

Genetic analysis of basophil function *in vivo*

Brandon M Sullivan^{1,2,8}, Hong-Erh Liang^{1,2,8}, Jennifer K Bando^{1,2}, Davina Wu^{1,2}, Laurence E Cheng³, James K McKerrow^{4,5}, Christopher D C Allen^{6,7} & Richard M Locksley^{1,2,6,7}

Contributions by basophils to allergic and helminth immunity remain incompletely defined. Using sensitive interleukin 4 (*Il4*) reporter alleles, we demonstrate here that basophil *Il4* production occurs by a CD4⁺ T cell-dependent process restricted to the peripheral tissues affected. We genetically marked and achieved specific deletion of basophils and found that basophils did not mediate T helper type 2 (T_H2) priming *in vivo*. Two-photon imaging confirmed that basophils did not interact with antigen-specific T cells in lymph nodes but engaged in prolonged serial interactions with T cells in lung tissues. Although targeted deletion of *Il4* and *Il13* in either CD4⁺ T cells or basophils had a minimal effect on worm clearance, deletion from both lineages demonstrated a nonredundant role for basophil cytokines in primary helminth immunity.

Helminth infections, which affect nearly 2.9 billion humans worldwide¹, elicit a stereotypical immune response with features shared by allergic and asthmatic reactions, including higher serum concentrations of immunoglobulin E (IgE), excessive mucus production and tissue inflammation dominated by polarized T helper type 2 (T_H2) cells, eosinophils, mast cells and basophils. The linked cytokines interleukin 4 (IL-4) and IL-13, as well as their shared signaling components, the IL-4 receptor α -chain and the transcription factor STAT6, are required for allergic and anti-helminth immunity^{2,3}. Although CD4⁺ T cells are required and T_H2 cells can produce these cytokines, innate sources of IL-4 and IL-13, such as basophils⁴ or lineage-negative IL-25- and IL-33-responsive cells^{5–7} (called ‘innate type 2 helper cells’ here) can contribute to primary anti-helminth immunity. Immunohistochemical techniques are not adequate at present to discern cytokine-producing cells *in situ* and thus a thorough assessment of their spatial and temporal relationships in tissues during the host response has been lacking.

Basophils are proposed to mediate B cell isotype switching and to drive the differentiation of CD4⁺ helper T cells^{8–10}. Several studies have indicated that basophils are antigen-presenting cells (APCs) that are necessary and sufficient for T_H2 priming^{11–13}, although this has been challenged by others^{14–17}. Despite such progress, the demonstration of basophil activation *in vivo* and a consensus understanding of their role in immunity are lacking, as attempts to mark these cells *in vivo* have not enabled unequivocal imaging of basophils in tissue^{16,18}.

Here we show that basophils were mobilized to many tissues during primary helminth infection, which confirmed the results of published studies^{19,20}, but that basophil IL-4 secretion was restricted to affected

organs. We used a newly developed basophil reporter strain, Basoph8, to demonstrate that basophils were not required for the initiation of allergic immunity. Two-photon microscopy showed that basophils did not interact with antigen-specific CD4⁺ T cells in inflamed lymph nodes but engaged in serial interactions with CD4⁺ T cells in affected tissues, a process that correlated with sites of basophil activation. Basophil depletion did not affect the clearance of *Nippostrongylus brasiliensis* in primary or secondary infection, but selective deletion of *Il4* and *Il13* from both basophils and CD4⁺ T cells demonstrated a nonredundant contribution of basophil-derived cytokines.

RESULTS

IL-4 production by basophils is restricted to tissues involved

We used mice with two independent markers targeted to the endogenous *Il4* locus to define which cells produce IL-4 during primary infection with *N. brasiliensis*²¹. These mice (KN2 $\times$ 4get) are heterozygous for the 4get allele²², which expresses green fluorescent protein (GFP) from an internal ribosomal entry site (IRES) downstream of the *Il4* stop site, and the KN2 allele, which expresses human CD2 from the *Il4* start site and replaces that gene with *CD2*. When stimulated, cells from KN2 $\times$ 4get mice reliably mark IL-4 competence by intracellular GFP expression and mark recent IL-4 protein secretion by the presence of human CD2 on the membrane²¹. After infection, basophils, eosinophils and a fraction of CD4⁺ T cells constituted the main GFP⁺ cells recruited to the lungs²⁰, where they begin to accumulate by day 3. On day 5, basophils and CD4⁺ T cells began to express human CD2, consistent with IL-4 secretion *in vivo* (Fig. 1a). We observed expression of human CD2 only in cells with enhanced GFP expression, which therefore provided internal confirmation that

¹Howard Hughes Medical Institute, University of California San Francisco, San Francisco, California, USA. ²Department of Medicine, University of California San Francisco, San Francisco, California, USA. ³Department of Pediatrics, University of California San Francisco, San Francisco, California, USA. ⁴Department of Pathology, University of California San Francisco, San Francisco, California, USA. ⁵The Sandler Center for Basic Research in Parasitic Diseases, University of California San Francisco, San Francisco, California, USA. ⁶Department of Microbiology/Immunology, University of California San Francisco, San Francisco, California, USA. ⁷The Sandler Asthma Basic Research Center, University of California San Francisco, San Francisco, California, USA. ⁸These authors contributed equally to this work. Correspondence should be addressed to R.M.L. (locksley@medicine.ucsf.edu) or C.D.C.A. (chris.allen@ucsf.edu).

Received 28 February; accepted 6 April; published online 8 May 2011; doi:10.1038/ni.2036

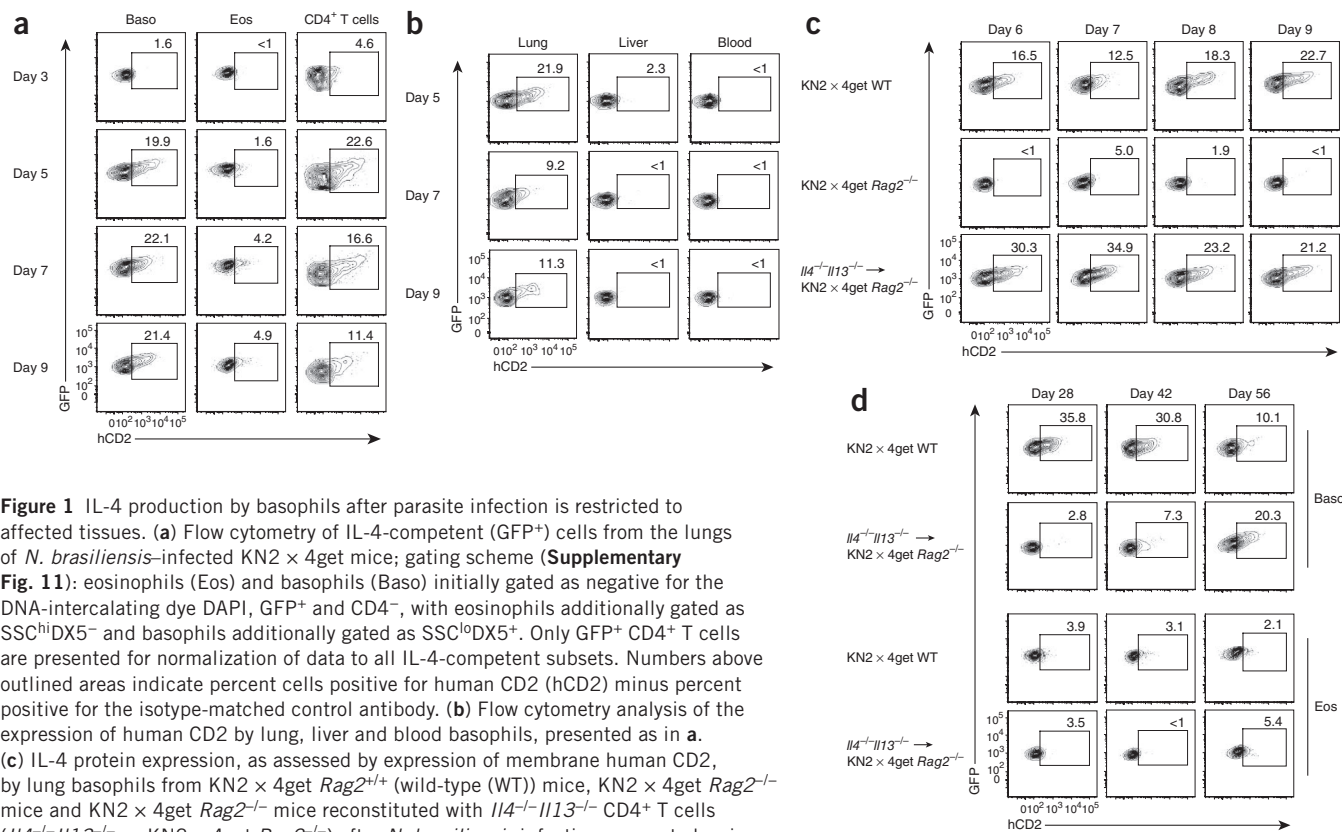


Figure 1 IL-4 production by basophils after parasite infection is restricted to affected tissues. **(a)** Flow cytometry of IL-4-competent (GFP⁺) cells from the lungs of *N. brasiliensis*-infected KN2 × 4get mice; gating scheme (**Supplementary Fig. 11**): eosinophils (Eos) and basophils (Baso) initially gated as negative for the DNA-intercalating dye DAPI, GFP⁺ and CD4⁺, with eosinophils additionally gated as SSC^{hi}DX5⁻ and basophils additionally gated as SSC^{lo}DX5⁺. Only GFP⁺ CD4⁺ T cells are presented for normalization of data to all IL-4-competent subsets. Numbers above outlined areas indicate percent cells positive for human CD2 (hCD2) minus percent positive for the isotype-matched control antibody. **(b)** Flow cytometry analysis of the expression of human CD2 by lung, liver and blood basophils, presented as in **a**. **(c)** IL-4 protein expression, as assessed by expression of membrane human CD2, by lung basophils from KN2 × 4get Rag2^{+/+} (wild-type (WT)) mice, KN2 × 4get Rag2^{-/-} mice and KN2 × 4get Rag2^{-/-} mice reconstituted with *Il4*^{-/-}*Il13*^{-/-} CD4⁺ T cells (*Il4*^{-/-}*Il13*^{-/-} → KN2 × 4get Rag2^{-/-}) after *N. brasiliensis* infection, presented as in **a**. **(d)** Flow cytometry of liver basophils and eosinophils from *S. mansoni*-infected KN2 × 4get mice and KN2 × 4get Rag2^{-/-} mice reconstituted with *Il4*^{-/-}*Il13*^{-/-} CD4⁺ T cells, presented as in **a**. Data are representative of three to five independent experiments.

activation of the 4get allele marked cells undergoing transcriptional activation of the *Il4* locus. The percentage of basophils and CD4⁺ T cells positive for human CD2 peaked on days 5–6 and then decreased (**Supplementary Fig. 1**).

