

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 immunity^{2,9-13}. 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 system^{14,15}. 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* studies^{2,15-17}. 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 characterized^{4,7,18,19}. 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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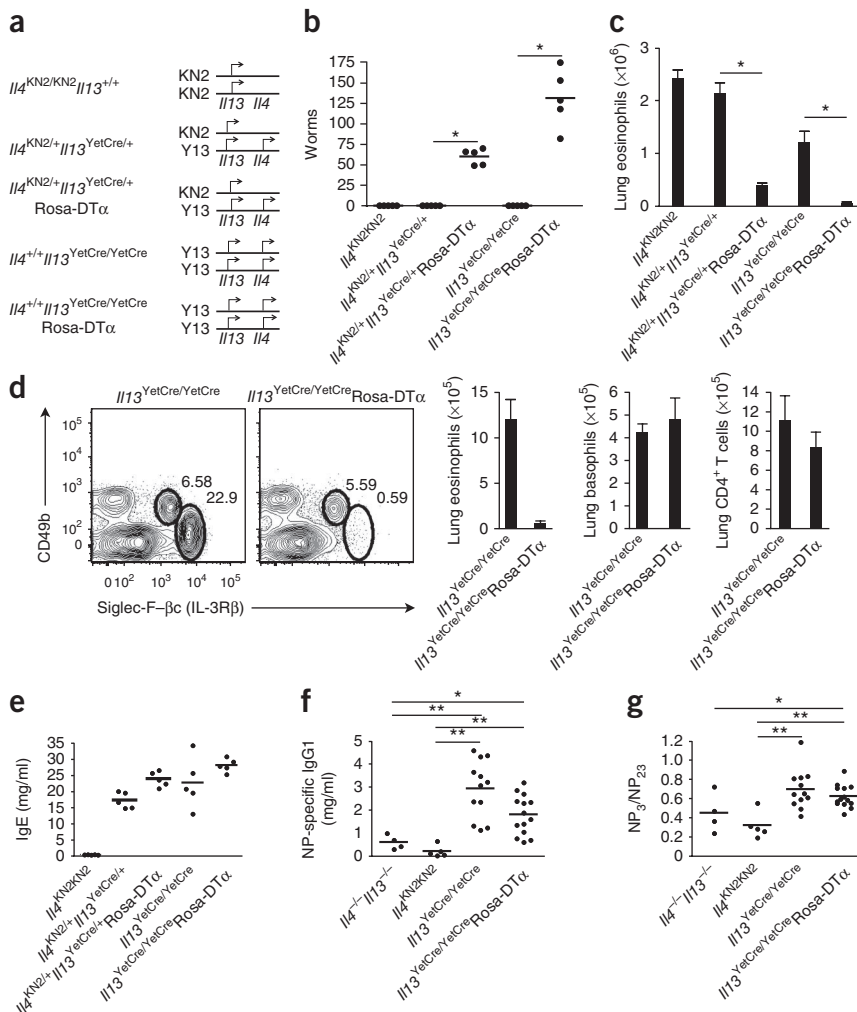


Figure 1 Eosinophil recruitment, worm expulsion and antibody production during infection with *N. brasiliensis* after depletion of IL-13-producing cells. **(a)** Active alleles in reporter mice. **(b)** Total worms in the small intestines of mice ($n = 5$ per group) 9 d after infection with *N. brasiliensis*. * $P < 0.001$ (two-tailed t -test). **(c)** Total eosinophils (Siglec-F^{hi}CD49b⁺) in the lungs of mice ($n = 2-3$ per group) 12 d after infection with *N. brasiliensis*. * $P < 0.01$ (two-tailed t -test). **(d)** Frequency (left) and total number (right) of eosinophils (Siglec-F⁺, with high expression of CD131 (common β -chain of the IL-3 receptor (IL-3R β); (β c^{hi})) and CD49b⁺), basophils (Siglec-F⁺ β c^{int}CD49b^{hi}) and CD4⁺ T cells in the lungs of mice ($n = 2-4$ per group) 9 d after infection with *N. brasiliensis*. Numbers adjacent to outlined areas indicate percent cells in each throughout. **(e)** IgE in serum of mice ($n = 5$ per group) 9 d after infection with *N. brasiliensis*. **(f)** Expression of high-affinity NP-specific IgG1 in serum of mice ($n = 4-15$ per group) 28 d after injection of NP-conjugated ovalbumin into the footpad. * $P < 0.05$ and ** $P < 0.005$ (two-tailed t -test). **(g)** Affinity maturation of serum NP-specific IgG1 in mice ($n = 4-15$ per group) after challenge with NP-conjugated ovalbumin, presented as the ratio of the binding of serum IgG1 to BSA conjugated to three moieties of NP to its binding to BSA conjugated to 23 moieties of NP (NP₃/NP₂₃). * $P < 0.05$ and ** $P < 0.005$ (two-tailed t -test). Each symbol represents an individual mouse; small horizontal lines indicate the mean (**b,e-g**). Data are representative of at least two independent experiments (**b,f**), at least three independent experiments (**c,e,g**) or two independent experiments (**d**; error bars (**b,d**), s.e.m.).

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*^{2,10}. 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 (huCD2) in place of the endogenous *Il4* sequence at the *Il4* start site (*Il4*^{KN2} mice). Cells activated to produce IL-4 in *Il4*^{KN2} mice express huCD2 on the cell membrane and provide an accurate report of IL-4-producing cells *in vitro* and *in vivo*^{20,21}. Homozygous *Il4*^{KN2/KN2} mice are IL-4 deficient, whereas heterozygous *Il4*^{KN2/+} mice are able to express IL-4 from the endogenous *Il4* 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 protein⁸. Cells from *Il13*^{YetCre/YetCre} mice are able to make both endogenous IL-4 and IL-13. Crossing *Il4*^{KN2} mice with *Il13*^{YetCre} 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-loxP-flanked stop sequence-diphtheria toxin- α (ROSA-DT α) allele allowed *in vivo* elimination of cells that had activated *Il13* expression, as expression of the eYFP-Cre fusion protein removed the loxP-flanked stop sequence, which enabled toxin expression to ensue^{8,22} (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 observations², IL-4-deficient *Il4*^{KN2/KN2} mice expelled worms normally. Immunity to *N. brasiliensis* was also intact in *Il4*^{KN2/+} mice and *Il13*^{YetCre/YetCre} mice (Fig. 1b). However, in *Il4*^{KN2/+} *Il13*^{YetCre/+} ROSA-DT α mice, worms were present at 9 d after infection in mice with one endogenous *Il13* allele and one YetCre13 allele (*Il13*^{YetCre/+}) and were twice as prevalent in *Il13*^{YetCre/YetCre} ROSA-DT α mice, in which two YetCre13 alleles were present to drive Cre-mediated deletion (Fig. 1b).

Although the absence of IL-4 in *Il4*^{KN2/KN2} 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-DT α (Fig. 1c). Eosinophils did not produce IL-13 because they did not

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

Genotype	Active <i>Il4</i> alleles	Deletion of IL-13-producing cells	Cytokines produced	Worm clearance	Eosinophil recruitment	IgE production
<i>Il4</i> ^{KN2/KN2} <i>Il13</i> ^{+/+}	0	No	IL-13	Yes	Yes	No
<i>Il4</i> ^{KN2/+} <i>Il13</i> ^{YctCre/+}	1	No	IL-4 + IL-13	Yes	Yes	Yes
<i>Il4</i> ^{KN2/+} <i>Il13</i> ^{YctCre/+} Rosa-DTα	1	Yes	IL-4	No	No	Yes
<i>Il4</i> ^{+/+} <i>Il13</i> ^{YctCre/YctCre}	2	No	IL-4 + IL-13	Yes	Yes	Yes
<i>Il4</i> ^{+/+} <i>Il13</i> ^{YctCre/YctCre} Rosa-DTα	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.

express eYFP in *Il13*^{YctCre} mice (as analyzed by flow cytometry), nor were these cells deleted from the blood, bone marrow or tissues of *Il13*^{YctCre/YctCre} Rosa-DTα mice (**Supplementary Fig. 2**). The deletion of IL-13-producing cells in *Il13*^{YctCre/YctCre} Rosa-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 *Il13*^{YctCre/YctCre} Rosa-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 mice^{2,9,11,23}.

IL-4 was required for IgE production, as *Il4*^{KN2/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 T_{HH} 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 the affinity of the anti-NP response after 28 d, as described before²¹. As expected, the absence of IL-4 in *Il4*^{KN2/KN2} mice and in mice with combined deficiency in IL-4 and IL-13 led to impaired expression of IgG1 protein and a diminished high-avidity anti-NP response (**Fig. 1f,g**). Although *Il13*^{YctCre/YctCre} Rosa-DTα mice, in which IL-13-expressing cells were deleted, had slightly less total NP-specific IgG1, the affinity maturation of anti-NP IgG1 was unaffected. As affinity maturation occurs in germinal centers, these findings were consistent with the absence of IL-13 expression in T_{HH} cells.

In sum, the studies described above confirmed the utility of the *Il4*^{KN2} and *Il13*^{YctCre} reporter strains in studying aspects of the expression of IL-13 and IL-4 during helminth infection. In addition, they suggested important roles for IL-4 in humoral responses but not tissue responses and supported the proposal of a fundamental role for IL-13 in tissue responses but not in high-affinity B cell responses. The deletion of IL-13-expressing cells had little effect on the activity of IL-4-expressing T_{HH} cells in lymph nodes, which suggested that the expression patterns of these two cytokines are distinct. Thus, IL-4 and IL-13 serve distinct and nonredundant functions in type 2 immunity.

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 *Il13*^{YctCre} 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 *Il13*^{Smart} mice to the

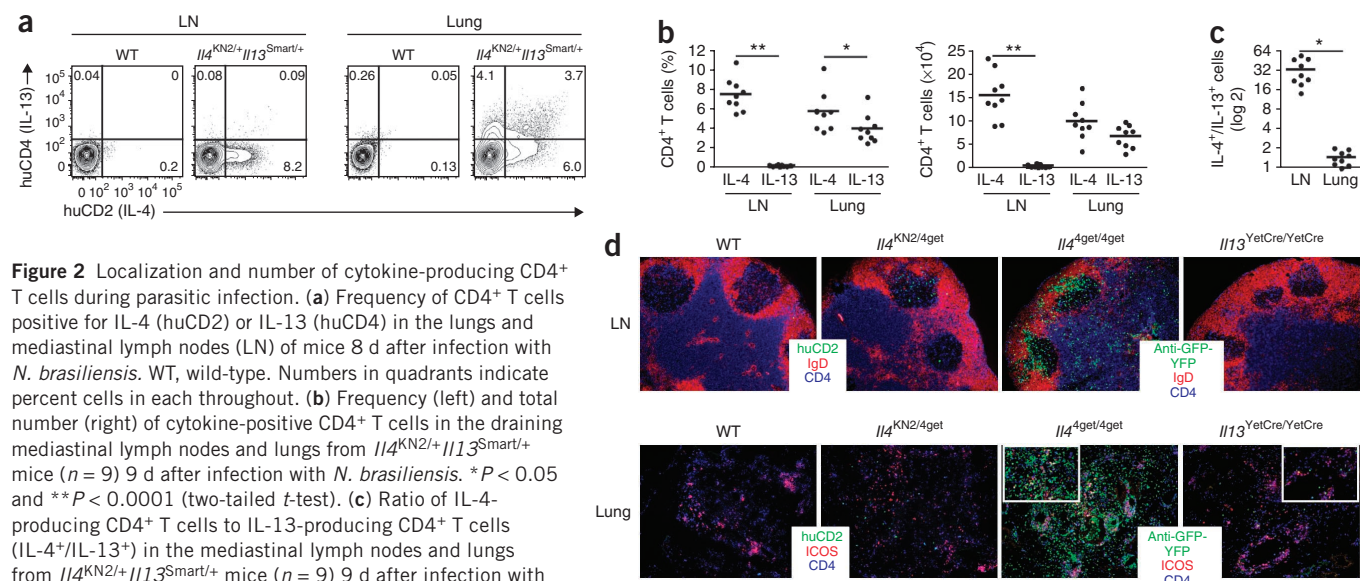
BALB/c background and then crossed them with *Il4*^{KN2} mice, which enabled simultaneous detection of the expression of both IL-4 and IL-13 at the single-cell level. After infecting *Il4*^{KN2/+} *Il13*^{Smart/+} mice with *N. brasiliensis*, we quantified IL-4- and IL-13-producing CD4⁺ T cells in the 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 T_{HH} 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 T_{H2} 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.

