

Systemically dispersed innate IL-13–expressing cells in type 2 immunity

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Type 2 immunity is a stereotyped host response to allergens and parasitic helminths that is sustained in large part by the cytokines IL-4 and IL-13. Recent advances have called attention to the contributions by innate cells in initiating adaptive immunity, including a novel lineage-negative population of cells that secretes IL-13 and IL-5 in response to the epithelial cytokines IL-25 and IL-33. Here, we use IL-4 and IL-13 reporter mice to track lineage-negative innate cells that arise during type 2 immunity or in response to IL-25 and IL-33 in vivo. Unexpectedly, lineage-negative IL-25 (and IL-33) responsive cells are widely distributed in tissues of the mouse and are particularly prevalent in mesenteric lymph nodes, spleen, and liver. These cells expand robustly in response to exogenous IL-25 or IL-33 and after infection with the helminth *Nippostrongylus brasiliensis*, and they are the major innate IL-13–expressing cells under these conditions. Activation of these cells using IL-25 is sufficient for worm clearance, even in the absence of adaptive immunity. Widely dispersed innate type 2 helper cells, which we designate lh2 cells, play an integral role in type 2 immune responses.

helminth infection | IL-25 | IL-33 | *Nippostrongylus brasiliensis*

Type 2 immune responses are important for the control of infections at mucosal barriers and the development of allergic inflammation. These responses are characterized by eosinophilia, elevated IgE, goblet cell metaplasia with enhanced mucus production, and smooth muscle hyperreactivity, all of which rely critically on production of the canonical type 2-associated cytokines IL-4, IL-5, and IL-13 (1, 2). Although adaptive Th2 cells and follicular T cells are important sources of these cytokines (3), various innate cells, including eosinophils, basophils, and mast cells, have also been implicated as producers of these cytokines in various model systems (1, 2, 4, 5). More recently, the cytokines IL-25 and IL-33, members of the IL-17 and IL-1 cytokine families, respectively, were found to induce type 2 cytokine production when administered to mice, implicating these cytokines in the initiation of type 2 immune responses (6, 7). IL-25 and IL-33 are expressed by epithelial cells, macrophages, and possibly other cell types (8), and they are expressed at elevated levels during infection with parasitic helminths (9, 10) or after challenge with allergens (9, 11). Administration of exogenous IL-25 or IL-33 to mice leads to markedly enhanced levels of IL-4, IL-5, and IL-13 and many of the tissue features of a type 2 immune response (6, 7). Conversely, deficiency in IL-25 leads to diminished IL-4, IL-5, and IL-13 production and variable delays in worm clearance in different helminth models (12, 13). Similarly, mice unable to respond to IL-33 because of deficiency in the T1–ST2 subunit of the IL-33 receptor display diminished Th2-associated cytokines and decreased granuloma formation after injection of *Schistosoma mansoni* eggs (14).

Some of the original descriptions of these cytokines as well as more recent reports have noted the capacity of exogenous IL-25, IL-33, or helminth infection to induce the proliferation of a novel non-T/non-B cell population (6, 9, 12, 15–17). Although the surface phenotype of these cells has not been firmly established, there seems to be a consensus that these cells are negative for

standard lineage markers and have a size and morphology that suggests a lymphoid origin. Multiple reports also describe a role for these cells in producing and secreting the Th2-associated cytokines IL-4, IL-5, and IL-13, and function marking using cytokine reporter mice has contributed directly to investigations of these cells (12, 16, 17).

Here, we characterize these non-T/non-B lineage-negative cells at rest, after administration of exogenous IL-25 and IL-33, and during the course of infection with the helminth *Nippostrongylus brasiliensis*. We show that these cells are present in many organs at rest, expand after the addition of cytokines or during infection, and possess a distinct surface phenotype and gene-expression pattern. Furthermore, we show that these lineage-negative cells are the major innate IL-13–producing cells in each of these models and thus, are poised to play a significant role in type 2 immune responses.

Results

Lineage-Negative Cells Are Systemically Distributed in Resting Mice.

We used IL-4 reporter mice (4get) (18) to determine the phenotype and distribution of IL-4–expressing cells in resting mice. In these mice, the 3'UTR of the *il4* gene was modified to include an internal ribosomal entry site (IRES) followed by *egfp*, resulting in 5' cap-independent translation of GFP when the locus is activated, thus effectively marking IL-4–competent cells in situ (19). Cell types that are constitutively GFP+ in these mice include eosinophils, basophils, mast cells, natural killer T (NKT) cells, and Th2 cells (4, 5, 20). On closer analysis, an additional GFP+ cell type was apparent in multiple tissues of resting mice (Fig. 1A). These cells were small, side-scatter low cells resembling lymphocytes and were negative for surface markers that characterize T and NKT cells (including CD3, CD4, and CD8), eosinophils (Siglec-F and CD11b), basophils (CD49b, IgE, and CD131), and mast cells (IgE) (Fig. 1A).