Published reports of studies using IL-4 reporter strains have described systemic recruitment of basophils to tissues after *N. brasiliensis* infection^{19,20}. Although we also observed more basophils in many tissues, activation of basophil IL-4 secretion was tissue restricted, as lung basophils expressed both GFP (IL-4 competence) and human CD2 (IL-4 secretion), whereas basophils from spleen, liver, lymph node, blood and bone marrow (data not shown) expressed GFP but not human CD2 (**Fig. 1b** and **Supplementary Fig. 2**). In contrast, CD4⁺ T cells that expressed both GFP and human CD2 were present in the spleen and lungs, consistent with their ability to function as both cytokine-secreting follicular helper T cells (in the spleen) and tissue effector T cells (T_H2 cells; in the lung)²³ (**Fig. 1b** and **Supplementary Fig. 2**). Basophils were also recruited to the small intestine (**Supplementary Fig. 3**). However, analysis of intestinal tissue was inconclusive between days 6 and 9 because of the substantial death of recovered cells, presumably a result of massive cellular activation, epithelial turnover and tissue injury. Eosinophils, despite their greater numbers and GFP⁺ status, did not express large amounts of human CD2 (**Fig. 1a**). Using a yellow fluorescent protein (YFP)-marked IL-13 reporter strain of mice, we did not find IL-13 production by basophils (data not shown). As shown elsewhere, innate type 2 helper cells are readily marked as IL-13-producing cells after *N. brasiliensis* infection, but these cells, in contrast to basophils and CD4⁺ T cells, do not produce IL-4 *in vivo*⁷. Thus, basophils represented the early non-T cell source of IL-4 during helminth infection,

in agreement with published studies^{19,24}, and their activation *in vivo*, as shown by the KN2 × 4get reporter, was sequestered within the affected tissues.

To define the requirements for basophil activation and the role of T_H2 cells, we generated KN2 × 4get mice on the recombination-activating gene 2-deficient (Rag2^{-/-}) background. Lung tissues from these mice developed less inflammation with fewer basophils after infection than did lung tissue from wild-type mice, consistent with published findings^{19,20}, and recruited basophils did not express human CD2 (**Fig. 1c**). When reconstituted with *Il4*^{-/-}*Il13*^{-/-} CD4⁺ T cells, however, lungs were infiltrated with more cells, including basophils that expressed human CD2 as a marker of recent IL-4 secretion (**Fig. 1c**). The infiltration and activation of basophils in the lungs of reconstituted mice was delayed by 1–2 d relative to that in wild-type mice, which either reflected effects of T cell reconstitution or exposed an amplification role for T_H2 cell-derived IL-4 and IL-13 *in vivo*. As expected, we detected no serum IgE in Rag2^{-/-} mice reconstituted with *Il4*^{-/-}*Il13*^{-/-} CD4⁺ T cells (data not shown).

We used an unrelated helminth model, infection with *Schistosoma mansoni*, to assess whether tissue-specific basophil activation could be visualized in the liver, which is a major site of the granulomatous response to trapped eggs²⁵. In contrast to basophils isolated from the liver in response to *N. brasiliensis* (**Fig. 1b**), basophils isolated from the livers of *S. mansoni*-infected KN2 × 4get Rag2^{-/-} mice that had been reconstituted with *Il4*^{-/-}*Il13*^{-/-} CD4⁺ T cells expressed human CD2, consistent with recent IL-4 secretion (**Fig. 1d**). Basophil activation in reconstituted mice was delayed relative to that in wild-type mice, which probably reflected, at least in part, the absence of IgE. As in primary *N. brasiliensis* infection, expression of innate IL-4

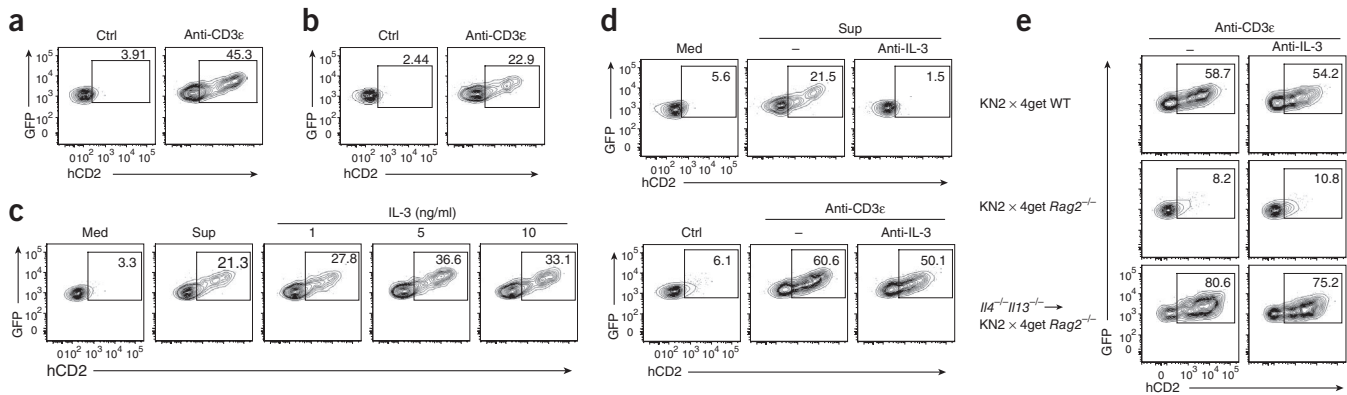


Figure 2 CD4⁺ T cell activation induces basophil IL-4 production *in vitro*. (a) IL-4 expression (assessed as in Fig. 1c) by splenic basophils without (control (Ctrl)) or after (Anti-CD3ε) stimulation of total splenocytes from *N. brasiliensis*-infected KN2 × 4get mice with anti-CD3ε. (b) IL-4 expression (assessed as in Fig. 1c) by basophils obtained by enrichment from the spleens of KN2 × 4get *Rag2*^{-/-} mice; basophils were cultured with, but separated by a Transwell insert from, purified CD4⁺ T cells left unstimulated (Ctrl) or stimulated with anti-CD3ε (Anti-CD3ε). (c) IL-4 expression (assessed as in Fig. 1c) by basophils enriched from the spleens of KN2 × 4get *Rag2*^{-/-} mice after overnight culture in medium alone (Med), supernatants of activated CD4⁺ T cells (Sup) or recombinant IL-3 (concentration, above plots). (d) Basophil IL-4 production during stimulation of total splenocytes with either medium or supernatants of activated CD4⁺ T cells (top row) or in the presence (Anti-CD3ε) or absence (Ctrl) of anti-CD3ε (bottom row), in the presence (Anti-IL-3) or absence (–) of neutralizing antibody to IL-3 (10 μg/ml). (e) IL-4 expression by basophils from the spleens of *N. brasiliensis*-infected KN2 × 4get mice (top), KN2 × 4get *Rag2*^{-/-} mice (middle) and KN2 × 4get *Rag2*^{-/-} mice reconstituted with *Il4*^{-/-} *Il13*^{-/-} CD4⁺ T cells (bottom), assessed after overnight stimulation of total splenocytes with anti-CD3ε in the presence or absence of neutralizing antibody to IL-3 as in d. Numbers above or in outlined areas indicate percent basophils positive for human CD2 (as in Fig. 1a). Data are representative of two to four independent experiments.

protein was restricted to basophils, and liver eosinophils expressed little human CD2 (Fig. 1a,d). Thus, basophils are activated by a CD4⁺ T cell-dependent but IL-4-, IL-13- and IgE-independent mechanism in parasite-involved tissues.

