Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity

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Interleukin 4 (IL-4) and IL-13 are critical for responses to parasitic helminthes. We used genetically engineered reporter mice to assess the temporal and spatial production of these cytokines in vivo. In lymph nodes, IL-4, but not IL-13, was made by follicular helper T cells (T FH cells). In contrast, tissue type 2 helper T cells (T H2 cells) produced both cytokines. There was also divergent production of IL-4 and IL-13 among cells of the innate immune system, whereby basophils produced IL-4, whereas innate helper type 2 cells (Ih2 cells) produced IL-13. IL-13 production by T H2 and Ih2 cells was dependent on the transcription factor GATA-3, which was present in large amounts in these cells, and in contrast to the small amount of GATA-3 in T FH cells and basophils.

The distinct localization and cellular expression of IL-4 and IL-13 explains their unique roles during allergic immunity.

Intestinal helminth infections represent one of the most prevalent chronic human infections worldwide1. Infection is associated with a polarized immune response, including the accumulation of type 2 helper T cells (T H2 cells), eosinophils and basophils in tissues, more serum immunoglobulin E (IgE), hyperplasia of mucosal mast cells, alternative activation of tissue macrophages and epithelial and smooth-muscle alterations that change the physiological milieu of the host-mucosal interface where the parasites mediate their damage2. Similar alterations occur in allergic diseases, such as allergic asthma, although in this case the immune response is focused inappropriately on ubiquitous environmental allergens3. These host responses are frequently referred to as ‘type 2 immunity’ and are critically dependent on interleukin 4 (IL-4) and IL-13. The identification of an additional cell of the innate immune response involved in these types of host responses has added further complexity to the functional organization of cytokine-producing cells in type 2 immunity4–8.

T H2 cells express IL-4 and IL-13, and the localization of these cytokines to sites of allergic immunity initiated efforts to link these cytokines themselves to mediating the diverse tissue manifestations associated with type 2 immunity. Indeed, whether administered exogenously, overexpressed or deleted, these two cytokines are necessary and sufficient to mediate most of the immunological and physiological aspects of type 2 immunity2,9–13. IL-4 and IL-13 are encoded by adjacent genes that share many cis-acting and trans-activating regulatory elements, and they transmit signals through a partially shared receptor and adaptor system14,15. Despite that, epigenetic and functional studies have suggested unique and non-redundant roles for these cytokines during immunity in vivo, although the mechanisms that underlie these distinctions are not readily apparent on the basis of in vitro studies2,15–17. Many innate and semi-invariant lymphocytes, including basophils, eosinophils, mast cells, innate helper type 2 cells (Ih2 cells) and natural killer T cells (NKT cells), can also be activated to produce IL-4 and IL-13 in vitro, but direct visualization of cytokine production by these cells in vivo has been incompletely characterized4,7,18,19. One model that could explain the disparate in vitro and in vivo data is compartmentalized expression and divergent regulation of these two cytokines in vivo, a process that is difficult to examine with the tools available at present. Further understanding of the organization of type 2 immunity in secondary lymphoid tissues such as lymph nodes and spleen and in nonlymphoid tissues such as lung, liver, skin and small intestine will require improved methods for assessing cytokine production in vivo and in situ.

We generated a series of new strains of mice with genetically altered Il4 and Il13 loci that allow detection of cytokine expression by individual cells in vivo. Through the use of infections that induce robust type 2 host responses, we compared the production of IL-4 and IL-13 in selected lymphoid and tissue compartments. We found unexpected parsing of expression of these two closely related cytokines among cells of both the adaptive and innate immune responses. IL-13 production was largely confined to T H2 cells and Ih2 cells in the lung and was associated with large amounts of cellular transcription factor GATA-3, which was necessary for sustaining the IL-13-producing phenotype. Conversely, follicular helper T cells (T FH cells) and basophils produced only IL-4 in vivo and did not have high expression of GATA-3.

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RESULTS

Compartmentalization of expression of IL-13 and IL-4

IL-4 and IL-13 share an overlapping spectrum of receptors, but studies with neutralizing reagents and mice deficient in individual cytokines and cytokine receptors have suggested a more prominent role for IL-13 in mediating the tissue responses that occur during infection with the helminth *Nippostrongylus brasiliensis*. Despite that information, precise knowledge about which cells produce IL-4 and IL-13 and their localization in vivo is lacking. We used a genetic approach to avoid caveats associated with restimulation of cells in vitro or ex vivo, such as the activation of all cytokine-competent cells, which can potentially obscure the physiological functions in situ. We used published IL-4 reporter mice that have a nonsignaling gene encoding human CD2 (hucDC2) in place of the endogenous Ifi4 sequence at the Ifi4 start site (Ifi4Kn2 mice). Cells activated to produce IL-4 in Ifi4Kn2 mice express hucCD2 on the cell membrane and provide an accurate report of IL-4-producing cells in vitro and in vivo.20,21 Homozygous Ifi4Kn2/Ifi4Kn2 mice are IL-4 deficient, whereas heterozygous Ifi4Kn2+ mice are able to express IL-4 from the endogenous Ifi4 allele. Additionally, we generated IL-13 reporter mice with sequence encoding a fusion protein of enhanced yellow fluorescent protein (eYFP) and humanized Cre recombinase behind an internal ribosomal entry site (IRES) element introduced immediately downstream of the endogenous Il13 stop codon and upstream of the 3′ untranslated sequence (YetCre13). This construct leaves endogenous Il13 intact while establishing a bicistronic link with the sequence encoding the eYFP-Cre fusion protein. Cells from II13YetCre/YetCre mice are able to make both endogenous IL-4 and IL-13. Crossing Il4Kn2/Il4Kn2 mice with II13YetCre mice allows simultaneous detection of IL-4- and IL-13-expressing cells (Fig. 1a, Table 1 and Supplementary Fig. 1). The introduction of an additional ROSA26-laxP-flanked stop sequence—diphtheria toxin-α (ROSA-DTα) allele allowed in vivo elimination of cells that had activated Il13 expression, as expression of the eYFP-Cre fusion protein removed the laxP-flanked stop sequence, which enabled toxin expression to ensue.22 (Fig. 1a, Table 1 and Supplementary Fig. 1).