Further examination of these cells revealed a surface phenotype characterized as lineage-negative for classic T, B, NK, myeloid, and dendritic cell markers (Fig. 1B) and c-kit low, Sca-1-negative, CD122 (IL-2Rβ) low, Ly5.2+, Thy1+, and CD44 high (Fig. 1C). As such, these resting cells resembled c-kit+ lineage-negative cells that were elicited in response to IL-25 or IL-33 in previous studies (6, 9, 12, 15, 16). In resting mice, we identified populations of these lineage-negative cells in all organs and tissues that we examined, except for the blood (Fig. 1D). The highest numbers of lineage-negative cells were present in the mesenteric lymph nodes, spleen, liver, and bone marrow, with fewer cells in the lung and peritoneum. Consistent with previous reports, lineage-

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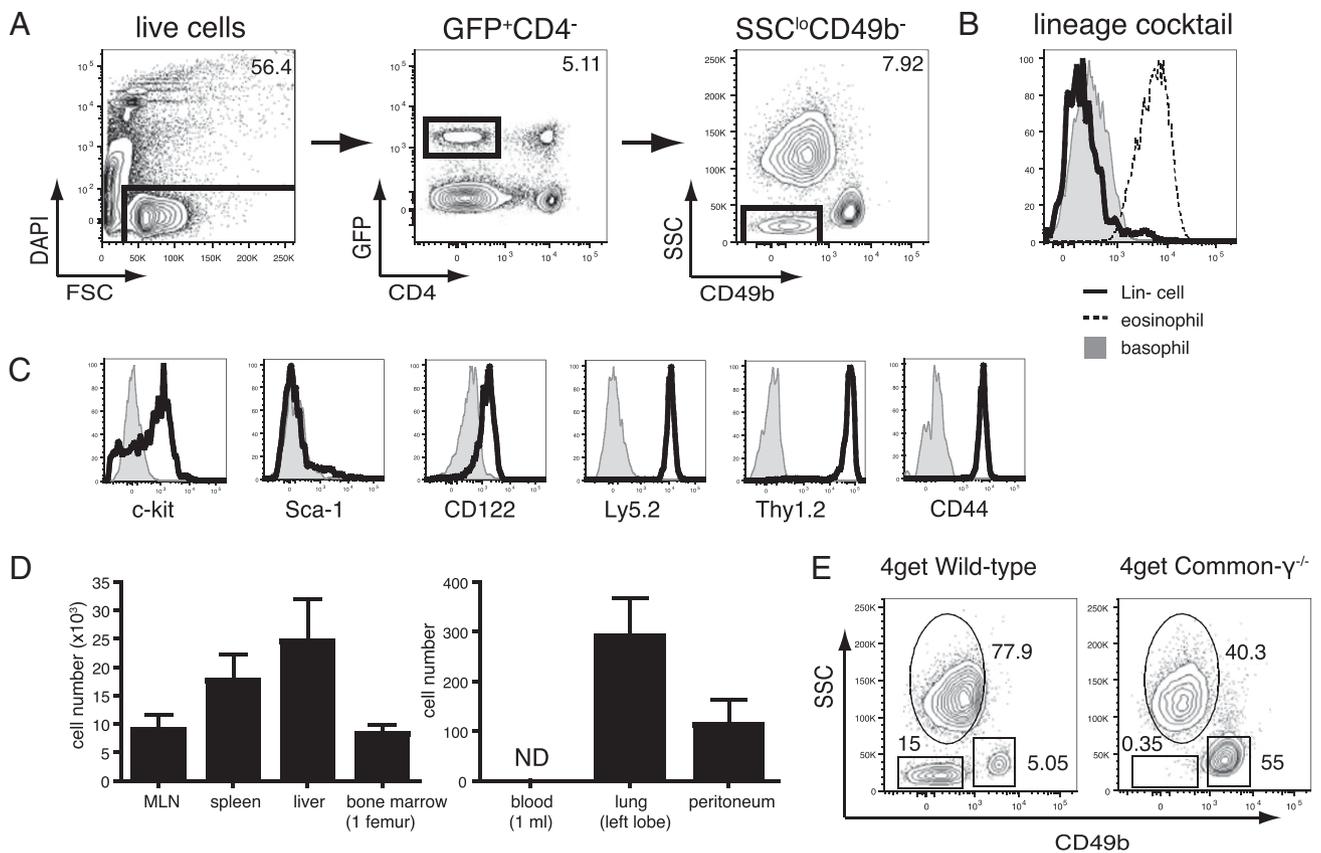


