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

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Abstract

Interleukin 4 (IL-4) and IL-13 are critical for responses against parasitic helminthes. Here we used genetically engineered reporter mice to assess the temporal and spatial production of these cytokine *in vivo*. In lymph nodes, IL-4 was confined to T follicular helper (T_{FH}) cells, however these cells did not make IL-13. In contrast, tissue type 2 helper T T_{H2} cells produce both cytokines. Divergent IL-4 and IL-13 production also occurred among innate immune cells, where basophils produced IL-4, while innate helper type 2 (Ih2) cells produced IL-13. IL-13 production by T_{H2} and Ih2 cells was dependent on high GATA-3 levels, in contrast to low GATA-3 levels in T_{FH} cells and basophils. Distinct localization and cellular expression of IL-4 and IL-13 explains their unique roles during allergic immunity.

Keywords

T_{H2} cells; follicular helper T cells; cytokines; IL-4; IL-13; Ih2 cells

Introduction

Intestinal helminth infections represent one of the most prevalent chronic human infections worldwide ¹. Infection is associated with a polarized immune response including the accumulation of T_{H2} cells, eosinophils and basophils in tissues, elevation of serum IgE, hyperplasia of mucosal mast cells, alternative activation of tissue macrophages and epithelial and smooth muscle alterations that change the physiologic milieu of the host-

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The authors declare no competing financial interests.

Author Contribution

H-E.L., R.L.R., and R.M.L. conceived 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 performed experiments. I.C.H. contributed critical reagents. R.L.R. and R.M.L. wrote the manuscript.

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 IL-4 and IL-13. Recent recognition of an additional innate immune cell 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 implicate the cytokines themselves in mediating the diverse tissue manifestations associated with type 2 immunity. Indeed, whether administered exogenously, over-expressed or deleted, these two cytokines are necessary and sufficient to mediate most of the immune and physiologic aspects of type 2 immunity^{2, 9-13}. IL-4 and IL-13 are encoded by adjacent genes that share a number of *cis*-acting and *trans*-activating regulatory elements and transmit signals through a partially shared receptor and adaptor system^{14, 15}. Despite this, epigenetic and functional studies have suggested unique and nonredundant roles for these cytokines during immunity *in vivo*, although the mechanisms underlying these distinctions are not readily apparent based on *in vitro* studies^{2, 15-17}. A number of innate and semi-invariant lymphocytes, including basophils, eosinophils, mast cells, innate helper 2 (Ih2) cells and natural killer T 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 which is difficult to examine with currently existing tools. Further understanding of the organization of type 2 immunity in secondary lymphoid tissues such as lymph nodes and spleen and in non-lymphoid 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 novel strains of mice with genetically altered loci at the *Il4* and *Il13* genes that allow detection of cytokine expression by individual cells *in vivo*. Using infections that induce robust type 2 host responses, we compared the production of IL-4 and IL-13 within selected lymphoid and tissue compartments. These studies reveal unexpected parsing of expression of these two closely related cytokines among cells in both the adaptive and innate immune cell populations. IL-13 production was largely confined to T_H2 cells and Ih2 cells in the lung, and was associated with high levels of cellular GATA-3, which was necessary for sustaining the IL-13-producing phenotype. Conversely, T_{FF} cells and basophils produced only IL-4 *in vivo* and did not express high levels of GATA-3.

RESULTS

Compartmentalization of expression for IL-13 and IL-4

IL-4 and IL-13 share an overlapping spectrum of receptors, but studies with neutralizing reagents and individual cytokine and cytokine receptor deficient mice suggest a more prominent role for IL-13 in mediating the tissue responses that occur during helminth infection with *Nippostrongylus brasiliensis*^{2, 10}. Despite this information, precise

knowledge regarding which cells produce IL-4 and IL-13 and their localization *in vivo* is lacking. We used a genetic approach in order 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 a previously described IL-4 reporter mice (IL-4^{KN2}), which contain a non-signaling human CD2 (huCD2) replacement gene at the IL-4 start site. Cells activated to produce IL-4 in IL-4^{KN2} mice express huCD2 on the cell membrane and accurately report IL-4-producing cells *in vitro* and *in vivo*^{20, 21}. Homozygous IL-4^{KN2/KN2} mice are IL-4-deficient, while heterozygous IL-4^{KN2/+} mice can express IL-4 from the endogenous *Il4* allele. Additionally, we generated IL-13 reporter mice containing a yellow fluorescent protein (YFP)-humanized Cre recombinase (YetCre13) fusion protein behind an IRES element introduced immediately downstream of the endogenous IL-13 stop codon and upstream of the 3' untranslated sequence. This construct leaves endogenous IL-13 intact while establishing a bicistronic link with the YFP-Cre fusion sequence⁸. Cells from IL-13^{YetCre/YetCre} mice are capable of making both endogenous IL-4 and IL-13. Crossing IL-4^{KN2} and IL-13^{YetCre} mice allows simultaneous detection of IL-4- and IL-13-expressing cells (Fig. 1a, Supplementary Fig. 1). The introduction of an additional ROSA26-flox-stop-diphtheria toxin α (ROSA-DT α) allele allowed *in vivo* elimination of cells that had activated IL-13 gene expression, as expression of the YFP-Cre fusion protein removes the flox-stop sequence enabling toxin expression to ensue (Fig. 1a, Supplementary Fig. 1)^{8, 22}.

To evaluate type 2 immunity in these various genetic strains of mice, we assessed the worm burden in the small intestine 9 days after infection with *N. brasiliensis* (Fig. 1a). Consistent with prior observations, IL-4-deficient IL-4^{KN2/KN2} mice expelled worms normally. Immunity against *N. brasiliensis* was also intact in IL-4^{KN2/+}IL-13^{YetCre/+} or IL-13^{YetCre/YetCre} (Fig. 1b). However, in IL-4^{KN2/+}IL-13^{YetCre13/+}ROSA-DT α mice, worms were present at 9 days post infection in mice with one YetCre13 allele and were twice as prevalent when two reporter alleles were present to drive Cre-mediated deletion (Fig. 1b).