To evaluate type 2 immunity in the various genetic strains of mice described above, we assessed the worm burden in the small intestine 9 d after infection with *N. brasiliensis* (Fig. 1a,b and Table 1). Consistent with published observations,23 IL-4–deficient Il4Kn2/Il4Kn2 mice expelled worms normally. Immunity to *N. brasiliensis* was also intact in Il4Kn2/Il13YetCre mice and II13YetCre mice (Fig. 1b). However, in II13YetCre/YetCre mice, worms were present at 9 d after infection in mice with one endogenous Il13 allele and one YetCre13 allele (II13YetCre/+) and were twice as prevalent in III13YetCre/YetCre ROSA-DTα mice, in which two YetCre13 alleles were present to drive Cre-mediated deletion (Fig. 1b).

Although the absence of IL-4 in II13YetCre/YetCre mice had no effect on the recruitment of eosinophils to tissues, deletion of IL-13-producing cells led to the attenuation of eosinophil accumulation in mice with one or two YetCre13 alleles paired with ROSA-DTα (Fig. 1c). Eosinophils did not produce IL-13 because they did not

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express eYFP in Il13YetCre mice (as analyzed by flow cytometry), nor were these cells deleted from the blood, bone marrow or tissues of Il13YetCreYetCreROSA-DTα mice (Supplementary Fig. 2). The deletion of IL-13-producing cells in Il13YetCreYetCreROSA-DTα mice led to the concomitant ablation of IL-5, as these cytokines were coexpressed in this system (Supplementary Fig. 3). It is likely that loss of both of these cytokines accounted for the lower eosinophilia in these mice. In contrast to the effects on eosinophils, the number of basophils and CD4+ T cells in the lungs of Il13YetCreYetCreROSA-DTα mice was unaffected by deletion of IL-13-expressing cells (Fig. 1d). Thus, IL-13-producing cells were required for sustaining tissue immunity to N. brasiliensis, and results obtained with these genetic variant strains confirmed published observations while confirming the utility of these reporter mice2,9,11,23.

IL-4 was required for IgE production, as Il4KN2/KN2 mice were unable to mount an IgE response (Fig. 1e). However, deletion of IL-13-producing cells caused no deficit in IgE production, which suggested that IL-4-expressing Tfh cells do not activate Il13 expression. To examine the effects of IL-13 expression on IL-4-mediated humoral responses, we immunized the various genetically marked mice with chicken ovalbumin conjugated to 4-hydroxy-3-nitrophenyl-acetyl (NP) in alum and assessed total IgG1 antibody to NP (anti-NP) and IL-13-expressing cells were used differently during immune responses, we intermingled in tissues at sites of allergic inflammation. Thus, IL-4- and IL-13-expressing cells were used differently during immune responses. CD4+ T cells in B cell–rich areas of the lymph nodes did not express IL-13. Tissue Tfh cells, however, expressed combinations of IL-4 and IL-13, with IL-5 production confined mainly to a subset of IL-13-expressing cells. To assess whether this divergent expression of IL-4 and IL-13 was a lung-specific phenomenon, we examined the intestines and mesenteric lymph nodes after infection with N. brasiliensis. Similar to results obtained for the lungs and lung-draining lymph nodes, CD4+ T cells isolated from the mesenteric lymph nodes produced substantial IL-4, whereas production of IL-13 was restricted to the small intestines (Supplementary Fig. 6). These findings showed that IL-4 and IL-13 served distinct functions during allergic immunity in part due to their restricted expression patterns in lymphoid and nonlymphoid tissues.

**Table 1 Genotypes and resulting phenotypes after infection with N. brasiliensis**

| Genotype | Active IL-4 alleles | Deletion of IL-13-producing cells | Cytokines produced | Worm clearance | Eosinophil recruitment | IgE production |
|----------|---------------------|-----------------------------------|--------------------|---------------|------------------------|---------------|
| Il4KN2/KN2 Il13+/+ | 0                   | No                                | IL-13              | Yes           | Yes                    | No            |
| Il4KN2/KN2 Il133Smart+/+ | 1                  | No                                | IL-4 + IL-13       | Yes           | Yes                    | Yes           |
| Il4KN2/KN2 Il133Smart+/+ | 1                  | Yes                               | IL-4              | No            | No                     | Yes           |
| Rosa-DTα Il4+/+ Il133Smart+/+ YetCre | 2                  | No                                | IL-4 + IL-13       | Yes           | Yes                    | Yes           |
| Rosa-DTα Il4+/+ Il133Smart+/+ YetCre | 2                  | Yes                               | IL-4              | No            | No                     | Yes           |

Cell fate, cytokine expression, worm clearance, eosinophil recruitment and IgE production in reporter mice 9 d after infection with N. brasiliensis. Data are representative of at least two experiments.

**Visualization of the expression of IL-4 and IL-13 in vivo**

We generated a second strain of IL-13 reporter mice by replacing the eYFP-Cre fusion protein used in the Il13YetCre reporter with a nonsignaling human CD4 marker, which provided a cell surface membrane-anchored reporter with which to identify IL-13-expressing cells (Smart13 (surface marker for the transcript of Il13); Supplementary Fig. 4). We backcrossed these Il133Smart13 mice to the BALB/c background and then crossed them with Il4KN2 mice, which enabled simultaneous detection of the expression of both IL-4 and IL-13 at the single-cell level. After infecting Il4KN2 Il133Smart+/+ mice with N. brasiliensis, we quantified IL-4- and IL-13-producing CD4+ T cells in the mediastinal lymph nodes and lungs directly after isolation (Fig. 2a,b).

In the lymph nodes, approximately 8% of CD4+ T cells expressed IL-4 and IL-13, and of those, 98% expressed only IL-4 and not IL-13. In the lungs, approximately 15% of CD4+ T cells expressed IL-4 and IL-13 alone or together, and the number of IL-4-expressing CD4+ T cells and IL-13-expressing CD4+ T cells was approximately equal (Fig. 2b). Thus, the ratio of IL-4-producing cells to IL-13-producing cells was significantly greater in the lymph nodes than in the lungs (Fig. 2c). Of note, after restimulation in vitro, lung IL-5-producing CD4+ T cells mostly coexpressed IL-13 but not IL-4 (Supplementary Fig. 3), which suggested that IL-13 and IL-5 share similar regulatory pathways that may be distinct from that of IL-4.

