived cytokines, IL-25 and IL-33, have been reported to be crucial for the induction and activation of ILC2 cells both in humans and in mice\textsuperscript{24,25}. IL-25 is a member of the IL-17 family that is associated with type 2 helper T cell (T\(H2\) cell)-like

\textsuperscript{1}Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. \textsuperscript{2}Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. \textsuperscript{3}Laboratory of Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. \textsuperscript{4}Diet, Genomics, and Immunology Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, USDA, Beltsville, Maryland, USA. Correspondence should be addressed to W.E.P. (wpaul@niaid.nih.gov).

Received 21 July; accepted 2 December; published online 22 December 2014; doi:10.1038/ni.3078
immune responses. IL-33 is a member of the IL-1 family, and its receptor, ST2, is expressed on both T112 cells and ILC2 cells, as well as on mast cells, basophils and eosinophils. IL-25 and IL-33 are important in anti-helmintic immunity and allergic inflammation. Infection with *N. brasiliensis* triggers epithelial cells to release IL-25 and IL-33, which in turn activate ILC2 cells, causing their population expansion and cytokine production. ILC2 populations with different tissue distribution and cytokine receptor-expression patterns have been reported to have different responsiveness to IL-25 or IL-33. Some ILC2 populations are thought to respond to either IL-25 or IL-33; and some, to both. However, the relationship between IL-25-responsive ILC2 cells and IL-33-responsive ILC2 cells is still unclear.

Here we report an IL-25-responsive ILC2 cell population that expressed large amounts of the activation marker KLRG1 and the IL-25 receptor (IL-7Rβ) but did not express ST2. These cells had a phenotype distinct from those of both MPP<sup>type2</sup> cells and conventional ILC2 cells in the lungs. They proliferated in response to IL-25 but not in response to IL-33. They developed into ST2<sup>+</sup> ILC2 cells both *in vitro* and *in vivo*. These KLRG1<sup>hi</sup> cells were elicited early in the course of infection with *N. brasiliensis*, before the proliferation of lung-resident ILC2 cells, and became ILC2-like cells during such infection. KLRG1<sup>hi</sup> cells also expressed intermediate amounts of RORγ<sup>+</sup>, whereas IL-33-responsive ILC2 cells did not. KLRG1<sup>hi</sup> cells had the potential to produce IL-17 and were able to develop into ILC3-like cells either under culture conditions like those that promote polarization into the TH17 subset of helper T cells or in response to IL-25 or IL-33. They proliferated in response to IL-25 in untreated mice, were present at an abundance of more than 4 × 10<sup>4</sup> cells per mouse in the lungs of IL-25-treated mice (*Fig. 1a*). In untreated mice, we observed only nILC2 (Lin<sup>−</sup>IL-7R<sup>α</sup>) cells, which were ST2<sup>+</sup> and responsive mainly to IL-33. Treatment with IL-25 did not elicit nILC2 cells in IL-25-deficient (Il17rb<sup>−/−</sup>) mice but did so in ST2-deficient (Il11r<sup>−/−</sup>) mice (*Fig. 1f*).

Previous studies have shown that 7 d of treatment with IL-25 elicits the myeloid population of MPP<sup>type2</sup> cells in MLNs. MPP<sup>type2</sup> cells are characterized as Lin<sup>−</sup>IL-7Rα<sup>−</sup>Thy-1<sup>−</sup>ST2<sup>−</sup> c-Kit<sup>+</sup>. To address whether MPP<sup>type2</sup> cells also appear in the lungs and to clarify the relationship among iILC2 cells, nILC2 cells and MPP<sup>type2</sup> cells, we assessed the presence of these three populations among lung leukocytes in untreated mice and mice treated with IL-25 daily for 3 d or 7 d (*Fig. 1g*).

In untreated mice, we observed only nILC2 (Lin<sup>−</sup>IL-7Rα<sup>+</sup> Thy-1<sup>−</sup>ST2<sup>−</sup> KLRG1<sup>hi</sup>) cells. 3 d of administration of IL-25 induced large numbers of iILC2 (Lin<sup>−</sup>IL-7Rα<sup>−</sup>Thy-1<sup>−</sup>ST2<sup>−</sup> KLRG1<sup>hi</sup>) cells but still no MPP<sup>type2</sup> cells. In mice treated for 7 d with IL-25, while iILC2 cells were still present, we observed the induction of MPP<sup>type2</sup> (Lin<sup>−</sup>IL-7Rα<sup>−</sup>Thy-1<sup>−</sup>ST2<sup>−</sup> c-Kit<sup>+</sup>) cells. Thus, IL-25 induced at least two cell populations in the lungs, lymphoid iILC2 cells and myeloid MPP<sup>type2</sup> cells, depending on the duration of treatment.

### RESULTS

#### Induction of a lineage-negative KLRG1<sup>hi</sup> cell population by IL-25

We treated wild-type mice intraperitoneally for 3 d with recombinant IL-33 or IL-25 and analyzed lung leukocytes for ILC surface markers (*Fig. 1a*). In naive mice, lung ILC2 cells, characterized as lacking lineage markers (Lin<sup>−</sup>) and ST2<sup>+</sup>, increased two- to threefold in number in response to IL-33 (*Fig. 1a–c*). A Lin<sup>−</sup>ST2<sup>−</sup> cell population, barely detectable in the lungs of untreated or IL-33-treated mice, appeared after treatment with IL-25 (*Fig. 1a*). This IL-25-induced cell population had abundant expression of KLRG1 (*Fig. 1a,b,*). Although KLRG1 was expressed on resident ILC2 cells, its intensity was substantially lower on those cells than on the IL-25-responsive population (*Fig. 1a,b,*).

We designated the Lin<sup>−</sup>ST2<sup>−</sup>KLRG1<sup>hi</sup> cells ‘iILC2 cells’ and the Lin<sup>−</sup>ST2<sup>−</sup>KLRG1<sup>hi</sup> cells ‘nILC2 cells’.