Fig. 1. Lineage-negative cells are systemically distributed at rest and possess a unique surface phenotype. (A) Gating scheme for lineage-negative cells in 4get mice. Example shown is resting liver. Box denotes lineage-negative gate. Numbers denote percentage of boxed cells of total. (B) Staining with a lineage-marker antibody mixture (CD3 ϵ , Ly-6G/Ly-6C, CD11b, CD45R, and TER-119). Eosinophils are used as a positive control, whereas basophils are used as a negative control. (C) Surface-marker antibody staining (black lines) compared with isotype control (gray) on lineage-negative cells from resting liver. (D) Numbers of lineage-negative cells in organs and tissues at rest. Experiment was independently repeated three times, and data were combined for graph ($n = 3\text{--}14$ mice per group). (E) Gating of lineage-negative cells in 4get mice compared with 4get \times common- $\gamma^{-/-}$ mouse liver. Lineage-negative cells collect in side-scatter (SSC)-lo, CD49b-negative gate. Numbers are percentages of total non-CD4 GFP-positive cells.

negative cells were readily recovered from tissues of Rag2 $^{-/-}$ mice lacking adaptive immune cells, Kit $^{W-sh/W-sh}$ mice lacking mast cells, and Δ dblGATA mice lacking eosinophils (Fig. S1). However, and as originally noted (6), these cells were completely absent in mice lacking the lymphocyte common- γ chain receptor, suggesting that these cells require signaling through a common- γ chain cytokine for their development and/or survival (Fig. 1E). Although more extensive analysis is required, we observed no cytokine expression or proliferation of these cells in vivo after administration of 500 ng of the individual γ c-binding cytokines IL-2, IL-4, IL-9, IL-13, IL-15, or IL-21 to 4get mice. Thus, lineage-negative IL-4-competent cells are distributed widely throughout tissues of resting mice and share phenotypic and developmental features with earlier described populations of lineage-negative innate immune cells that expand in response to IL-25.

Lineage-Negative Cells Expand in Response to Exogenous IL-25 or IL-33 and During Helminth Infection. The previously described lineage-negative cells were identified initially in lung and spleen (6, 9) and later in mesentery (12) by their capacity to expand in vivo in response to exogenous IL-25 or IL-33 and release IL-13 and IL-5 implicated in epithelial goblet cell metaplasia and eosinophilia, respectively. It was unclear whether the cells we describe in multiple organs share this common effector capacity. We administered 500 ng IL-25 or IL-33 on 4 consecutive d to 4get mice and monitored the kinetics of cell expansion and eosinophil accumulation in the respective tissues. After four doses of cytokine,

we documented dramatic increases in the numbers of lineage-negative cells and eosinophils in all organs examined (Fig. 2A). With kinetic studies, we showed that lineage-negative cells peaked in the mesenteric lymph node, spleen, and liver between days 3 and 4, whereas eosinophils continued to increase in the various organs throughout the 8-d time course (Fig. S2), suggesting that eosinophil recruitment is likely downstream of activation of these lineage-negative cells and presumably, reflects their IL-5- and IL-13-producing capacity. Importantly, these studies link these widely dispersed lineage-negative cells by their common responsiveness to IL-25 and IL-33 and by their effector capacity as revealed by eosinophil recruitment into multiple tissues throughout the body.

N. brasiliensis is an intestinal helminth that models the migratory pathway of human hookworm (21); s.c. injected larvae enter the vasculature, migrate to the lungs, molt, penetrate the alveoli, and ascend the trachea, where the worms are swallowed to complete maturation in the small bowel. Adult worms of this rat parasite are cleared in immunocompetent mice after 8–10 d by a Th2-orchestrated type 2 immune response (22). After infection with *N. brasiliensis*, the lineage-negative GFP+ cell population also increased in all organs examined, peaking in the mesenteric lymph node 5 d after infection and in the spleen and lung 2 d later (Fig. 2B). Thus, lineage-negative cells respond to both exogenous cytokines and helminth challenge by marked increase in numbers, consistent with a shared lineage for all of these cells, even in disparate organs. Furthermore, the eosinophil infiltration

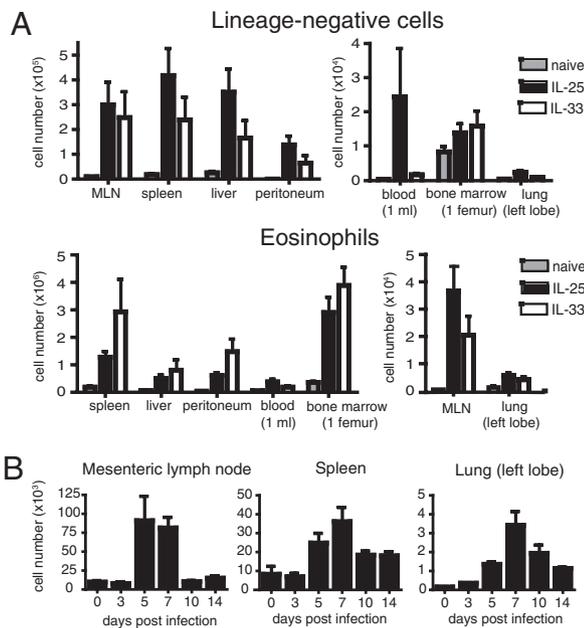


Fig. 2. Lineage-negative cells increase in number after IL-25 administration and during infection. (A) Numbers of lineage-negative cells and eosinophils in designated organs after four daily doses of 500 ng IL-25 or IL-33. (B) Numbers of lineage-negative cells after infection with *N. brasiliensis*. Experiments were repeated three times, and data were compiled for graphs ($n = 3-14$ mice per group). Bars are means with SEM.

induced in each of these organs in response to IL-25 or helminth infection supports their systemic activation to a common functional state after these challenges.