Immunohistochemical staining confirmed the higher ratio of IL-4-producing cells to IL-13-producing cells in the lymph nodes than in the lungs (Fig. 2d and Supplementary Fig. 5). As reported before21, IL-4-producing hUCD2+ cells were Tfh cells confined to follicular and germinal center B cell–rich areas. The few IL-13-producing cells observed after acute infection with N. brasiliensis or more chronic infection with Leishmania major resided almost exclusively in the T cell zones and parafollicular areas of the lymph node (Fig. 2d and Supplementary Fig. 5). In contrast, IL-4- and IL-13-producing CD4+ T cells were intermingled in tissues at sites of allergic inflammation. Thus, IL-4- and IL-13-expressing cells were used differently during immune responses. CD4+ T cells in B cell–rich areas of the lymph nodes did not express IL-13. Tissue Tfh2 cells, however, expressed combinations of IL-4 and IL-13, with IL-5 production confined mainly to a subset of IL-13-expressing cells. To assess whether this divergent expression of IL-4 and IL-13 was a lung-specific phenomenon, we examined the intestines and mesenteric lymph nodes after infection with N. brasiliensis. Similar to results obtained for the lungs and lung-draining lymph nodes, CD4+ T cells isolated from the mesenteric lymph nodes produced substantial IL-4, whereas production of IL-13 was restricted to the small intestines (Supplementary Fig. 6). These findings showed that IL-4 and IL-13 served distinct functions during allergic immunity in part due to their restricted expression patterns in lymphoid and nonlymphoid tissues.

**Tfh cells are distinct from Tfh2 cells and do not produce IL-13**

We next crossed each of the Il4KN2 reporter mice and the Il133Smart reporter mice to the 4get IL-4–reporter strain of mice (Il44get), which allows transcription of bicistronic mRNA encoding IL-4, followed by an IRES and mRNA encoding enhanced green fluorescent protein (eGFP), and translation of both IL-4 and eGFP from the same mRNA. The progeny that result from these crosses allow marking of IL-4- or IL-13-producing cells among the larger subset of IL-4- and IL-13–competent cells24. At 8 d after infection with N. brasiliensis, approximately half of the ‘competent’ eGFP+ CD4+ T cells in the mediastinal lymph nodes of Il4KN2/4get mice expressed IL-4 *in situ* (huCD2+), and about 40% of those IL-4–producing cells had high expression of the Tfh1 cell markers CD279 (PD-1) and CXCR5 (refs. 25–27; Fig. 3a,b). In contrast, few of the eGFP+ ‘competent’ CD4+ T cells expressed IL-13 in the lymph nodes and none of those cells expressed IL-13 and IL-5 together (Fig. 3a,b). Thus, the ratio of IL-4-producing cells to IL-13-producing cells was significantly greater in the lymph nodes than in the lungs (Fig. 2c). Of note, after restimulation in vitro, lung IL-5–producing CD4+ T cells mostly coexpressed IL-13 but not IL-4 (Supplementary Fig. 3), which suggested that IL-13 and IL-5 share similar regulatory pathways that may be distinct from that of IL-4.
IL-13-producing cells expressed T<sub>FH</sub> cell markers. Among the IL-13-expressing CD4<sup>+</sup> T cells in the lung, none had expression of CD279 (PD-1) and CXCR5 similar to that of lymph node IL-4-producing T<sub>FH</sub> cells. Thus, IL-13-expressing T cells did not express T<sub>FH</sub> markers and did not localize to B cell areas. Furthermore, CD4<sup>+</sup> T cells with markers characteristic of T<sub>FH</sub> cells were not present among the cytokine-expressing T<sub>H2</sub> cells in the lung.

To more completely characterize the differences between cytokine-expressing T<sub>FH</sub> cells and T<sub>H2</sub> cells, we sorted populations of cytokine-competent and cytokine-producing cells from the mediastinal lymph nodes and lungs of Il4<sup>−/−</sup>Il13<sup>−/−</sup> mice after infection with N. brasiliensis and analyzed IL-4 and IL-13 transcripts by quantitative PCR. Although IL-4-producing cells taken from the lymph nodes expressed less IL-4 and IL-13 mRNA than did their lung counterparts, they expressed more IL-21, a cytokine associated with TFH cells. Consistent with published reports on cytokine production by T<sub>FH</sub> cells during parasitic and viral infections, IL-4-producing cells from the lymph node had substantial expression of both IL-4 and IL-21 mRNA (Fig. 3c), which suggested that T<sub>FH</sub> cells are poised to make IL-4 as well as IL-21 during a type 2 immune response. IL-4-producing cells from the lymph nodes also had much higher expression of Bcl-6, a key transcription factor in T<sub>FH</sub> cell development, than did IL-4-producing cells isolated from the lungs. Conversely, lung IL-4-producing cells 'preferentially' expressed Blimp-1, a negative regulator of Bcl-6. Bcl-6 expression was inversely correlated not only with Blimp-1 transcripts but also with GATA-3 transcripts. Whether assessed by 'competence' (eGFP expression from the 4get allele) or 'expression' (huCD2 expression from the Il4<sup>kn2</sup> allele), IL-4-expressing lymph node CD4<sup>+</sup> T cells had much lower GATA-3 mRNA expression and a greater abundance of Bcl-6 transcripts than did tissue IL-4- or IL-13-expressing T<sub>H2</sub> cells. We also found that inverse relationship in protein expression by intracellular staining and flow cytometry. Bcl-6<sup>+</sup> cells were present only in lymph nodes, and Bcl-6<sup>+</sup> CD4<sup>+</sup> T cells were almost completely absent from lung tissue (Fig. 3d). The GATA-3<sup>hi</sup> cells did not express Bcl-6 in either tissue (Fig. 3d). Furthermore, most IL-4-producing cells in the lymph nodes had high expression of Bcl-6, which confirmed their identity as T<sub>FH</sub> cells, but had little GATA-3, whereas IL-13-producing cells were uniformly GATA-3<sup>-hi</sup> and Bcl-6<sup>-lo</sup> (Supplementary Fig. 7).