The lungs of naive mice contain 4 × 10<sup>3</sup> to 5 × 10<sup>3</sup> nILC2 cells (*Fig. 1c*). Treatment with IL-33 increased that to ~1 × 10<sup>4</sup>, while IL-25 caused a statistically insignificant increase in lung nILC2 cells (*Fig. 1c*). In contrast, iILC2 cells, undetectable in the lungs of untreated or IL-33-treated mice, were present at an abundance of more than 4 × 10<sup>4</sup> cells per mouse in the lungs of IL-25-treated mice (*Fig. 1c*). ILC2 cells were all positive for the proliferation marker Ki67 (*Fig. 1d*), which indicated that they had proliferated very rapidly in the IL-25-treated mice. We also detected iILC2 cells in spleen, mesenteric lymph nodes (MLNs) and liver, but detected few in the bone marrow (*Supplementary Fig. 1*).

Phenotypically, iILC2 cells were c-Kit<sup>−</sup>CD44<sup>+</sup> and had lower expression of the receptor IL-7Rα and the alloantigen Thy-1 than did nILC2 cells (*Fig. 1e*). Most iILC2 cells lacked the lineage marker Sca-1, which was uniformly expressed on nILC2 cells (*Fig. 1e*).

Notably, iILC2 cells were IL-17RB<sup>+</sup>, whereas nILC2 cells had much lower expression of IL-17RB (*Fig. 1e*). Thus, iILC2 cells were ST2<sup>−</sup> IL-17RB<sup>−</sup> and responded to IL-25 but not to IL-33, whereas nILC2 cells were ST2<sup>+</sup> and responded mainly to IL-33. Treatment with IL-25 did not elicit iILC2 cells in IL-17RB-deficient (Il17rb<sup>−/−</sup>) mice but did so in ST2-deficient (Il11r<sup>−/−</sup>) mice (*Fig. 1f*).

#### iILC2 development depends on γc and IL-7Rα

We assessed whether iILC2 cells exist in mice deficient in the recombinase component RAG-2 (Rag2<sup>−/−</sup>) mice) or in Rag2<sup>−/−</sup> mice also deficient in γc (Rag2<sup>−/−</sup>Il7ra<sup>−/−</sup> mice). Rag2<sup>−/−</sup> mice treated with IL-25 produced iILC2 cells in the lungs and MLNs (*Fig. 2a*). Many more iILC2 cells were elicited by IL-25 in Rag2<sup>−/−</sup> mice than in wild-type mice: in the lungs, ~5 × 10<sup>4</sup> iILC2 cells appeared in Rag2<sup>−/−</sup> mice treated with IL-25 (*Fig. 2a*), compared with fewer than 5 × 10<sup>4</sup> in wild-type mice (*Fig. 1c*). MLNs are much smaller in RAG-2-deficient mice than in wild-type mice; however, MLNs of Rag2<sup>−/−</sup> mice become enlarged after IL-25 treatment. 67% of the total MLN cells in Rag2<sup>−/−</sup> mice were iILC2 cells, with numbers in excess of 1 × 10<sup>6</sup> (*Fig. 2a*). In contrast, iILC2 cells were totally absent in Rag2<sup>−/−</sup>Il7ra<sup>−/−</sup> mice treated with IL-25 (*Fig. 2b*), which indicated that iILC2 cells were γc dependent.

All ILCs, other than classic IL-7Rα<sup>−</sup>NK1.1<sup>−</sup> natural killer cells, require IL-7Rα for their development. Treating IL-7Rα<sup>−</sup>-deficient (Il7ra<sup>−/−</sup>) mice with IL-25 led to substantially fewer iILC2 cells than did treatment of wild-type mice (*Fig. 2c,d,*), which indicated that IL-7Rα was critical for the induction of iILC2 cells by IL-25. Notably, while we detected no nILC2 cells in Il7ra<sup>−/−</sup> mice, iILC2 cells were not totally abolished in IL-25-treated Il7ra<sup>−/−</sup> mice (*Fig. 2d*), which suggested that iILC2 cells and nILC2 cells might arise through somewhat different developmental pathways or that more than one cell population might have been present in the iILC2 ‘gate’.

We cultured the sorted iILC2 (Lin<sup>−</sup>ST2<sup>−</sup> KLRG1<sup>hi</sup>) cells from IL-25-treated wild-type mice in IL-2 plus IL-7 or, alternatively, in IL-3 plus the cytokine stem cell factor (SCF). iILC2 cells survived and proliferated in the presence of IL-2 plus IL-7 but died in the presence of IL-3 plus SCF (*Fig. 2e*). This pattern of responsiveness differed from that reported for MPP<sup>type2</sup> cells, which further indicated that iILC2 cells were distinct from MPP<sup>type2</sup> cells.

#### Production of type 2 cytokines by iILC2 cells

To quantify type 2 cytokine-producing cells, we generated 4C13R dual reporter mice, with transgenic expression (via a bacterial artificial chromosome) of the cyan fluorescent protein AmCyan under the control of Il4 regulatory elements and of the red fluorescent protein dsRed under the control of Il13 regulatory elements; in these mice, IL-4...
production is reported by AmCyan and IL-13 production is reported by destabilized DsRed (Supplementary Fig. 2a). These mice allow detection of IL-4- or IL-13-producing cells without in vitro stimulation. In naive 4C13R mice, ~2–9% of lung nILC2 cells produced IL-13, but few if any made IL-4 (Fig. 3a and Supplementary Fig. 2b). After the administration of IL-25, the frequency of IL-13-producing nILC2 cells rose to ~14%, but we observed no IL-4 production (Fig. 3b).

Among iILC2 cells from IL-25-treated mice, ~31% were DsRed+ (Fig. 3b), which indicated that they were producing IL-13. A few of those cells (~2%) were AmCyan+ (Fig. 3b). Thus, iILC2 cells shared with nILC2 cells the ability to make type 2 cytokines.

To further address the cytokine-producing ability of iILC2 cells, we purified them from IL-25-treated wild-type mice, cultured them for 3 d in IL-2, IL-7 and IL-25 and then stimulated the cells with IL-25, IL-33 or the phorbol ester PMA plus ionomycin (Fig. 3c).