Lineage-Negative Cells Express a Distinct Transcriptome Compared with Other Type 2 Immune Cytokine-Secreting Cells. Prior investigations have identified Th2 cells and basophils as the major

type 2 cytokine-expressing cells in tissues from mice challenged with helminths (23). We sorted Th2 cells, basophils, and lineage-negative cells from tissues of *N. brasiliensis*-infected 4get mice and used microarray to compare their transcriptional profiles (Fig. 3A). By this analysis, lineage-negative cells could be independently grouped as a separate lineage and were readily distinct from Th2 cells or basophils. Each population had sets of uniquely expressed transcripts not expressed by the other lineages (Fig. 3B and C). Conversely, using stringent criteria, some effector populations also shared a core set of transcripts, consistent with their concordant activities in type 2 immune responses.

Lineage-Negative Cells Are the Major Innate IL-13-Expressing Cells After Administration of IL-25 and During *N. brasiliensis* Infection.

Despite the constitutive GFP fluorescence of the lineage-negative cells from 4get mice, it remained unclear whether these cells produce the cytokine IL-4 in vivo. To address this question, we used 4get \times KN2 mice, which express one 4get allele and one allele in which a modified human CD2 gene replaces the *il4* gene at the endogenous IL-4 start site (24). Cells from these mice can be used to track IL-4-secreting cells in vivo without the need for restimulation. Whether assessed at rest, after IL-25 challenge, or during the course of *N. brasiliensis* infection, however, we were unable to document significant human CD2 expression on lineage-negative cells from 4get \times KN2 mice, despite our ability to show robust human CD2 expression by Th2 cells collected from the same tissues (Fig. S3). Thus, we infer that lineage-negative cells do not produce IL-4 in vivo under physiologic conditions.

IL-13 plays an important and nonredundant role in type 2 immune responses (22, 25). After infection with *N. brasiliensis*, mice deficient in IL-4 have diminished Th2 cytokine responses but are able to clear worms (26). In contrast, mice deficient in IL-13 have a more profound deficit in worm clearance and show defects in goblet cell hyperplasia (22, 27). To gain insight into the expression and distribution of IL-13-producing cells in vivo, we generated IL-13 reporter mice, designated YFP-enhanced transcript with Cre recombinase at the *il13* gene (YetCre-13), by introducing an IRES followed by YFP-Cre recombinase fusion