CD4⁺ T cells mediate basophil IL-4 production *in vitro*

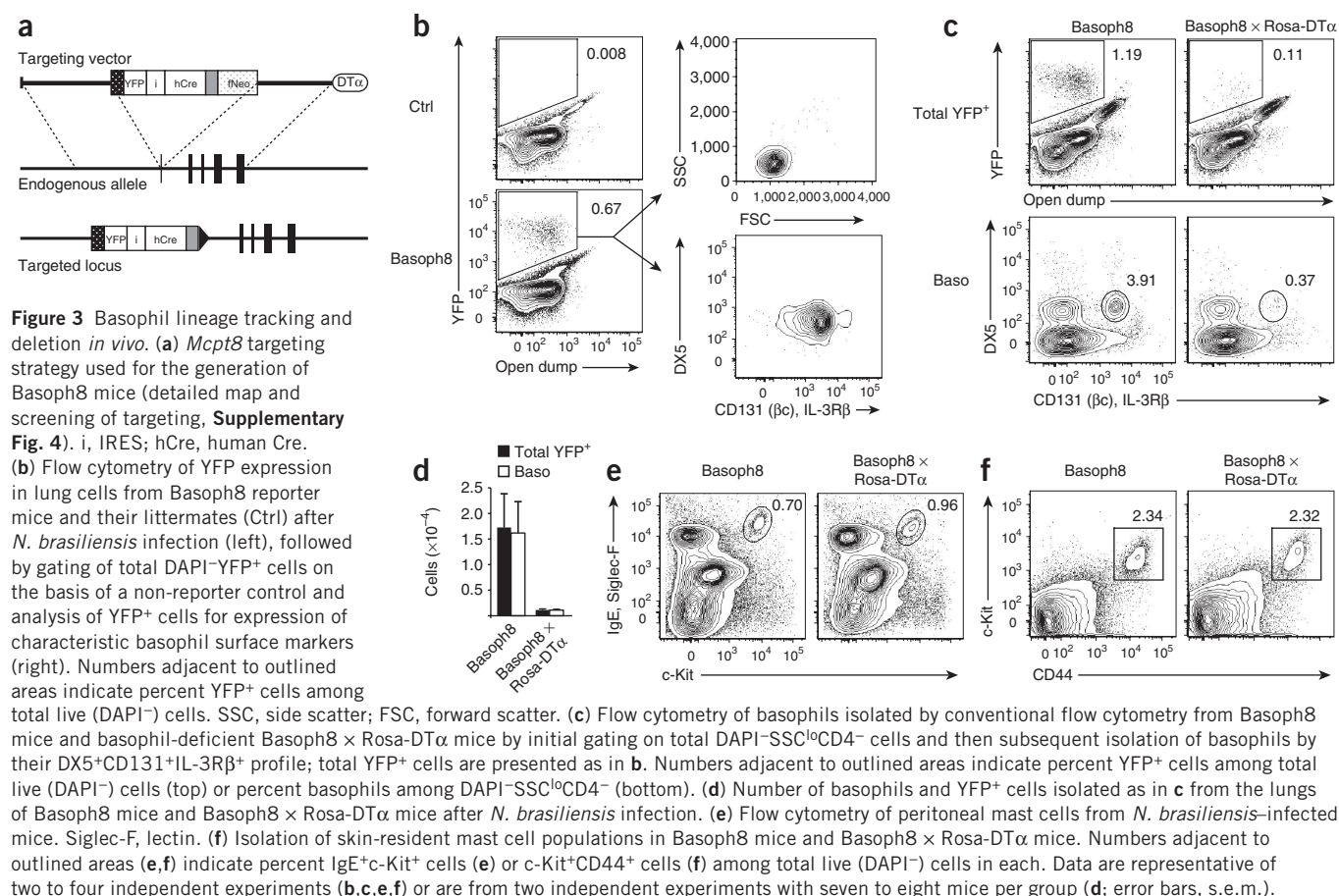
We sought to characterize the CD4⁺ T cell-derived factor(s) responsible for basophil activation in affected tissue. Although they were recruited to the spleen in relatively large numbers after *N. brasiliensis* infection, splenic basophils did not express human CD2 (Supplementary Fig. 2), which perhaps reflected an absence of interactions between spleen basophils and T cells during infection. To overcome potential restrictions *in vivo*, we isolated spleen cells from *N. brasiliensis*-infected KN2 × 4get mice and stimulated them overnight on plate-bound antibody to CD3ε (anti-CD3ε) to promote T cell activation. The next day, splenic basophils expressed human CD2, consistent with their activation to produce IL-4 (Fig. 2a). We next assessed whether basophil activation required direct contact with stimulated CD4⁺ T cells or whether it could be provided by soluble, secreted factors from activated CD4⁺ T cells. We purified CD4⁺ T cells from the spleens of *N. brasiliensis*-infected mice (5–6 d after infection), followed by enrichment of basophils from the spleens of resting KN2 × 4get *Rag2*^{-/-} mice. To prevent cell-cell contact, we used a Transwell insert to separate the purified CD4⁺ T cells from the basophils. As assessed by induction of human CD2, basophils without contact with CD4⁺ T cells were activated to produce IL-4 (Fig. 2b), although to a lesser degree than were basophils in contact with activated T cells (Fig. 2a). Furthermore, supernatants collected after the overnight stimulation of CD4⁺ T cells from infected mice were sufficient to promote the expression of human CD2 by basophils (Fig. 2c). IL-3 has been linked to the promotion of basophil population expansion, survival and activation in the context of crosslinking of surface-bound IgE^{26–28}. IL-3 was present in the supernatants of stimulated CD4⁺ T cells (where it had an average concentration of 2.5 ng/ml), and IL-3 at those concentrations activated the production of IL-4 by basophils

in vitro (Fig. 2c). Additionally, neutralizing antibody to IL-3 inhibited the production of IL-4 by basophils after incubation with the supernatants of activated CD4⁺ T cells. However, blockade of IL-3 did not block basophil activation when basophils were cultured together with CD4⁺ T cells (Fig. 2d). Consistent with our findings obtained *in vivo*, CD4⁺ T cell-derived IL-4 and IL-13 and IgE were not required for basophil activation *in vitro*, as *Il4*^{-/-} *Il13*^{-/-} CD4⁺ T cells mediated basophil activation in the absence of antibodies (Fig. 2e). These data, as well as our findings in the context of *N. brasiliensis* infection, support a model in which the production of IL-4 by basophils is restricted to the tissues involved and is dependent on antigenic stimulation of CD4⁺ T cells by a process that optimally requires both direct contact with CD4⁺ T cells and IL-3.

The Basoph8 basophil reporter mouse strain

To track and delete basophils with high specificity, we replaced the gene encoding mast cell protease 8 (*Mcpt8*) at the start site by inserting a reporter cassette containing sequence encoding YFP followed by an IRES and sequence encoding humanized Cre recombinase (Fig. 3a and Supplementary Fig. 4). *Mcpt8* is a basophil gene in the conserved chymase locus, which has expanded in rodents through gene duplications to contain many species-specific genes^{29,30}. *Mcpt8* is basophil specific in mice and is absent from humans, which suggests that it is not required for development of the basophil lineage. Published reports targeting this locus have confirmed the specificity of gene expression in basophils^{16,18}.

We analyzed tissues at the peak of the inflammatory response after *N. brasiliensis* infection and confirmed that all YFP⁺ cells were basophils, characterized as SSC^{lo}, CD4⁺, C-Kit⁺, positive for CD49b (DX5), positive for CD131 (cytokine receptor common β-chain subunit (βc), IL-3Rβ⁺ and IgE⁺ (Fig. 3b and data not shown). Furthermore, isolation of basophils from Basoph8 mice by conventional flow cytometry confirmed that all basophils from all tissues expressed YFP (Fig. 3c,d). The only discrepancy in the expression of YFP and conventional basophil markers occurred in the bone marrow and was due to variegated expression of CD49b (DX5) in the Basoph8⁺ population,



which probably highlights the developmental regulation of this adhesion molecule during basophil differentiation (**Supplementary Fig. 5**).

To delete basophils, we crossed Basoph8 mice with Rosa-DTα mice, which have the gene encoding diphtheria toxin α-chain (DTα) inserted into the ubiquitous *Rosa26* locus downstream of a loxP-flanked transcriptional stop site^{7,31}. The resultant Basoph8 × Rosa-DTα mice demonstrated highly efficient deletion of basophils, as shown by deletion of YFP⁺ cells and of basophils, assessed by conventional staining (CD49b⁺CD131⁺IL-3Rβ⁺SSC^{lo}; **Fig. 3c,d**). Deletion was approximately 96% efficient after *N. brasiliensis* infection, and surviving cells had noticeably attenuated YFP fluorescence (**Fig. 3c,d**). The number of mast cells isolated after peritoneal lavage of *N. brasiliensis*-infected mice (**Fig. 3e**) and from the skin of older mice was unaffected, despite deletion of basophils from blood and tissues (**Fig. 3f** and **Supplementary Fig. 6**). Eosinophils and CD4⁺ T cells likewise remained unaffected in Basoph8 × Rosa-DTα mice (discussed below).

No requirement for basophils in the priming of CD4⁺ T cells

Studies suggest that basophils serve a necessary and sufficient role as APCs in T_H2-associated immunity^{11–13}. Mechanistically, a model has been proposed in which basophils transiently migrate to draining lymph nodes, present peptide in the context of major histocompatibility complex class II and provide lineage-determining cytokines, all of which are necessary for efficient T_H2 priming. Although antigen-presenting function has been attributed to basophils, other studies with different approaches have questioned the role of basophils in this process^{14–17}. To assess the requirement for basophils in T_H2 priming *in vivo*, we crossed basophil-deficient Basoph8 × Rosa-DTα mice

onto the KN2 background. In this way, we were able to resolve activation of IL-4 secretion from CD4⁺ T cells by flow cytometry in the presence or absence of basophils without the need for restimulating the cells *ex vivo*.

Immunization with *S. mansoni* eggs induces T_H2 priming in the draining lymph node within 3–4 d (ref. 11). Using established protocols, we injected 2,500 eggs into the footpads of Basoph8 × KN2 mice and Basoph8 × Rosa-DTα × KN2 mice and monitored the kinetics of the accumulation of basophils and IL-4-secreting T cells positive for human CD2. Basophils were recruited to the draining popliteal lymph node in Basoph8 × KN2 mice after immunization, with a peak of approximately 3,000–4,000 cells at 48 h; basophils were almost completely undetectable in Basoph8 × Rosa-DTα mice after similar challenge (**Fig. 4a,b**). Consistent with our observations obtained with *N. brasiliensis*-infected mice, basophils did not express IL-4 in the draining lymph node at any time after immunization with *S. mansoni* eggs (**Supplementary Fig. 7**). In confirmation of the results of published studies¹¹, IL-4 secretion by lymph node CD4⁺ T cells, as assessed by the surface expression of human CD2, was first present at day 3 and peaked at day 4 (**Fig. 4c,d**). Over 99% of basophils were deleted in similarly challenged Basoph8 × Rosa-DTα × KN2 mice, but there was no decrease in the number of IL-4-secreting lymph node CD4⁺ T cells (**Fig. 4c,d**).