While the absence of IL-4 in IL-4^{KN2/KN2} mice had no effect on eosinophil tissue recruitment, depletion of IL-13-producing cells led to 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 express YFP in IL-13^{YetCre} mice (as analyzed by flow cytometry), nor were these cells deleted from the blood, bone marrow or tissues in IL-13^{YetCre/YetCre}ROSA-DT α mice (Supplementary Fig. 2). The deletion of IL-13-producing cells in IL-13^{YetCre/YetCre}ROSA-DT α mice led to the concomitant ablation of IL-5, as these cytokines are co-expressed in this system (Supplementary Fig. 3). It is likely that loss of both of these cytokines accounts for the reduced eosinophilia in these mice. In contrast to the effects on eosinophils, the numbers of basophils and CD4⁺ T cells in the lung of IL-13^{YetCre/YetCre}ROSA-DT α mice were unaffected by deletion of IL-13-expressing cells (Fig. 1d). Thus, IL-13-producing cells are required for sustaining tissue immunity against *N. brasiliensis* and these genetic variant strains corroborate earlier observations while validating the use of these reporter mice^{2, 9, 11, 23}.

IL-4 was required for IgE production, because IL-4^{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, suggesting that IL-4-expressing T_{FH} cells do not activate IL-13 gene expression. To examine the effects of IL-13 expression on IL-4-mediated humoral responses we immunized the various genetically-marked mice with 4-hydroxy-3-nitrophenyl-acetyl (NP)-ovalbumin in alum and assessed total anti-NP IgG1 antibody levels and anti-NP affinity after 28 days as described previously²¹. As expected, the absence of IL-4 in IL-4^{KN2/KN2} and in combined IL-4-IL-13-deficient mice led to impaired IgG1 protein expression and reductions in the high-avidity anti-NP response (Fig. 1f, g). Although total NP-specific IgG1 was somewhat reduced in IL-13^{Y_{et}Cre/Y_{et}Cre}ROSA-DT α mice, in which IL-13-expressing cells were deleted, the affinity maturation of anti-NP IgG1 was unaffected. As affinity maturation occurs in germinal centers, the findings are consistent with the absence of IL-13 expression in T_{FH} cells.

In sum, these studies validated the use of the IL-4^{KN2} and IL-13^{Y_{et}Cre} reporter strains to study aspects of IL-13 and IL-4 expression during helminth infection. In addition, they suggested important roles for IL-4 in humoral responses, but not tissue responses and supported a fundamental role for IL-13 in tissue responses, but not high-affinity B cell responses. Because deletion of IL-13-expressing cells had little effect on the activities of IL-4 expressing T_{FH} cells in lymph nodes, it suggests that the expression pattern of these two cytokines is distinct. As such, IL-4 and IL-13 serve distinct and non-redundant functions in type 2 immunity.

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

We generated a second strain of IL-13 reporter mice by replacing the YFP-Cre fusion protein used in the IL-13^{Y_{et}Cre} reporter with a non-signaling human CD4 marker, thus providing a cell surface membrane-anchored reporter to identify IL-13-expressing cells (Supplementary Fig. 4). These mice, designated Surface MARKer for the Transcript of IL-13 (Smart13 or IL-13^{Smart}), were backcrossed to the BALB/c background and then crossed to IL-4^{KN2} mice, thus enabling the simultaneous detection of both IL-4 and IL-13 cytokine expression at the single-cell level. After infection of IL-4^{KN2/+}IL-13^{Smart/+} mice with *N. brasiliensis*, mediastinal lymph nodes and lung were analyzed for the numbers of IL-4- and IL-13-producing CD4⁺ T cells directly after isolation (Fig. 2a, b). In the lymph nodes, approximately 8% of CD4⁺ T cells expressed IL-4 and IL-13, and of these, 98% expressed only IL-4 and not IL-13. In the lung, approximately 15% of CD4⁺ T cells expressed IL-4 and IL-13 as single or double-expressers, and the numbers of IL-4- and IL-13-expressing CD4⁺ T cells were approximately equal (Fig. 2b). Thus, the ratio of IL-4- to IL-13-producing cells is significantly greater in the lymph node as compared to the lung (Fig. 2c). Of note, after restimulation *in vitro*, lung IL-5-producing CD4⁺ T cells largely co-expressed IL-13, but not IL-4, suggesting that IL-13 and IL-5 share similar regulatory pathways that may be distinct from IL-4 (Supplementary Fig. 3).

Immunohistochemical staining corroborated the increased ratio of IL-4- to IL-13-producing cells in the lymph nodes as compared to the lung (Fig. 2d, Supplementary Fig. 5). As previously reported, IL-4-producing huCD2⁺ cells are T_{FH} cells confined to follicular and germinal center B cell-rich areas²¹. The few IL-13-producing cells observed after acute *N. brasiliensis* or a more chronic *Leishmania major* infection resided almost exclusively in the

T cell zones and parafollicular areas of the lymph node (Fig. 2d, 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 are differentially employed 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 predominantly confined 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 intestine and mesenteric lymph nodes after *N. brasiliensis* infection. Similar to the lung and lung-draining lymph nodes, CD4⁺ T cells isolated from the mesenteric lymph nodes produced substantial amounts IL-4, while production of IL-13 was restricted to the small intestine (Supplementary Fig. 6). These findings show that IL-4 and IL-13 serve distinct functions during allergic immunity in part due to their restricted expression patterns in lymphoid and non-lymphoid tissues.

T_{FH} cells are distinct from T_H2 cells and do not produce IL-13

We next crossed each of the IL-4^{KN2} reporter mice and the IL-13^{Smart} reporter mice to the 4get IL-4-reporter strain of mice (IL-4^{4get}), which allows for transcription of a bicistronic IL-4-IRES-eGFP mRNA and translation of both IL-4 and eGFP from the same mRNA. The resulting crosses allowed the marking of IL-4- or IL-13-producing cells among the larger subset of IL-4- and IL-13-competent cells²⁴. Eight days after *N. brasiliensis* infection, approximately half of the 'competent' GFP⁺ CD4⁺ T cells in the mediastinal lymph node of IL-4^{KN2/4get} mice expressed IL-4 *in situ* (huCD2⁺), and about 40% of these IL-4-producing cells expressed high levels of the T_{FH} cell markers, PD-1 (CD279) and CXCR5 (Fig. 3a,b)^{25–27}. In contrast, few of the GFP⁺ competent CD4⁺ T cells expressed IL-13 in the lymph nodes and none of these IL-13-producing cells expressed T_{FH} cell markers. Among the IL-13-expressing CD4⁺ T cells in the lung, none expressed levels of PD-1 and CXCR5 comparable to lymph node IL-4-producing T_{FH} cells. Thus, IL-13-expressing T cells do not express T_{FH} markers and are not localized to B cell areas. Further, 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} and T_H2 cells, we sorted populations of cytokine-competent and cytokine-producing cells from the mediastinal lymph nodes and lungs of IL-4^{KN2/4get} after *N. brasiliensis* infection and analyzed IL-4 and IL-13 transcripts using quantitative PCR. Although IL-4-producing cells taken from the lymph nodes expressed less IL-4 and IL-13 mRNA than their lung counterparts, they expressed more IL-21, a cytokine associated with T_{FH} cells. Consistent with previous reports on cytokine production by T_{FH} cells during parasitic and viral infection^{2128, 2927}, IL-4-producing cells from the lymph node express significant amounts of both IL-4 and IL-21 mRNA, suggesting that T_{FH} cells are poised to make IL-4 as well as IL-21 during a type 2 immune response (Fig 3c). IL-4-producing cells from the lymph nodes also expressed substantially higher levels of Bcl-6, a key transcription factor in T_{FH} development, as compared to IL-4-producing cells isolated from the lung^{26, 30, 31}. Conversely, lung IL-4-producing cells preferentially expressed Blimp-1, a negative regulator of Bcl-6. Bcl-6 expression inversely correlated not only with Blimp-1, but also with