T_{HH} cells are distinct from T_{H2} cells and do not produce IL-13

We next crossed each of the *Il4*^{KN2} reporter mice and the *Il13*^{Smart} reporter mice to the 4get IL-4-reporter strain of mice (*Il4*^{4get}), 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 *Il4*^{KN2/4get} mice expressed IL-4 *in situ* (huCD2⁺), and about 40% of those IL-4-producing cells had high expression of the T_{HH} 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



IL-13-producing cells expressed T_{FH} cell markers. Among the IL-13-expressing CD4⁺ T cells in the lung, none had expression of CD279 (PD-1) and CXCR5 similar to that of lymph node IL-4-producing T_{FH} cells. Thus, IL-13-expressing T cells did not express T_{FH} markers and did not localize to B cell areas. Furthermore, CD4⁺ T cells with markers characteristic of T_{FH} cells were not present among the cytokine-expressing T_{H2} cells in the lung.

To more completely characterize the differences between cytokine-expressing T_{FH} cells and T_{H2} cells, we sorted populations of cytokine-competent and cytokine-producing cells from the mediastinal lymph nodes and lungs of *Il4*^{KN2/4get} 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 T_{FH} cells. Consistent with published reports on cytokine production by T_{FH} cells during parasitic and viral infection^{21,27–29}, 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_{FH} 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_{FH} development, than did IL-4-producing cells isolated from the lungs^{26,30,31}. 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*^{KN2} allele), IL-4-expressing lymph node CD4⁺ 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_{H2} cells. We also found that inverse relationship in protein expression by intracellular staining and flow cytometry. Bcl-6⁺ cells were present only in lymph nodes, and Bcl-6⁺ CD4⁺ T cells were almost completely absent from lung tissue (Fig. 3d). The GATA-3^{hi} 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

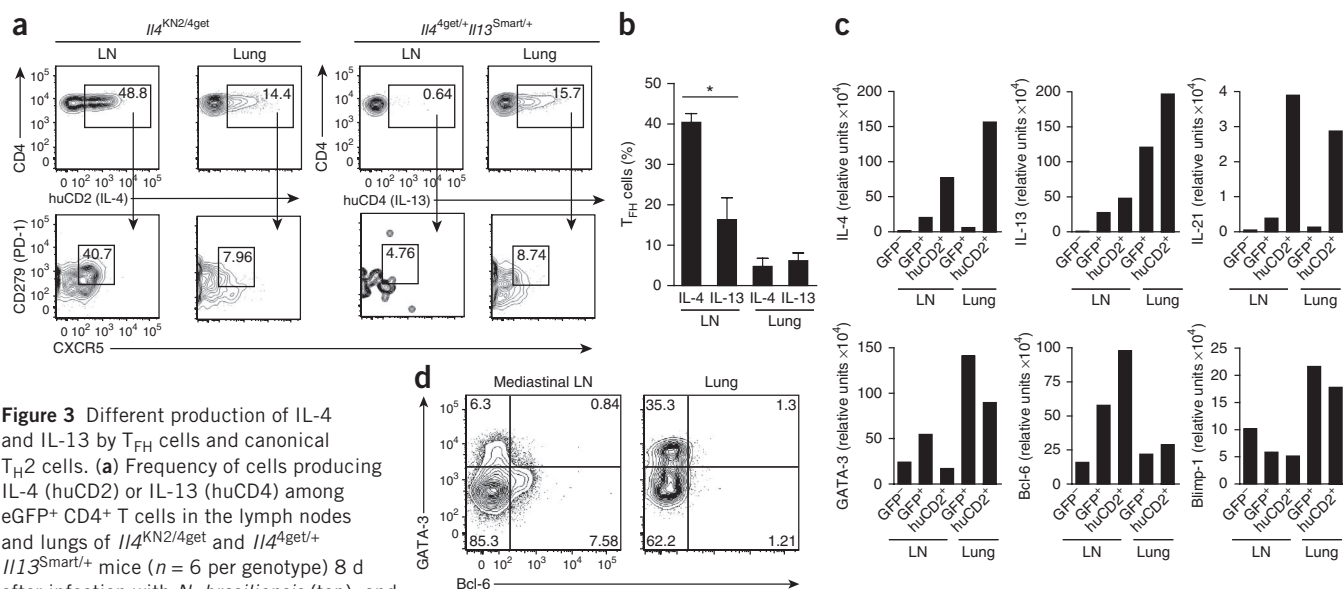
identity as T_{FH} cells, but had little GATA-3, whereas IL-13-producing cells were uniformly GATA-3^{hi} and Bcl-6[−] (Supplementary Fig. 7). These results suggested that T_{FH} cells and T_{H2} cells have distinct and nonoverlapping profiles of transcription factor expression and probably use different transcription factors in different ways to achieve cytokine activation from the *Il4* and *Il13* loci.

Cytokine-producing T_{H2} cells are GATA-3^{hi}

Although GATA-3 was originally linked to the production of IL-4 by T_{FH} cells during helminth infection²⁹, studies of mRNA expression during infection with lymphocytic choriomeningitis virus²⁷ 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_{FH} and T_{H2} cells, we isolated T_{FH} and T_{H2} cells from *Il4*^{KN2/+}/*Il13*^{Smart/+} 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⁺ T cells were predominantly GATA-3^{lo}. 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^{hi} 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⁺ 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^{hi} regardless of whether the CD4⁺ T cells were isolated from the lymph nodes (in which there were very few cells) or the lungs. Thus, IL-4 expression by T_{FH} cells was associated with low expression of GATA-3, whereas expression of IL-13 by T_{H2} 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⁺ 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



cells in a GATA-3^{hi} cytokine-expressing state³². To investigate whether cytokine signaling was required for higher GATA-3 expression and IL-13 production, we generated *Il4*^{KN2/+} *Il13*^{Smart/+} double-reporter mice on a background deficient in the transcription factor STAT6. There was a lower frequency of GATA-3^{hi} CD4⁺ T cells in both the lymph nodes and the lungs (Fig. 5a), and we noted this effect in both the IL-4-expressing CD4⁺ T cells and IL-13-expressing CD4⁺ T cells of the dual-reporter mice (Fig. 5b). We obtained similar results with *Il4*^{KN2/4get} mice and *Il4*^{4get/+} *Il13*^{Smart/+} mice deficient in IL-4 receptor- α expression (Supplementary Fig. 9). Of note, although we observed a lower frequency of IL-4-producing CD4⁺ T cells in the lymph nodes and lungs of *Il4*^{KN2/+} *Il13*^{Smart/+} mice than in those of wild-type mice, there was no difference in the number of total IL-4-producing cells at 8 d after infection with *N. brasiliensis* (Fig. 5c). Similarly, we found no significant effect of STAT6 deficiency on the frequency or number of IL-13-producing CD4⁺ T cells in the lungs of *Il4*^{KN2/+} *Il13*^{Smart/+} mice (Fig. 5c). Furthermore, when we reconstituted sublethally irradiated wild-type mice and mice deficient in IL-4 and IL-13 with either

congenic IL-4–IL-13–deficient or wild-type naive CD4⁺ T cells, and then infected the mice with *N. brasiliensis*, we observed similar proportions of GATA-3^{hi} cells among activated (ICOS⁺) donor T cells (Supplementary Fig. 10), which suggested that IL-4 and IL-13 derived from T cells or from host cells were not necessary to drive GATA-3 expression to large amounts. Thus, high GATA-3 expression associated with tissue cytokine expression, particularly IL-13, was optimized by the presence of signaling from IL-4 and IL-13, but neither cytokine was required for the development of T_H2 cells into a GATA-3^{hi} cytokine-expressing state *in vivo*. IL-4 production in lymphoid tissues occurred independently of both STAT6 and higher GATA-3 expression.

IL-13 is associated with high GATA-3 expression in Ih2 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 T_H2 cells and T_H cells but also natural NKT cells, mast cells, basophils, eosinophils and Ih2 cells, all of which express eGFP constitutively in 4get mice because of the permissive

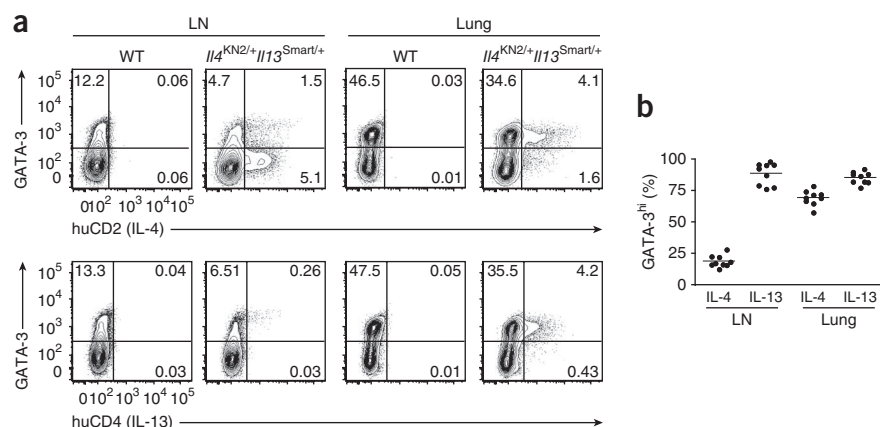


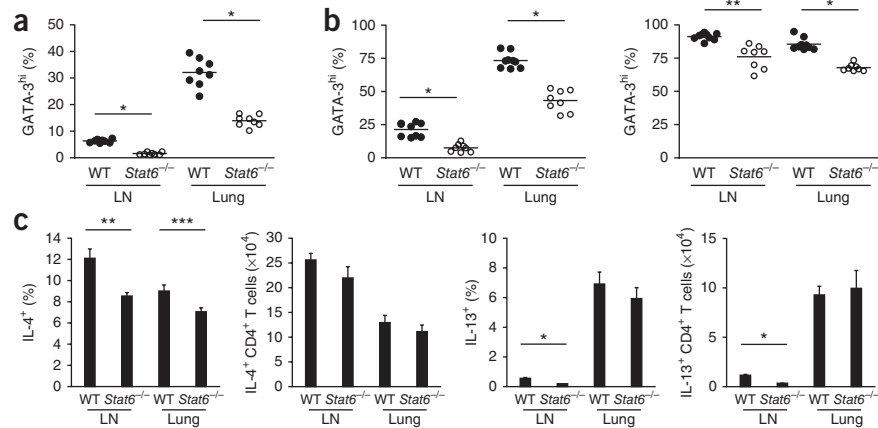
Figure 4 GATA-3 expression in IL-4- and IL-13-producing CD4⁺ T cells after infection with *N. brasiliensis*. (a) Frequency of intracellular GATA-3^{hi} CD4⁺ T cells and CD4⁺ T cells producing IL-4 (huCD2) and IL-13 (huCD4) from the draining lymph nodes and lungs of wild-type or *Il4*^{KN2/+} *Il13*^{Smart/+} mice 9 d after infection with *N. brasiliensis*. (b) Frequency of GATA-3^{hi} cells among total CD4⁺ T cells producing IL-4 (huCD2) or IL-13 (huCD4) in the mediastinal lymph nodes and lungs of mice 9 d after infection with *N. brasiliensis*. Each symbol represents an individual mouse; small horizontal lines indicate the mean. Data are representative of at least five independent experiments with three to four mice each (a) or are from three independent experiments (b).

Figure 5 GATA-3 expression and cytokine production by CD4⁺ T cells in the absence of STAT6. (a) Frequency of GATA-3^{hi} cells among total CD4⁺ T cells from STAT6-sufficient (WT) and STAT6-deficient (*Stat6*^{-/-}) *Il4*^{KN2/+}/*Il13*^{Smart/+} mice (*n* = 8 per genotype) 8 d after infection with *N. brasiliensis*.