To assess the requirement for basophils in allergic responses to soluble stimuli, we challenged similar groups of mice with the protease papain, which can induce the influx of basophils into the draining lymph node, followed 24 h later by a T cell IL-4 response⁹. In similar conditions, however, the frequency and number of IL-4-producing CD4⁺ T cells were unaffected by the deletion of basophils in Basoph8 ×

Figure 4 Priming of CD4⁺ T cells in the absence of basophils. (a) Flow cytometry of total live (DAPI⁻) lymph node cells from the draining popliteal nodes 48 h after injection of *S. mansoni* eggs into the footpad. Numbers adjacent to outlined areas indicate percent YFP⁺ cells. Data are representative of three experiments. (b) Total basophils in the draining lymph node after injection of *S. mansoni* eggs into the footpad. Data are from three experiments with four to five mice per group at each time point (mean $\pm$ s.e.m.). (c) Flow cytometry of cytokine-positive DAPI⁻ CD4⁺ T cells (assessed by surface expression of human CD2 as in **Supplementary Fig. 11**) in the draining node 4 d after immunization with *S. mansoni* eggs. Numbers in outlined areas indicate percent CD4⁺ T cells positive for human CD2. Data are representative of three experiments. (d) Frequency of human CD2⁺, cytokine-producing (Cyt⁺) CD4⁺ T cells among total CD4⁺ T cells (top) and the total cytokine-positive CD4⁺ T cells (bottom) on days 3 and 4 after immunization as in c. Data are from three independent experiments (mean and s.e.m.). (e) Flow cytometry of human CD2⁺ CD4⁺ T cells as in c at 72 h after immunization of footpads with papain (50 μ g). Numbers in outlined areas indicate percent CD4⁺ T cells positive for human CD2. Data are representative of three experiments. (f) Frequency of cytokine-positive CD4⁺ T cells after papain immunization. Data are from three independent experiments with four to seven mice per group at each time point (mean and s.e.m.).

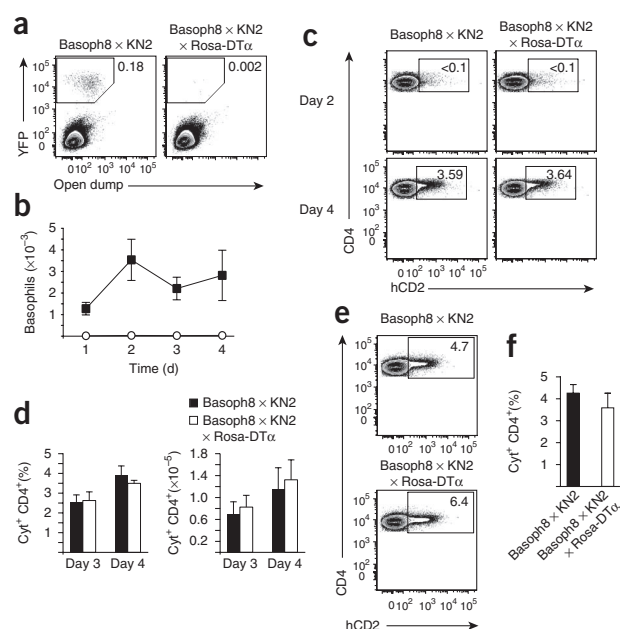
Rosa-DT α $\times$ KN2 mice compared with those of basophil-replete Basoph8 $\times$ KN2 mice (**Fig. 4e,f**). Thus, in these reporter strains of mice, we did not find a requirement for basophils in the CD4⁺ T cell responses to particulate or soluble IL-4-inducing stimuli.

Imaging of interactions between basophils and CD4⁺ T cells

The data reported above indicated that basophils are dispensable for CD4⁺ T cell priming in lymph nodes but that CD4⁺ T cells are essential for basophil activation in peripheral tissues, which suggested that the mode of interactions between basophils and CD4⁺ T cells differs in lymph nodes versus peripheral tissues. To visualize these interactions, we took advantage of the Basoph8 mice, in which *Mcp18* drives abundant YFP fluorescence selectively in basophils. We visualized CD4⁺ T cells responding to immunization by adoptively transferring ovalbumin (OVA)-specific OT-II CD4⁺ T cells expressing the red fluorescent protein DsRed or labeled with an orange dye. By modifying immunization models described above to include OVA, we were able to directly visualize the dynamics of the interactions between antigen-specific CD4⁺ T cells and basophils by time-lapse imaging with two-photon microscopy.

We observed robust population expansion of OT-II T cells and recruitment of basophils to the draining lymph nodes 2 d after subcutaneous immunization with *S. mansoni* eggs mixed with OVA. Between days 2 and 3, basophils occasionally contacted OT-II T cells, but these contacts were of very short duration and no stable conjugates were formed (**Fig. 5a–c** and **Supplementary Video 1**). We obtained similar results 2–3.5 d after subcutaneous immunization with papain mixed with OVA (**Supplementary Video 2**). The lack of stable interactions between CD4⁺ T cells and basophils suggested that basophils do not act as APCs for CD4⁺ T cells in primary immune responses in lymph nodes.

For comparison, we visualized interactions between classical APCs and CD4⁺ T cells within lymph nodes. As reported for other systems³², we confirmed that prolonged interactions of OT-II T cells with dendritic cells could be observed within the first day after immunization with papain mixed with OVA, but these interactions subsided by days 2–3, when basophils were recruited (data not shown). As T cell–B cell interactions typically occur during this time³³, we transferred OT-II T cells and cyan fluorescent protein-expressing Hy10 B cells, specific for avian egg lysozyme, together into Basoph8 recipient mice. After subcutaneous immunization with papain mixed with a conjugate of duck egg lysozyme and OVA



(DEL-OVA), we observed basophils, OT-II T cells and Hy10 B cells together in the imaging field. Interactions between OT-II T cells and Hy10 B cells were much longer than those between OT-II T cells and basophils, and we observed many stable conjugates between OT-II T cells and Hy10 B cells, some of which exceeded the duration of the recording (**Fig. 5a–c** and **Supplementary Video 3**). These prolonged T cell–B cell interactions were similar to those that occurred in mice immunized with DEL-OVA mixed with a classical adjuvant monophosphoryl lipid A and trehalose dicorynomycolate (data not shown). Thus, at the time at which basophils were recruited to the lymph node, CD4⁺ T cells were engaged in prolonged interactions with B cells that did not involve basophils.

To visualize the interaction dynamics of OT-II T cells and basophils in the lungs, we infected mice with *N. brasiliensis* and then administered OVA intranasally. In explanted lung slices, we found basophils and OT-II T cells together in small regions of the lung near hemorrhagic sites where *N. brasiliensis* larvae had penetrated the blood vessels. We observed interactions between OT-II T cells and basophils in the lung that were significantly longer than the brief contacts in lymph nodes (**Fig. 5a–c** and **Supplementary Video 4**). To confirm that basophils also interacted with nontransgenic T cells, we bred Basoph8 mice to transgenic mice expressing DsRed under the control of the human CD2 promoter, which illuminated the entirety of polyclonal T cells, and observed very similar interactions in the lungs after *N. brasiliensis* infection (**Supplementary Video 5**).

Unexpectedly, in several cases in both systems, we observed that an individual T cell and basophil in the lung came in contact with each other for a few minutes, disengaged and then came back in contact, in a repetitive way (**Fig. 5a** and **Supplementary Videos 4** and **5**). Unlike the stable T cell–B cell conjugates observed in lymph nodes, in which T cells closely track B cell movements³⁴, the T cells and basophils in the lungs did not closely follow each other's movements (**Supplementary Fig. 8**). Instead, the T cells and basophils alternately pulled away from each other and moved toward each other even while remaining in contact. This distinction was further reflected by quantification of the distances between the centroids (centers of mass) of the cells (**Fig. 5d,e**). The median distances between centroids of T cells and basophils were significantly greater than those