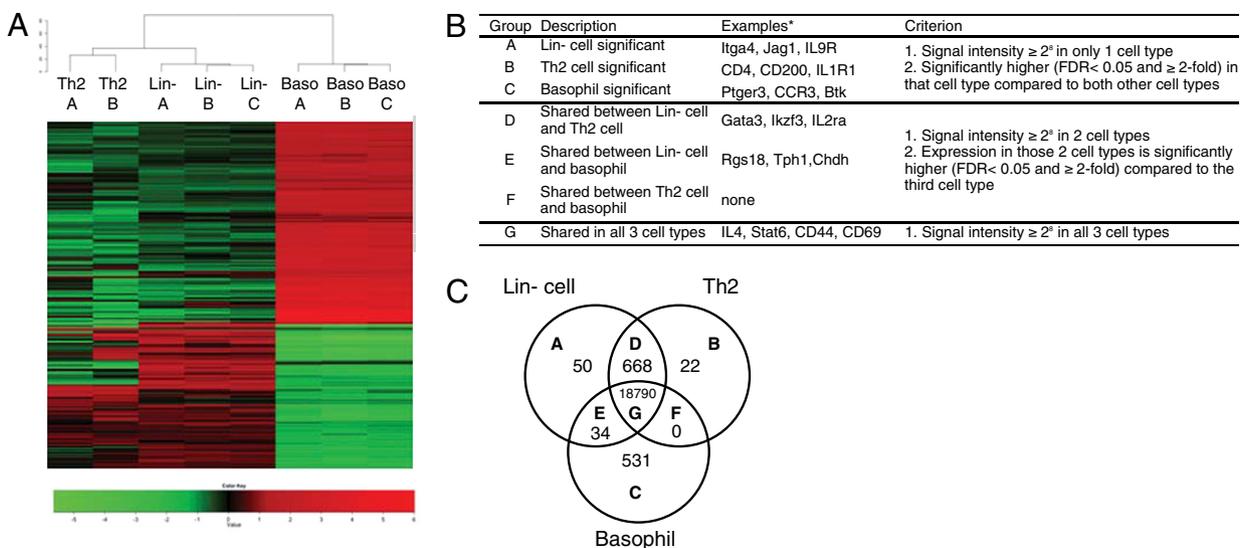


Fig. 3. Lineage-negative cells are a distinct Th2-associated cell type. (A) Relative gene expression in Th2 cells (two samples), lineage-negative cells (three samples), and basophils (three samples) isolated from *N. brasiliensis*-infected mice. Heat map shows log₂ (sample intensity/mean intensity for all eight samples) for all microarray probes with differential expression (false discovery rate (FDR) < 0.05) in any pairwise comparison between cell types. (B) Grouping of microarray probes based on their expression in lineage-negative cells, Th2 cells, and basophils. Signal intensity cutoff of 2⁸ is ~2³-fold higher than the median signal obtained with randomized negative-control probes. *, *Itga4*, integrin alpha 4; *Jag1*, Jagged 1; *IL9R*, IL-9 receptor; *IL1R1*, IL-1 receptor type 1; *Ptger3*, prostaglandin E receptor 3; *CCR3*, chemokine receptor 3; *Btk*, Bruton agammaglobulinemia tyrosine kinase; *Gata3*, GATA binding protein 3; *Ikzf3*, IKAROS family zinc finger 3 (*Aiolos*); *IL2ra*, IL-2 receptor alpha chain; *Rgs18*, regulator of G protein signaling 18; *Tph1*, tryptophan hydrolase 1; *Chdh*, choline dehydrogenase; *Stat6*, signal transducer and activator of transcription 6 (C). Diagram showing the numbers of probes in each of the seven groups listed in B.

protein at the start of the 3'UTR of the *il13* gene. To enhance detection of lineage-negative cells that activated the IL-13 locus, we crossed YetCre-13 mice to Rosa-floxed-Stop-YFP mice (28). In this way, any cells having activated the YFP-Cre fusion protein at any time will flox and activate the Rosa-YFP in a constitutive fashion, thus leaving a lineage mark that defines the prior activation of the *il13* gene.

Using the YetCre13 Rosa-YFP amplifier reporter mice to follow IL-13-expressing cells in response to IL-25, we observed only small numbers of YFP+ CD4+ T cells and substantial populations of innate YFP+ cells in multiple organs with the same surface and size phenotype as the lineage-negative cells defined using the 4get allele (Fig. 4A and B). Induction of IL-13 + lineage-negative cells also occurred after infection with *N. brasiliensis*, with similar kinetics and numbers of cells as previously shown using 4get mice (Fig. 4C). Using these IL-13 reporter mice, we found that, after IL-25 administration or during *N. brasiliensis* infection, the only significant population of innate cells that expressed IL-13 besides the lineage-negative cells was a minor population of mast cells in the peritoneum (Fig. 4D). Eosinophils and basophils did not express the IL-13 marker under these conditions (Fig. S4). Taken together, these data suggest that a substantial portion of the lineage-negative cells express IL-13 when activated in vivo during type 2 immune responses and that these cells are the major innate IL-13-producing cells in multiple tissues in the mouse. Additionally, when taken directly from mice after expansion and assayed for in-

tracellular IL-5 production, a substantial proportion of lineage-negative cells also produced IL-5, consistent with their effects on tissue eosinophils after activation (Fig. S5).

Lineage-Negative Cells Contribute to Worm Clearance and Tissue Eosinophil Accumulation. To confirm that IL-13 production constitutes a stereotyped response by the lineage-negative cells in all organs, we crossed YetCre-13 mice to Rosa-flox-Stop-diphtheria toxin α (Rosa-DTA) mice (29). In cells from these animals, activation of *il13* transcription generates YFP-Cre fusion protein that will excise the flox-stop cassette and lead to the production of diphtheria toxin α , thus killing IL-13-producing cells. These mice were additionally crossed onto a heterozygous 4get background, enabling the tracking of the total numbers of lineage-negative cells using surface markers and GFP fluorescence. After administration of IL-25, we noted a substantial decrease in the numbers of recovered lineage-negative cells in the YetCre13-Rosa-DTA mice compared with littermate controls in all organs assayed, including the mesenteric lymph nodes, spleen, liver, and peritoneum (Fig. 5A and B). Furthermore, the decrease in lineage-negative cells was accompanied by a marked decrease in the numbers of infiltrating eosinophils. Although the deleter allele will similarly delete IL-13-producing Th2 cells, the small numbers of these cells induced after IL-25 suggest that lineage-negative cells are the major mediators of eosinophil recruitment after administration of this cytokine.