These results suggested that T<sub>FH</sub> cells and T<sub>H2</sub> cells have distinct and nonoverlapping profiles of transcription factor expression and probably use different transcription factors in different ways to achieve cytokine activation from the Il4 and Il13 loci.

**Cytokine-producing T<sub>H2</sub> cells are GATA-3<sup>-hi</sup>**

Although GATA-3 was originally linked to the production of IL-4 by T<sub>FH</sub> cells during helminth infection, studies of mRNA expression during infection with lymphocycte choriomeningitis virus have suggested that this might not be the case. To further explore the relationship between intracellular expression of GATA-3 and expression of IL-4 and IL-13 in T<sub>FH</sub> and T<sub>H2</sub> cells, we isolated T<sub>FH</sub> and T<sub>H2</sub> cells from Il4<sup>kn2</sup>Il13<sup>3mat</sup> dual reporter mice after infection with N. brasiliensis and assessed the expression of intracellular GATA-3 relative to in vivo cytokine expression at the single-cell level. In lymph nodes, the IL-4-producing CD4<sup>+</sup> T cells were predominantly GATA-3<sup>-hi</sup>. Only 20% of the total IL-4-producing cells had high expression of intracellular GATA-3 in the lymph nodes (Fig. 4). However, this was not the case in the lungs, in which over 70% of IL-4 production was associated with a GATA-3<sup>-hi</sup> population. We also observed this difference in the expression of GATA-3 in lymphoid and nonlymphoid tissues among gut and mesenteric lymph node–resident IL-4-producing CD4<sup>+</sup> T cells (Supplementary Fig. 8). The relationship between IL-13 production and GATA-3 was distinctly different because over 90% of the IL-13-producing cells were GATA-3<sup>−hi</sup> regardless of whether the CD4<sup>+</sup> T cells were isolated from the lymph nodes (in which there were very few cells) or the lungs. Thus, IL-4 expression by T<sub>FH</sub> cells was associated with low expression of GATA-3, whereas expression of IL-13 by T<sub>H2</sub> cells was associated with large amounts of intracellular GATA-3 (Figs. 3c,d and 4) independently of the tissue origin of the IL-13-expressing CD4<sup>+</sup> T cells.

**IL-4 and IL-13 are not required for high GATA-3 expression**

Signals from IL-4 and IL-13 can directly drive GATA-3 expression, leading to autoactivation and a feed-forward loop that sustains the

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**Figure 2.** Localization and number of cytokine-producing CD4<sup>+</sup> T cells during parasitic infection. (a) Frequency of CD4<sup>+</sup> T cells positive for IL-4 (huCD2) or IL-13 (huCD4) in the lungs and mediastinal lymph nodes (LN) of mice 8 d after infection with N. brasiliensis. WT, wild-type. Numbers in quadrants indicate percent cells in each throughout. (b) Frequency (left) and total number (right) of cytokine-positive CD4<sup>+</sup> T cells in the draining mediastinal lymph nodes and lungs from Il4<sup>kn2</sup>Il13<sup>3mat</sup> mice (n = 9) d after infection with N. brasiliensis. *P < 0.05 and **P = 0.0001 (two-tailed t-test). (c) Ratio of IL-4-producing CD4<sup>+</sup> T cells to IL-13-producing CD4<sup>+</sup> T cells (IL-4+/IL-13+) in the mediastinal lymph nodes and lungs from Il4<sup>kn2</sup>Il13<sup>3mat</sup> mice (n = 9) d after infection with N. brasiliensis. *P < 0.0001 (two-tailed t-test). Each symbol represents an individual mouse; small horizontal lines indicate the mean (b,c). (d) Immunohistochemistry of popliteal lymph nodes 21 d after infection with L. major (top) and of lungs 8 d after infection with N. brasiliensis (bottom). Wild-type tissues serve as negative controls for huCD2 and fluorescent reporter staining. Original magnification, ×100 (main images) or ×200 (insets). Data are representative of at least four independent experiments with three to five mice each (a), are from two independent experiments (b,c) or are representative of two independent experiments with three to four mice.
cells in a GATA-3hi cytokine-expressing state. To investigate whether cytokine signaling was required for higher GATA-3 expression and IL-13 production, we generated Il4kn2a4get and Il4a4get, Il13smaart mice (n = 6 per genotype) 8 d after infection with N. brasiliensis (top), and frequency of cells positive for CD279 (PD-1) and CXCR5 (T FH cells) among the IL-4- or IL-13-producing cells (bottom). (c) Expression of mRNA from CD4+ T cells isolated from the lymph nodes and lungs of Il13a4get mice 9 d after infection with N. brasiliensis, presented relative to expression of mRNA for the housekeeping gene Gapdh. (d) Frequency of intracellular GATA-3 and Bcl-6 in CD4+ T cells isolated from the mesenteric lymph nodes and lungs of Il13a4get mice (n = 5) 8 d after infection with N. brasiliensis. Data are representative of two (a,d) or at least three (c) independent experiments, or are from two independent experiments (b; error bars, s.e.m.).

IL-13 is associated with high GATA-3 expression in Th2 cells

The use of 4get mice has facilitated the recognition of many cell types competent to produce IL-4 (as assessed by eGFP expression), including not only Tfh2 cells and Tfh cells but also natural NKT cells, mast cells, basophils, eosinophils and Th2 cells, all of which express eGFP constitutively in 4get mice because of the permissive nature immunology.