P* < 0.0001 (two-tailed *t*-test). (b) Frequency of GATA-3^{hi} cells among IL-4- or IL-13-secreting CD4⁺ T cells from STAT6-sufficient and STAT6-deficient *Il4*^{KN2/+}/*Il13*^{Smart/+} 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 *Il4*^{KN2/+}/*Il13*^{Smart/+} 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 IRES^{4,8,18,19,33,34}. To assess the contributions of these various cells to tissue responses in type 2 immunity, we assessed IL-4-expressing eGFP⁺huCD2⁺ cells in *Il4*^{KN2/4get} mice and IL-13-expressing eGFP⁺huCD4⁺ cells in *Il4*^{4get/+}/*Il13*^{Smart/+} 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 helminth^{23,35–38}. 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 *Il13*^{YecCre} mice with *N. brasiliensis*. As observed for infected *Il4*^{4get/+}/*Il13*^{Smart/+} 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

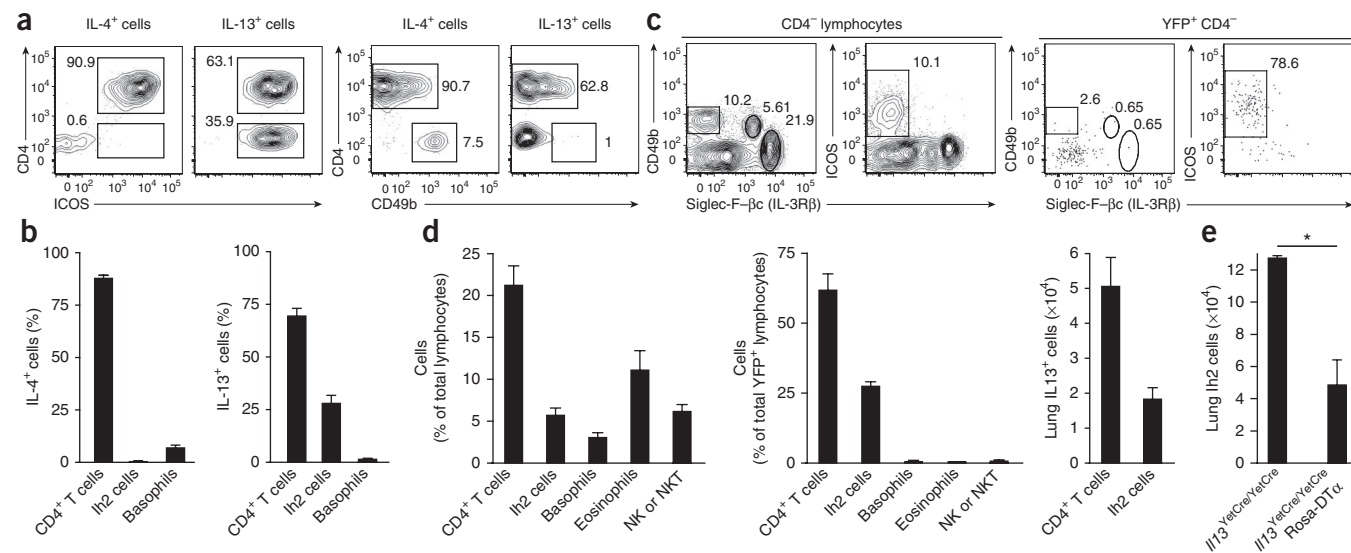


Figure 6 Production of IL-4 and IL-13 by innate type 2 effector cells after infection with *N. brasiliensis*. (a) Frequency of ICOS⁺CD4⁺ and ICOS⁺CD4⁻ cells (top) or CD4⁺CD49b⁻ and CD4⁺CD49b⁺ cells (bottom) among total IL-4- or IL-13-producing lung cells from *Il4*^{KN2/4get} or *Il4*^{4get/+}/*Il13*^{Smart/+} mice 8 d after infection with *N. brasiliensis*. Gates indicate CD4⁺ T cells (CD4⁺CD49b⁻CD278⁺), Ih2 cells (CD4⁺CD49b⁻CD278⁺) or basophils (CD4⁺CD49b⁻CD278⁻). (b) Frequency of CD4⁺ T cells, Ih2 cells or basophils among total IL-4- or IL-13-producing cells in the lungs of *Il4*^{KN2/4get} or *Il4*^{4get/+}/*Il13*^{Smart/+} mice (*n* = 3 per genotype) 8 d after infection with *N. brasiliensis*. (c) Frequency of eosinophils (CD4⁻Siglec-F^{hi}CD49b⁻), basophils (CD4⁻Siglec-F^{hi}CD49b^{hi}) and Ih2 cells (CD4⁻Siglec-F^{hi}CD49b⁻ICOS^{hi}) among CD4⁻ cells in the lungs of *Il13*^{YecCre/YecCre} mice (*n* = 9) 9 d after infection with *N. brasiliensis*. (d) Frequency and total number of total and eYFP-expressing CD4⁺ T cells, Ih2 cells, basophils, eosinophils and NK or NKT cells among total or eYFP⁺ (IL-13-producing) lymphocytes in the lungs of *Il13*^{YecCre/YecCre} mice (*n* = 3) 9 d after infection with *N. brasiliensis*. (e) Total Ih2 cells (Siglec-F^{hi}CD49b⁻DX5⁻CD4⁻ICOS^{hi}) in the lungs of *Il13*^{YecCre/YecCre} mice (*n* = 2–4) 12 d after infection with *N. brasiliensis*. **P* < 0.05 (two-tailed *t*-test). Data are representative of at least three independent experiments (a,b) or two (c,e) or three (d) independent experiments (error bars (b,d), s.e.m.).

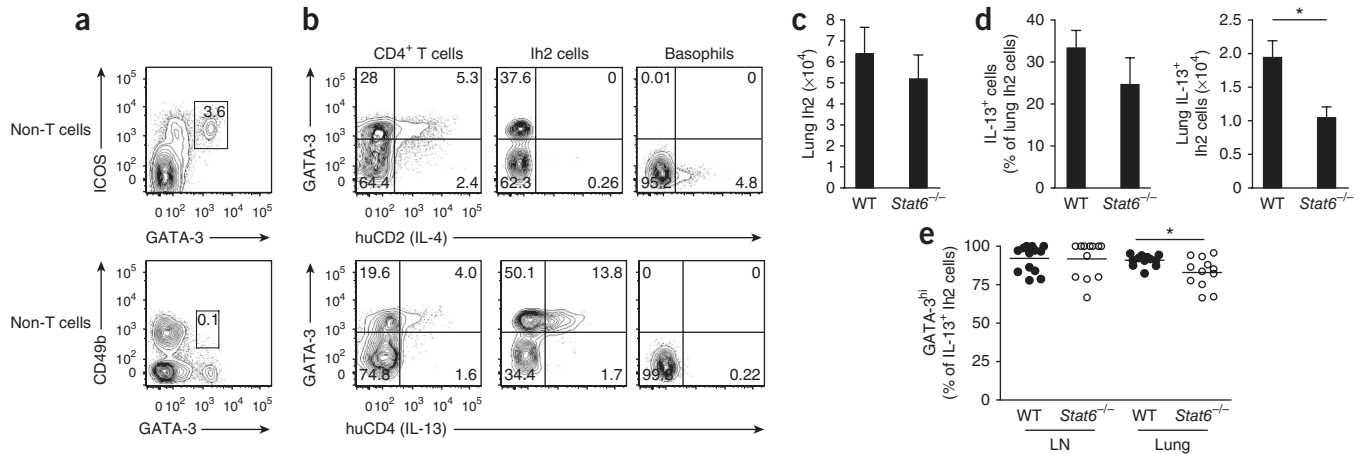


Figure 7 Requirement for STAT6 and GATA-3 in cytokine production by innate type 2 effector cells. **(a)** Frequency of intracellular GATA-3^{hi} Ih2 cells (ICOS⁺) and basophils (CD49b⁺) among total non-T cells from the lungs of *Il4*^{KN2/4get} and *Il4*^{get/+}*Il13*^{Smart/+} mice 9 d after infection with *N. brasiliensis*. **(b)** Frequency of GATA-3^{hi} CD4⁺ T cells producing IL-4 (huCD2) or IL-13 (huCD4), Ih2 cells and basophils from the lungs of *Il4*^{KN2/4get} mice (top row) and *Il4*^{get/+}*Il13*^{Smart/+} mice (bottom row) 8 d after infection with *N. brasiliensis*. **(c)** Total Ih2 cells (ICOS⁺CD4⁺CD8⁺CD19⁺) in the lungs of STAT6-sufficient (WT) and STAT6-deficient *Il4*^{KN2/+}*Il13*^{Smart/+} mice (*n* = 8 per genotype). **(d)** Frequency and total number of Ih2 cells secreting IL-13 (huCD4) in the lungs of STAT6-sufficient and STAT6-deficient *Il4*^{KN2/+}*Il13*^{Smart/+} mice (*n* = 8 per genotype) 8 d after infection with *N. brasiliensis*. **P* < 0.01 (two-tailed *t*-test). **(e)** Frequency of GATA-3^{hi} IL-13-producing Ih2 cells in the mediastinal lymph nodes and lungs of STAT6-sufficient and STAT6-deficient *Il4*^{KN2/+}*Il13*^{Smart/+} mice (*n* = 8 per genotype) 8 d after infection with *N. brasiliensis*. Each symbol represents an individual mouse; small horizontal lines indicate the mean. **P* < 0.01 (two-tailed *t*-test). Data are representative of at least three independent experiments with three to four mice each (**a**,**b**) or are from two independent experiments (**c**–**e**; error bars (**c**,**d**), s.e.m.).

CD49b (DX5) and positive for ICOS, also produced IL-13 at 9 d after infection (Fig. 6c,d). Ih2 cells were substantially deleted in *Il13*^{Y^{et}Cre}*ROSA-DTα* 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 T_H2 cells and Ih2 cells in the small intestines of *N. brasiliensis*-infected *Il4*^{get/+}*Il13*^{Smart/+} mice (Supplementary Fig. 6).

IL-4 expression by basophils and IL-13 expression by Ih2 cells resembled the distinctive cytokine patterns of T_{FH} cells and T_H2 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-3^{hi} 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. brasiliensis* and did not express GATA-3. Intracellular GATA-3 staining in *Il4*^{KN2/4get} and *Il4*^{get/+}*Il13*^{Smart/+} reporter mice showed that GATA-3^{hi} T_H2 cells expressed both IL-4 and IL-13 *in vivo*, whereas GATA-3^{hi} Ih2 cells or GATA-3^{lo} basophils expressed only IL-13 or IL-4, respectively (Fig. 7b). Thus, as in T cells, IL-13 was expressed only in GATA-3^{hi} innate cells, which during infection with *N. brasiliensis* were Ih2 cells.