GATA-3 transcripts. Whether assessed by ‘competence’ (GFP expression from the 4get allele) or ‘expression’ (huCD2 expression from the KN2 allele), IL-4-expressing lymph node CD4⁺ T cells had substantially lower GATA-3 mRNA expression and elevated levels of Bcl-6 transcripts as compared to tissue IL-4- or IL-13-expressing T_H2 cells. This inverse relationship was also found in protein expression using intracellular staining and flow cytometry. Bcl-6-positive cells were only present in lymph nodes and Bcl-6-positive CD4⁺ T cells were virtually absent from lung tissue (Fig. 3d). The GATA-3^{high} cells did not express Bcl-6 in either tissue (Fig. 3d). Furthermore, the majority of IL-4-producing cells in the lymph nodes expressed high levels of Bcl-6, confirming their identity as T_{FH} cells, but little GATA-3, while IL-13-producing cells were uniformly GATA-3^{high} and Bcl-6 negative (Supplementary Fig. 7). These results suggest that T_{FH} and T_H2 cells express distinct and non-overlapping transcription factor profiles, and likely utilize different transcription factors in different ways to achieve cytokine activation from the IL-4 and IL-13 locus.

Cytokine-producing T_H2 cells are GATA-3^{high}

Although GATA-3 was originally implicated in production of IL-4 by T_{FH} cells during helminth infection ²⁹, recent mRNA expression studies during LCMV infection ²⁷ suggested that this might not be the case. To further explore the relationship between intracellular GATA-3 expression and IL-4 and IL-13 expression in T_{FH} and T_H2 cells, we isolated T_{FH} and T_H2 cells from IL-4^{KN2/+}IL-13^{Smart13/+} dual reporter mice after *N. brasiliensis* infection and assessed the amount of intracellular GATA-3 in relation to *in vivo* cytokine expression at the single-cell level. In lymph nodes, the IL-4-producing CD4⁺ T cells were predominantly GATA-3^{low}. Only 20% of the total IL-4-producing cells expressed high levels of intracellular GATA-3 in the lymph nodes (Fig. 4a, b). However, this was not the case in the lung, where over 70% of IL-4 production was associated with a GATA-3^{high} population. This differential expression of GATA-3 in lymphoid and non-lymphoid tissues was also observed 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^{high}, regardless of whether the CD4⁺ T cells were isolated from the lymph nodes (where there were very few cells) or the lung. Thus, IL-4 expression by T_{FH} cells is associated with low expression of GATA-3, whereas expression of IL-13 by T_H2 cells is associated with high levels of intracellular GATA-3 (Fig 3c, d and Fig. 4), independent 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

IL-4 and IL-13 signals can directly drive GATA-3 expression leading to auto-activation and a feed-forward loop that sustains the cells in a GATA-3^{high}, cytokine-expressing state ³². To investigate whether cytokine signaling was required for increased GATA-3 expression and IL-13 production, we generated IL-4^{KN2/+}IL-13^{Smart/+} double-reporter mice on a STAT6-deficient background. There was a decrease in the percentages of GATA-3^{high} CD4⁺ T cells in both the lymph nodes and the lungs (Fig. 5a), and this effect was seen in both the IL-4-expressing and the IL-13-expressing CD4⁺ T cells of the dual-reporter mice (Fig. 5b). Similar results were obtained in IL-4^{KN2/4get} and IL-4^{4get/+}IL-13^{Smart/+} mice deficient in IL-4 receptor-α expression (Supplementary Fig. 9). Of note, although we observed a

reduction in the percentages of IL-4-producing CD4⁺ T cells in the lymph nodes and lungs of IL-4^{KN2/+}IL-13^{Smart/+} mice as compared to wild-type mice, there was no difference in the numbers of total IL-4-producing cells at 8 days after worm infection (Fig. 5c). Similarly, we found no significant effect of STAT6-deficiency on the percentages or numbers of IL-13-producing CD4⁺ T cells in the lung of IL-4^{KN2/+}IL-13^{Smart/+} mice (Fig. 5c). Furthermore, when sub-lethally irradiated wild-type and IL-4-IL-13-deficient mice were reconstituted using either congenic IL-4-IL-13-deficient or wild-type naïve CD4⁺ T cells, and then infected with *N. brasiliensis*, similar proportions of GATA-3^{high} cells were observed among activated (ICOS⁺) donor T cells, suggesting that IL-4 and IL-13 derived from T cells or from host cells were not necessary to drive GATA-3 expression to high levels (Supplementary Fig. 10). Thus, high GATA-3 expression levels associated with tissue cytokine expression, particularly IL-13, are optimized by the presence of IL-4 and IL-13 signaling, but neither cytokine is required for the development of T_H2 cells to a GATA-3^{high}, cytokine-expressing state *in vivo*. IL-4 production in lymphoid tissues occurs independently of both STAT6 and increased 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 multiple cell types with competence (GFP expression) to produce IL-4, including T_H2 and T_{FH} cells, but also NKT cells, mast cells, basophils, eosinophils and innate helper type-2 (Ih2) cells, all of which express GFP constitutively in 4get mice due to the permissive translational features of the IRES^{4, 8, 18, 19, 33, 34}. To assess the contributions by these various cells to tissue responses in type 2 immunity, nine days after infection of IL-4^{KN2/4get} and IL-4^{4get/+}IL-13^{Smart/+} mice with *N. brasiliensis*, IL-4-expressing GFP⁺huCD2⁺ cells in IL-4^{KN2/4get} mice and IL-13-expressing GFP⁺huCD4⁺ cells in IL-4^{4get/+}IL-13^{Smart/+} mice were assessed in the lung (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 could not directly visualize IL-13 expression in other cytokine-competent cell populations, including eosinophils, NKT cells and basophils, which is 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 is capable of expressing IL-4 and IL-13 after stimulation *in vitro*, this study reveals that expression *in vivo* is regulated in a much more restricted fashion than previously anticipated.