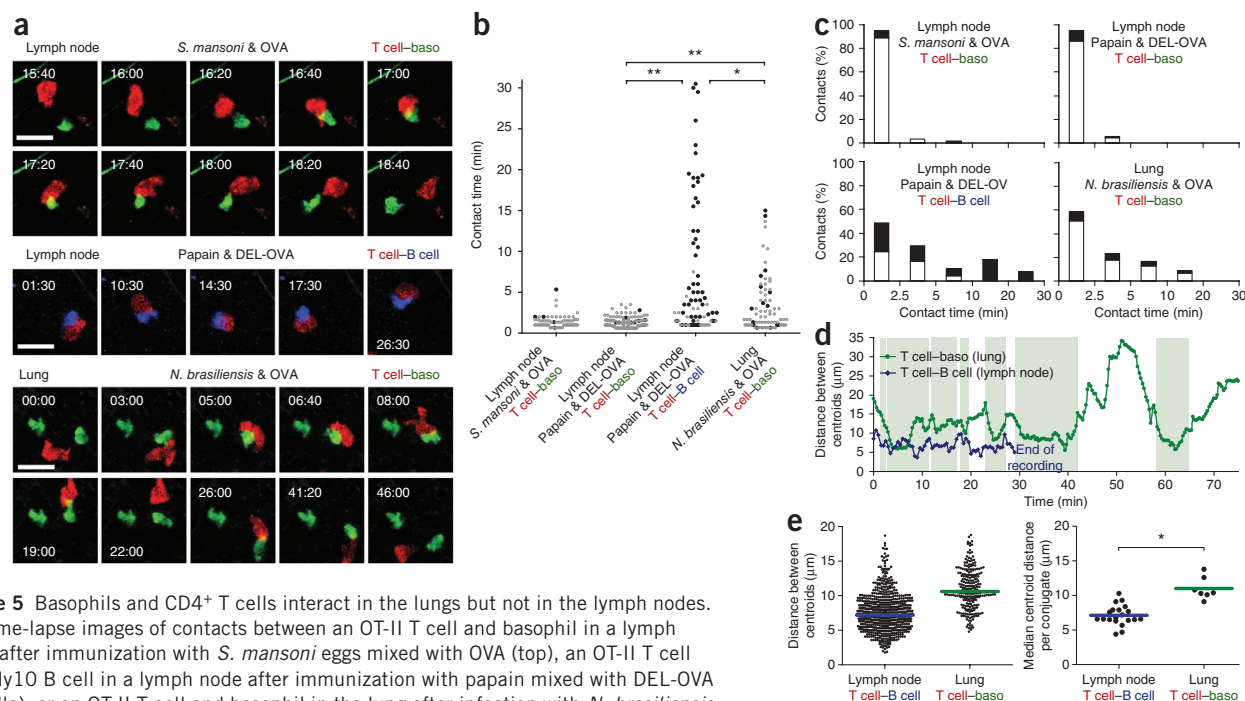


Figure 5 Basophils and CD4⁺ T cells interact in the lungs but not in the lymph nodes. (a) Time-lapse images of contacts between an OT-II T cell and basophil in a lymph node after immunization with *S. mansoni* eggs mixed with OVA (top), an OT-II T cell and Hy10 B cell in a lymph node after immunization with papain mixed with DEL-OVA (middle), or an OT-II T cell and basophil in the lung after infection with *N. brasiliensis* and immunization with OVA. Images are z-projections of approximately 30 μ m and maximum intensity. Contacts in the middle and bottom rows are further characterized in **d** and **Supplementary Figure 8**; bottom row corresponds to **Supplementary Video 4**. Numbers in images indicate elapsed time (minutes:seconds). Scale bars, 20 μ m. (b) Duration of contacts between cells (as in a). Each symbol represents a single contact: gray circles, contacts completely observed from start to finish; black dots, contacts not tracked for their entire duration, typically because the conjugate entered or exited the imaging volume or exceeded the duration of the recording (thus values for black dots are underestimates). * $P < 0.05$ and ** $P < 0.01$ (Kruskal-Wallis test). (c) Compilation of the data in **b**; black shading indicates conjugates not tracked for their entire duration. (d) Distance between cell centroids for the cell contacts in **a**. Green shading indicates time periods during which the T cell and basophil were in contact; the T cell-B cell conjugate persisted for the entire duration of a 30-minute recording. (e) Distance between cell centroids at time points at which cells were in contact, for cells that remained in contact for over 8 min. Each dot indicates the distance at a separate time point (left) or the median distance for each cell conjugate (right); horizontal bars, mean. * $P = 0.0003$ (Mann-Whitney U-test). Data are from one experiment (11 recordings; *S. mansoni* and OVA), five experiments (26 recordings; papain and DEL-OVA) or three experiments (16 recordings; *N. brasiliensis* and OVA).

for T cells and B cells while the cells were in contact. Together these data indicate that CD4⁺ T cells and basophils engage in multiple serial interactions in affected lungs of short to moderate duration, in contrast to the very brief contacts observed in lymph nodes, but also in a manner distinct from the close stable conjugates formed between T cells and B cells.

Basophil-derived cytokines contribute to worm clearance

Published studies have suggested modest contributions by basophils to the host response to *N. brasiliensis*¹⁶. In Basoph8 mice and Basoph8 $\times$ Rosa-DT α mice, we found no effect of basophil deletion on worm clearance, total lung cellularity, number of infiltrating CD4⁺ T cells or eosinophils, or serum IgE concentrations during primary infection (Fig. 6a–c). Secondary infection of mice with *N. brasiliensis* results in a greatly accelerated response, and the few worms that reach the intestine are expelled within 5 d. Using KN2 $\times$ 4get mice to assess the secondary response, we observed more IL-4-producing basophils in the lungs at earlier time points (Supplementary Fig. 9a). In contrast to the primary response, the accelerated secondary response was almost entirely due to IL-4-dependent immunoglobulins, as mice reconstituted with *Il4*^{−/−}*Il13*^{−/−} CD4⁺ T cells, which lack IgE and high-affinity IgG1, did not manifest this augmented basophil cytokine response despite the development of tissue inflammation and basophilia (Supplementary Fig. 9a,b). Indeed, when we collected serum from *N. brasiliensis*-infected wild-type or *Rag2*^{−/−} mice and adoptively transferred it into KN2 $\times$ 4get *Rag2*^{−/−} mice that

had been reconstituted with *Il4*^{−/−}*Il13*^{−/−} CD4⁺ T cells, we found that the augmented basophil cytokine response was dependent on reconstitution by wild-type serum but was not dependent on reconstitution by *Rag2*^{−/−} serum. In contrast to *Rag2*^{−/−} serum, wild-type serum resulted in ‘decoration’ of the basophil surface with IgE (Supplementary Fig. 9c). In contrast to findings obtained with another basophil-deficient strain¹⁶, we observed no deficits in worm clearance or infiltration of tissue by eosinophils by basophil-deficient Basoph8 $\times$ Rosa-DT α mice during secondary infection with *N. brasiliensis* (Supplementary Fig. 10).

Although we discerned no requirements for basophils, as assessed by their deletion, the activation of IL-4 secretion by basophils in tissues raised the possibility that cytokines produced by these cells are redundant in the context of infection. As both T_H2 cells and basophils secreted cytokines in affected tissues (Fig. 1a,b), we generated mice with loxP sites engineered into the *Il4*-*Il13* locus (*Il4*-*Il13*^{fl/fl}) such that Cre-mediated recombination would result in deletion of both genes³⁵. We crossed the homozygous *Il4*-*Il13*^{fl/fl} mice with *Il4*^{−/−}*Il13*^{−/−} mice, mice expressing Cre driven by *Cd4* and/or Basoph8 mice to facilitate lineage-specific deletion of the cytokines from CD4⁺ T cells (*Cd4*-Cre $\times$ *Il4*-*Il13*^{fl/fl}) or basophils (Basoph8 $\times$ *Il4*-*Il13*^{fl/fl}) or both (*Cd4*-Cre $\times$ Basoph8 $\times$ *Il4*-*Il13*^{fl/fl}) to assess their contributions to the host cytokine response. Deletion of IL-4 and IL-13 from CD4⁺ T cells did not have much of an effect on the ability to expel worms during primary infection despite the absence of IgE (Fig. 6d,e). Deletion of IL-4 and IL-13 from basophils also did not affect worm clearance but, conversely,

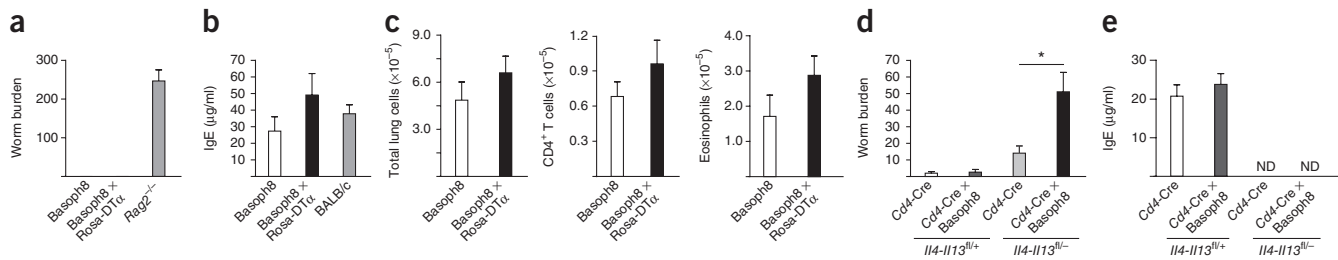


Figure 6 Basophil-derived cytokines contribute to anti-helminth immunity. **(a)** Worm burden in the small intestine 10 d after primary infection with *N. brasiliensis*. **(b)** Serum IgE concentrations of the mice in **a** and *N. brasiliensis*-infected BALB/c control mice. **(c)** Inflammatory response (cellularity) in the lung of the mice in **a**, assessed by flow cytometry. **(d)** Worm burden in the small intestine as in **a**. * $P = 0.0036$ (Student's *t*-test). **(e)** Serum IgE concentrations in the mice in **d**. ND, not detected. Data are from three independent experiments with three to five mice per group (**a–c**) or two experiments with five to eight mice per group (**c,d**; mean and s.e.m.).

had no effect on serum IgE concentrations (data not shown). In contrast, simultaneous deletion of IL-4 and IL-13 from both CD4⁺ T cells and basophils resulted in a significantly larger intestinal worm burden (Fig. 6d,e), which indicated that cytokines from both of these lineages contribute to the host response to migratory helminths.

DISCUSSION

Although they were described by Paul Ehrlich in 1879 and are conserved in all vertebrates, including fish, birds and reptiles, basophils remain enigmatic and precise delineation of their role has remained elusive because of the lack of adequate reagents with which to address their specific contributions³⁶. Seminal studies in the 1990s first identified a non-B, non-T cell as a major source of IL-4 after parasite challenge that was identified by crosslinking of the receptor FcεRI and augmented by IL-3 (refs. 37–40). Subsequently, two different IL-4 reporter mice were used to demonstrate that basophils accumulate in tissues through a CD4⁺ T cell-dependent but STAT6-independent mechanism^{19,20}. Attempts to achieve depletion of basophils with various antibodies^{11,12,17,41–43} have resulted in controversy about the sensitivity and specificity of the targeted deletion¹⁵; two groups have targeted the basophil-specific gene *Mcpt8* to achieve lineage marking^{16,18}. Here we have combined IL-4 reporter mice with the newly generated Basoph8 basophil reporter mice; this uses a different targeting strategy that allows direct visualization of basophils in living tissues as well as the generation of basophil-deficient mice. Using these mice we have obtained several findings, including the CD4⁺ T cell-dependent localization of basophil IL-4 production to tissues invaded by parasites; replication of the CD4⁺ T cell-dependent ability to activate IL-4 production from basophils *in vitro* through a contact- and IL-3-dependent but IgE-independent process; and evaluation of basophil dynamics *in vivo* that confirmed the interaction of basophils with antigen-specific CD4⁺ T cells in tissues but not in draining lymph nodes. Finally, by lineage-specific deletion of *Il4* and *Il13* from CD4⁺ T cells and basophils, we have shown cooperation between these cells in the primary response to migratory helminths.

The signals from CD4⁺ T cells that recruit and license basophils to secrete IL-4 during the primary response are of much interest. IL-3 is an important factor for the differentiation and growth of basophils that is able to expand basophil populations from the bone marrow *in vitro* and *in vivo*. IL-3 from activated CD4⁺ T cells was important in mobilizing basophils from the bone marrow during infection with *N. brasiliensis*, as Rag2^{-/-} mice reconstituted with IL-3-deficient CD4⁺ T cells had fewer bone marrow and blood basophils than did mice reconstituted with wild-type T cells²⁸. Additionally, IL-3 mediated the recruitment of basophils to lymph nodes after helminth infection⁴⁴. As shown here, recombinant IL-3 or IL-3 that accumulated in

supernatants of activated CD4⁺ T cells stimulated IL-4 production by basophils *in vitro*. However, maximal IL-4 production occurred when basophils were cultured together with activated CD4⁺ T cells, which suggests that direct cell contact probably contributes additional signals. Indeed, IL-3-neutralizing antibody did not block basophil IL-4 production *in vitro*, and optical imaging studies of live tissues demonstrated interactions between CD4⁺ T cells and basophils in the lungs but not in the lymph nodes, which supports the idea of a contact-driven requirement for tissue basophil activation. More work is needed to determine the parameters of this process, but the tools we have generated will facilitate further study.