As with T_H2 cells, the total number of ICOS^{hi} GATA-3^{hi} Ih2 cells and the frequency of IL-13-producing Ih2 cells in the lung were not

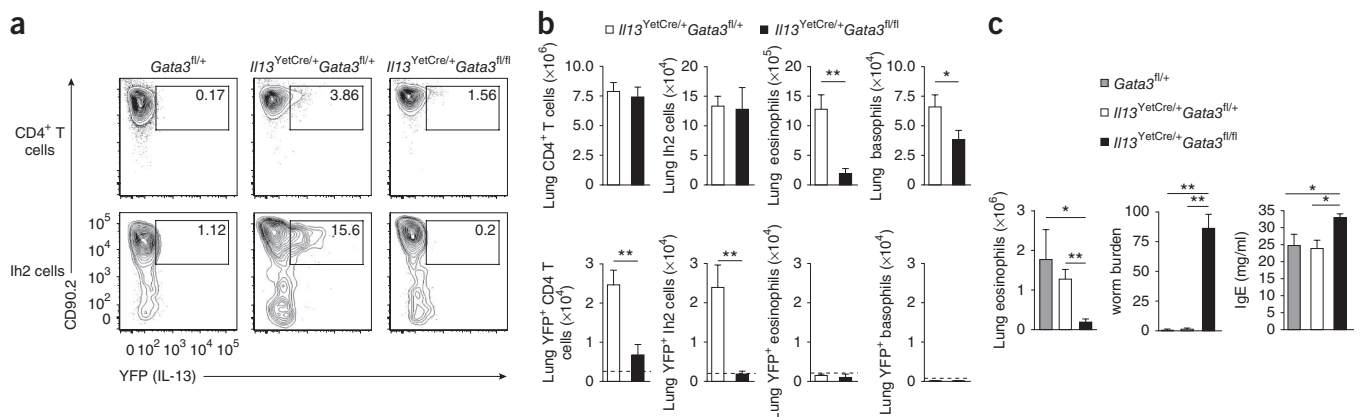


Figure 8 GATA-3 is required for IL-13 expression, the recruitment of cells of the innate immune system and worm expulsion, but not for IgE production. **(a)** Frequency of CD4⁺ T cells and Ih2 cells producing IL-13 (eYFP) in the lungs of *Gata3*^{fl/+}, *Il13*^{Y^{et}Cre/+}*Gata3*^{fl/+} and *Il13*^{Y^{et}Cre/+}*Gata3*^{fl/fl} mice 9 d after infection with *N. brasiliensis*. **(b)** Total or IL-13 (eYFP)-producing basophils (CD4⁺Siglec-F⁺CD49b⁺CD90.2^{int}), eosinophils (CD4⁺Siglec-F⁺CD49b⁺ and high side scatter), CD4⁺ T cells (CD4⁺ICOS^{hi}CD90.2⁺) and Ih2 cells (CD4⁺Siglec-F⁺CD49b⁺ICOS^{hi}CD90.2⁺) in the lungs of *Il13*^{Y^{et}Cre/+}*Gata3*^{fl/+} or *Il13*^{Y^{et}Cre/+}*Gata3*^{fl/fl} mice (*n* = 7–9 per group) 9 d after infection with *N. brasiliensis*. Dashed lines indicate the limit of detection for the reporter-negative wild-type control. **P* < 0.05 and ***P* < 0.005 (two-tailed *t*-test). **(c)** Eosinophil recruitment, worm expulsion and total serum IgE in *Il13*^{Y^{et}Cre/+}*Gata3*^{fl/+} or *Il13*^{Y^{et}Cre/+}*Gata3*^{fl/fl} mice (*n* = 4–5 per group) 9 d after infection with *N. brasiliensis*. **P* < 0.05 and ***P* < 0.005 (two-tailed *t*-test). Data are representative of two independent experiments with seven to nine mice per group (**a**), are a composite of two independent experiments (**b**) or are representative of two experiments (**c**; error bars (**b**,**c**), s.e.m.).

significantly lower in the absence of STAT6 (Fig. 7c,d). However, the lungs of STAT6-deficient $Il4^{KN2/+}Il13^{Smart/+}$ mice had 50% as many total IL-13-producing $Ih2$ cells as the lungs of wild-type $Il4^{KN2/+}Il13^{Smart/+}$ 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 T_H2 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 $Il13^{YecCre}$ mice with mice with loxP-flanked *Gata3* alleles ($Gata3^{fl/fl}$) to generate mice in which GATA-3 would be deleted only in IL-13-producing cells³⁹. At 9 d after infection with *N. brasiliensis*, IL-13 production, as assessed by eYFP expression from the $Il13^{YecCre}$ allele, was significantly lower in $CD4^+$ T cells and was absent from $Ih2$ cells isolated from the lungs of $Il13^{YecCre/+}Gata3^{fl/fl}$ mice relative to its production in control $Il13^{YecCre/+}Gata3^{fl/+}$ mice (Fig. 8a,b). Ablation of GATA-3 in IL-13-producing cells resulted in a phenocopy of our results with $Il13^{YecCre}ROSA-DT\alpha$ 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 T_{FH} cell–IL-4 arm of allergic immunity, which supports antibody production, is distinct from the T_H2 – $Ih2$ 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. T_{FH} cells expressed IL-4, whereas T_H2 cells expressed a spectrum of either IL-4 or IL-13, or both IL-4 and IL-13. In confirmation of published studies^{8,11,21,23}, deletion of IL-4 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 T_{FH} cells constitute the most prevalent IL-4-producing cells in the lymph nodes during allergic immunity²¹ 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, T_{FH} cells and T_H2 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 IL-4 and IL-13 (refs. 40,41). Our *in vivo* results confirmed those findings by showing an obligatory role for GATA-3 in IL-13 production by canonical T_H2 cells, but they also suggested that the regulation of IL-4 and IL-13 in $CD4^+$ T cells *in vivo* is more complicated, as T_{FH} 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 T_H2 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^{15,32}. Although the absence of STAT6 or IL-4 receptor- α had some effect on the ultimate number of GATA-3^{hi} T_H2 effector cells that developed in tissues, the ability of $CD4^+$ T cells to achieve a GATA-3^{hi} IL-13-producing phenotype was not lost, a finding consistent with published studies⁴². Further studies are needed to explore the pathways that enable STAT6-independent differentiation of T_H2 cells *in vivo*.

We speculate that large amounts of Bcl-6 in T_{FH} 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^{30,43,44}. Because Bcl-6 overexpression can induce a T_{FH} phenotype²⁶, 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 T_H2 differentiation in the periphery^{21,25}. 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 T_H2 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^{2,11}. 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 T_H2 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^{6,8,23}. 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 such 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

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.

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ONLINE METHODS

Mice. Dual IL-4 reporter mice ($Il4^{KN2/4get}$ mice) have been described²⁰; 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. $Il4^{KN2}$ 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 surface²⁰. Mice with eYFP-enhanced transcripts with Cre recombinase at *Il13* (YetCre13) have been described⁸. 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 $Il13^{YetCre/YetCre}ROSA-DT\alpha$ mice⁸ or $Il13^{YetCre/+}Gata3^{fl/fl}$ mice³⁹, respectively, were generated. Cells expressing IL-13 and the eYFP-Cre fusion protein express diphtheria toxin- α , which kills the cell. 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 described¹¹. 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 described²³. 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 immunized subcutaneously in the footpad with 50 μ g chicken ovalbumin conjugated to 15 moieties of NP (Biosearch Technologies) emulsified in Alum Inject (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 F(ab')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 μ m 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-IgD (AMS9.1; 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-diamidino-2'-phenylindole dihydrochloride; Roche) in PBS before being mounted on coverslips. Digital images in the fluorescein isothiocyanate, indocarbocyanine and indocarbocyanine 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 $Il13^{YetCre}$ mice, eYFP⁺ cells were analyzed with phycoerythrin-anti-Siglec-F (E50-2440; BD Pharmingen), phycoerythrin-anti-CD131 (J0R050; BD Pharmingen), phycoerythrin-anti-CD49b (DX5; eBioscience), allophycocyanin-anti-CD278 (C398.4A; Biolegend), allophycocyanin-eFluor 780-anti-CD4 (RM4-5; eBioscience), peridinin chlorophyll protein-cyanine 5.5-anti-CD8 (53-6.7; BD Pharmingen), peridinin chlorophyll protein-cyanine 5.5-anti-CD19 (ID3; BD Pharmingen); for detection of the IL-13 and IL-4 reporters in $Il4^{4get/+}Il13^{Smart/+}$ and $Il4^{KN2/4get}$ 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-cyanine 5.5-anti-CD19 (ID3; BD Pharmingen), peridinin chlorophyll protein-cyanine 5.5-anti-CD8 (53-6.7; BD Pharmingen), allophycocyanin-biotin-anti-ICOS (D10.G4.1; Biolegend), phycoerythrin-indotricarbocyanine-anti-CD49b (DX5; eBioscience); T_{HH} cells were stained with phycoerythrin-indotricarbocyanine-anti-PD-1 (RMP1-30) and biotin-anti-CXCR5 (2G8; BD Pharmingen) followed by streptavidin-peridinin chlorophyll protein; for detection of IL-4 and IL-13 reporters in $Il4^{KN2/+}Il13^{Smart/+}$ mice, CD4⁺ cells were stained with phycoerythrin-indotricarbocyanine- or fluorescein isothiocyanate-anti-huCD4 (RPA-T4; eBioscience), phycoerythrin-anti-huCD2 (S5.5; Caltag), allophycocyanin-eFluor780-anti-CD4 (RM4-5; eBioscience), peridinin chlorophyll protein-cyanine 5.5-anti-CD19 (ID3; BD Pharmingen), peridinin chlorophyll protein-cyanine 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 α and CD19 labeled with peridinin chlorophyll protein-cyanine 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. $Il4^{KN2/+}Il13^{Smart/+}$, $Il4^{KN2/4get}$ or $Il4^{4get/+}Il13^{Smart/+}$ 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-indotricarbocyanine-, fluorescein isothiocyanate- or phycoerythrin-anti-huCD4 (RPA-T4; eBioscience), phycoerythrin- or allophycocyanin-anti-CD2 (Caltag), allophycocyanin-anti-anti-CD278 (C398.4A; Biolegend), allophycocyanin-eFluor 780-anti-CD4 (RM4-5; eBioscience), peridinin chlorophyll protein-cyanine 5.5-anti-CD8 (53-6.7; BD Pharmingen), peridinin chlorophyll protein-cyanine 5.5-anti-CD19 (ID3; BD Pharmingen) and phycoerythrin-indotricarbocyanine-anti-CD49b (DX5; eBioscience). After surface staining, cells were stained with Violet Live/Dead fixable stain (Invitrogen) for the exclusion of dead cells. Cells were fixed, made permeable and stained intracellularly with phycoerythrin- or allophycocyanin-anti-GATA-3 (TWAJ; eBiosciences) and Alexa Fluor 647-conjugated rabbit polyclonal antibody to Bcl-6 (7D1; Santa Cruz) with intracellular Fix/Permeabilization Buffers according to the manufacturer's instructions (00-5523; eBioscience). Anti-Bcl-6 was conjugated to Alexa Fluor 647 with an Alexa Fluor 647 Monoclonal Antibody Labeling Kit (A-20186; Invitrogen). Live lymphocytes were gated on size and granularity on the basis of forward and side scatter. A 'dump channel' was used as described above.

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 QuantiTect Primer Assay, and specific PCR products were verified by melting-curve and gel analysis.

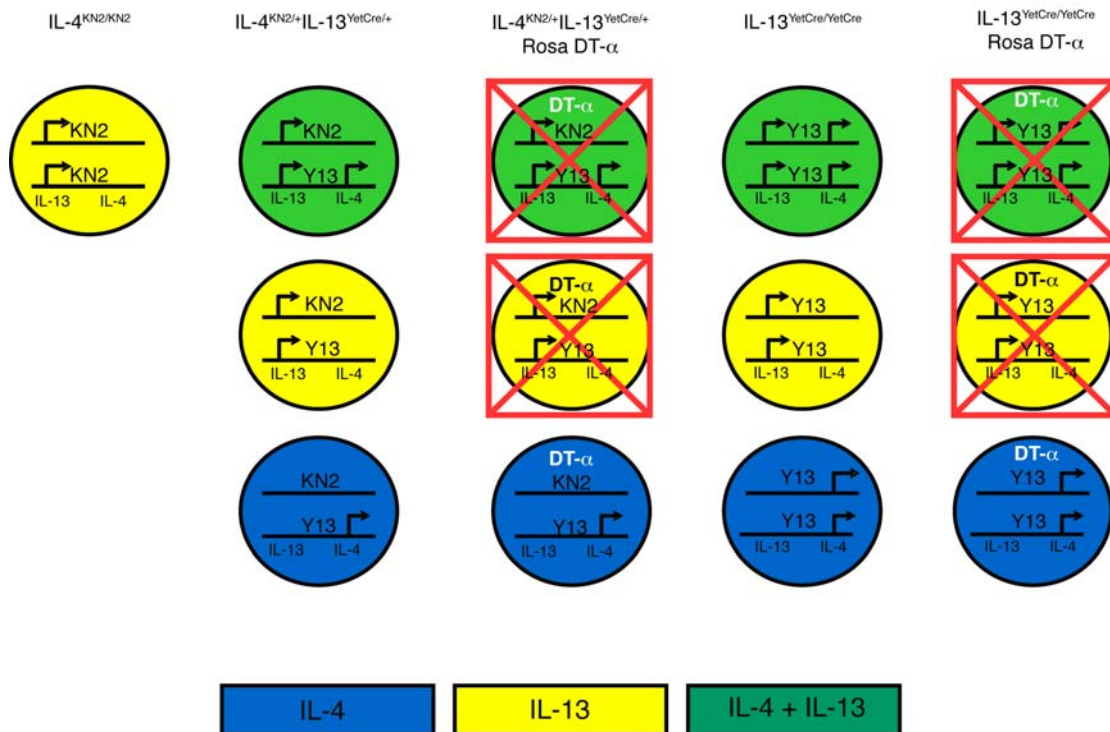


Enzyme-linked immunosorbent assay. First, 96-well plates coated with NP₂₃-BSA or NP₃-BSA (Biosearch) were incubated with serial fivefold dilutions of serum. Then, IgG1 NP-specific antibodies were detected by incubation with biotinylated antibody to mouse IgG1 (RMG1-1; BioLegend) followed by streptavidin-horseradish 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).