CD4⁺ T cells were the only cells that produced both IL-4 and IL-13 in the lung (Fig. 6a, b). We confirmed the restricted pattern of IL-13 expression among Ih2 and CD4⁺ T cells by infecting IL-13^{Y^{et}Cre} mice with *N. brasiliensis*. As observed in infected IL-4^{4get/+}IL-13^{Smart/+} mice, CD4⁺ T cells were the predominant IL-13-producing subset. However, Ih2 cells, marked as Lineage (markers for common lineage determinants for T cells, B cells, NK cells, dendritic cells or other myeloid cells⁷)-Siglec-F⁻CD131(common β -chain)⁻CD49b⁻ICOS⁺ cells, also produced IL-13 nine days post-infection (Fig. 6c, d). Ih2 cells were substantially deleted in IL-13^{Y^{et}Cre}ROSA-DT α mice, indicating that these cells are a relevant population of IL-13-expressing cells in this infectious model (Fig. 6e). IL-13

expression restricted to T_H2 cells and Ih2 cells was also observed in the small intestine of *N. brasiliensis*-infected IL4^{4get/+}IL-13^{Smart/+} mice (Supplementary Fig. 6).

IL-4 expression by basophils and IL-13 expression by Ih2 cells resembled the distinctive cytokine patterns of T_{FFH} and T_H2 cells, which express IL-4 or IL-13 respectively in a manner associated with distinct amounts of GATA-3. Lineage⁻ICOS⁺ Ih2 cells comprise the only non-T cell GATA-3 high population in the lung (Fig. 7a). In contrast, basophils, assessed by CD49b (DX5) expression, are the only IL-4-producing innate cells in the lung at 9 days post *N. brasiliensis* infection and do not express GATA-3. Intracellular GATA-3 staining in IL-4^{KN2/4get} and IL4^{4get/+}IL-13^{Smart/+} reporter mice showed that GATA-3 high T_H2 cells express both IL-4 and IL-13 *in vivo*, whereas GATA-3-high Ih2 cells and GATA-3 low basophils express only IL-13 or IL-4, respectively (Fig. 7b). Thus, as in T cells, IL-13 expression occurs only in GATA-3-high innate cells, which during *N. brasiliensis* infection are Ih2 cells.

As with T_H2 cells, the total numbers of ICOS^{high} GATA-3^{high} Ih2 cells and the percentages of IL-13-producing Ih2 cells in the lung were not significantly reduced in the absence of STAT6 (Fig. 7c, d). However, there was a 2-fold reduction in the total numbers of IL-13-producing Ih2 cells in the lung of STAT6-deficient, IL-4^{KN2/+}IL-13^{Smart/+} mice on day 8 after *N. brasiliensis* infection. Greater than 95% of the Ih2 cells producing IL-13 also expressed high levels of intracellular GATA-3 (Fig. 7e). Similar findings were observed in 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, suggesting a role for IL-4 receptor signaling in their maintenance or survival.

GATA-3 is required for IL-13 production and worm clearance

To test whether GATA-3 is required for IL-13 production, we crossed IL-13^{Y_{et}Cre} mice to floxed-GATA-3 mice to generate mice in which GATA-3 would be deleted only in IL-13-producing cells³⁹. Nine days after *N. brasiliensis* infection, IL-13 production, as assessed by YFP expression from the IL-13^{Y_{et}Cre} allele, was significantly reduced in CD4⁺ T cells and absent from Ih2 cells isolated from the lung of IL-13^{Y_{et}Cre/+}GATA-3^{fl/fl} mice as compared to control IL-13^{Y_{et}Cre/+}GATA-3^{fl/+} mice (Fig. 8a,b). Ablation of GATA-3 from IL-13-producing cells phenocopied our previous results using the IL-13^{Y_{et}Cre}ROSA-DT α mice, in that both eosinophil recruitment to the lung and worm clearance were compromised (Fig. 8b,c). IgE production was unaffected in these mice (Fig. 8c), confirming that the T_{FFH}-IL-4 arm of allergic immunity, which supports antibody production, is distinct from the T_H2-Ih2 driven IL-13 arm responsible for the peripheral manifestations of type 2 immune responses, including tissue eosinophil recruitment and worm expulsion.

Discussion

We used novel lines of mice to expose an unexpected partitioning of IL-4 and IL-13, cytokines previously implicated in mediating allergic and helminth immunity, among tissues and cell types. T_{FFH} cells express IL-4, whereas T_H2 cells express a spectrum of either or both cytokines. Corroborating earlier studies^{8, 11, 21, 23}, deleting IL-4 led to profound

deficiencies in humoral immunity with minimal effects on tissue responses, whereas deleting IL-13-expressing cells led to profound effects on tissue immunity with minimal effects on humoral responses. Among innate cells, basophils expressed IL-4 but not IL-13 whereas Th2 cells expressed IL-13 but not IL-4. We extend our prior observations that T_{FH} cells constitute the most prevalent IL-4-producing cells in the lymph nodes during allergic immunity²¹ by showing that these cells do not activate IL-13 gene expression, as assessed by direct marker analysis and through lineage-mediated deletion. Thus, despite a 'competent' locus for production of the linked type 2 cytokines, T_{FH} and Th2 cells show differential display of these cytokines *in vivo*.

The mechanisms that prevent spreading of cytokine activation across both IL-4 and IL-13 genes remain of interest. Although low levels of GATA-3 are required in naïve CD4⁺ T cells, chromatin modifications and the accessibility of GATA-3 for select sites within the IL-4 and IL-13 locus play an important regulatory role in the differential expression of IL-4 and IL-13^{40, 41}. Our *in vivo* results corroborate these findings by showing an obligatory role for GATA-3 in IL-13 production by canonical Th2 cells, but also suggest that the regulation of IL-4 and IL-13 in CD4⁺ T cells *in vivo* is more complicated, since T_{FH} cells do not require high levels of GATA-3 for IL-4 production and do not express IL-13. We considered the possibility that IL-4 and/or IL-13 from innate immune cells in the periphery might contribute to terminal differentiation of Th2 cells by directly up-regulating GATA-3 levels to thresholds required to facilitate IL-13 (and IL-5) expression by binding to the respective promoters in these genes^{15, 32}. Although the absence of STAT6 or IL-4R α had some effects on the ultimate numbers of GATA-3^{high} Th2 effector cells developing in tissues, the ability of CD4⁺ T cells to achieve a GATA-3^{high}, IL-13-producing phenotype was not lost, a finding consistent with prior studies⁴². Further studies are necessary to explore the pathways that enable STAT6-independent differentiation of Th2 cells *in vivo*.