A notable aspect of basophil biology has been reports suggesting a role for basophils as primary APCs in T_H2-dominated immunity in various systems^{11–13}. Those studies used nonspecific monoclonal antibodies to achieve basophil depletion, and subsequent studies have raised questions about the specificity of the antibodies, suggesting that off-target deletion of other critical immune cells, such as dendritic cells, may have occurred^{14,15,17}. Clearly, lineage tracking of basophils is needed to investigate this process, and two groups have reported targeting the *Mcpt8* locus in mice^{16,18}, as we have reported here. *Mcpt8* is a basophil-specific protease in mice whose enzymatic substrate remains unknown^{29,45}. In the first reporter strain (*Mcpt8*^{DTR}), a downstream IRES is followed by a diphtheria toxin receptor-enhanced GFP fusion protein that allows diphtheria-mediated depletion of basophils that is sustained up to 5 d after injection of toxin¹⁸. These mice have been used to demonstrate a requirement for basophils to impede tick re-feeding on mice by a process dependent on antibody and basophil Fc receptors¹⁸. In the second reported strain, mice transgenic for a bacterial artificial chromosome were generated with replacement of *Mcpt8* with sequence encoding Cre recombinase, which resulted in unexpected constitutive deletion of approximately 90% of basophils by a process believed to be mediated by Cre-dependent toxicity from five- to seven-copy integration events¹⁶. These mice have been used to demonstrate no requirement for basophils in various allergic immune models, including papain- and OVA-induced allergy models, although the results support the idea of a role for basophils in IgE-mediated chronic dermatitis, in confirmation of earlier studies^{16,46}. Additionally, a role for basophils has been suggested in secondary, but not primary, *N. brasiliensis* infection, as recruitment of eosinophils and T_H2 cells to the lungs and worm clearance are diminished¹⁶.

In the Basoph8 mice we have reported here, *Mcpt8* was replaced by a YFP-IRES-Cre cassette, which established bright YFP fluorescence in basophils and allowed conditional deletion of the cells or their cytokines. Studies with these mice allowed us to assess the positioning of basophils in tissues, their relationships with the CD4⁺ T cells on which they depend for functional activity and the consequence

of specific basophil-restricted deletion of IL-4 and IL-13 to assess directly the contributions of these cytokines from basophils. In terms of the entry of basophils into inflamed lymph nodes, we confirmed basophil entry with a soluble stimulus (papain) and a particulate stimulus (schistosome eggs) but found no requirement for basophils in the activation of IL-4 production by naive CD4⁺ T cells, as assessed in the Basoph8 × KN2 × Rosa-DTα deleter strain. That observation was independently confirmed by flow cytometry of cells from mice with sensitive *Il4* reporter alleles to demonstrate that basophils did not produce IL-4 in lymphoid tissues. Additionally, our imaging studies showed that basophils did not interact with CD4⁺ T cells in a manner consistent with their proposed role as APCs. Finally, we specifically removed IL-4 and IL-13 from basophils and confirmed that these cytokines did not contribute to host IgE or immunity to *N. brasiliensis* when CD4⁺ T cells were intact. We conclude through these multiple confirmatory yet independent lines of investigation that basophils are not required for the initiation of primary cellular or humoral immunity under the conditions examined.

Published studies have indicated that CD4⁺ T cells are necessary for the accumulation of basophils^{19,20}. Using the KN2 × 4get reporter strain, we have demonstrated that CD4⁺ T cells were needed to mediate basophil secretion of IL-4, which was sequestered to tissues invaded by parasites and infiltrated by activated CD4⁺ T cells. Although neither IL-4 nor IL-13 from either CD4⁺ T cells or basophils was alone needed to expel intestinal *N. brasiliensis* after primary infection, combined deletion of IL-4 and IL-13 from both cell types resulted in a significant and reproducible deficit in this response, which demonstrated a contribution by basophil-derived IL-4 to this process. IgE responses were completely dependent on CD4⁺ T cell-derived IL-4 and IL-13. In contrast to a published study¹⁶, we found no deficit in immunity to secondary *N. brasiliensis* infection in basophil-deficient mice. Under the conditions examined here, augmented basophil IL-4 production in secondary infection was entirely immunoglobulin dependent, as demonstrated by adoptive transfer of serum. Thus, our data are most consistent with models of tick infestation and chronic allergic dermatitis in identifying interactive roles for basophils and immunoglobulin^{16,18}. Thus, basophils, despite their identification as cells of the innate immune response, are unexpectedly dependent on cells of the adaptive immune response, including CD4⁺ T cells and antibodies, for robust generation of IL-4 in these model systems.

Although combined deletion of IL-4 and IL-13 from CD4⁺ T cells and basophils reproducibly impaired intestinal worm clearance, the number of intestinal worms was consistently lower in *Cd4-Cre* × Basoph8 × *Il4-Il13*^{fl/fl} mice than in infected *Rag2*^{-/-} mice or in *Il4*^{-/-}*Il13*^{-/-} mice^{47,48}. The contributions of other cells, variably referred to as natural helper cells, nuocytes and innate type 2 helper cells, have been demonstrated^{5–7}. These cells produce high concentrations of IL-13 and IL-5 but, like basophils, require CD4⁺ T cells for optimal activity⁵. We speculate that innate type 2 helper cells are responsible for the partial worm clearance in the setting of deficiency in IL-4 and IL-13 in CD4⁺ T cells and basophils, although we note the overlapping effects of IL-4, IL-13, IL-5 and IL-9, as assessed by stepwise deletion of the genes encoding these cytokines during analysis of the effects on worm immunity⁴⁹. Basoph8 mice represent a new tool with which to explore the role of basophils in infectious and inflammatory models in addition to those explored here and to place these cells in the context of additional cell types and cytokines linked to allergic immunity and the response to helminths.

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.

ACKNOWLEDGMENTS

We thank K.C. Lim and N. Flores for technical support; E. Thornton and M. Krummel for training in lung-slice preparation; D. Kioussis (Medical Research Council National Institute for Medical Research) for huCD2-DsRed transgenic mice; and J. Cyster (University of California, San Francisco) for mice and two-photon microscope use. Supported by the US National Institutes of Health (AI026918 and AI077439), the Howard Hughes Medical Institute and the Sandler Asthma Basic Research Center at the University of California San Francisco, San Francisco.

AUTHOR CONTRIBUTIONS

B.M.S., H.-E.L., C.D.C.A. and R.M.L. conceived of the work; H.-E.L. generated Basoph8 reporter mice; B.M.S. designed and did most experiments; C.D.C.A. contributed two-photon imaging data; J.K.B. and B.M.S. analyzed basophils in the small intestine; D.W. and B.M.S. generated data from mice infected with *S. mansoni* cercariae; L.E.C. analyzed mast cells in the skin in Basoph8 mice; J.K.M. provided *S. mansoni* cercariae and eggs; and B.M.S., C.D.C.A. and R.M.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at <http://www.nature.com/natureimmunology/>.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

- Chan, M.S. The global burden of intestinal nematode infections—fifty years on. *Parasitol. Today* **13**, 438–443 (1997).
- Urban, J.F. Jr. Maliszewski, C.R., Madden, K.B., Katona, I.M. & Finkelman, F.D. IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice. *J. Immunol.* **154**, 4675–4684 (1995).
- Urban, J.F. Jr. et al. IL-13, IL-4Rα, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity* **8**, 255–264 (1998).
- Voehringer, D., Reese, T.A., Huang, X., Shinkai, K. & Locksley, R.M. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J. Exp. Med.* **203**, 1435–1446 (2006).
- Neill, D.R. et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367–1370 (2010).
- Moro, K. et al. Innate production of T_H2 cytokines by adipose tissue-associated c-Kit⁺Sca-1⁺ lymphoid cells. *Nature* **463**, 540–544 (2010).
- Price, A.E. et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci. USA* **107**, 11489–11494 (2010).
- Oh, K., Shen, T., Le Gros, G. & Min, B. Induction of Th2 type immunity in a mouse system reveals a novel immunoregulatory role of basophils. *Blood* **109**, 2921–2927 (2007).
- Sokol, C.L., Barton, G.M., Farr, A.G. & Medzhitov, R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* **9**, 310–318 (2008).
- Yanagihara, Y. et al. Cultured basophils but not cultured mast cells induce human IgE synthesis in B cells after immunologic stimulation. *Clin. Exp. Immunol.* **111**, 136–143 (1998).
- Perrigou, J.G. et al. MHC class II-dependent basophil-CD4⁺ T cell interactions promote T_H2 cytokine-dependent immunity. *Nat. Immunol.* **10**, 697–705 (2009).
- Sokol, C.L. et al. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* **10**, 713–720 (2009).
- Yoshimoto, T. et al. Basophils contribute to T_H2-IgE responses *in vivo* via IL-4 production and presentation of peptide-MHC class II complexes to CD4⁺ T cells. *Nat. Immunol.* **10**, 706–712 (2009).
- Phythian-Adams, A.T. et al. CD11c depletion severely disrupts Th2 induction and development *in vivo*. *J. Exp. Med.* **207**, 2089–2096 (2010).
- Hammad, H. et al. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* **207**, 2097–2111 (2010).
- Ohnmacht, C. et al. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* **33**, 364–374 (2010).
- Tang, H. et al. The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. *Nat. Immunol.* **11**, 608–617 (2010).
- Wada, T. et al. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J. Clin. Invest.* **120**, 2867–2875 (2010).
- Min, B. et al. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* **200**, 507–517 (2004).
- Voehringer, D., Shinkai, K. & Locksley, R.M. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* **20**, 267–277 (2004).
- Mohrs, K., Wakil, A.E., Killeen, N., Locksley, R.M. & Mohrs, M. A two-step process for cytokine production revealed by IL-4 dual-reporter mice. *Immunity* **23**, 419–429 (2005).
- Mohrs, M., Shinkai, K., Mohrs, K. & Locksley, R.M. Analysis of type 2 immunity *in vivo* with a bicistronic IL-4 reporter. *Immunity* **15**, 303–311 (2001).