Figure 3 Different production of IL-4 and IL-13 by Tfh cells and canonical Tfh2 cells. (a) Frequency of cells producing IL-4 (huCD2) or IL-13 (huCD4) among eGFP+ CD4+ T cells in the lymph nodes and lungs of Il4kn2a4get and Il4a4get, Il13smaart mice (n = 6 per genotype) 8 d after infection with N. brasiliensis (top), and frequency of cells positive for CD279 (PD-1) and CXCR5 (T FH cells) among the IL-4- or IL-13-producing cells (bottom). (b) Frequency of Tfh cells among total IL-4- or IL-13-producing CD4+ T cells in the lymph nodes or lungs of Il4kn2a4get and Il4a4get, Il13smaart mice (n = 4 per genotype) 8 d after infection with N. brasiliensis. *P < 0.01 (two-tailed t-test). (c) Expression of mRNA from CD4+ T cells isolated from the lymph nodes and lungs of Il13a4get mice 9 d after infection with N. brasiliensis, presented relative to expression of mRNA for the housekeeping gene Gapdh. (d) Frequency of intracellular GATA-3 and Bcl-6 in CD4+ T cells isolated from the mediastinal lymph nodes and lungs of Il13a4get mice (n = 5) 8 d after infection with N. brasiliensis. Data are representative of two (a,d) or at least three (c) independent experiments, or are from two independent experiments (b; error bars, s.e.m.).
Figure 5 GATA-3 expression and cytokine production by CD4+ T cells in the absence of STAT6. (a) Frequency of GATA-3hi cells among total CD4+ T cells from STAT6-sufficient (WT) and STAT6-deficient (Stat6−/−) Il4Δkn2/Δkn2; Il13Δsmart/+ mice (n = 8 per genotype) 8 d after infection with N. brasiliensis. *P < 0.0001 (two-tailed t-test), (b) Frequency of GATA-3hi cells among IL-4- or IL-13-secreting CD4+ T cells from STAT6-sufficient and STAT6-deficient Il4Δkn2/Δkn2; Il13Δsmart/+ mice (n = 8 per genotype) 8 d after infection with N. brasiliensis. *P < 0.0001 and **P < 0.0002 (two-tailed t-test). Each symbol represents an individual mouse; small horizontal lines indicate the mean (a,b). (c) Frequency and total number of IL-4- and IL-13-producing CD4+ T cells in the lymph nodes and lung of STAT6-sufficient and STAT6-deficient Il4Δkn2/Δkn2; Il13Δsmart/+ mice (n = 8 per genotype) 8 d after infection with N. brasiliensis. *P < 0.001, **P < 0.01 and ***P < 0.05 (two-tailed t-test). Data are from two independent experiments (error bars c, s.e.m.).

translational features of the IRES4,8,18,19,33,34. To assess the contributions of these various cells to tissue responses in type 2 immunity, we assessed IL-4-expressing eGFP+huCD2+ cells in Il4Δkn2/Δkn2; Il13Δsmart/+ mice and IL-13-expressing eGFP+huCD4+ cells in Il4Δkn2/Δkn2; Il13Delta/+ mice in the lungs 9 d after infecting the mice with N. brasiliensis (Fig. 6a,b). At this time point, CD4+ T cells and basophils represented the only IL-4-expressing cells in the lung, and CD4+ T cells and Ih2 cells were the only IL-13-expressing cells. We were unable to directly visualize IL-13 expression in other cytokine-competent cell populations, including eosinophils, NKT cells and basophils, which was consistent with the lack of an immune phenotype after deletion of these cell types during primary infection with this helminth23,35–38. Of note, invariant NKT cells, although present in the lung at this time, made little IL-13 (Supplementary Fig. 11). Thus, although each of these cells was able to express IL-4 and IL-13 after stimulation in vitro, this study showed that expression in vivo was regulated in a much more restricted way than previously anticipated.

CD4+ T cells were the only cells that produced both IL-4 and IL-13 in the lungs (Fig. 6a,b). We confirmed the restricted pattern of IL-13 expression among Ih2 and CD4+ T cells by infecting Il13FetCre mice with N. brasiliensis. As observed for infected Il4Δkn2/Δkn13Δsmart/+ mice, CD4+ T cells were the predominant IL-13-producing subset. However, Ih2 cells, marked as lineage negative (Lin−; lacking markers for common lineage determinants for T cells, B cells, NK cells, dendritic cells or other myeloid cells7) and negative for the sialoadhesin CD169 (Siglec-F), CD131 (common β-chain of the receptor for IL-3) and the integrin...
CD49b (DX5) and positive for ICOS, also produced IL-13 at 9 d after infection (Fig. 6c,d). Ih2 cells were substantially deleted in Il13cre/+ROSA-DT+ reporter mice (Fig. 6e), which indicated that these cells were a relevant population of IL-13-expressing cells in this infectious model. We also observed IL-13 expression restricted to T1h2 cells and Ih2 cells in the small intestines of N. brasilensis–infected Il4+/+; Il13+/+ mice (Supplementary Fig. 6).

IL-4 expression by basophils and IL-13 expression by Ih2 cells resembled the distinctive cytokine patterns of T1h cells and T1h2 cells, which expressed IL-4 and IL-13, respectively, in a manner associated with distinct amounts of GATA-3. Lin−ICOS+ Ih2 cells constituted the only non-T cell GATA-3hi population in the lung (Fig. 7a). In contrast, basophils, assessed by expression of CD49b (DX5), were the only IL-4–producing innate cells in the lungs at 9 d after infection with N. brasilensis and did not express GATA-3. Intracellular GATA-3 staining in Il4+/+; Il13+/+ or Il4+/+; Il13−/− reporter mice showed that GATA-3hi Ih2 cells expressed both IL-4 and IL-13 in vivo, whereas GATA-3hi Ih2 cells or GATA-3hi basophils expressed only IL-13 or IL-4, respectively (Fig. 7b). Thus, as in T cells, IL-13 was expressed only in GATA-3hi innate cells, which during infection with N. brasilensis were Ih2 cells.

As with T1h2 cells, the total number of ICOS+ GATA-3hi Ih2 cells and the frequency of IL-13–producing Ih2 cells in the lung were not
significantly lower in the absence of STAT6 (Fig. 7c,d). However, the lungs of STAT6-deficient Il4RKOIl13smart/+ mice had 50% as many total IL-13-producing Ih2 cells as the lungs of wild-type Il4RKOIl13smart/+ Il13smart/+ mice had on day 8 after infection with N. brasiliensis. Over 95% of the Ih2 cells that produced IL-13 also had high intracellular expression of GATA-3 (Fig. 7e). We obtained similar results with IL-4 receptor-α-deficient mice (Supplementary Fig. 9). Thus, as in Ih2 cells, GATA-3 expression and IL-13 production occurred independently of STAT6, but optimal numbers of Ih2 cells required STAT6-dependent signals, which suggested a role for signaling via the IL-4 receptor in their maintenance or survival.