Consistent with the results obtained with 4C13R mice, some iILC2

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**Figure 1** IL-25 induces a Lin−ST2−KLRG1hi cell population distinct from nILC2 or MPPtype2 cells. (a) Flow cytometry analyzing the expression of ST2, KLRG1 and lineage (Lin) markers (CD3ε, CD5, CD19, B220, TCRy6, NK1.1, CD11b, CD11c, Gr-1, FcrR1 and Ter119) in leukocytes from the lungs of wild-type C57BL/6 (B6) mice treated intraperitoneally daily for 3 d with PBS, IL-33 or IL-25 (200 ng per mouse per day for each cytokine). Numbers adjacent to outlined areas indicate percent Lin−ST2− cells (left) or Lin−ST2+ cells (right) (top row) or Lin−KLRG1hi cells (bottom row). (b) Expression of ST2 and KLRG1 on Lin− cells in the lungs of the mice in a; outlined areas indicate nILC2 cells (red) or iILC2 cells (blue). (c) Quantification of nILC2 cells and iILC2 cells in the lungs of mice treated as in a. (d) Ki67 expression on lung leukocytes from IL-25-treated mice as in b; right: red, nILC2 cells gated on Lin−ST2−KLRG1int; blue, iILC2 cells gated on Lin−ST2−KLRG1hi; gray, cells gated on Lin−ST2−KLRG1−. Outlined area indicates Ki67+ cells. (e) Expression of ILC2 markers (horizontal axes; right) on Lin− cells (gated at left) from the lungs of IL-25-treated B6 mice: red, nILC2 cells; blue, iILC2 cells; gray shaded curves, negative control for each marker (for example, Lin−IL-7Rα− cells were gated as a negative control for IL-7Rα expression). (f) Expression of lineage markers, Thy-1 and KLRG1 by lung leukocytes from wild-type (WT), II17rb−/− or II1rL1−/− mice treated for 3 d with IL-25, analyzed by flow cytometry with gating on Lin−. Numbers adjacent to outlined areas indicate percent Thy-1−KLRG1int cells (top left) and Thy-1−KLRG1hi cells (bottom right). (g) Expression of ILC markers (along axes) by lung leukocytes from wild-type B6 mice left untreated (UT) or treated for 3 or 7 d (left margin) with IL-25, analyzed by flow cytometry with gating on Lin− Thy−1−IL-7Rα−ST2−c-Kit− (MPPtype2 cells), Lin−Thy−1−IL-7Rα−ST2−KLRG1int (nILC2 cells) or Lin−Thy−1−IL-7Rα−ST2−KLRG1hi (iILC2 cells); numbers adjacent to outlined areas indicate percent cells in those gates. NS, not significant; * P ≤ 0.05 (unpaired two-tailed t test).

Data are representative of three (a-e) or two (f,g) independent experiments with n = 3 mice per group in each (a-c); mean and s.e.m. in (c), n = 2 mice in each (d,e) or n = 2 mice per group in each (f,g).
cells produced IL-13 even without stimulation. PMA plus ionomycin induced the great majority of iILC2 cells to produce IL-4, IL-13 and IL-5. Unexpectedly, cultured iILC2 cells failed to respond to IL-25 but did produce IL-13 and IL-5 in response to IL-33; no IL-4 was produced in response to IL-33. Since iILC2 cells were sorted as ST2−IL-17RB+ cells, these results suggested that iILC2 cells might have changed their expression pattern of ST2 and IL-17RB when cultured in vitro. These results emphasized that iILC2 cells, while they produced only IL-13 and IL-5 in response to cytokines, were able to produce IL-4 when stimulated with PMA plus ionomycin.

Development of iILC2 cells into ST2+ nILC2-like cells

The altered responsiveness of iILC2 cells to IL-33 and IL-25 suggested they might have upregulated ST2 expression and downregulated IL-17RB expression during culture. We sorted iILC2 cells from IL-25-treated Rag2−/− donors, cultured them in various cytokine combinations and measured ST2 and IL-17RB expression on day 7. The starting cell population was ST2−IL-17RB+ (Fig. 4a,b). 7 d of culture in IL-2 and IL-7 resulted in the great majority becoming ST2+ (Fig. 4a). The addition of IL-25 or IL-33 or both to the culture led to even higher expression of ST2 among those cells (Fig. 4a). Culture in IL-2 and IL-7

Figure 2 iILC2 development depends on γc and IL-7Rα. (a) Expression of lineage markers and KLRG1 by leukocytes from the lungs or MLNs of Rag2−/− mice treated for 3 d with PBS or IL-25, analyzed by flow cytometry with gating of iILC2 cells as Lin−KLRG1hi (numbers adjacent to outlined areas indicate percent cells in that gate) (left), and quantification of total lymphocytes and iILC2 cells in the lungs (top) or MLNs (bottom) of those mice (right). (b,c) Expression of lineage markers and KLRG1 by lung leukocytes from IL-25-treated wild-type and Rag2−/−Il2rg−/− mice (b) or wild-type and Il7ra−/− mice (c), analyzed by flow cytometry (numbers adjacent to outlined areas (c), as in a). (d) Quantification of iILC2 cells and nILC2 cells from mice as in e. (e) Viability (right) of Lin−ST2−KLRG1hi cells purified by sorting (left) from the lungs of IL-25-treated wild-type mice and cultured for 3 d either ST2+ or IL-7+ in IL-3 plus SCF (10 ng/ml each cytokine), assessed by staining with LIVE/DEAD viability dye. Numbers adjacent to outlined areas indicate percent LIVE/DEAD− (viable) cells. SSA, side scatter. Data are representative of more than five (a) or three (b–d) or two (e) independent experiments with n = 3 mice per group in each (a–d; mean and s.e.m. in a,d) or n = 2 wells per condition in each (e).