23. Reinhardt, R.L., Liang, H.E. & Locksley, R.M. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat. Immunol.* **10**, 385–393 (2009).
24. van Panhuys, N. *et al.* Basophils are the major producers of IL-4 during primary helminth infection. *J. Immunol.* **186**, 2719–2728 (2011).
25. Pearce, E.J. & MacDonald, A.S. The immunobiology of schistosomiasis. *Nat. Rev. Immunol.* **2**, 499–511 (2002).
26. Lantz, C.S. *et al.* Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. *Nature* **392**, 90–93 (1998).
27. Lantz, C.S. *et al.* IL-3 is required for increases in blood basophils in nematode infection in mice and can enhance IgE-dependent IL-4 production by basophils in vitro. *Lab. Invest.* **88**, 1134–1142 (2008).
28. Shen, T. *et al.* T cell-derived IL-3 plays key role in parasite infection-induced basophil production but is dispensable for in vivo basophil survival. *Int. Immunol.* **20**, 1201–1209 (2008).
29. Poorafshar, M., Helmbj, H., Troye-Blomberg, M. & Hellman, L. MMCP-8, the first lineage-specific differentiation marker for mouse basophils. Elevated numbers of potent IL-4-producing and MMCP-8-positive cells in spleens of malaria-infected mice. *Eur. J. Immunol.* **30**, 2660–2668 (2000).
30. Gallwitz, M. & Hellman, L. Rapid lineage-specific diversification of the mast cell chymase locus during mammalian evolution. *Immunogenetics* **58**, 641–654 (2006).
31. Voehringer, D., Liang, H.E. & Locksley, R.M. Homeostasis and effector function of lymphopenia-induced 'memory-like' T cells in constitutively T cell-depleted mice. *J. Immunol.* **180**, 4742–4753 (2008).
32. Henrickson, S.E. & von Andrian, U.H. Single-cell dynamics of T-cell priming. *Curr. Opin. Immunol.* **19**, 249–258 (2007).
33. Cyster, J.G. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu. Rev. Immunol.* **23**, 127–159 (2005).
34. Okada, T. *et al.* Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.* **3**, e150 (2005).
35. Voehringer, D., Wu, D., Liang, H.E. & Locksley, R.M. Efficient generation of long-distance conditional alleles using recombineering and a dual selection strategy in replicate plates. *BMC Biotechnol.* **9**, 69 (2009).
36. Sullivan, B.M. & Locksley, R.M. Basophils: a nonredundant contributor to host immunity. *Immunity* **30**, 12–20 (2009).
37. Ben-Sasson, S.Z., Le Gros, G., Conrad, D.H., Finkelman, F.D. & Paul, W.E. Cross-linking Fc receptors stimulate splenic non-B, non-T cells to secrete interleukin 4 and other lymphokines. *Proc. Natl. Acad. Sci. USA* **87**, 1421–1425 (1990).
38. Conrad, D.H., Ben-Sasson, S.Z., Le Gros, G., Finkelman, F.D. & Paul, W.E. Infection with *Nippostrongylus brasiliensis* or injection of anti-IgD antibodies markedly enhances Fc-receptor-mediated interleukin 4 production by non-B, non-T cells. *J. Exp. Med.* **171**, 1497–1508 (1990).
39. Le Gros, G. *et al.* IL-3 promotes production of IL-4 by splenic non-B, non-T cells in response to Fc receptor cross-linkage. *J. Immunol.* **145**, 2500–2506 (1990).
40. Seder, R.A. *et al.* Purified FcεR⁺ bone marrow and splenic non-B, non-T cells are highly enriched in the capacity to produce IL-4 in response to immobilized IgE, IgG2a, or ionomycin. *J. Immunol.* **147**, 903–909 (1991).
41. Denzel, A. *et al.* Basophils enhance immunological memory responses. *Nat. Immunol.* **9**, 733–742 (2008).
42. Obata, K. *et al.* Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* **110**, 913–920 (2007).
43. Ohnmacht, C. & Voehringer, D. Basophil effector function and homeostasis during helminth infection. *Blood* **113**, 2816–2825 (2009).
44. Kim, S. *et al.* Cutting edge: Basophils are transiently recruited into the draining lymph nodes during helminth infection via IL-3, but infection-induced Th2 immunity can develop without basophil lymph node recruitment or IL-3. *J. Immunol.* **184**, 1143–1147 (2010).
45. Gallwitz, M., Enoksson, M. & Hellman, L. Expression profile of novel members of the rat mast cell protease (rMCP)-2 and (rMCP)-8 families, and functional analyses of mouse mast cell protease (mMCP)-8. *Immunogenetics* **59**, 391–405 (2007).
46. Mukai, K. *et al.* Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* **23**, 191–202 (2005).
47. Finkelman, F.D. *et al.* Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu. Rev. Immunol.* **15**, 505–533 (1997).
48. Finkelman, F.D. *et al.* Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol. Rev.* **201**, 139–155 (2004).
49. Fallon, P.G. *et al.* IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity* **17**, 7–17 (2002).

ONLINE METHODS

Mice. KN2 × 4get, IL-4 dual reporter mice have been described²¹. KN2 and 4get mice were bred individually onto a *Rag2*^{-/-} background and were intercrossed to derive KN2 × 4get *Rag2*^{-/-} mice on the BALB/c background. *Il4-Il13*^{fl/fl} and Rosa-DTα mice have been described^{31,35}. *Cd4*-Cre mice and OT-II.2 (subline 425-2) mice on a *Rag1*^{-/-} C57BL/6 background were from Taconic. Hemizygous OT-II.2 (subline 426-6) mice⁵⁰ were bred repeatedly to human CD2-DsRed transgenic mice⁵¹ until homozygous for the transgene encoding the fluorescent reporter. Hy10 (formerly VD9/k5) mice⁵² were bred to β-actin–cyan fluorescent protein mice (Tg(ACTB-ECFP)1Nagy/J; 004218; The Jackson Laboratory). Interactions of T cells with DCs were imaged after adoptive transfer of OT-II T cells into CD11c-YFP–transgenic mice on the C57BL/6 background⁵³. Mice were maintained according to institutional guidelines in specific pathogen-free facilities of the University of California San Francisco, San Francisco.

Generation of Basoph8 mice. A composite fluorescent reporter–Cre recombinase cassette was assembled by standard cloning procedures in the following order: genomic sequence of the rabbit β-globin gene partial exon 2–3 served as an artificial intron and splicing donor–acceptor; the gene encoding enhanced YFP (Clontech) served as the fluorescent reporter; encephalomyocarditis virus IRES; P1 phage Cre recombinase cDNA with codon use optimized for mammalian cells (humanized Cre); bovine growth hormone poly(A); a *loxP*-flanked neomycin-resistance cassette derived from pL2neo2 placed 3′ of the entire cassette. This 4.8-kilobase reporter-recombinase cassette was cloned into a basal targeting construct pKO915-DT (Lexicon) containing sequence encoding DTα for negative selection. The 5′ and 3′ homologous arms used to flank the reporter-recombinase cassette were obtained by high-fidelity PCR amplification of the *Mcpt8* locus from 129/SvJ genomic DNA. The 5′ arm consists of a 2.9-kilobase fragment covering the promoter region and 5′ untranslated region. The 3′ arm consists of a 2.6-kilobase fragment spanning the endogenous start codon to exon 5 of *Mcpt8*. The construct was made linear by digestion with NotI and then was transfected by electroporation into PrmCre embryonic stem cells, which express Cre recombinase driven by the protamine promoter. Embryonic stem clones resistant to the aminoglycoside G418 were screened for homologous recombination by Southern blot analysis. Two independent clones were injected into C57BL/6 blastocysts to generate chimeras. The neomycin-resistance cassette was deleted from the male germline by Cre-mediated recombination after breeding of male chimeras to wild-type C57BL/6 female mice. Mice carrying the Basoph8 allele were backcrossed to the C57BL/6 or BALB/c background.

Parasites and papain immunization. Maintenance and infection with *N. brasiliensis* have been described²⁰. *S. mansoni* were maintained as described, and mice were infected by subcutaneous injection of 200 or 260 cercariae⁵⁴. Liver samples were processed for flow cytometry or were fixed in 4% (wt/vol) paraformaldehyde for histology. *S. mansoni* eggs were collected from hamster livers, and 2,500 eggs (in a volume of 50 μl) were injected subcutaneously into the footpads of mice as described¹¹. Mice were immunized with 50 μg papain (Calbiochem) as described⁹. For lymph node imaging, *S. mansoni* eggs or papain were mixed with OVA (0.5–1 mg/ml; albumin from chicken egg white, grade VII; Sigma-Aldrich) or DEL-OVA (0.1–0.33 mg/ml)⁵². Mice were immunized subcutaneously on both lower flanks and bilaterally proximal to the base of the tail with a volume of 25 μl per site. This subcutaneous immunization resulted in the recruitment of basophils to the draining inguinal lymph nodes similar to their recruitment to the popliteal lymph nodes after footpad immunization (data not shown). In control experiments, an equivalent dose of DEL-OVA was mixed with the Sigma Adjuvant System containing monophosphoryl lipid A and trehalose dicorynomycolate (Sigma-Aldrich). For activation of OT-II T cells in the lungs after *N. brasiliensis* infection, mice were lightly anesthetized with isoflurane, and 50 μg OVA in 50 μl PBS was administered intranasally on days 1 and 6 after infection.