SUPPLEMENTARY MATERIALS

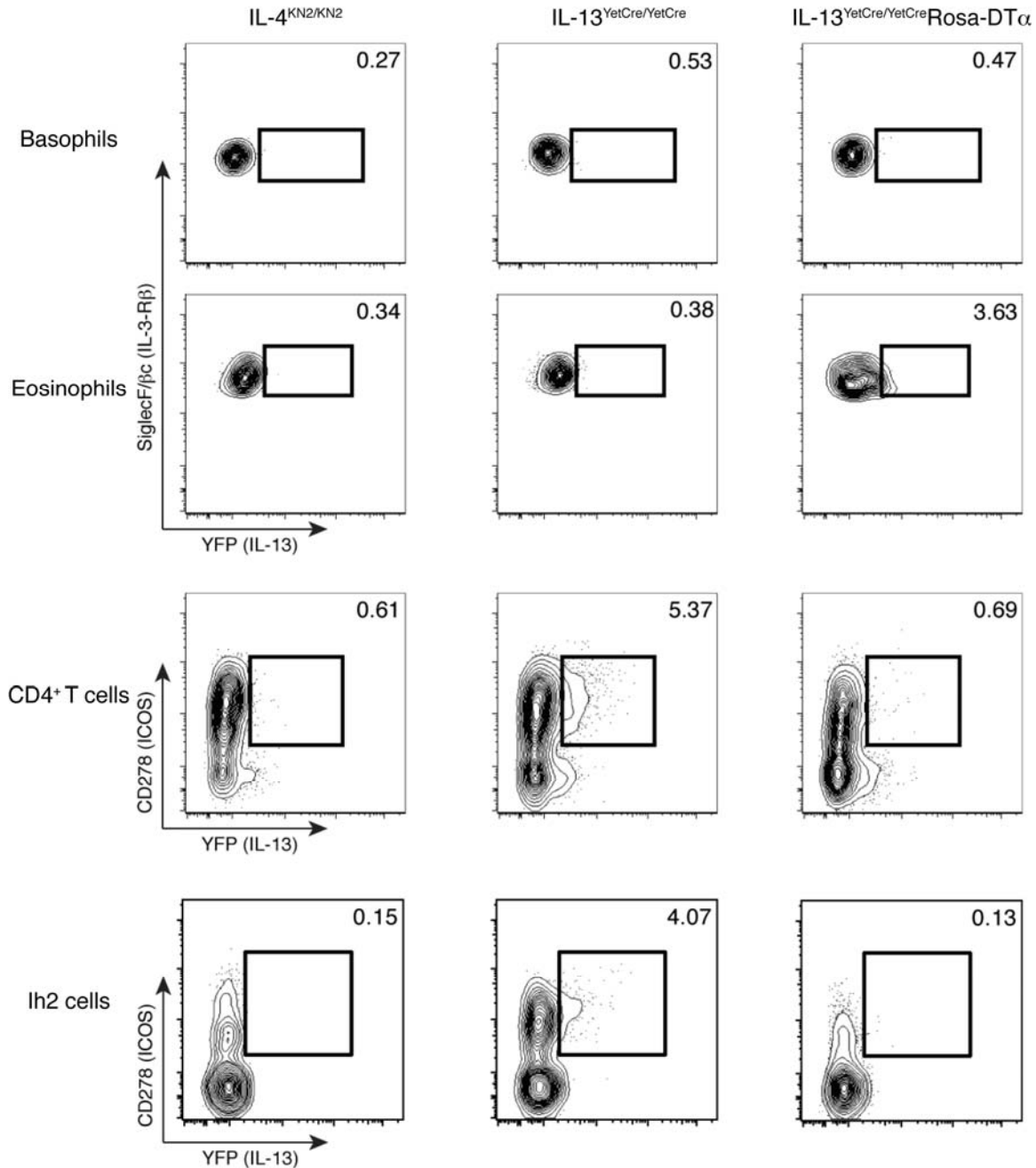
Supplementary Figures



Supplementary Figure 1

Genotype and Phenotype of various cytokine reporter mice

Model diagram of genotype, cytokine expression, and Rosa-diphtheria toxin expression of various reporter mice used in this study. Circles represent the possible cell fate and cytokine expression patterns within the indicated genotype assuming biallelic expression of cytokine genes. Colors represent cytokines expressed in the indicated genotypes after *N. brasiliensis* infection. Yellow = IL-13 alone, Blue = IL-4 alone, Green = IL-4 + IL-13. Red cross represents cytokine expression pattern resulting in diphtheria toxin production and cell death.

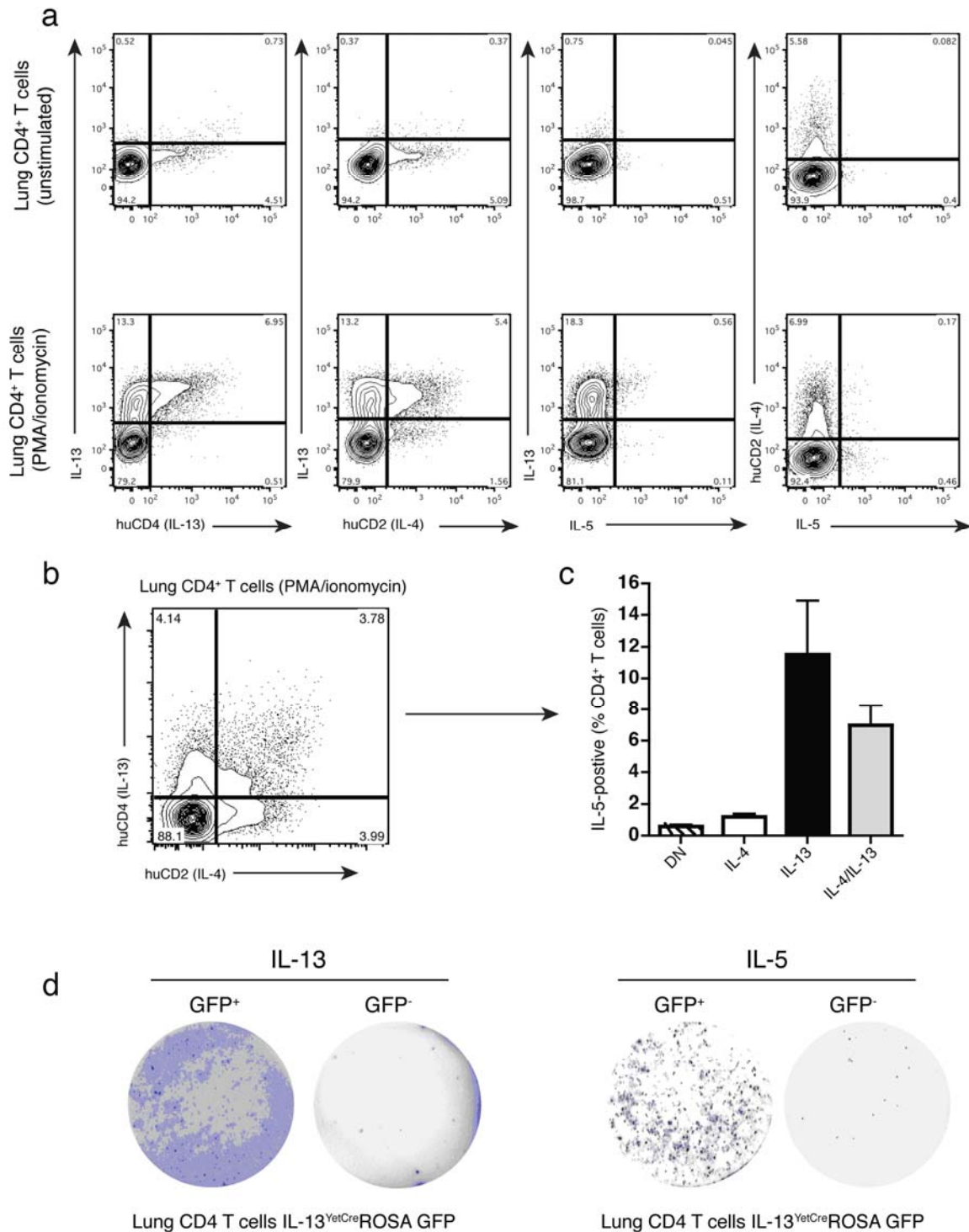


Supplementary Figure 2.

IL-13 expression by immune cells after *N. brasiliensis* infection

Flow cytometry of lung from indicated cytokine reporter mice infected 8 days previously with *N. brasiliensis*. Plots represent DAPI⁻, CD19⁻, CD8⁻ lymphocytes stained with various surface markers to discriminate the indicated subsets.

Basophils (CD4^{neg}, Siglec-F^{neg}, CD49b^{pos}), Eosinophils (CD4^{neg}, Siglec-F^{pos}, CD49b^{neg}, SSC^{high}), CD4 cells (CD4^{pos}, ICOS^{high}), Ih2 cells (CD4^{neg}, Siglec-F^{neg}, CD49b^{neg}, ICOS^{high}). The numbers represent the percent of total YFP⁺ cells in the gate. Plots are representative of 3 independent experiments.