To establish that lineage-negative cells alone could reconstitute functional aspects of antihelminth immunity, we corroborated prior findings (12, 16) that exogenous IL-25 could mediate worm

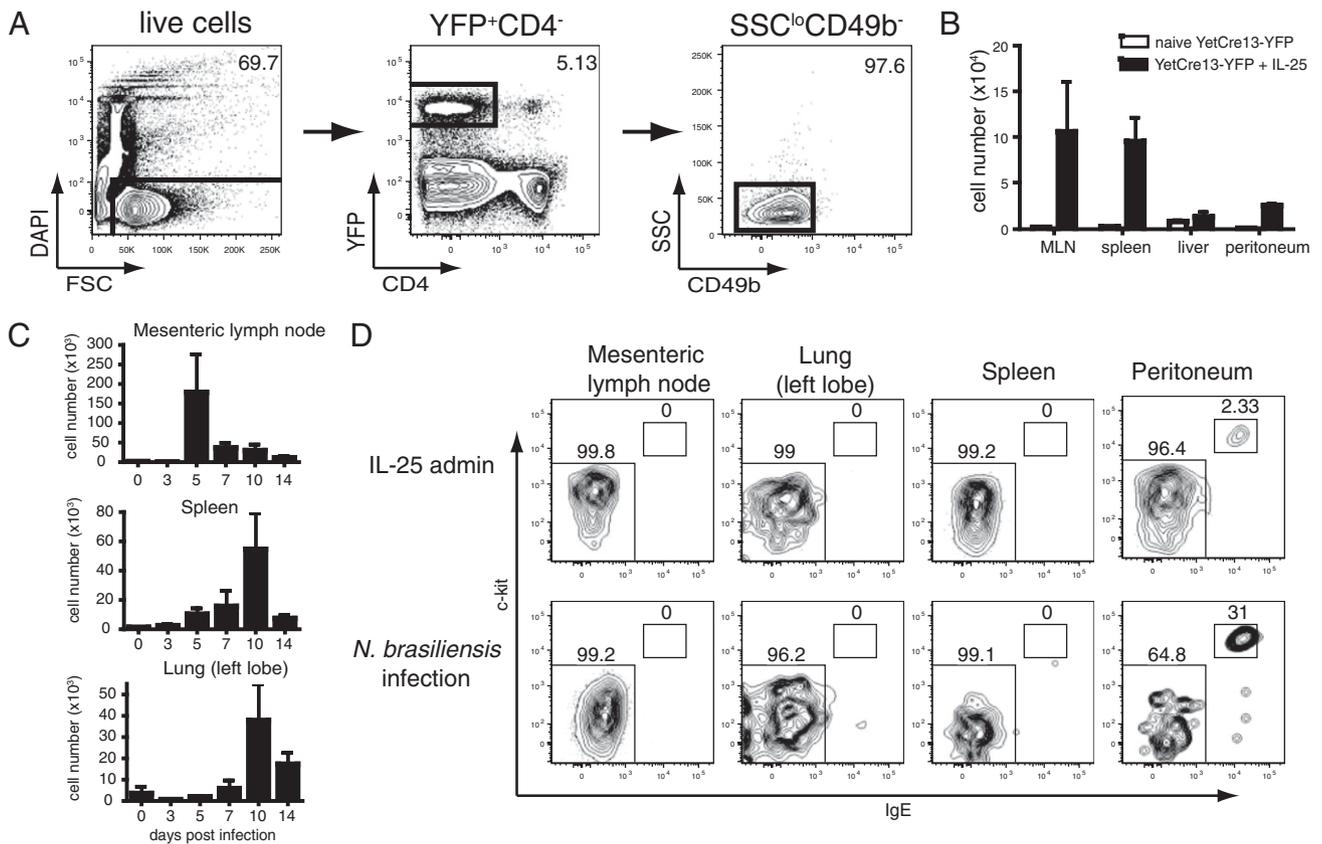


Fig. 4. Lineage-negative cells are major innate IL-13-expressing cells. (A) Gating of YFP+ lineage-negative cells from YetCre13 Rosa-YFP amplifier mice. Example shown is of liver after administration of IL-25. Gates and percentages are as in Fig. 1. (B) Numbers of YFP+ lineage-negative cells after IL-25 administration. Experiment was repeated two times, and results were compiled ($n = 2-3$ mice per group). Bars are means with SEM. (C) Time course showing YFP+ lineage-negative cells from designated organs during infection with *N. brasiliensis*. Experiment was repeated two times, and results were compiled ($n = 3-5$ mice per group). Bars are means with SEM. (D) Gating of non-CD4+ YFP+ cells showing percentages of mast cells (c-kit^{hi}IgE⁺) and lineage-negative (c-kit^{lo}IgE⁻) cells in multiple organs after IL-25 administration (Upper) or after infection with *N. brasiliensis* (Lower).