We speculate that high levels of Bcl-6 in T_{FH} cells restrict GATA-3 to levels insufficient to activate the IL-13 (and likely IL-5) genes. Although Bcl-6 is a direct transcriptional repressor for many genes, it might suppress GATA-3 at a post-transcriptional level, perhaps through miRNA-mediated suppression^{30, 43, 44}. Because Bcl-6 over-expression can induce a T_{FH} phenotype²⁶, it is tempting to speculate that decay of Bcl-6 or expression of Blimp-1 in T cells upon leaving secondary lymphoid tissues is a prerequisite for relieving repression of the genetic programs, such as homing and extended cytokine expression, necessary to complete Th2 differentiation in the periphery^{21, 25}. Alternatively, Bcl-6 may be required to sustain expression of cytokines and chemokines required for efficient interactions with B cells. In this way, Th2 effector development is delayed until entry into 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 eosinophil tissue infiltration. The finding that deletion of these cells phenocopies the loss of IL-13 suggests that the major 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 find that specific deletion of GATA-3 in the IL-13-producing cells is sufficient to inhibit the peripheral manifestations of type 2 immunity. As assessed by direct visualization *in vivo*,

only T_H2 cells and I_H2 cells express IL-13 in tissues during this infection, suggesting that either or both of these cells are required for optimal tissue immunity^{6, 8, 23}. The role for IL-13 from I_H2 cells may be of particular importance when considering T-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 have utilized knock-in approaches that leave endogenous expression intact from a locus regulated by the endogenous *cis*-acting elements. Regardless, introduction of exogenous genetic material unavoidably will alter genomic distances and perhaps impact 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 behave similarly in all aspects as the endogenous genes in wild-type genomes with the caveat that the half-lives of the markers, particularly membrane proteins such as CD2 and CD4, will be distinct from the half-lives of the secreted cytokine proteins.

Following our observations, therapeutic targeting of IL-4 or IL-13 would be predicted to have markedly different effects depending on whether the intended targets reflect aspects of humoral or cellular responses. Together, these studies reveal previously unappreciated regulation of these duplicated cytokines, as suggested by the differences in dependence on and expression of GATA-3 between IL-4- and IL-13-expressing cells, providing fruitful opportunities for further understanding of the nuances underpinning gene regulation in immune cells.

Methods

Mice

Dual IL-4 reporter mice (IL-4^{KN2/4get} mice) have been described²⁰. In brief, 4get mice were generated by introducing an IRES-eGFP construct after the stop codon of IL-4 by homologous recombination, which leads to transcription of a 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 GFP expression without the need for restimulation. KN2 mice were generated by introducing a human *CD2* cDNA at the start site of the *Il4* gene. After appropriate stimulation, IL-4 secretion becomes marked by the appearance of huCD2 on the cell surface²⁰. YFP-enhanced transcript with Cre recombinase at the *Il13* gene (YetCre13) have been described⁸. Briefly, cells from these mice contain an IRES followed by YFP-Cre recombinase fusion protein at the start of the 3'UTR of the *Il13* gene. To delete cells that activate IL-13 transcription and translation or delete GATA-3, we generated homozygous IL-13^{YetCre/YetCre}-ROSA-diphtheria toxin- α mice⁸ or IL-13^{YetCre/+GATA-3^{fl/fl}} mice³⁹, respectively. Cells expressing IL-13 and the YFP-Cre fusion protein will express DT- α , thus killing the cell. SMART13 (Surface MARKer for the Transcript of IL-13) mice were generated by introducing a truncated human-*CD4* cDNA fragment (with a F43I point mutation to abrogate mouse MHC-II binding) preceded by an IRES between the stop codon of the *Il13* gene and the 3'UTR by homologous recombination. BALB/c IL-4/IL-13-deficient mice have been described¹¹. Mice were maintained in the UCSF specific pathogen-free animal facility in accordance with the

guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center.

Infections, immunizations and worm clearance

L. major strain WHOM/IR-/173 was prepared and injected as $0.5-1 \times 10^6$ metacyclic promastigotes in the hind footpad of mice. Third-stage larvae (L3) of *N. brasiliensis* were prepared and worm counts were assayed from the small intestine as described²³. Where indicated, mice received a sub-lethal dose of irradiation (450 rads) prior to transfer of donor T cells in order to create space in the recipient mice. Transferred CD4⁺ T cells were allowed to undergo homeostatic expansion for 5–7 days before mice were infected. Mice were immunized with 50 µg NP-conjugated chicken ovalbumin (OVA-NP(15)) (Biosearch Technologies) emulsified in Alum Imject (Pierce) subcutaneously in the footpad.

Tissue immunohistochemistry

For detection of eGFP and YFP, the signal was amplified using tyramide amplification on paraformaldehyde (PFA)-fixed tissue and huCD2 was detected on acetone-dehydrated slides after incubation with biotinylated anti-human CD2 (RPA-2.10, eBioscience). GFP and YFP were detected using rabbit anti-GFP polyclonal antibody (Ab 6556, Novus Biologicals) followed by biotinylated donkey anti-rabbit F(ab')₂ (Jackson ImmunoResearch). In brief, lymph nodes were isolated at indicated times and either frozen directly in O.C.T. embedding compound (Sakura Finetek U.S.A., Inc.) or after 2 h incubation in 1–4% PFA followed by 30% sucrose. 6–8 µm sections were cut using a Leica CM 3050S cryomicrotome (Leica Microsystems Inc.). Sections were treated with FITC-tyramide from the TSATM-fluorescein kit according to manufacturer's instructions (Perkin Elmer). Multiple biotinylated antibodies could be detected on the same slide by repeated quenching and blocking of peroxidase and biotin followed by another round of tyramide-Alexafluor 555 (Invitrogen), tyramide-Cy5 or tyramide-biotin (Perkin Elmer) amplification. Other biotinylated antibodies used were anti-IgD, CD23 (B3B4, BD Pharmingen), anti-CD4 (RM4–5, Biolegend) and anti-CD278 (C398.4A, Biolegend). Nuclei were counterstained with 4', 6-diamidino-2'-phenylindole dihydrochloride (DAPI; Roche) in PBS prior to mounting the coverslip. Digital images in the FITC, Cy3 and Cy5 channels were collected using a Nikon Eclipse E800 fluorescence microscope equipped with SimplePCI software (Compix Inc.). Images were converted to RGB, colored and overlaid using Adobe Photoshop CS2 software (Adobe systems Inc.).