Supplementary Figure 3

IL-5 production is restricted to IL-13-producing Th2 cells in the lung

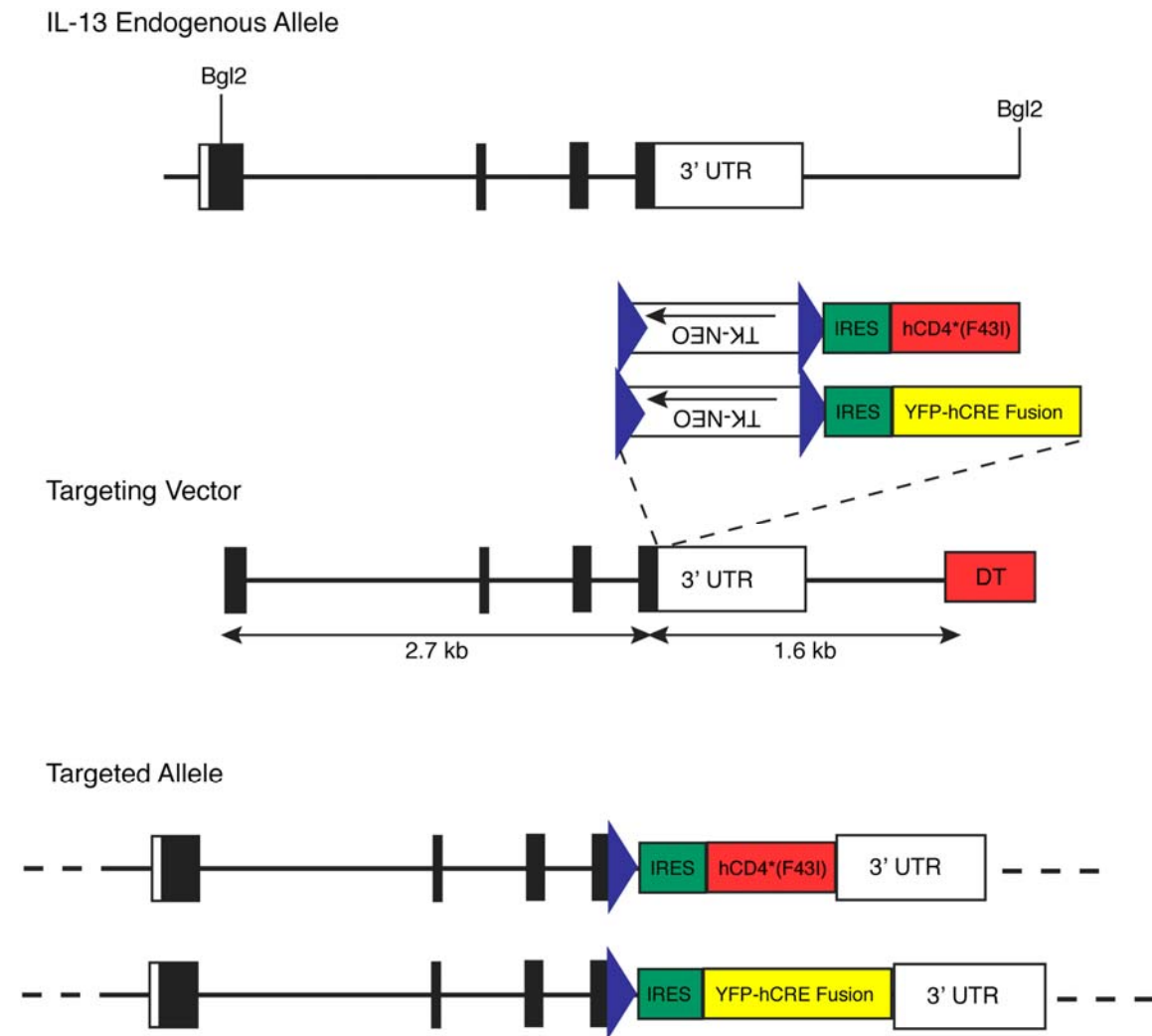
(a) CD4⁺ T cells were isolated from the lungs of IL-4^{KN2/+} IL-13^{Smart/+} mice seven days post infection with *N. brasiliensis*. Single cells suspensions were left untreated (Top panel) or pulsed for 2 hours with PMA/ionomycin (bottom panel). Cells were harvested and assessed using intracellular cytokine staining. Gates represent CD4 T cells (Live/dead dye^{neg}, CD8^{neg}, CD19^{neg},

CD11b^{neg}, CD4^{pos} cells) stained as indicated for cytokine reporters huCD2 (IL-4) and huCD4 (IL-13) and intracellular IL-5 and IL-13.

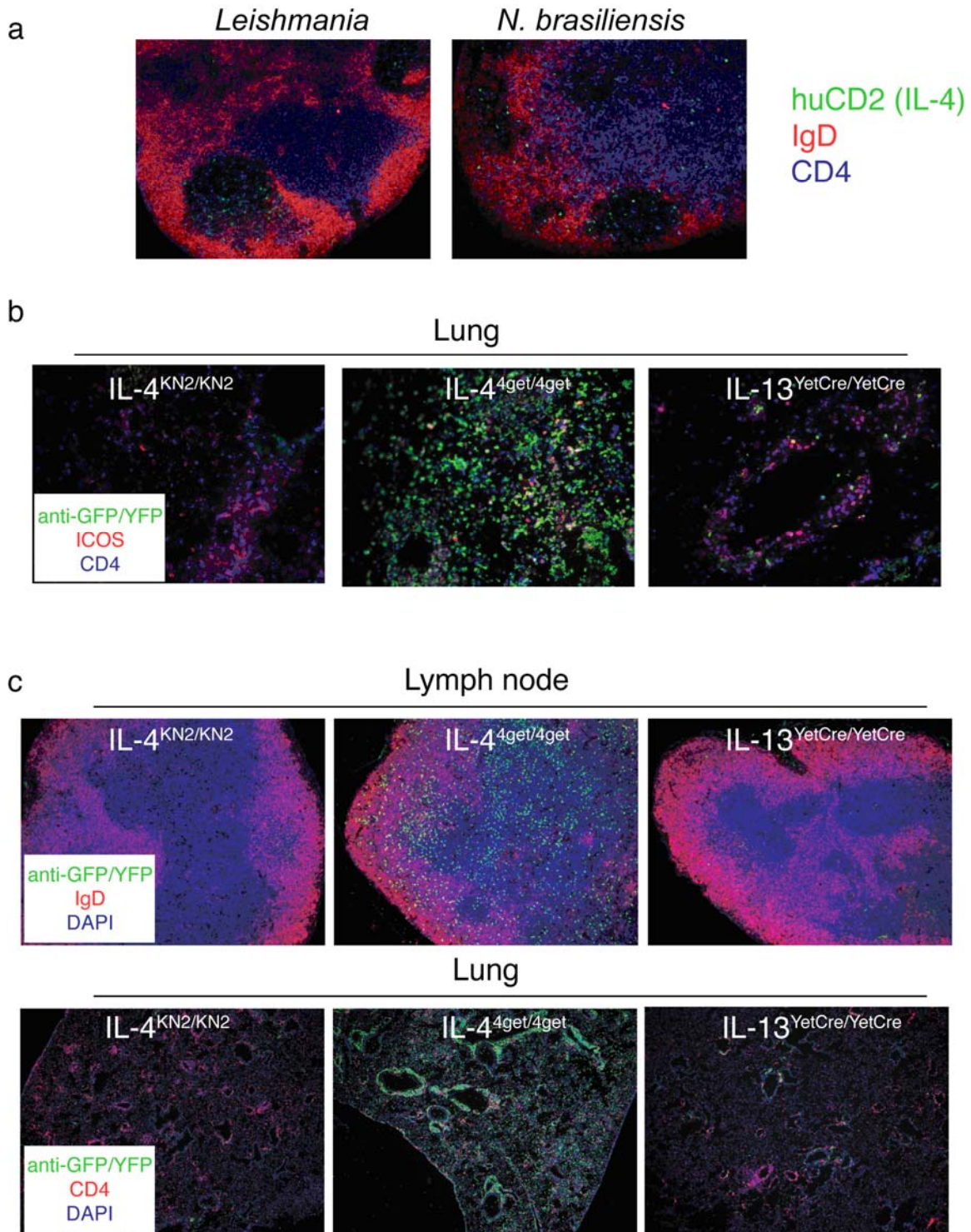
(b) Plots represent PMA/ionomycin-pulsed lung cells as described in (a). Number in each quadrant represents the percentage of CD4 T cells expressing huCD2 (IL-4-reporter) and huCD4 (IL-13-reporter).

(c) Graph represents the percentage of cells in each quadrant from (b) that express IL-5 $\pm$ S.E.M. by intracellular cytokine staining. (n = 3 mice)

(d) Photos represent one well of an ELISPOT. GFP⁺ or GFP⁻ CD4 T cells were isolated and sorted from lungs 9 days after *N. brasiliensis* infection. Cells were plated at 4,500 cells per well and stimulated for 24 hours with PMA/ionomycin. ELISPOTS were developed using biotinylated anti-IL-13 and anti-IL-5 antibodies as indicated followed by streptavidin-Alkaline Phosphatase and BCIP substrate.



Supplementary Figure 4
Schematic for Smart13 (IL-13^{Smart}) and YetCre13 (IL-13^{YetCre}) alleles.



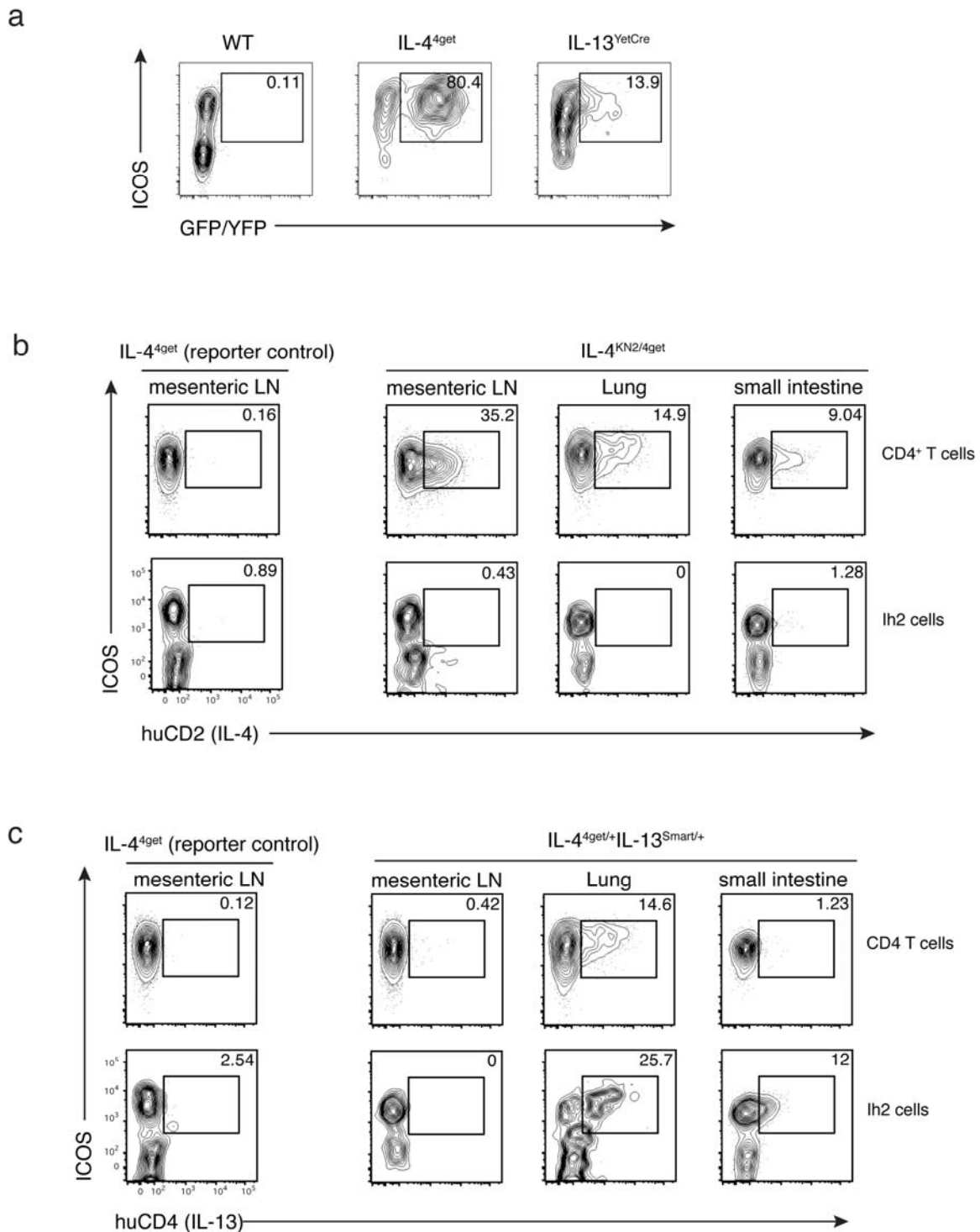
Supplementary Figure 5

IL-13 expression in the lung and lymph nodes of mice after parasite infection

(a) IL-4^{KN2/4get} mice were infected for 21 days with *Leishmania major* (left) and 8 days with *N. brasiliensis* (right), and popliteal lymph nodes were harvested and directly frozen. Six micron sections were obtained and dehydrated in acetone prior to staining for huCD2 (green), IgD (red), and CD4 (blue). Magnification 100x. Images are representative of at least 2 independent experiments.

(b) Lungs from indicated cytokine reporter mice infected 8 days previously with *N. brasiliensis* were dissected, fixed and sectioned as described in material and methods. Six micron sections of lungs from the indicated mice were stained for anti-GFP (green), ICOS (Red) and CD4 (blue). IL-4^{KN2/KN2} lungs served as a negative control because these mice lack GFP and YFP expression. Magnification 200x. Images are representative of at least 2 independent experiments.