Figure 3 iILC2 cells produce type 2 cytokines. (a) Expression of lineage markers, KLRG1 and ST2 (left) and AmCyan (IL-4) and DsRed (IL-13) (right) by lung leukocytes isolated from naive 4C13R mice or non-transgenic B6 mice (WT), analyzed by flow cytometry with nILC2 cells (right) gated as Lin−ST2−KLRG1int (left). Numbers in quadrants indicate percent cells in each throughout. (b) Analysis as in a of lung leukocytes from 4C13R mice treated intraperitoneally for 3 d with IL-25, with nILC2 cells gated as Lin−ST2−KLRG1int (top right) and iILC2 cells gated as Lin−ST2+KLRG1hi (bottom right). (c) Intracellular staining IL-13, IL-4 and IL-5 in iILC2 cells sorted from IL-25-treated wild-type B6 mice and cultured for 3 d with IL-2, IL-7 and IL-25 (10 ng/ml for each cytokine), then left unstimulated (Mock) or stimulated for 6 h with PMA plus ionomycin (PMA + iono), IL-25 (50 ng/ml) or IL-33 (50 ng/ml), assessed by flow cytometry. Numbers adjacent to outlined areas indicate percent cytokine-positive cells. FSC, forward scatter. Data are representative of three independent experiments with n = 2–5 mice per group in each (a), n = 2 mice in each (b) or n = 3 wells per condition in each (c).
iILC2 cells sorted from IL-25-treated lungs were sorted into an nILC2 phenotype. Mesenteric nILC2 cells have been reported to respond to IL-2 and IL-25. Numbers in quadrants (left) indicate percent CD45.1+ cells (top left) or CD45.2+ cells (bottom right). Isotype (key, right), isotype-matched control antibody. (d) Flow cytometry assessing the expression of KLRG1 and ST2 by iILC2 cells from the lungs of transferred cells in the lungs had become ST2+ nILC2-like cells (Fig. 1g). This result suggested that iILC2 cells gave rise to cells of the two cell populations expanded to the same extent but also established that iILC2 cells converted to cells with a nILC2 phenotype.

While the majority of KLRG1hi cells were ST2+ at day 3 after in vivo treatment with IL-25, a small proportion of these cells were ST2+ or ST2lo (Fig. 1g). By day 7 of in vivo IL-25 treatment, ST2- and ST2+ cells were present in equal proportions among the KLRG1hi population (Fig. 1g). This result suggested that iILC2 cells gave rise to cells of an nILC2 phenotype in vivo. To address this possibility, we transferred sorted iILC2 cells into Rag2−/−Il2rg−/− mice. 8 d later, more than 60% of transferred cells in the lungs had become ST2+ nILC2-like cells (Fig. 4d). We propose that IL-25-induced iILC2 cells are transient progenitors of nILC2 cells or of cells similar to nILC2 cells.

Regulation of anti-helminth immunity by iILC2 cells

We investigated whether helminth infection elicits iILC2 cells. We infected wild-type mice (Fig. 5a) or 4C13R reporter mice (Supplementary Fig. 4a) with N. brasiliensis and analyzed lung ILCs at various times. iILC2 cells first appeared on day 5 after infection; however, at that time, nILC2 cells had not yet proliferated (Fig. 5a and Supplementary Fig. 4a). Thereafter, the number of iILC2 cells decreased, while nILC2 cells increased in number (Fig. 5a and Supplementary Fig. 4a). On day 14, most iILC2 cells had disappeared, but nILC2 cells had increased three- to fourfold compared with their abundance in uninfected mice (Fig. 5a). N. brasiliensis–activated nILC2 cells produced IL-13, and some produced IL-4, but iILC2 cells were better cytokine producers (Supplementary Fig. 4a).

N. brasiliensis–induced iILC2 cells were IL-25 dependent; they were absent in infected Il17rb−/− mice (Fig. 5b,c). The population expansion of nILC2 cells was also significantly impaired in Il17rb−/− mice (Fig. 5d), which suggested that iILC2 cells made a substantial contribution to the number of nILC2 cells during infection with N. brasiliensis. Intestinal worm burden was substantially greater in Il17rb−/− mice than in wild-type mice (Fig. 5e), which indicated that the IL-25-dependent iILC2 cells were important for worm expulsion.

The results reported above were consistent with the possibility that the N. brasiliensis–induced iILC2 cells were a transient progenitor population that developed into nILC2-like cells and participated in the expulsion of helminths. To assess this possibility, we transferred CD45.1+ nILC2 cells and CD45.2+ iILC2 cells together into...
Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice and infected the recipient mice with *N. brasiliensis*. 14 d later, the ratio of CD45.1<sup>+</sup> cells to CD45.2<sup>+</sup> cells remained similar to that in the injected cell populations, but the iILC2 cells had developed into IL-7R<sup>+</sup>Thy-1<sup>−</sup>ST2<sup>+</sup>KLGR1<sup>hi</sup> nILC2-like cells (Fig. 5f). In addition, the transferred iILC2 cells developed into nILC2-like cells in the absence of nILC2 cells (Supplementary Fig. 4b). These results indicated that iILC2 cells were indeed transient progenitors of nILC2 cells during helminth infection.

We sought to determine whether iILC2 cells could protect mice against infection with *N. brasiliensis*. Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice cannot expel worms of this parasite because of the absence of T cells and ILCs. Between day 7 and day 11 after infection of Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice with *N. brasiliensis*, we detected many eggs in mouse feces (Fig. 5g), and we observed adult worms in the intestine on day 14 (Fig. 5h). The transfer of iILC2 cells into Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice resulted in a significantly lower number of eggs in the feces and the worm burden in the intestine, comparable to that observed by the transfer of a similar number of nILC2 cells (Fig. 5g,h), which indicated that iILC2 cells and/or their descendants were able to limit worm expansion.

**Development of iILC2 cells into IL-17 producers**

We compared the expression of GATA-3 and RORγt by lung iILC2 cells, lung nILC2 cells and small intestine lamina propria (siLP) ILC3 cells (Fig. 6a). Both iILC2 cells and ILC3 cells expressed GATA-3, as reported<sup>20</sup> (Fig. 6a). As expected, nILC2 cells expressed more GATA-3 than did ILC3 cells; iILC2 cells expressed even more GATA-3 than did nILC2 cells (Fig. 6a). iILC2 cells also expressed an intermediate amount of RORγt, less than ILC3 cells, but significantly distinct from the GATA-3 expression of nILC2 cells (Fig. 6a). In keeping with the presence of RORγt, a small proportion of freshly isolated IL-13-producing iILC2 cells also produced IL-17 upon stimulation with PMA plus ionomycin (Fig. 6b).