Flow cytometric analysis

Indicated mice were perfused with 20 ml PBS and mediastinal lymph nodes and lung were isolated. Single-cell suspensions were prepared and labeled with antibodies as listed: for IL-13 reporter detection in IL-13^{Y_{et}Cre} mice, YFP⁺ cells were analyzed for expression of Siglec-F, (E50–2440, PE-anti-Siglec-F, BD Pharmingen), CD131 (J0R050, PE-anti-IL-3-Rβ, BD Pharmingen), CD49b (DX5, PE-Cy7-anti-CD49b, eBioscience), CD278 (C398.4A, APC-anti-ICOS, Biolegend), CD4 (RM4–5, APC-780-anti-CD4, eBioscience), CD8 (53-6.7, PerCP-Cy5.5-anti-CD8, BD Pharmingen), CD19 (ID3, percp-Cy5.5-anti-CD19, BD Pharmingen); for IL-13 and IL-4 reporter detection in IL-4^{4get/+}IL-13^{Smart/+}, and IL-4^{KN2/4get}, mice, GFP-expressing cells were analyzed for huCD4 (RPA-T4, PE-anti-

human CD4, eBioscience), huCD2, (PE- α -CD2, Caltag), CD4 (RM4-5, APC-780-anti-CD4, eBioscience), CD19 (1D3, PerCP-Cy5.5- α -CD19, BD-Pharmingen), CD8 (53-6.7, PerCP-Cy5.5-anti-CD8, BD Pharmingen), ICOS (D10.G4.1, APC, Biotin- α -CD278, Biolegend), CD49b (DX5, PE-Cy7-anti-CD49b, eBioscience); for T_{FH} staining, PD-1 (RMP1-30, PE-Cy7-anti-CD279) and CXCR5 (2G8-biotin-anti-CXCR5, BD-Pharmingen) followed by streptavidin-PerCP were also included as indicated; for detection of IL-4 and IL-13 reporters in IL-4^{KN2/+}IL-13^{Smart/+} mice, CD4 cells were stained for huCD4 (RPA-T4, PE-Cy7 or FITC-anti-human CD4, eBioscience), huCD2, (PE- α -CD2, Caltag), CD4 (RM4-5, APC-780-anti-CD4, eBioscience), CD19 (1D3, PerCP-Cy5.5- α -CD19, BD-Pharmingen), CD8 (53-6.7, PerCP-Cy5.5-anti-CD8, BD Pharmingen), ICOS (D10.G4.1, APC, Biotin- α -CD278, Biolegend), as indicated. CD1d tetramer was obtained from the NIH tetramer facility. Samples were analyzed on a LSR-II (BD Biosciences). A dump channel of PerCP-Cy5.5-labeled CD8 α and CD19 was used to reduce non-specific staining. Live lymphocytes were gated by DAPI exclusion, size and granularity based on forward- and side-scatter. Cell counts were performed using Vi-Cell 2.02 (Beckman-Coulter).

Flow cytometric analysis of GATA-3 and Bcl-6

IL-4^{KN2/+}IL-13^{Smart/+}, IL-4^{KN2/4get}, or IL-4^{4get/+}IL-13^{Smart/+} mice were perfused with 20 ml PBS, and mediastinal lymph nodes and lung were collected. Single-cell suspensions were prepared and labeled with antibodies against surface molecules as listed: huCD4 (RPA-T4, PE-Cy7, FITC, or PE-anti-human CD4, eBioscience), huCD2, (PE or APC- α -CD2, Caltag), CD278 (C398.4A, APC-anti-ICOS, Biolegend), CD4 (RM4-5, APC-780-anti-CD4, eBioscience), CD8 (53-6.7, PerCP-Cy5.5-anti-CD8, BD Pharmingen), CD19 (ID3, PerCP-Cy5.5-anti-CD19, BD Pharmingen), CD49b (DX5, PE-Cy7-anti-CD49b, eBioscience). After surface staining, cells were stained with Violet Live/Dead fixable stain (Invitrogen) to exclude dead cells. Cells were fixed, permeabilized and stained for intracellular GATA-3 (TWAJ, PE- or APC-anti-GATA-3, eBiosciences) and BCL-6 (Rabbit Polyclonal, Alexa-647-anti-BCL-6, Santa Cruz) using intracellular Fix/Permeabilization Buffers (ebioscience) according to manufacturer's instructions. BCL-6 antibodies were conjugated to Alexa-647 using Alexa-647 Monoclonal Antibody Labeling Kit (Invitrogen). Live lymphocytes were gated on size and granularity based on forward- and side-scatter. A dump channel of PerCP-Cy5.5-labeled CD8 α and CD19 was used to reduce non-specific staining.

Real-time RT-PCR

Cells were isolated from mediastinal lymph nodes and lungs of *N. brasiliensis*-infected mice and viable GFP⁺ or GFP⁺huCD2⁺ CD4⁺ T cells were sorted based on DAPI⁻, CD4⁺, CD19⁻, CD8⁻ staining. Cells were lysed and reverse transcribed using Cells Direct cDNA synthesis kit (Invitrogen). Transcripts were quantified using platinum SYBR green incorporation using a StepOnePlus Real-Time PCR System (Applied Biosystems) and plotted relative to expression of GAPDH. Real-time primers were obtained from Qiagen as listed, and specific PCR products verified by melting-curve and gel analysis.

Enzyme linked immunosorbent assay (ELISA)

96-well plates were coated with NP(23)-BSA or NP(3)-BSA (Biosearch) and incubated with serial 5-fold dilutions of serum. IgG1 NP-specific antibodies were detected by incubation with biotinylated anti-mouse IgG1 followed by streptavidin-HRP and o-phenylenediamine (OPD). Affinity maturation was determined as described²¹. Briefly, the concentration of serum anti-NP antibodies bound to NP(3)-BSA was divided by the concentration of anti-NP antibodies bound to NP(23)-BSA. As the affinity of the antibodies increase, the ratio approaches one. The concentrations of anti-NP IgG1 were determined by comparison to standard curves generated using IgG1 from the anti-NP IgG1 clone H33L γ 1/ λ 1 (kindly provided by G. Kelsoe, Duke University). To detect total IgE from serum of infected mice 96-well plates were coated with anti-IgE (B1E3, anti-IgE) and incubated with 2–100-fold dilutions of serum, depending on day of infection. Bound IgE was detected using a biotinylated anti-IgE (EM95, biotin-anti-IgE) followed by streptavidin-alkaline-phosphatase and substrate NPP. Samples were read in duplicate or triplicate and concentrations were obtained according to the IgE standard using an ELISA plate reader (Delta Soft).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Eosinophil recruitment, worm expulsion and antibody production during *N. brasiliensis* infection after depletion of IL-13-producing cells