**IL-13 production and worm clearance require GATA-3**

To determine whether GATA-3 is required for IL-13 production, we crossed Il13YctCre mice with mice with loxP-flanked Gata3 alleles (Gata3fl/fl) to generate mice in which GATA-3 would be deleted only in IL-13-producing cells. At 9 d after infection with N. brasiliensis, IL-13 production, as assessed by eYFP expression from the Il13YctCre allele, was significantly lower in CD4+ T cells and was absent from Ih2 cells isolated from the lungs of Il13YctCre/Gata3fl/fl mice relative to its production in control Il13YctCre/Gata3fl/+ mice (Fig. 8a,b). Ablation of GATA-3 in IL-13-producing cells resulted in a phenocopy of our results with Il13YctCre/Rosa-DTA mice, in that both the recruitment of eosinophils to the lung and worm clearance were compromised (Fig. 8b,c). IgE production was unaffected in these mice (Fig. 8c), which confirmed the proposal that the Tfh cell–IL-4–arm of allergic immunity, which supports antibody production, is distinct from the Ih2–Ih2 cell–driven IL-13 arm responsible for the peripheral manifestations of type 2 immune responses, including the recruitment of eosinophils to tissues and worm expulsion.

**DISCUSSION**

Here we used new lines of mice to expose an unexpected partitioning of IL-4 and IL-13, cytokines linked to the mediation of allergic immunity and to helminths, among tissues and cell types. Tfh cells expressed IL-4, whereas Ih2 cells expressed a spectrum of either IL-4 or IL-13, or both IL-4 and IL-13. In confirmation of published studies, deletion of Il4 led to profound deficiencies in humoral immunity with minimal effects on tissue responses, whereas deletion of IL-13–expressing cells led to profound effects on tissue immunity with minimal effects on humoral responses. Among cells of the innate immune system, basophils expressed IL-4 but not IL-13, whereas Ih2 cells expressed IL-13 but not IL-4. We extended published observations demonstrating that Tfh cells constitute the most prevalent IL-4–producing cells in the lymph nodes during allergic immunity by showing that these cells did not activate Il13 expression, as assessed by direct marker analysis and lineage-mediated deletion. Thus, despite having a locus ‘competent’ for production of the linked type 2 cytokines, Tfh cells and Ih2 cells produced these cytokines differently in vivo.

The mechanisms that prevent the concordant activation of both Il4 and Il13 remain of interest. Although small amounts of GATA-3 are required in naive CD4+ T cells, chromatin modifications and the accessibility of GATA-3 to select sites in the Il4 and Il13 loci have an important regulatory role in the different expression of Il4 and Il13 (refs. 40,41). Our in vivo results confirmed those findings by showing an obligatory role for GATA-3 in IL-13 production by canonical Tfh2 cells, but they also suggested that the regulation of IL-4 and IL-13 in CD4+ T cells in vivo is more complicated, as Tfh2 cells did not require large amounts of GATA-3 for IL-4 production and did not express IL-13. We considered the possibility that IL-4 and/or IL-13 from cells of the innate immune system in the periphery might contribute to the terminal differentiation of Tfh2 cells by directly upregulating GATA-3 to thresholds needed to facilitate the expression of Il13 (and Il5) by binding to the respective promoters of these genes.

Although the absence of STAT6 or IL-4 receptor-α had some effect on the ultimate number of GATA-3+=Tfh2 effector cells that developed in tissues, the ability of CD4+ T cells to achieve a GATA-3+=IL-13-producing phenotype was not lost, a finding consistent with published studies. Further studies are needed to explore the pathways that enable STAT6-independent differentiation of Tfh2 cells in vivo.

We speculate that large amounts of Bcl-6 in Tfh cells restrict GATA-3 to concentrations insufficient to activate Il13 (and probably Il5). Although Bcl-6 is a direct transcriptional repressor for many genes, it might suppress GATA-3 at a post-transcriptional level, perhaps through suppression mediated by microRNA.

Bcl-6 overexpression can induce a Tfh phenotype, we are tempted to speculate that decay of Bcl-6 or expression of Blimp-1 in T cells after they leave secondary lymphoid tissues is a prerequisite for relieving repression of the genetic programs, such as homing and extended cytokine expression, necessary for the completion of Tfh2 differentiation in the periphery. Alternatively, Bcl-6 may be needed to sustain the expression of cytokines and chemokines required for efficient interactions with B cells. In this way, the development of effector Tfh2 cells is delayed until the cells enter peripheral tissues, where the local cytokine milieu can be sensed, thus enabling plasticity in the response.

The use of a Cre fusion protein allowed us to demonstrate that deletion of IL-13–producing cells was sufficient to block peripheral manifestations of type 2 immunity, including helminth expulsion and the infiltration of eosinophils into tissues. The finding that deletion of these cells resulted in a phenocopy of the loss of IL-13 suggested that the main role of these cells in type 2 immunity is the tissue distribution of IL-13, at least as assessed in this model. Furthermore, we found that specific deletion of GATA-3 in the IL-13–producing cells was sufficient to inhibit the peripheral manifestations of type 2 immunity. As assessed by direct visualization in vivo, only Tfh2 cells and Ih2 cells expressed IL-13 in tissues during this infection, which suggested that either or both of these cells is (are) required for optimal tissue immunity. The role for IL-13 from Ih2 cells may be of particular importance in considering T cell–independent responses, perhaps during challenges with less noxious antigens, such as allergens, or during tissue repair.

It is important to consider some of the caveats associated with the introduction of markers at the cytokine genes. We used knock-in approaches that left endogenous expression intact from a locus regulated by the endogenous cis-acting elements. Regardless of similar regulation, the introduction of exogenous genetic material unavoidably alters genomic distances and perhaps affects the speed of transcription or modify long-distance interactions that are dependent on precise spacing elements. To the best of our ability, we have documented that these markers acted similarly to the endogenous genes in wild-type genomes in all aspects, with the caveat that the half-lives of the markers, particularly membrane proteins such as CD2 and CD4, are distinct from the half-lives of the secreted cytokine proteins.