Naive CD4<sup>+</sup> T cells can differentiate into diverse helper T cell subsets, either in response to distinct pathogenic pressure in *vivo* or under polarized culture conditions in *vitro*. We mimicked in *vitro* conditions for helper T cell differentiation in our cultures of iILC2 cells, although we omitted antigen-presenting cells. More than 50% of iILC2 cells cultured with IL-4, anti-IFN-γ and anti-IL-12 (Th12 conditions), in addition to the usual iILC2 culture conditions, produced IL-13 and, upon stimulation with PMA plus ionomycin, ~90% of the cells produced IL-13 (Fig. 6c). Among iILC2 cells cultured with the addition of IL-12 and anti-IL-4 (Th11 conditions) or the cytokine TGF-β and IL-6 (Th17 conditions), fewer produced IL-13 with or without PMA plus ionomycin (Fig. 6c). This difference was particularly pronounced for IL-4 production induced by PMA plus ionomycin (Fig. 6c). iILC2 cells cultured under any conditions failed to produce IFN-γ (Fig. 6c). Impressively, 50% of iILC2 cells in “Th11” conditions produced IL-17 in response to PMA plus ionomycin, while fewer iILC2 cells cultured under “Th11” or “Th12” conditions were able to make IL-17 (Fig. 6c). Most IL-17 producers also produced IL-13 (Fig. 6c). Some cells cultured under “Th11” conditions produced both IL-17 and IL-13.

**Deviation of IL-18 producers**

Development of iILC2 cells into IL-18 producers was assessed by RNA-Seq analysis of day 5 iILC2 cells (Fig. 6d). Most of the gene expression changes observed by RNA-Seq analysis were shared between GATA-3 and RORγt. When we compared day 5 iILC2 cells to day 3 iILC2 cells, 63% of the gene expression changes observed by RNA-Seq were shared between GATA-3 and RORγt. When we compared day 5 iILC2 cells to day 5 nILC2 cells, 69% of the gene expression changes observed by RNA-Seq were shared between GATA-3 and RORγt. These results indicated that iILC2 cells and nILC2 cells expressed similar gene expression profiles, which may have been due to the initial IL-7R<sup>+</sup> mounting as well as the GATA-3 and RORγt expression profile.
conditions produced IL-17 even without stimulation with PMA plus ionomycin (Fig. 6c), a result we confirmed by measuring secreted IL-17 protein in the supernatants of cultured cells (Fig. 6d).

To determine whether the potential of iILC2 cells to become double producers of both IL-13 and IL-17 was limited to a subset of these cells or was a general property, we sorted DsRed+ and DsRed− iILC2 cells from IL-25-treated 4C13R mice and cultured them under ‘T\(\gamma\)1’ or ‘T\(\gamma\)17’ conditions. After culture, both groups were equivalent in their capacity to produce IL-17, although those cultured under ‘T\(\gamma\)17’ conditions were superior to those cultured under ‘T\(\gamma\)1’ conditions (Fig. 6e). We also used IL-17–RFP reporter mice (in which expression of red fluorescent protein (RFP) indicates expression of IL-17) in similar experiments. Both cells that produced IL-17 and those that did not produce IL-17, after stimulation PMA plus ionomycin, had the same potential to become cells that produced both IL-13 and IL-17 upon culture (Supplementary Fig. 5). This finding was consistent with the homogenous expression of RORγ in freshly isolated iILC2 cells (Fig. 6a). Thus, iILC2 cells expressed both GATA-3 and RORγ. They produced IL-13 and were also able to produce IL-17 under certain culture conditions in vitro.

We also investigated whether nILC2 cells had such plasticity as well. When cultured under ‘T\(\gamma\)1’, ‘T\(\gamma\)2’ or ‘T\(\gamma\)17’ conditions, only ~2% to 5% of nILC2 cells produced IL-17 upon stimulation with PMA plus ionomycin (Supplementary Fig. 6). This result was independent of culture conditions (Supplementary Fig. 6), which indicated that nILC2 cells had less capacity to develop into IL-17 producers than did iILC2 cells.

**Figure 6 iILC2 cells express RORγ and have the ability to develop into IL-17-producing cells.** (a) Flow cytometry analyzing the expression of GATA-3 and RORγ on nILC2 cells and iILC2 cells from the lungs of IL-25-treated wild-type B6 mice and on ILC3 cells (Lin−IL-7RααRORγt) from siLP of naive wild-type B6 mice. (b) Intracellular staining of IL-17 and IL-13 in iILC3 cells freshly sorted from IL-25-treated Rag2−/− mice and left unstimulated (Mock) or stimulated for 6 h with PMA plus ionomycin, analyzed by flow cytometry. (c) Intracellular staining of IL-4, IL-13, IFN-γ and IL-17 in iILC2 cells freshly sorted from IL-25-treated Rag2−/− mice and cultured for 7 d in ‘T\(\gamma\)1’ or ‘T\(\gamma\)17’ conditions (left margin), then stimulated and analyzed as in b. (d) Enzyme-linked immunosorbent assay of IL-17 in supernatants of iILC2 cells cultured and stimulated as in c. (e) Intracellular staining of IL-13 and IL-17 in iILC2 cells from IL-25-treated 4C13R mice, sorted as DsRed+ and DsRed− populations and cultured for 7 d in ‘T\(\gamma\)2’ or ‘T\(\gamma\)17’ conditions, followed by 6 h of stimulation with PMA plus ionomycin. Data are representative of three (a–c) or two (d,e) independent experiments (n = 2 mice per group in each (a) or n = 2 wells per condition in each (b,c,e)); mean ± s.e.m. of n = 3 wells per condition in each (d)).