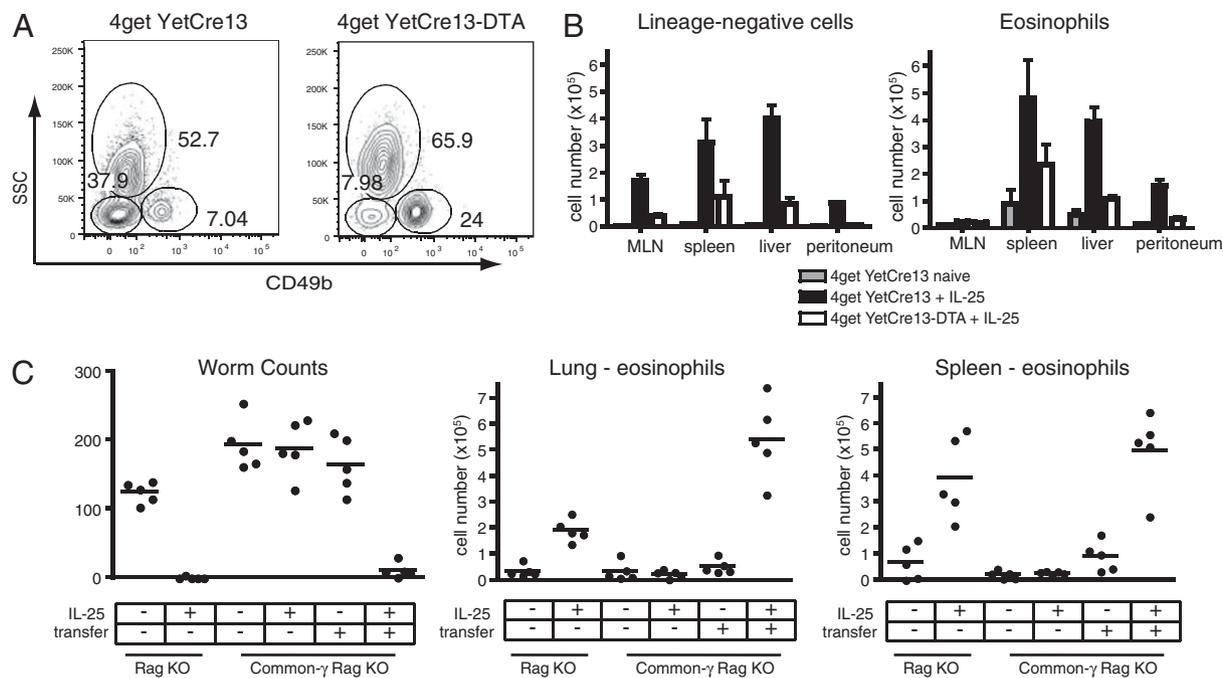


Fig. 5. Lineage-negative cells contribute to eosinophilia and mediate IL-25-dependent worm clearance. (A) Gating of GFP⁺CD4⁻ cells in IL-25-treated 4get YetCre13-Rosa-DTA deleter mice or 4get YetCre13 littermates. Percentages shown are eosinophils (SSC^{lo}CD49b^{lo}), basophils (SSC^{lo}CD49b^{hi}), and lineage-negative cells (SSC^{hi}CD49b⁻). (B) Numbers of lineage-negative cells and eosinophils in multiple organs in 4get YetCre13-DTA deleter mice or 4get YetCre13 controls after administration of IL-25. Experiment was repeated two times, and results were compiled (n = 3–5 mice per group). Bars denote means with SEM. (C) Rag2^{-/-}, common-γ^{-/-} × Rag2^{-/-}, or common-γ^{-/-} × Rag2^{-/-} mice adoptively transferred with 500,000 lineage-negative cells 2 d previously were infected with *N. brasiliensis*. Five mice from each group received IL-25 on days 0–4. Worm counts from the small intestine and numbers of eosinophils (SSC^{hi}CD11b⁺Siglec-F⁺) were quantified on day 5. Experiment was repeated two times, and a representative experiment is shown.

clearance and eosinophil tissue infiltration in Rag 2^{-/-} mice infected with *N. brasiliensis* (Fig. 5C). In contrast, administration of IL-25 to common-γ^{-/-} × Rag2^{-/-} mice, which lack both adaptive immunity and lineage-negative cells did not lead to worm clearance or significant increases in tissue eosinophils. To confirm that the failure to mediate these effects was caused by the absence of lineage-negative cells, we adoptively transferred 5 × 10⁵ lineage-negative cells into the common-γ^{-/-} × Rag 2^{-/-} mice 2 d before infection with *N. brasiliensis*. When assessed 5 d after infection, lineage-negative cells alone had no effect on worm numbers or eosinophil tissue infiltration, consistent with the need for an additional signal regulating their activation during infection. Indeed, adoptively transferred lineage-negative cells substantially rescued IL-25-dependent worm clearance and eosinophil tissue infiltration in common-γ^{-/-} × Rag 2^{-/-} mice, showing that these cells are necessary and sufficient to integrate IL-25 signals that mediate these downstream pathways.