(c) Mediastinal lymph nodes (top) and lungs (bottom) from indicated cytokine reporter mice infected 8 days previously with *N. brasiliensis* were prepared as in (b). Lungs from indicated mice were stained for anti-GFP (green), ICOS (Red), and CD4 (blue). IL-4^{KN2/KN2} lungs served as a negative control because these mice lack GFP and YFP expression. Magnification 200x. Images are representative of at least 2 independent experiments.

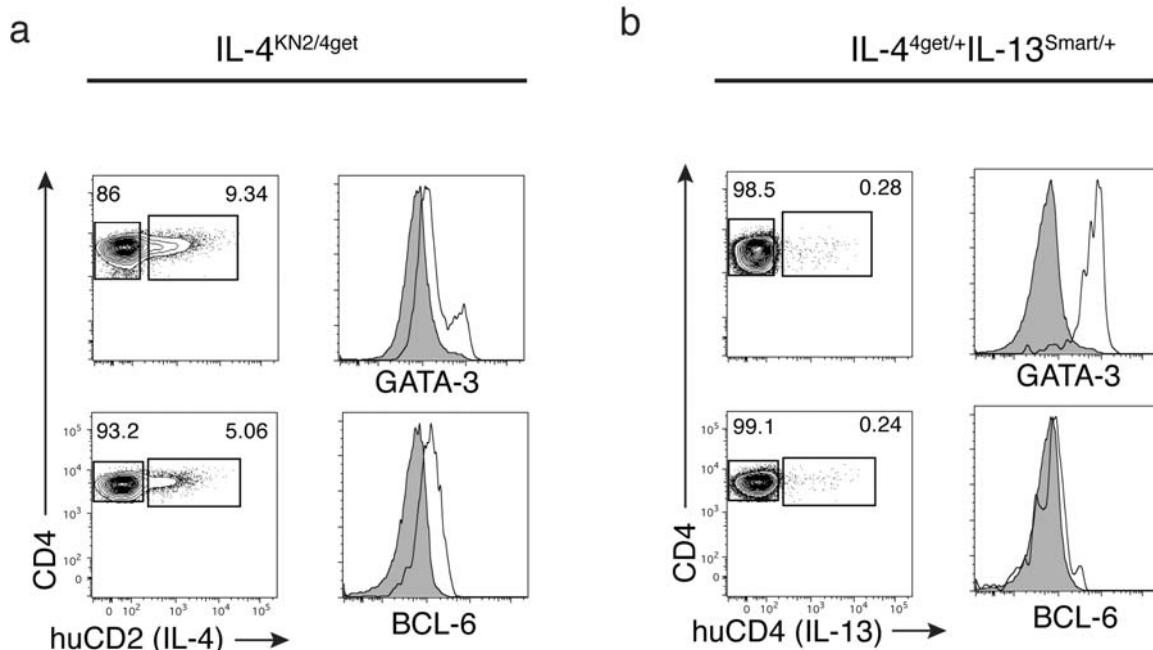
**Supplementary Figure 6****IL-4 and IL-13 expression among CD4 T cells and Ih2 cells in the Gut**

Flow cytometry of cells isolated from indicated cytokine reporter mice infected 9 days previously with *N. brasiliensis*.

(a) Plots represent the percentage of GFP (4get) or YFP (YetCre13) positive cells among CD4 T cells and Lineage negative Ih2 cells.

(b) The plots are gated on GFP⁺ CD4 Tcells (top panel) and lineage negative Ih2 cells (bottom panel) from IL-4^{KN2/4get} mice infected for nine days with *N. brasiliensis*. The cells were isolated from the indicated organs. Plots are representative of 3 independent experiments.

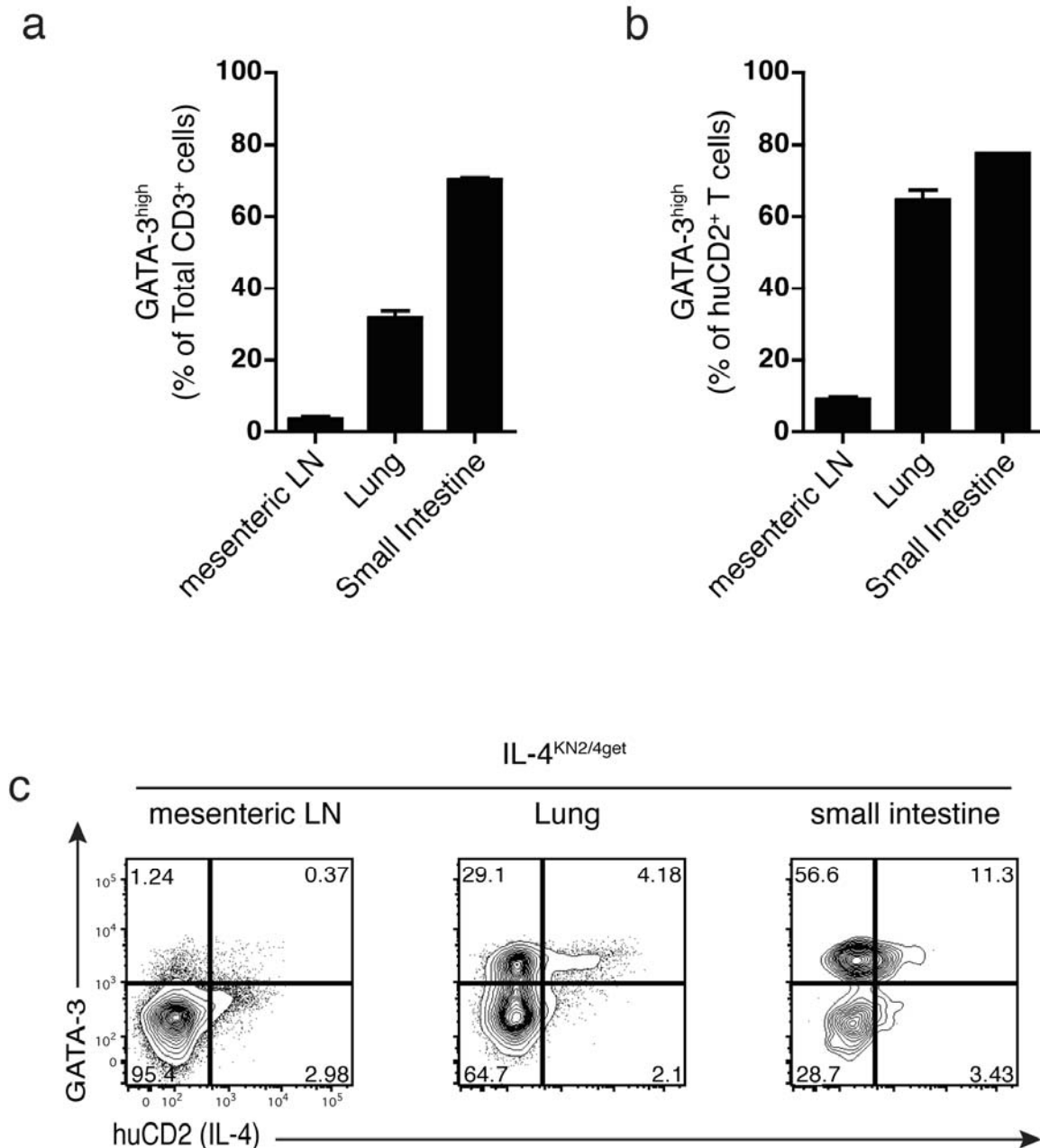
(c) The plots are gated on GFP⁺ CD4 Tcells (top panel) and lineage negative Ih2 cells (bottom panel) from IL-4^{4get/+}IL-13^{Smart/+} mice infected for nine days with *N. brasiliensis*. The cells were isolated from the indicated organs. Plots are representative of 3 independent experiments.



Supplementary Figure 7

GATA-3 and BCL-6 expression among IL-4 and IL-13 expressing cells

Flow cytometry of cells isolated from indicated cytokine reporter mice infected 8 days previously with *N. brasiliensis*. **(a)** Plots represent the percentage of huCD2^{pos} or huCD2^{neg} CD4 T cells from draining mediastinal lymph nodes of IL-4^{KN2/4get} mice. Histograms mark the expression of GATA-3 (top panel) and BCL-6 (bottom panel) from huCD2 negative (filled histogram) or huCD2⁺ (black line) CD4 T cells. **(b)** Plots represent the percentage of huCD4^{pos} or huCD4^{neg} CD4 T cells from draining mediastinal lymph nodes of IL-4^{4get/+}IL-13^{Smart/+} mice. Histograms mark the expression of GATA-3 (top panel) and BCL-6 (bottom panel) from huCD4 negative (filled histogram) or huCD4⁺ (black line) CD4 T cells.



Supplementary Figure 8

IL-4 expression is restricted to CD4 T cells expressing high-levels of GATA-3 in the gut

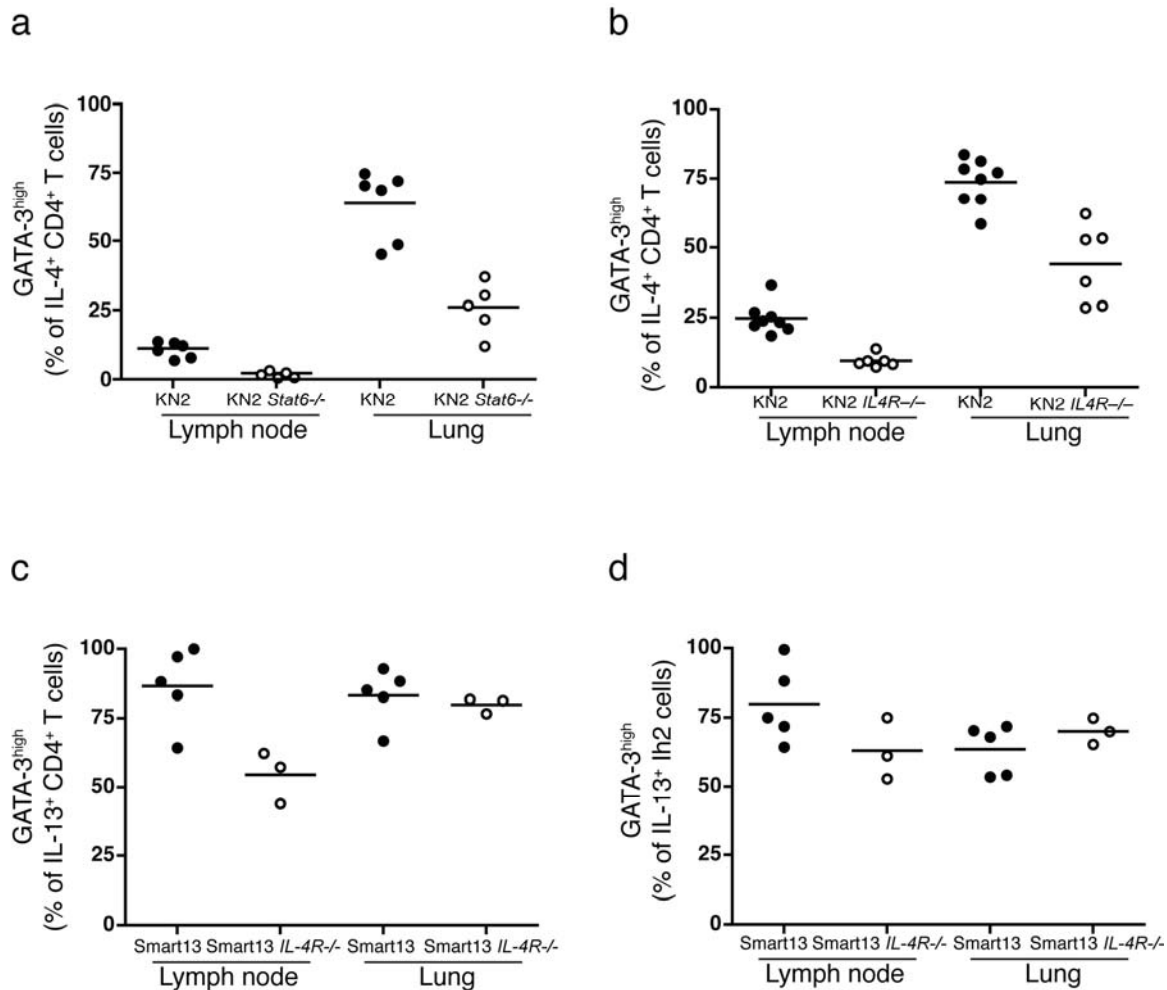
IL-4^{KN2/4get} mice were infected with *N. brasiliensis* and draining lymph nodes, lungs, and small intestine were harvested 9 days after infection.