**Contribution of iILC2 cells to antifungal immunity**

Since iILC2 cells were able to develop into IL-17 producers in vitro, we sought to determine whether they had a role in controlling IL-17-sensitive pathogens. A published study has shown that IL-17-producing ILC3 cells are important for protection after oral infection with *C. albicans*28. We infected wild-type, Rag2−/− and Il2rg−/− mice sublingually with *C. albicans*. Both wild-type and Rag2−/− mice lost ~10% of their body weight by 1–2 d after infection but recovered completely (Fig. 7a). In contrast, Rag2−/−Il2rg−/− mice displayed continued weight loss and all died by day 7 of infection (Fig. 7a,d). On day 6, we detected a large number of *C. albicans* in the tongue tissue of Rag2−/−Il2rg−/− mice but not in that of wild-type or Rag2−/− mice (Fig. 7b). ILCs from tongue-draining lymph nodes of infected Rag2−/− mice proliferated much more than did those of uninfected mice (Supplementary Fig. 7), which indicated that after oral infection with *C. albicans*, γδ-dependent cells, presumably ILCs, had a major role in the control of infection. In addition, ~35% of the ILCs expressed IL-17RB, and a portion of these cells were also KLRG1+ (Supplementary Fig. 7), which suggested that the IL-25-responsive ILCs might have had some role in the innate lymphocyte response to infection with *C. albicans*.

We transferred 0.5 × 10⁶ or 1.2 × 10⁶ iILC2 cells into Rag2−/−Il2rg−/− mice, followed by sublingual infection of the recipients with *C. albicans*. 

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iILC2 cells significantly diminished the weight loss of infected mice, with the transfer of 1.2 x 10^6 iILC2 cells limiting weight loss by about one-third (Fig. 7c). iILC2 cells also improved survival by 4 days (Fig. 7d). The abundance of C. albicans in the tongue tissue was lower in the two groups that received iILC2 cells than in mice that did not receive cells, with the group that had received 9 x 10^5 cells in another experiment showing a lower number of C. albicans in the tongue (Fig. 7e); this confirmed that iILC2 cells were able to provide partial protection against the pathogen.

There was no detectable IL-17 mRNA in Rag2−/− Il2rg−/− mice that did not receive cells; in contrast, IL-17 mRNA was readily detectable in the tongues of mice that had received iILC2 cells, being greater in the tongues of mice that had received a larger number of iILC2 cells (Fig. 7f). However, the transfer of iILC2 cells did not enhance IL-13 expression in the tongue tissue (Fig. 7f). We also analyzed the cytokine production by transferred iILC2 cells in the lungs of infected mice by flow cytometry. 5 days after transfer into C. albicans−infected mice, iILC2 cells were producing IL-17 but not IL-13 (Fig. 7g), which indicated that they had become ILC3-like cells. Thus, iILC2 cells displayed a degree of in vivo plasticity and were able to provide partial protection against C. albicans.

**DISCUSSION**

ILCs are a first line of defense in many infectious models. They reside in barrier tissues and respond quickly to infections. Transient activation and expansion in the number of ILCs occurs upon stimulation with cytokines or pathogens. ILC2 cells show cytokine-based activation and population expansion in response to IL-25 and/or IL-33. Whether the same cell populations respond to the two different cytokines had not been clarified. In the original report of nuocytes[5], their heterogeneous expression of ST2 and IL-17RB could have been due to a mixture of separate cell populations: IL-17RB-expressing ILC2 cells and ST2-expressing ILC2 cells. Here we have presented evidence that responsiveness to IL-25 and IL-33 is a property of distinct cell types and propose that two ILC2 developmental pathways exist: a natural or homeostatic ILC2 (nILC2) pathway and an inflammatory ILC2 (iILC2) pathway.

In naïve mice, ST2+ nILC2 cells were resident in the lungs and other tissues and became cytokine producers and expanded moderately in number in response to IL-33 stimulation. These cells initiated IL-13 production quickly after infection with N. brasiliensis, although they showed little expansion in number until after day 5. A second population of IL-13-producing ILCs arose in response to treatment with IL-25. These IL-17RB+ cells lacked ST2 and expressed large amounts of KLRG1. We have designated these ‘iILC2 cells’. iILC2 cells were undetectable in the lungs and in most other peripheral tissues in naïve mice but showed massive elicitation in response to treatment with IL-25, a result that was even greater in Rag2−/− mice. iILC2 cells appeared by day 5 of infection with N. brasiliensis, before the induction of T1h cells or the population expansion of nILC2 cells. Expansion in the number of nILC2 cells in N. brasiliensis−infected mice was significantly impaired in I17rb−/− mice, which lacked iILC2 cells. On the basis of their phenotypic changes in transfer experiments, we concluded that iILC2 cells became nILC2-like cells during infection with N. brasiliensis. Thus, IL-25−induced iILC2 cells acted as transient progenitors of nILC2-like cells. Since the number of nILC2 cells at barrier surfaces at steady state was low and their proliferation was both slow and limited, iILC2 cells seem to be an important source of ILC2 cells to combat helminth infection.

The development of iILC2 cells was dependent on the expression of γc; iILC2 cells expressed IL-7Ra, as did nILC2 cells. iILC2 cells were induced normally in mice that lacked ST2, and nILC2 numbers were normal in mice that lacked IL-17RB. This indicated that the development of these two populations was independent of the ‘alternative’ cytokines.

The cytokine receptor expression and responsiveness pattern of iILC2 cells was dynamic. *In vitro*, highly purified iILC2 cells quickly acquired responsiveness to ST2 and IL-33 while they lost responsiveness...
to IL-25 and expression of IL-17RB, particularly when cultured in the presence of IL-33. When iILC2 cells were transferred into untreated mice, they became ST2+ nILC2-like cells in the lungs.

GATA-3 expression and activation of the transcription factors STAT5 and NF-kB are essential for inducing T cell antigen receptor–independent ST2 expression in Tp2 cells. A similar requirement probably exists in ILC2 cells. IL-25-induced iILC2 cells expressed very large amounts of GATA-3, as did nILC2 cells; IL-7 activates STAT5 in both cell types; and both IL-25 and IL-33 trigger activation of NF-kB. Thus, in vitro induction of ST2 expression on iILC2 cells in response to the STAT5 activators IL-2 and IL-7 and in response to the addition of either IL-25 or IL-33 would seem reasonable.