Discussion

IL-4 and IL-13 play central roles in the orchestration of anti-helminth immunity and allergic responses. Originating from a gene duplication, these cytokines bind to shared and disparate receptors and mediate many overlapping downstream effector pathways (30, 31). Despite these observations, these cytokines also have more dedicated functions during a type 2 response, with IL-4 facilitating humoral IgG1 and IgE production (3) and IL-13 contributing to epithelial hyperplasia and eosinophil recruitment in peripheral tissues (32). As such, efforts to understand where and how IL-4 and IL-13 are produced in tissues will be important in understanding the dichotomous roles for these cytokines in barrier and allergic immunity.

Here, we use knockin reporter mice to identify IL-4- and IL-13-expressing cells that accumulate in tissues in response to ex-

ogenous cytokines, IL-25 and IL-33, and intestinal worm infection. We confirm prior findings that a unique lineage-negative cell is a target of IL-25 and IL-33, that activation of these cells is accompanied by their expansion and their expression of IL-13 and IL-5, and that activation of these cells is sufficient to mediate the major peripheral effects of IL-25, including eosinophilia and worm clearance (6, 9, 12, 15, 16). We extend these findings to show that cells of this same phenotype populate many organs in the resting mouse and are particularly prevalent in the mesenteric lymph node, spleen, and liver. Importantly, all of these populations share a similar surface phenotype and functional response, suggesting that they represent a common lineage of cells that is distributed throughout the body where they can integrate signals mediated by IL-25, IL-33, and possibly other cytokines. In deference to the original observations of these cells by investigators at the prior DNAX Research Institute as well as the historical designation of Th1 and Th2 cells at that institute, we designate these cells innate helper type 2 cells (Ih2 cells), although alternative names have been suggested for similar cells by other investigators (15–17).

Despite the potent production of IL-13 and IL-5, we could not show production of IL-4 by Ih2 cells in vivo. We used sensitive reporter mice to show that these cells do not produce IL-4 in vivo, even under conditions where Th2 cells in tissues can be readily shown to be IL-4-secreting. After administration of IL-25, IL-33, or during *N. brasiliensis* infection, Ih2 cells were the predominant IL-13-expressing cells in all tissues examined. As such, the widespread effects of IL-13 deficiency on tissue manifestations of type 2 immunity may be explained by the functional deficit conferred on these peripherally arrayed Ih2 cells.

The widespread distribution of Ih2 cells raises questions regarding their trafficking and survival in peripheral tissues. Despite a sensitive marker for these cells, we did not detect Ih2 cells

in blood, consistent with rapid transit through blood to tissue or local expansion from tissue-restricted precursors. Further work will be required to address these possibilities. Additionally, the sources of IL-25 or IL-33, or possibly other cytokines, that lead to activation of these cells during physiologic responses will be important areas of investigation. Lastly, the potential exists for a fundamental homeostatic role for these cells in regulating the status of peripheral organs and tissues by cytokine-mediated interactions with resident and recruited cells. As such, aberrant activation of tissue-resident Th2 cells may contribute to the chronic nature of allergic diseases such as atopy and asthma, and further assessment of their role in such diseases will be aided through use of the reporter mice that we describe.

Finally, it will be important to determine the relationship of Th2 cells to innate cells associated with type 2 immunity defined in prior reports. The functional identification of Th2 cells as a major IL-13-producing population is consistent with the original description of these cells (6) as well as more recent studies using IL-13 reporter mice (16). We have not noted organization of Th2 cells into the adipose-associated structures reported to be present in the mesentery (15), although further study will be required to more precisely determine the localization of Th2 cells in peripheral tissues. A report by Saenz et al. (17) described cells with a similar surface phenotype in mesenteric lymph nodes that may have a precursor relationship with myeloid lineages involved in type 2 immunity. In contrast, the IL-13-producing

cells we and others describe have properties suggesting a lymphoid lineage, including, as shown here, the shared expression of Aiolos with Th2 cells (33). Further work is certainly needed to understand the interrelationships between these intriguing innate cell populations.

Methods

Mice. IL-4 reporter (4get, KN2) (18, 24), mast cell-deficient Kit^{W-sh/W-sh} (34), and eosinophil-deficient Δ dblGATA (35) mice have been described. Rag2^{-/-} and common- γ ^{-/-}Rag2^{-/-} mice on the C57BL/6 background were purchased (Taconic) and bred to the 4get background as noted. Detailed information on the generation of IL-13 reporter mice is in *SI Methods*.

Cytokine Injections. Mice were injected intraperitoneally on 4 consecutive d with 500 ng IL-25 or IL-33 (R&D Systems) where designated.

***N. brasiliensis* Infection.** Infection with *N. brasiliensis* third-stage larvae (L3) was as described (4). Where noted, mice received an i.v. injection of 5×10^5 lineage-negative cells sorted from IL-25-stimulated mice 2 d before infection and/or were treated with daily i.p. injections of 500 ng IL-25 over the first 4 d of infection.

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