(a) Graph represents the percentage of total CD3⁺ T cells expressing high levels of GATA-3 from IL-4^{KN2/4get} mice in the indicated organs. Data points are combined from two independent experiments. (n = 6 mice per group; gut cells pooled).

(b) Graphs represent the percentage of IL-4-secreting CD4 T cells expressing high levels of GATA-3 from CD3⁺, huCD2⁺, IL-4^{KN2/4get} mice in the indicated

organs. Data points are from one experiment representative of two independent experiments (n=3 mice per group, gut cells pooled).

(c) Live, CD4⁺, CD3⁺, CD8⁻, CD11b⁻, CD19⁺ T cells were stained for huCD2 (IL-4 secretion) followed by intracellular staining for GATA-3. Plots are representative of 2 independent experiments.



Supplementary Figure 9

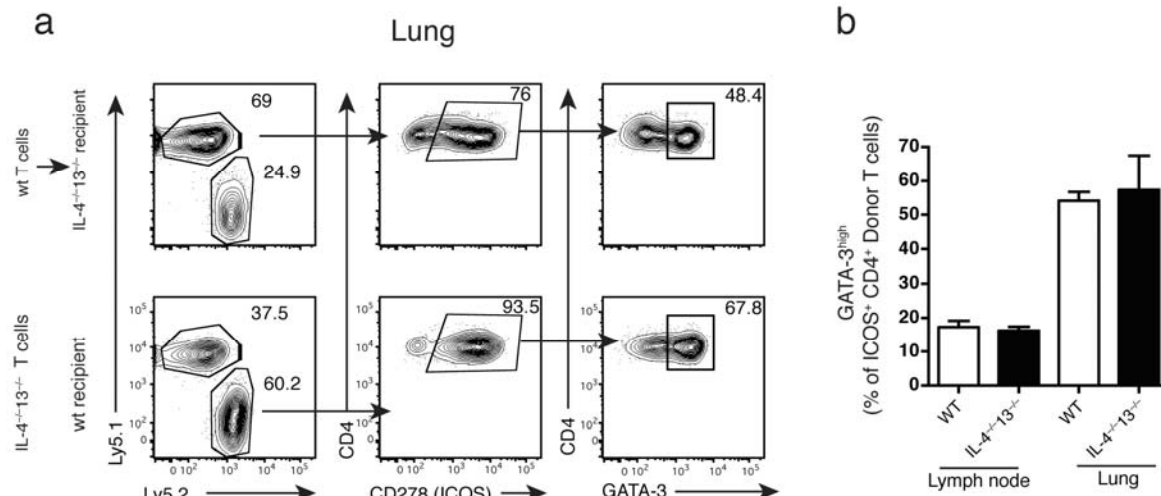
IL-4 and IL-13 signaling is not required for increased GATA-3 expression

(a) Wild-type and *Stat6*-deficient IL-4^{KN2/4get} mice were infected with *N. brasiliensis*, and lymph nodes and lungs were harvested 8 days after infection. Graph represents the percentage of IL-4-producing (huCD2⁺) CD4⁺ T cells expressing high levels of GATA-3 from wild-type and *Stat6*-deficient IL-4^{KN2/4get} mice. Wild-type CD4 T cells (filled circles); *Stat6*-deficient CD4⁺ T cells (white circles). Each data point represents an individual mouse combined from two separate experiments.

(b) Wild-type and IL-4 receptor-deficient IL-4^{KN2/4get} mice were infected with *N. brasiliensis*, and lymph nodes and lungs were harvested 8 days after infection. Graph represents the percentage of IL-4-producing (huCD2⁺) CD4⁺ T cells expressing high levels of GATA-3 from wild-type and IL-4 receptor-deficient IL-4^{KN2/4get} mice. Wild-type CD4 T cells (filled circles); IL-4 receptor-deficient CD4⁺ T cells (white circles). Each data point represents an individual mouse combined from two separate experiments.

(c, d) Wild-type and IL-4 receptor-deficient IL-4^{4get/+} IL-13^{Smart/+} mice were infected with *N. brasiliensis*, and lymph nodes and lungs were harvested 8 days after infection. Graph represents the percentage of IL-13-producing (huCD4⁺) CD4 T cells **(c)** or Ih2 cells **(d)** expressing high levels of GATA-3 from wild-type and IL-4 receptor-deficient IL-4^{4get/+} IL-13^{Smart/+} mice. Wild-type CD4 T cells (filled circles); IL-4 receptor-deficient CD4 T cells

(white circles). Each data point represents an individual mouse and is representative of two independent experiments.



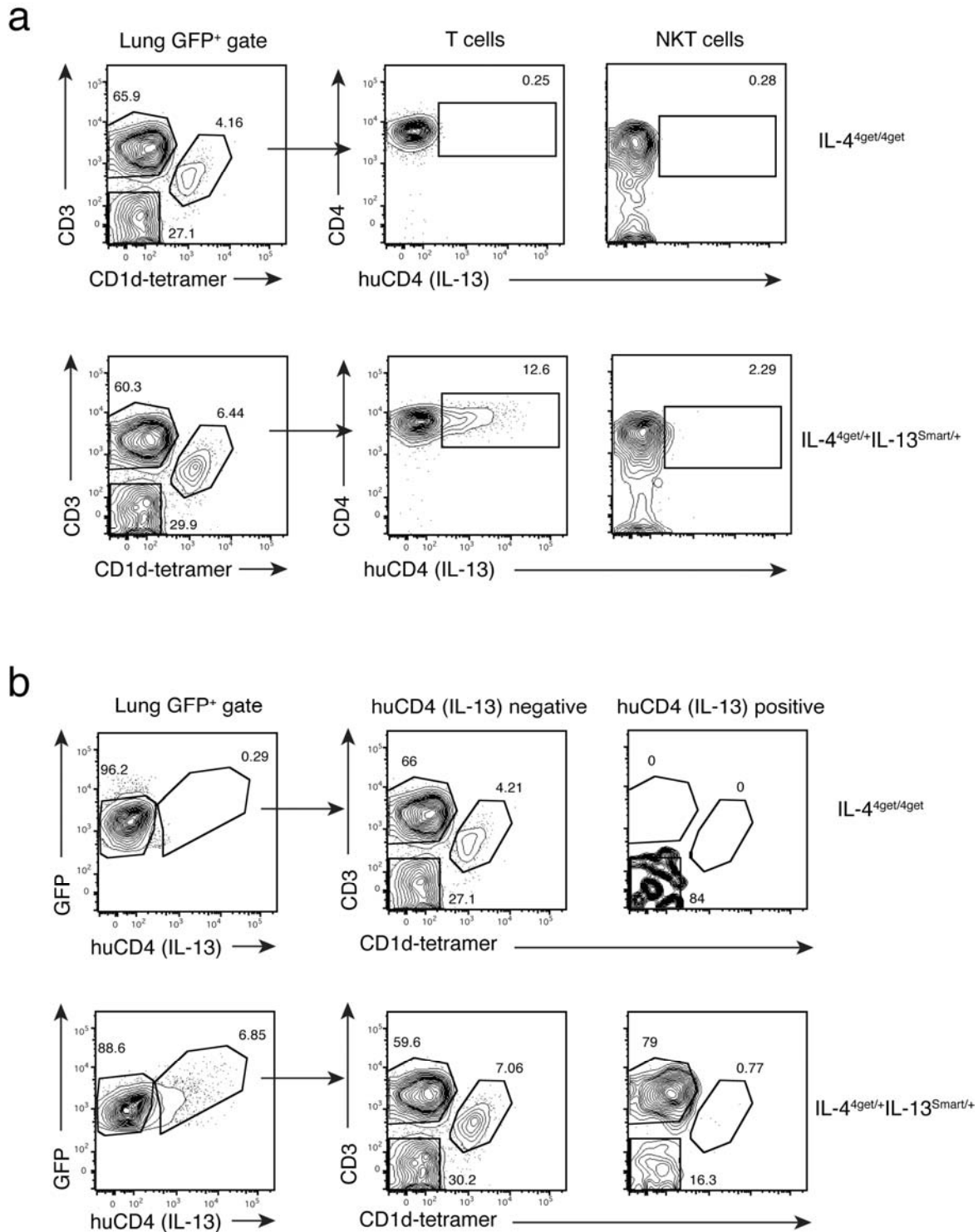
Supplementary Figure 10

Regardless of the cellular source, neither IL-4 nor IL-13 is required for the development of GATA-3^{hi} T_H2 cells

(a) Wild-type and IL-4^{-/-}IL-13^{-/-} deficient mice were lightly irradiated and transferred with 3-5 x 10⁶ donor CD4 T cells. Wild-type CD45.1 mice received T cells from IL-4^{-/-}IL-13^{-/-} CD45.2 mice and IL-4^{-/-}IL-13^{-/-} recipients received T cells from congenic CD45.1 wild-type mice. Mice were rested for 7 days before being infected with *N. brasiliensis*.

Mediastinal lymph nodes and lungs were harvested 8 days after infection and donor activated CD4 T cells were stained for GATA-3. Top Panel: Wild-type CD45.1 T cells into IL-4^{-/-}IL-13^{-/-} recipients. Arrows mark the gating for the percentage of GATA-3^{high} cells among total ICOS⁺ CD4⁺, CD8⁻, CD19⁻, CD45.1⁺, CD45.2⁻ donor T cells. Bottom Panel: IL-4^{-/-}IL-13^{-/-} T cells into wild-type CD45.1 recipients. Arrows mark the percentage of GATA-3^{high} cells among total ICOS⁺, CD4⁺, CD8⁻, CD19⁻, CD45.1⁺, CD45.2⁺ donor T cells. Gates are representative of two independent experiments. (n = 3-4 mice per experiment).

(b) Graph represents the percentage of GATA-3^{high} donor T cells within the lymph nodes and lung 8 days after *N. brasiliensis* infection. Data is representative of two independent experiments. (Percentage of GATA-3^{high} cells ± S.E.M.; n = 3-4 mice per experiment).



Supplementary Figure 11

IL-13 expression from invariant NKT (iNKT) cells is negligible after *N. brasiliensis* infection

$IL-4^{4get/4get}$ and $IL-4^{4get/+}IL-13^{Smart/+}$ mice were infected with *N. brasiliensis*. Lungs were harvested 8 days after infection and were stained for the expression of huCD4 (IL-13) and CD1d-tetramer to assess IL-13 production within T cells and invariant NKT (iNKT) cells.

(a) Gates in left plot represent CD3^{pos} T cells (CD3^{pos}, CD1d-tetramer^{neg}) or iNKT cells (CD3^{pos}, CD1d-tetramer^{pos}) among total GFP^{pos}, DAPI^{neg}, CD11b^{neg}, CD8^{neg}, CD19^{neg} cells isolated from

4get (top panel) or 4get-Smart13 (bottom panel) lungs. Arrows indicate CD4 and huCD4 (IL-13) staining within the indicated T cells (left plot) and iNKT cells (right plot) gates. Number within the gate represents the percentage of CD4^{pos}, huCD4^{pos} cells within the plot.

(b) Gates in left plot represent huCD4^{neg} or huCD4^{pos} cells among total GFP^{pos}, DAPI^{neg}, CD11b^{neg}, CD8^{neg}, CD19^{neg} cells from IL-4^{4get/4get} (top panel) or IL-4^{4get/+}IL-13^{Smart/+} (bottom panel) lungs. Arrows indicate CD3 and CD1d-tetramer staining within the indicated IL-13-negative (left plot) and IL-13-positive (right plot) gates. Number within the gate represents the percentage of T cells (CD3^{pos}, CD1d-tetramer^{neg}), iNKT cells (CD3^{pos}, CD1d-tetramer^{pos}) within each gate. Plots are representative of two independent experiments. (n = 2-3 mice per experiment).