Given our observations, therapeutic targeting of IL-4 or IL-13 would be predicted to have very different effects depending on whether the intended targets reflect aspects of humoral or cellular responses. Together our studies have shown previously unappreciated regulation of these duplicated cytokines, as suggested by the differences between IL-4- and IL-13–expressing cells in dependence on and expression of GATA-3. This should provide fruitful opportunities for further understanding of the nuances that underpin gene regulation in cells of the immune system.
Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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Author Contributions
H.-E.L., R.L.R. and R.M.L. conceived of the work; H.-E.L. generated IL-13 reporter mice; H.-E.L., R.L.R., J.K.B. and B.M.S. designed and/or did experiments; I.-C.H. contributed reagents; R.L.R. and R.M.L. wrote the manuscript.

Competing Financial Interests
The authors declare no competing financial interests.

Methods
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15.分布
**ONLINE METHODS**

**Mice.** Dual IL-4 reporter mice (Il4RαN2/4get mice) have been described; these were generated by the introduction of an IRES-eGFP construct after the stop codon of Il4 by homologous recombination, which leads to transcription of bicistronic IL-4–IRES–eGFP mRNA and translation of both IL-4 and eGFP from the same mRNA. This allows analysis of IL-4–competent cells in vivo by detection of eGFP expression without the need for restimulation. Il4RαN2/4get mice were generated by the introduction of cDNA encoding human CD2 at the start site of Il4. After the appropriate stimulation, IL-4 secretion is reported by the appearance of huCD2 on the cell surface. Mice with eYFP-enhanced transcripts with Cre recombinase at Il13 (YetCre) have been described. Cells from these mice have an IRES followed by sequence encoding a eYFP-Cre recombinase fusion protein at the start of the 3′ untranslated region of Il13. For deletion of cells that activate Il13 transcription and translation or deletion of GATA-3, homozygous Il13YetCre/YetCre ROSA26 DTαCre mice or Il13YetCre/Gata3ΔβTII mice, respectively, were generated. Cells expressing IL-13 and the eYFP-Cre fusion protein express diphtheria toxin α, which kills the cells. Smart13 mice were generated by the introduction of a cDNA fragment encoding truncated human CD4 (with a point mutation that results in the substitution of isoleucine for phenylalanine at position 43 of the protein, to abrogate binding of mouse major histocompatibility complex class II) preceded by an IRES between the stop codon of Il13 and the 3′ untranslated region, by homologous recombination. BALB/c mice deficient in IL-4 and IL-13 have been described. Mice were maintained in the University of California San Francisco specific pathogen-free animal facility in accordance with the guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center.

**Infection, immunization and worm clearance.** *L. major* strain WHOM/IR/–173 was prepared and injected as 0.5×10^6 to 1×10^6 metacyclic promastigotes in the hind footpads of mice. Third-stage larvae of *N. brasiliensis* were prepared and worms were counted in the small intestine as described. Where indicated, mice received a sublethal dose of irradiation (450 rads) before transfer of donor T cells to create space in the recipient mice. Transferred CD4+ T cells were allowed to undergo homeostatic expansion for 5–7 d before mice were infected. Mice were infected subcutaneously in the footpad with 50 µg chicken ovalbumin conjugated to 15 moieties of NP (Biosearch Technologies) emulsified in Alum Imject (Pierce).

**Tissue immunohistochemistry.** For detection of eGFP and eYFP, signals were amplified by tyramide amplification on paraformaldehyde-fixed tissues, and huCD2 was detected on acetone-dehydrated slides after incubation with biotinylated antibody to human CD2 (RPA-2.10; eBioscience). Both eGFP and eYFP were detected with rabbit polyclonal antibody reactive to eGFP and eYFP (Ab 6556; Novus Biologicals) followed by biotinylated Fab(′)2, donkey antibody to rabbit (711-066-152; Jackson Immunoresearch). Lymph nodes were isolated at the appropriate time and were either frozen immediately in optimum cutting temperature embedding compound (Sakura Finetek USA) or were frozen after 2 h of incubation in 1–4% (vol/vol) paraformaldehyde followed by 30% (vol/vol) sucrose. Sections 6–8 µm in thickness were cut with a Leica CM 3050S cryomicrotome (Leica Microsystems). Sections were treated with fluorescein isothiocyanate–tyramide from the TSA Fluorescein System according to the manufacturer’s instructions (NEL 701001KT; PerkinElmer). Multiple biotinylated antibodies could be detected on the same slide by repeated quenching and blocking of peroxidase and biotin followed by another round of amplification with tyramide–Alexa Fluor 555 (Invitrogen), tyramide-indocarbocyanine or tyramide-biotin (PerkinElmer). Other biotinylated antibodies used were anti-Idg (AM591; BD Pharmingen), anti-CD23 (B3B4; BD Pharmingen), anti-CD4 (RM4-5; Biolegend) and anti-CD278 (anti-ICOS; C398.4A; Biolegend). Nuclei were counterstained with DAPI (4′, 6-diamidine-2′-phenylindole dihydrochloride; Roche) in PBS before being mounted on coverslips. Digital images in the fluorescein isothiocyanate, indocarbocyanine and indodicarbocyanine channels were collected with a Nikon Eclipse E800 fluorescence microscope equipped with SimplePCI software (Compix). Images were converted to red-green-blue, then were colored and overlaid with Photoshop CS2 software (Adobe Systems).