Important differences between iILC2 cells and nILC2 cells were that iILC2 cells expressed an intermediate level (between the amounts for nILC2 and ILC3) of RORyt, whereas nILC2 cells did not. Moreover, we identified nILC2 cells in the lungs and fat-associated lymphoid tissues but did not identify as many in the spleen or liver, while ILC2 cells were present in many sites, including lung, MLNs, spleen, liver and bone marrow, after treatment with IL-25.

Effector CD4+ helper T cells have the ability to alter their cytokine-producing phenotype in response to infectious challenges or cytokine exposure. Whether various types of ILCs also have plasticity or flexibility in their cytokine-producing potential is unknown. Generally, ILCs are considered to be terminally differentiated cells. Most are resident in barrier tissues, and some constitutively produce cytokines. It has been reported that deficiency in Gfi1 leads ILC2 cells to lose their restricted cytokine-production phenotype; these cells can produce both IL-13 and IL-17 upon stimulation with PMA plus ionomycin, which suggests that ILCs may have plasticity in their cytokine-producing potential. We found here that ILC2 cells displayed a degree of plasticity or multipotentiality. In contrast to nILC2 cells, they expressed both GATA-3 and RORyt and had the ability to develop into either nILC2-like cells or ILC3-like cells.

We propose that there are two ILC2 developmental pathways: one that gives rise to tissue-resident nILC2 cells, and one that gives rise to iILC2 cells. We have clarified the relationship between IL-33-responsive ILC2 cells and IL-25-responsive ILC2 cells and demonstrated that IL-17RB-expressing ILC2 cells and their immediate progenitors were precursors of an ST2-expressing ILC2 population during inflammation and infection. We have also reported that iILC2 cells displayed the ability to be converted into distinct cytokine producers. Although ILCs lack antigen-specific receptors, they ‘read’ and distinguish types of microbial pathogens through epithelial cell–derived cytokines to react properly and to orchestrate immune responses.

METHODS

Methods and any associated references are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank I. Zhu for critical reading of the manuscript; J. Edwards for assistance in the preparation of sorter–purified cells; L. Feigenbaum of the SAIC Laboratory Animal Sciences Program for injection of the recombinant bacterial artificial chromosome into oocytes, transfer into pseudopregnant females and screening of pups; C. Dong (MD Anderson Cancer Center) for IL-17F–RFP reporter mice; A. McKenzie (MRC Laboratory of Molecular Biology, Cambridge) for Il17F–cre mice; and members of Laboratory of Immunology at NIAID for discussions. Supported by the NIAID Division of Intramural Research (US National Institutes of Health).

AUTHOR CONTRIBUTIONS

Y.H. and W.E.P. designed and interpreted the experiments and wrote the manuscript; I.G. assisted with the experiments and read the manuscript; Y.H. did the experiments; J.Q. did the C. albicans oral infection; X.G. generated 4C13R mice; J.H. assisted with cell culture and flow cytometry; U.S. provided Il17r+/- mice; P.R.W. assisted in the design and interpretation of experiments with C. albicans; and J.F.U. provided N. brasiliensis and helped to design and interpret experiments with N. brasiliensis.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**4C13R mice.** Mice with transgenic expression of AmCyan under the control of regulatory elements of Il4 and destabilized DsRed (DsRed-DR) under the control of regulatory elements of Il13 (4C13R mice) were generated through the use of bacterial artificial chromosome (BAC) recombination-mediated engineering technology with selection via galK (which encodes galactose kinase). The BAC clone RP79.23H11 containing the Tg2 locus-control region and Il13, Il4 and Kif3a was obtained from Children’s Hospital of Oakland Research Institute. The plasmids p-AmCyan-1-N1 and p-DsRed-Express-DR were obtained from Clontech. The start codon ATG of Il4 in the BAC was targeted with a galK construct containing homology arms at both the 5′ and 3′ ends of galK. galK was subsequently targeted with sequence encoding AmCyan. Then, similarly, the ATG of Il13 in the ‘AmCyan-IL-4 BAC’ construct was replaced with sequence encoding DsRed-DR via galK selection. The final BAC was fully sequenced and then linearized by digestion with AscI. Microinjection of the linear construct into B6 oocytes was followed by transfer into pseudopregnant foster mothers. The pups were screened to identify the mice containing both AmCyan and DsRed-DR by Southern blot analysis. Correlation of the expression of AmCyan and DsRed-DR with simultaneous expression of Il-4 and Il-13 was shown by culture of CD4+ T cells from the transgenic mice for 3 d under Tg2 conditions, followed by stimulation for 4 h with PMA (phorbol 12-myristate 13-acetate) plus ionomycin, plus ionomycin, after which cells were stained for Il-4 and Il-13. The in vivo expression of AmCyan and DsRed-DR was also examined in Schistosoma mansoni–infected mice by flow cytometry.

**Mice.** Wild-type C57BL/6 mice were obtained from Taconic or Jackson Laboratory. B6SJL (CD45.1 congenic) mice, Rag2−/− mice, Rag2−/− mice and Rag2−/−tgε−/− mice were from Taconic. Il7ra−/− mice were from Jackson Laboratory. IL-17-RFP reporter mice were from C. Dong, Il11r1−/− mice were from A. McKenzie. Il17rb−/− mice were generated in-house (in the laboratory of U.S.). All mice used for experiments were between 6 and 18 weeks of age. For infection with *N. brasiliensis* or *C. albicans*, mice were females at 8–10 weeks of age. All animal experiments were performed under the approval of the NIAID Animal Care and Use Committee.