(a) The cell fate, cytokine expression, worm clearance, eosinophil recruitment and IgE production in the indicated reporter mice 9 days post *N. brasiliensis* infection.[IV1] (b) Total number of worms in the small intestine of *N. brasiliensis* infected mice, 9 days post infection. ($n = 5$ mice per group; $*P < 0.001$, Two-tailed t test). Graph representative of at least 2 independent experiments. (c) Total number of eosinophils (Siglec-F^{hi}, CD49b^{neg}) in the lung of *N. brasiliensis* infected mice, 12 days post-infection. ($n = 2-3$ mice per group;

* $P < 0.01$, Two-tailed t test). Graph representative of at least 3 independent experiments. **(d)** The percentage and total number of eosinophils (SiglecF/Bc^{hi}, DX5^{neg}), basophils (SiglecF/Bc^{int}, DX5^{high}) and CD4⁺ T cells in the lung of indicated mice on day 9 after *N. brasiliensis* infection. Plots are representative of two independent experiments ($n = 2-4$ mice). **(e)** IgE level in the serum of indicated mice 9 days after *N. brasiliensis* infection. ($n = 5$ mice). Graph is representative of at least 3 independent experiments. **(f)** Expression of high-affinity NP-specific IgG1 in serum of indicated mice 28 days after OVA-NP(15) injection in the footpad ($n = 4-15$ mice; * $P < 0.05$, ** $P < 0.005$, Two-tailed t test). Graph is representative of at least 2 independent experiments. **(g)** Affinity maturation of serum anti-NP-specific IgG1 antibodies from the indicated mice following OVA-NP(15) challenge. Ratio depicts binding of serum IgG1 to BSA-NP(3)/BSA-NP(23) ($n = 4-15$ mice; * $P < 0.05$, ** $P < 0.005$, Two-tailed t test). Graph is representative of at least 3 independent experiments. Each data point represents an individual mouse (b, e, f, g) .

Figure 2. The localization and enumeration of cytokine-producing CD4⁺ T cells during parasitic infection

(a) The percentage of IL-4- (huCD2) or IL-13 (huCD4)-positive CD4⁺ T cells in the lung and mediastinal lymph nodes from indicated mice infected 8 days previously with *N. brasiliensis*. Plots are representative of at least 4 independent experiments (n = 3–5 mice per experiment). (b) Percentage and total number of cytokine-positive CD4⁺ T cells in the lung and draining mediastinal LN 9 days after *N. brasiliensis* infection. Graphs represent data points combined from two independent experiments (n = 9; *P < 0.05; **P < 0.0001, two-

tailed t test). Each data point represents an individual mouse. **(c)** Ratio of IL-4/IL-13 producing-CD4⁺ T cells within the mediastinal lymph nodes and lung 9 days post infection with *N. brasiliensis*. Graph represents data points combined from two independent experiments (n = 9; $**P < 0.0001$, two-tailed t test). Data points represent individual mice. **(d)** Immunohistochemistry of popliteal lymph nodes (Top panel) 21 days after *L. major* infection and lung (bottom panel) 8 days after *N. brasiliensis* infection. Wild-type tissues served as negative controls for huCD2 and fluorescent reporter staining. Image magnification is 100x; insets magnification 200x. Images are representative of tissues from 3–4 animals.

Figure 3. Differential IL-4 and IL-13 production by T_{H1} and canonical T_{H2} cells

(a) The percentage of IL-4- (huCD2) or IL-13 (huCD4)-producing cells within GFP⁺ CD4⁺ T cells in the lymph nodes and lung of IL-4^{KN2/4get} and IL-4^{4get/+}IL-13^{Smart/+} mice 8 days post *N. brasiliensis* infection. Arrow indicates the percentage of T_{H1} cells among the IL-4- or IL-13-producing cells. Plots are representative of two independent experiments (n = 6 mice). (b) The percentage of (PD-1⁺ CXCR5⁺) T_{H1} cells among total IL-4- or IL-13-producing CD4⁺ T cells in the lymph node or lung from IL-4^{KN2/4get} and IL-4^{4get/+}IL-13^{Smart/+} mice 8 days after *N. brasiliensis* infection (Percentage of cells ±

S.E.M. ($n = 4$; $*P < 0.01$, two-tailed t test). Data points combined from two independent experiments. **(c)** Relative expression of mRNA transcripts plotted against housekeeping gene GAPDH for the indicated genes from CD4⁺ T cells isolated from the lymph nodes and lungs of IL-4^{KN2/4get} mice 9 days post infection with *N. brasiliensis*. Data are representative of at least 3 independent experiments. **(d)** Percentage of intracellular GATA-3 and Bcl-6 in CD4⁺ T cells isolated from the mediastinal lymph node and lung from IL-4^{KN2/4get} mice 8 days post *N. brasiliensis* infection. Plots are representative of 5 mice from 2 independent experiments.

Figure 4. GATA-3 expression in IL-4- and IL-13-producing CD4⁺ T cells after *N. brasiliensis* infection

(a) The percentage of intracellular GATA-3^{high} and IL-4- (huCD2) and IL-13 (huCD4)-producing CD4⁺ T cells from the draining lymph nodes and Lung of WT or IL-4^{KN2/+}IL-13^{Smart/+} mice 9 days after *N. brasiliensis*. Plots are representative of at least 5 independent experiments (n = 3–4 mice per experiment). **(b)** percentage of GATA-3^{high} cells among total IL-4- (huCD2) and IL-13- (huCD4) producing CD4⁺ T cells in the mediastinal lymph nodes and lung 9 days after *N. brasiliensis* infection.

Figure 5. GATA-3 expression and cytokine production by CD4⁺ T cells in the absence of STAT6

(a) Percentage of GATA-3^{high} CD4⁺ T cells from wild-type and STAT6-deficient IL-4^{KN2/+}IL-13^{Smart/+} mice 8 days post *N. brasiliensis* infection. Data points are combined from two independent experiments. (n = 8; *P < 0.0001, two-tailed t test). Each data point represents an individual mouse. **(b)** Percentage of GATA-3^{high} IL-4- or IL-13-secreting CD4⁺ T cells from wild-type and Stat6-deficient IL-4^{KN2/+}IL-13^{Smart/+} mice 8 days post *N. brasiliensis* infection. Data points are combined from two independent experiments. (n = 8; *P < 0.0001; **P < 0.0002, two-tailed t test). Each data point represents an individual mouse.