**Flow cytometry.** Mice were perfused with 20 ml PBS and mediastinal lymph nodes and lungs were isolated. Single-cell suspensions were prepared and labeled with the following antibodies: for detection of the IL-13 reporter in Il13YetCre mice, eYFP+ cells were analyzed with phycoerythrin–anti-Siglec-F (E50-2440; BD Pharmingen), phycoerythrin–anti-CD131 (10B8; BD Pharmingen), phycoerythrin–anti-CD49b (DX5; eBioscience), allophycocyanin–anti-CD278 (C398.4A; Biolegend), allophycocyanin–eFluor 780–anti-CD4 (RM4-5; eBioscience), peridinin chlorophyll protein–cytacine 5.5–anti-CD8 (53-6.7; BD Pharmingen), peridinin chlorophyll protein–cytacine 5.5–anti-CD19 (ID3; BD Pharmingen); for detection of the IL-13 and IL-4 reporters in Il4RαN2/4get mice, eGFP-expressing cells were analyzed with phycoerythrin–anti-huCD4 (RPA-T4; eBioscience), phycoerythrin–anti-huCD2 (S5.5; Caltag), allophycocyanin–eFluor 780–anti-CD4 (RM4-5; eBioscience), peridinin chlorophyll protein–cytacine 5.5–anti-CD19 (ID3; BD Pharmingen), peridinin chlorophyll protein–cytacine 5.5–anti-CD8 (53-6.7; BD Pharmingen), allophycocyanin–biotin–anti-ICOS (D10.G4.1; Biolegend), phycoerythrin-indocarbocyanine–anti-CD49b (DX5; eBioscience); T_HEL cells were stained with phycoerythrin-indodicarbocyanine–anti-PD-1 (RMP1-30) and biotin-anti-CXCR5 (2G8; BD Pharmingen) following streptavidin–peridinin chlorophyll protein; for detection of IL-4 and IL-13 reporters in Il4RαN2/4get Il13Smart+ mice, CD4+ cells were stained with phycoerythrin-indocarbocyanine–or fluorescein isothiocyanate–anti-huCD4 (RPA-T4; eBioscience), phycoerythrin–anti-huCD2 (S5.5; Caltag), allophycocyanin–eFluor 780–anti-CD4 (RM4-5; eBioscience), peridinin chlorophyll protein–cytacine 5.5–anti-CD19 (ID3; BD Pharmingen), peridinin chlorophyll protein–cytacine 5.5–anti-CD8 (53-6.7; BD Pharmingen), allophycocyanin–biotin–anti-ICOS (D10.G4.1; Biolegend). The CD1d tetramer was obtained from the US National Institutes of Health tetramer facility. Samples were analyzed on an LSR II (BD Biosciences). A ‘dump channel’ of CD8+ and CD19 labeled with peridinin chlorophyll protein–cytacine 5.5 was used to diminish nonspecific staining. Live lymphocytes were gated by DAPI exclusion, size and granularity based on forward and side scatter. Cells were counted with Vi-Cell 2.02 (Beckman-Coulter).

**Analysis of GATA-3 and Bcl-6 by flow cytometry.** Il4RαN2/4get Il13Smart+ or Il4RαN2/4get Il13Δbeta1 mice were perfused with 20 ml PBS, and mediastinal lymph nodes and lungs were collected. Single-cell suspensions were prepared and labeled with antibodies to surface molecules as follows: phycoerythrin-indodicarbocyanine–, fluorescein isothiocyanate– or phycoerythrin–anti-huCD4 (RPA-T4; eBioscience), phycoerythrin– or allophycocyanin–anti-CD2 (Caltag), allophycocyanin–anti-CD131 (C398.4A; Biolegend), allophycocyanin–eFluor 780–anti-CD4 (RM4-5; eBioscience), peridinin chlorophyll protein–cytacine 5.5–anti-CD19 (ID3; BD Pharmingen), peridinin chlorophyll protein–cytacine 5.5–anti-CD8 (53-6.7; BD Pharmingen), allophycocyanin–biotin–anti-ICOS (D10.G4.1; Biolegend). The CD1d tetramer was obtained from the US National Institutes of Health tetramer facility. Samples were analyzed on an LSR II (BD Biosciences). A ‘dump channel’ of CD8+ and CD19 labeled with peridinin chlorophyll protein–cytacine 5.5 was used to diminish nonspecific staining. Live lymphocytes were gated by DAPI exclusion, size and granularity based on forward and side scatter. Cells were counted with Vi-Cell 2.02 (Beckman-Coulter).

**Real-time RT-PCR.** Cells were isolated from mediastinal lymph nodes and lungs of *N. brasiliensis*–infected mice, and viable eGFP+ or eGFP-huCD2+ CD4+ T cells were sorted on the basis of DAPI CD4+CD19+ CD8– staining. Cells were lysed, followed by reverse transcription with SuperScript III CellsDirect cDNA synthesis kit (Invitrogen). Transcripts were quantified by incorporation of Platinum SYBR Green with a StepOnePlus Real-Time PCR System (Applied Biosystems), and results are presented relative to the expression of Gapdh (encoding glyceraldehyde phosphate dehydrogenase). Real-time primers were obtained from Qiagen as provided by Quantitec Primer Assay, and specific PCR products were verified by melting-curve and gel analysis.
Enzyme-linked immunosorbent assay. First, 96-well plates coated with NP23-BSA or NP3-BSA (Biosearch) were incubated with serial fivefold dilutions of serum. Then, IgG1 NP-specific antibodies were detected by incubation with biotinylated antibody to mouse IgG1 (RMG1-1; BioLegend) followed by streptavidin–horseradish peroxidase and o-phenylenediamine. Affinity maturation was assessed as described21. The concentration of serum anti-NP bound to NP3-BSA was divided by the concentration of anti-NP bound to NP23-BSA. As the affinity of the antibodies increases, the ratio approaches one. The concentrations of anti-NP IgG1 were determined by comparison with standard curves generated with IgG1 from the anti-NP IgG1 clone H33Lγ1/λ1 (provided by G. Kelsoe). For the detection of total IgE from serum of infected mice, 96-well plates were coated with anti-IgE (553413; BD Pharmingen) and were incubated with serum at increasing dilutions (2- to 100-fold) as the time for serum collection after infection increased. Bound IgE was detected with a biotinylated anti-IgE (553419; BD Pharmingen), followed by streptavidin–alkaline phosphatase and the substrate p-nitro phenyl phosphate. Samples were assessed in duplicate or triplicate, and concentrations were measured according to the IgE standard with an enzyme-linked immunosorbent assay plate reader (DeltaSoft).