**Antibodies and reagents.** The following fluorochrome-conjugated antibodies were used for flow cytometry. Anti-CD3e (145-2C11), anti-CD5 (53-7.3), anti-CD19 (1D3), anti-CD20 (RA3-6B2), anti-CD11b (M1/70), anti-CD11c (N418), anti-NK1.1 (PK136), anti-TCRβ (eBioGL3), anti-Gr-1 (RB6-8C5), anti-FcγRI (AR71), anti-FcγRIII (CD16), anti-CD44 (eBio16-1F9), anti-TER119 (TER-119), anti-IL-7Rα (A7R34), anti-γδ TCR (XMG1.2), anti-IL-12 (107D12), anti-IL-17A (eBio17B7), anti-IL-17F–RFP (AFJK5-9) and anti-Ki67 (B56) were from eBioscience. Anti-KLRG1 (2F1), anti-IL-4 (11B11), anti-Ki67 (B56) and anti-GATA-3 (A20) were from BD Biosciences. Anti-ST2 (D7), anti-KLRG1 (2F1), anti-IL-4 (11B11), anti-CD44 (eBio16-1F9), anti-TER119 (TER-119), anti-IL-7Rα (A7R34), anti-γδ TCR (XMG1.2), anti-IL-17A (eBio17B7), anti-IL-17F–RFP (AFJK5-9) and anti-T-bet (4B10) were from eBioscience. Anti-KLRG1 (2F1), anti-IL-4 (11B11), anti-Ki67 (B56) and anti-GATA-3 (A20) were from BD Biosciences. Anti-ST2 (D7) was from MD Bioproducts. Anti-IL-17RB (752101) was from R&D Systems. Recombinant IL-25 and IL-33 were from R&D Systems. For in vitro stimulation with PMA plus ionomycin, then supernatants of cultured cells were collected and diluted. The concentration of secreted IL-17 protein was determined with a mouse IL-17AF (heterodimer) ELISA Ready-Set-Go kit (eBioscience).

**Flow cytometry and cell sorting.** Non-specific binding in cells in PBS solution with 3% PBS was blocked with anti-CD16/CD32 (2.4G2; Harlan Laboratories), then cells were incubated with fluorochrome-conjugated antibodies (identified above) and LIVE/DEAD fixable dead cell stain kit. Staining and washing were performed at 4 °C. Cells were analyzed on an LSR II flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (version 10.6.6). For cell sorting, cells were stained and washed in a PBS solution with 10% FBS, but LIVE/DEAD dye was omitted. Cells were purified on FACSaria cell sorter (BD Biosciences).

**Intracellular staining.** For cytokine staining, monensin was added to the culture medium for the final 2 h during stimulation. After surface staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS solution, and then were incubated with fluorochrome-conjugated cytokine-specific antibodies (identified above). For staining of GATA-3, RORγt and Ki67, a Foxp3/Transcription Factor Staining Buffer Set was used according to the manufacturer’s instruction (eBioscience).

**Isolation of leukocytes from lung tissue.** Lung tissues were harvested after perfusion and were disrupted into small pieces, then were digested for 20 min at 37 °C with Liberase TM (Roche) plus DNase I (Roche). Tissue pieces were strained into single cells, and leukocytes were purified by centrifugation with 40% Percoll (GE Healthcare Life Sciences) in PBS solution, followed by treatment with ACK Solution (Life Technologies).

**In vitro ‘polarization’ of ILC2 cells.** ILC2 cells purified by cell sorting from the lungs of IL-25-treated Rag2−/− mice were cultured for 7 d in various conditions as follows (reagents added to culture medium): ‘Th1’ conditions, IL-7 (10 ng/ml), IL-2 (10 U/ml), IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml); ‘Th2’ conditions, IL-7 (10 ng/ml), IL-2 (10 U/ml), IL-33 (10 ng/ml), IL-4 (100 U/ml), anti-IFN-γ (10 μg/ml) and anti-IL-12 (10 μg/ml); and ‘Th17’ conditions, IL-7 (10 ng/ml), IL-2 (10 U/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml), IL-23 (20 ng/ml), TGF-β (5 ng/ml), anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml) and anti-IL-12 (10 μg/ml). The culture medium was refreshed every 2 or 3 d.

**N. brasiliensis infection.** Mice were given subcutaneous injection of 300 third-stage *N. brasiliensis* larvae, and cell transfer was performed on the same day if required. Mouse feces were collected from each individual mouse on day 7 to day 9 after infection, and eggs in feces were counted. Worm burden in small intestines were measured on day 14.

**Oral infection with C. albicans.** Cotton swabs were immersed for 5 min in a solution of 1 × 10⁶ colony-forming units of *C. albicans* (strain SC5314) and then were placed sublingually in the mice for 45 min while mice were anesthetized. Cell transfer was performed the day before if required. Mice were weighed daily and death and survival were monitored. For determination of fungal colony-forming units, tongues were disrupted in PBS solution and were cultured for 2 d on YPD (yeast-peptone-dextrose) plates.

**Quantitative PCR.** One-third of the mouse tongue was disrupted in TRIZol reagent, and total RNA was purified according to the manufacturer’s protocol (Life Technologies). Reverse transcription was performed with oligo(dT)₂₀ primers. TaqMan probes were used for measurement of the expression of Il13 (Mm00439240_m1; Life Technologies) and Il17a (Mm00439618_m1; Life Technologies), and mRNA expression was adjusted relative to the expression of Gapdh (Mm03302249_g1; Life Technologies).

**Enzyme-linked immunosorbent assay.** Sorted cells were cultured under various conditions in 96-well plates, with 2 × 10⁵ cells per well, with or without stimulation with PMA plus ionomycin, then supernatants of cultured cells were collected and diluted. The concentration of secreted IL-17 protein was determined with a mouse IL-17AF (heterodimer) ELISA Ready-Set-Go kit (eBioscience).

**Statistical analysis.** Sample or experiment sizes were determined empirically for sufficient statistical power. No statistical tests were used to predetermine the size of experiments. No samples were excluded specifically from analysis, and no randomization or blinding protocol was used. GraphPad Prism 6 software was used for statistical analysis. Survival curves were analyzed according to the Kaplan–Meier estimator, and the difference between two groups was determined by the log-rank (Mantel–Cox) test. Statistical differences for other experiments were determined by a two-tailed t-test. P values of ≤0.05 were considered to represent means with a statistically significant difference. Statistical analysis was performed on groups with similar variance. Limited variance was observed within sample groups.