(c) Percentage and total number of IL-4- and IL-13-producing CD4⁺ T cells in the lymph nodes and lung of wild-type and Stat6-deficient IL-4^{KN2/+}IL-13^{Smart/+} mice 8 days post *N. brasiliensis* infection. Data points are combined from two independent experiments. (Percentage of cytokine-positive cells or total number ± S.E.M.; n = 8; *P < 0.001; **P < 0.01; ***P < 0.05, two-tailed t test).

Figure 6. IL-4 and IL-13 production by innate type 2 effector cells after *N. brasiliensis* infection
(a) Top panel shows the percent of ICOS⁺ CD4⁺ or ICOS⁺ CD4⁻ cells among total IL-4- or IL-13-producing lung cells. Bottom panel shows the percentage of CD4⁺ CD49b⁻ or CD4⁻ CD49b⁺ cells among total IL-4- or IL-13-producing lung cells from IL-4^{KN2/4get} or IL-4^{4get/+}IL-13^{Smart/+} mice 8 days post *N. brasiliensis* infection. Gates represent CD4⁺ T cells (CD4⁺, CD49b⁻, CD278⁺), Ih2 cells (CD4⁻, CD49b⁻, CD278⁺), or basophils (CD4⁻, CD49b⁺, CD278⁻). Plots are representative of at least three independent experiments. **(b)** Percentage of CD4⁺ T cells, Ih2 cells, or basophils among total IL-4- and IL-13-producing

cells in the lung of IL-4^{KN2/4get} or IL-4^{get/+}IL-13^{Smart/+} mice 8 days post *N. brasiliensis*. Graphs are representative of at least 3 independent experiments, percentage of cells $\pm$ S.E.M. (n = 3). **(c)** Percentage of eosinophils (CD4^{neg}, SiglecF/ β c^{hi}, DX5^{neg}), basophils (CD4^{neg}, SiglecF/ β c^{int}, DX5^{high}), and Ih2 cells (CD4^{neg}, SiglecF/ β c^{neg}, DX5^{neg}, ICOS^{high}) among CD4⁻ cells in the lung of IL-13^{Y^{et}Cre/Y^{et}Cre} 9 days after *N. brasiliensis* infection. Plots are representative two independent experiments (n = 9 lungs). **(d)** The percentage and total number of total and YFP-expressing CD4⁺ T cells, Ih2 cells, basophils, eosinophils, and NK/NKT cells among total or YFP-positive (IL-13-producing) lymphocytes in the lung of IL-13^{Y^{et}Cre/Y^{et}Cre} mice 9 days post *N. brasiliensis* infection. Graphs are representative of 3 independent experiments, percentage of cells $\pm$ S.E.M. (n = 3). **(e)** The total number of Ih2 cells (SiglecF/Bc^{neg}, DX5^{neg}, CD4^{neg}, ICOS^{high}) in the lung of IL-13^{Y^{et}Cre/Y^{et}Cre} mice 9 days after *N. brasiliensis* infection. Plots are representative two independent experiments (n = 2–4 lungs).

Figure 7. Requirements for STAT6 and GATA-3 on cytokine production by innate type 2 effector cells

(a) Percentage of intracellular GATA-3^{high} Ih2 (ICOS⁺) cells and basophils (CD49b⁺) among total non-T cells in the lung of IL-4^{KN2/4get} and IL-4^{get/+}IL-13^{Smart/+} mice 9 days post *N. brasiliensis* infection. Plots are representative of at least three independent experiments (n = 3–4 mice per experiment).

(b) Percentage GATA-3^{high} IL-4 (huCD2) and IL-13 (huCD4)-producing CD4⁺ T cells, Ih2 cells, and basophils from the lungs 8 days after *N. brasiliensis* infection.

(c) Total number of Ih2 cells (ICOS⁺, CD4⁺, CD8⁺, CD19⁺) in the lung of wild-type and Stat6-deficient IL-4^{KN2/+}IL-13^{Smart/+} mice. Data points are combined from two independent experiments. (Total Ih2 cells ± S.E.M.; n = 8).

(d) The percentage and total number of IL-13 (huCD4)-secreting Ih2 cells in the lungs of wild-type and Stat6-deficient IL-4^{KN2/+}IL-13^{Smart/+} mice 8 days after *N. brasiliensis* infection. Data points are combined from two independent experiments. (Total number or percentage of cells ± S.E.M.; n = 8; *P < 0.01, two-tailed t test).

(e) Percentage of GATA-3^{high} IL-13-producing Ih2 cells in the mediastinal lymph nodes and lung of wild-type and Stat6-deficient IL-4^{KN2/+}IL-13^{Smart/+} mice 8 days post *N. brasiliensis* infection. Data points are combined from two independent

experiments. ($n = 8$; $*P < 0.01$, two-tailed t test). Each data point represents an individual mouse.

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Figure 8. GATA-3 is required for IL-13 expression, innate cell recruitment and worm expulsion, but not IgE production

(a) Percentage of IL-13- (YFP) producing CD4⁺ T cells and Ih2 cells in the lung of GATA-3^{fl/+}, IL-13^{YetCre/+}GATA-3^{fl/+}, IL-13^{YetCre/+}GATA-3^{fl/fl} mice 9 days post *N. brasiliensis* infection. Plots are representative of 2 independent experiments with 7–9 mice per group. (b) The number of Total or IL-13-(YFP) producing basophils (CD4^{neg}, Siglec-F^{neg}, CD49b^{pos}, CD90.2^{int}), eosinophils (CD4^{neg}, Siglec-F^{pos}, CD49b^{neg}, SSC^{high}), CD4⁺ T cells (CD4^{pos}, ICOS^{high}, CD90.2^{pos}), and Ih2 cells (CD4^{neg}, Siglec-F^{neg}, CD49b^{neg},

ICOS^{high}, CD90.2^{pos}) in the lung of IL-13^{Y^{et}Cre/+GATA-3^{fl/+}} (white bars) or IL-13^{Y^{et}Cre/+GATA-3^{fl/fl}} (black bars) of mice 9 days post *N. brasiliensis* infection. Graphs are a composite of two independent experiments (+/- SEM, n = 7–9 mice per group, **P* < 0.05, ***P* < 0.005, two-tailed t test). Dashed line represents the limit of detection as scored by the reporter negative wild-type control. (c) Eosinophil recruitment, worm expulsion, and total serum IgE assessed in IL-13^{Y^{et}Cre/+GATA-3^{fl/+}} (white bars) or IL-13^{Y^{et}Cre/+GATA-3^{fl/fl}} (black bars) mice 9 days post *N. brasiliensis* infection. (+/- SEM, n = 4–5 mice per group, **P* < 0.05, ***P* < 0.005, two-tailed t test).