Flow cytometry. Phycoerythrin–anti-CD131 (JRO50), allophycocyanin–biotin–anti-c-Kit (2B8) and allophycocyanin–anti-CD44 (IM7) were from BD Biosciences; allophycocyanin–eFluor780–anti-CD4 (RM4-5), allophycocyanin–anti-CD49b (DX5) and allophycocyanin–eFluor780–anti-c-Kit

(DX5) were from eBioscience; and phycoerythrin-conjugated antibody to human CD2 (S5.5) and phycoerythrin-conjugated antibody to mouse IgG2a (MG2A04) were from Invitrogen. Cells were incubated with anti-CD16–CD32 (2.4G2; BD Biosciences), then were stained with the appropriate antibodies and labeled with the vital dye DAPI (4′,6-diamidino-2-phenylindole; 1 μg/ml; Roche). Cells were counted with Caltag Counting Beads (Invitrogen). The gating strategy used for human CD2 staining is in **Supplementary Figure 11**. The frequency of B cells binding hen egg lysozyme was determined by flow cytometry as described⁵². The frequency of T cells with transgenic expression of the T cell antigen receptor was determined by flow cytometry⁵² with the following antibodies: rat antibody to mouse T cell antigen receptor α-chain variable region 2 (conjugated to peridinin chlorophyll protein–cyanine 5.5; B20.1; BioLegend), mouse antibody to mouse T cell antigen receptor β-chain variable region 5.1 and 5.2 (conjugated to fluorescein isothiocyanate; MR9-4; BD Biosciences), rat antibody to mouse CD4 (conjugated to allophycocyanin-H7; GK1.5; BD Biosciences), hamster antibody to mouse CD3 (conjugated to Alexa Fluor 647; 145-2C11; BioLegend) or rat antibody to mouse CD3 (conjugated to Pacific Blue; 17A2; BioLegend). Cells were analyzed on an LSRII (Becton Dickinson) and data were analyzed with FlowJo software (Treestar).

Cell purification, labeling and adoptive transfer. For reconstitution of KN2 × 4get *Rag2*^{-/-} mice with CD4⁺ T cells, the axial, brachial, cervical, inguinal, popliteal and mesenteric lymph nodes were isolated from *Il4*^{-/-}*Il13*^{-/-} mice. CD4⁺ T cells were purified from single-cell suspensions by negative depletion with antibody-coated magnetic beads (Miltenyi Biotec), and 1 × 10⁷ cells were transferred into KN2 × 4get *Rag2*^{-/-} mice by injection into the tail vein. Mice were infected with *N. brasiliensis* 1 week later.

Details of cell purification and labeling for the imaging experiments are in the **Supplementary Methods**. B cells were purified by CD43 and CD11c negative selection. CD4⁺ T cells were purified with the Dynabeads FlowComp Mouse CD4 kit (Invitrogen). For some transfers of small numbers of cells (<2.5 × 10⁴ T cells or <5.0 × 10⁴ B cells per recipient), no purification was needed, and approximately 250 μl blood was collected from the retro-orbital plexus of anesthetized donor mice via heparinized capillary tubes (Fisher Scientific) into microtainer tubes with EDTA (BD). Erythrocytes were lysed with ACK Lysing Buffer (Quality Biological) and cells were resuspended in PBS. Cells were adoptively transferred into the retro-orbital plexus of anesthetized Basoph8 mice 12–24 h before immunization.

In vitro basophil activation. Spleens were obtained from mice 5–6 d after infection with *N. brasiliensis*. Total splenocytes (5 × 10⁶ cells per ml) were stimulated overnight on plate-bound antibody to mouse CD3ε (2 μg/ml; 145-2C11; BD Biosciences). Splenic CD4⁺ T cells were isolated from *N. brasiliensis*-infected mice by positive magnetic bead selection (L3T4, Miltenyi Biotec) and were incubated overnight at a density of 2 × 10⁶ cells per ml on plate-bound antibody to mouse CD3ε. Supernatants were collected, followed by cytokine analysis by enzyme-linked immunosorbent assay. Basophils were enriched from the spleens of KN2 × 4get *Rag2*^{-/-} mice by selection of CD49b⁺ cells with negative beads (Miltenyi Biotec). Enriched basophils (40 × 10⁴ to 60 × 10⁴) were stimulated overnight in 1 ml supernatant of activated CD4⁺ T cells or recombinant IL-3 (R&D Systems), and expression of human CD2 by basophils was assessed by flow cytometry as described.

Two-photon microscopy and data analysis. Details of the lung slice and lymph node explant preparation and imaging setup are in the **Supplementary Methods**. Lung slices were prepared for imaging by a modification of established methods (E. Thornton and M. Krummel, personal communication)⁵⁵. Lungs were inflated with agarose, which was allowed to solidify, and then thick slices were cut on a vibrating-blade microtome. Lung slices and explanted lymph nodes were perfused in an imaging chamber with warmed RPMI medium 'bubbled' with 95% O₂ and 5% CO₂; the temperature was maintained at 35–37 °C.

Two-photon microscopy was done with an upright LSM 7 MP laser-scanning microscope (Carl Zeiss MicroImaging) outfitted with a W Plan-Apochromat water-immersion 20× objective (numerical aperture, 1.0), an acousto-optical modulator, a motorized stage (Prior Scientific), a Chameleon Ultra II laser (Coherent) and ZEN 2009 software. Each xy plane was collected at a resolution



of 512×512 pixels with bidirectional scanning and variable zoom. The spacing between planes in the z dimension was set to three to five times the xy pixel size for a given zoom setting. The fluorescence of both Basoph8 YFP and cyan fluorescent protein was detected with a sensitive gallium arsenide phosphide (GaAsP) detector, and the YFP fluorescence and cyan fluorescent protein fluorescence were spectrally separated by switching of the laser excitation wavelength (details, **Supplementary Methods**). The fluorescence of DsRed and the dye CellTracker Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) was collected on a standard non-descanned detector through an ET605/70 emission filter (Chroma).

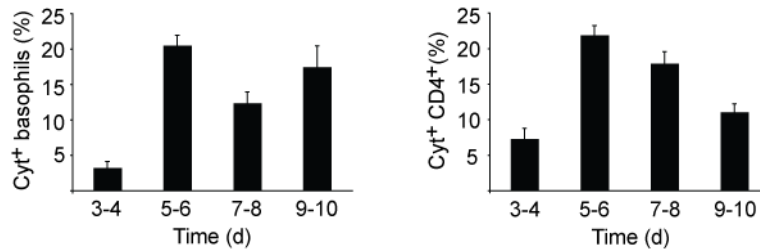
Contact times were determined by a semiautomated measurement task with Volocity 5.2.1 or 5.4.2 software (PerkinElmer) and data were analyzed with Microsoft Excel 2003 and GraphPad Prism (details, **Supplementary Methods**). Maximum intensity z -projection still images and time-lapse sequences were prepared with Metamorph software version 7.7 (Molecular Devices) and ZEN 2009 Light Edition software (Carl Zeiss MicroImaging), respectively. Linear adjustments were made for brightness and contrast, and in some cases

photobleaching was partially corrected by interpolation. Images were processed with low pass or median noise filters. Adobe After Effects 7.0 was used for annotation and final compilation of videos. QuickTime format videos were compressed with a JPEG-2000 codec.

50. Barnden, M.J., Allison, J., Heath, W.R. & Carbone, F.R. Defective TCR expression in transgenic mice constructed using cDNA-based α - and β -chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* **76**, 34–40 (1998).
51. Veiga-Fernandes, H. *et al.* Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. *Nature* **446**, 547–551 (2007).
52. Allen, C.D., Okada, T., Tang, H.L. & Cyster, J.G. Imaging of germinal center selection events during affinity maturation. *Science* **315**, 528–531 (2007).
53. Lindquist, R.L. *et al.* Visualizing dendritic cell networks *in vivo*. *Nat. Immunol.* **5**, 1243–1250 (2004).
54. Davies, S.J. *et al.* Modulation of blood fluke development in the liver by hepatic CD4⁺ lymphocytes. *Science* **294**, 1358–1361 (2001).
55. Delmotte, P. & Sanderson, M.J. Ciliary beat frequency is maintained at a maximal rate in the small airways of mouse lung slices. *Am. J. Respir. Cell Mol. Biol.* **35**, 110–117 (2006).

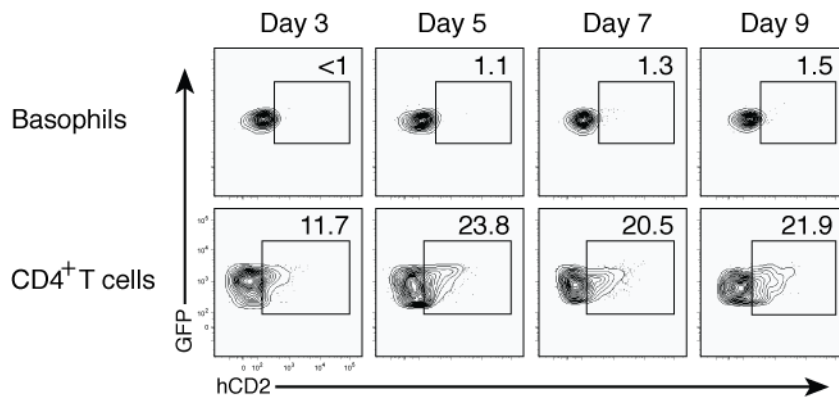
SUPPLEMENTARY MATERIALS

Supplementary Figures



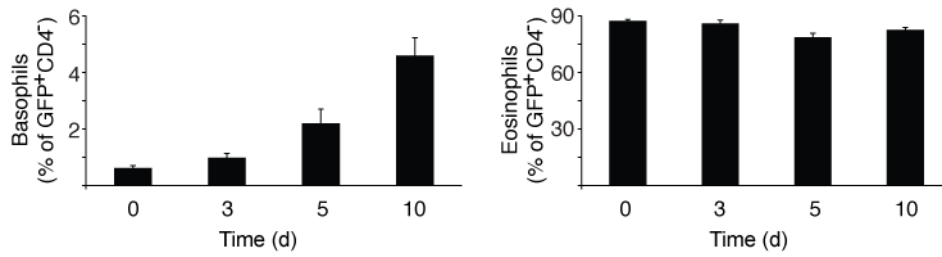
Supplementary Figure 1 IL-4-producing basophils and CD4⁺ T cells over the course of *N. brasiliensis* infection.

Graphs represent the percent of cytokine producing basophils and IL-4-competent (GFP⁺) CD4⁺ T cells, as assessed by surface hCD2 expression, from the lungs of *N. brasiliensis*-infected KN2 x 4get mice. Lung tissues were harvested at the indicated timepoints. Data is presented as the mean $\pm$ SEM, compiled from 7 independent experiments with a total of 10-15 mice per timepoint.



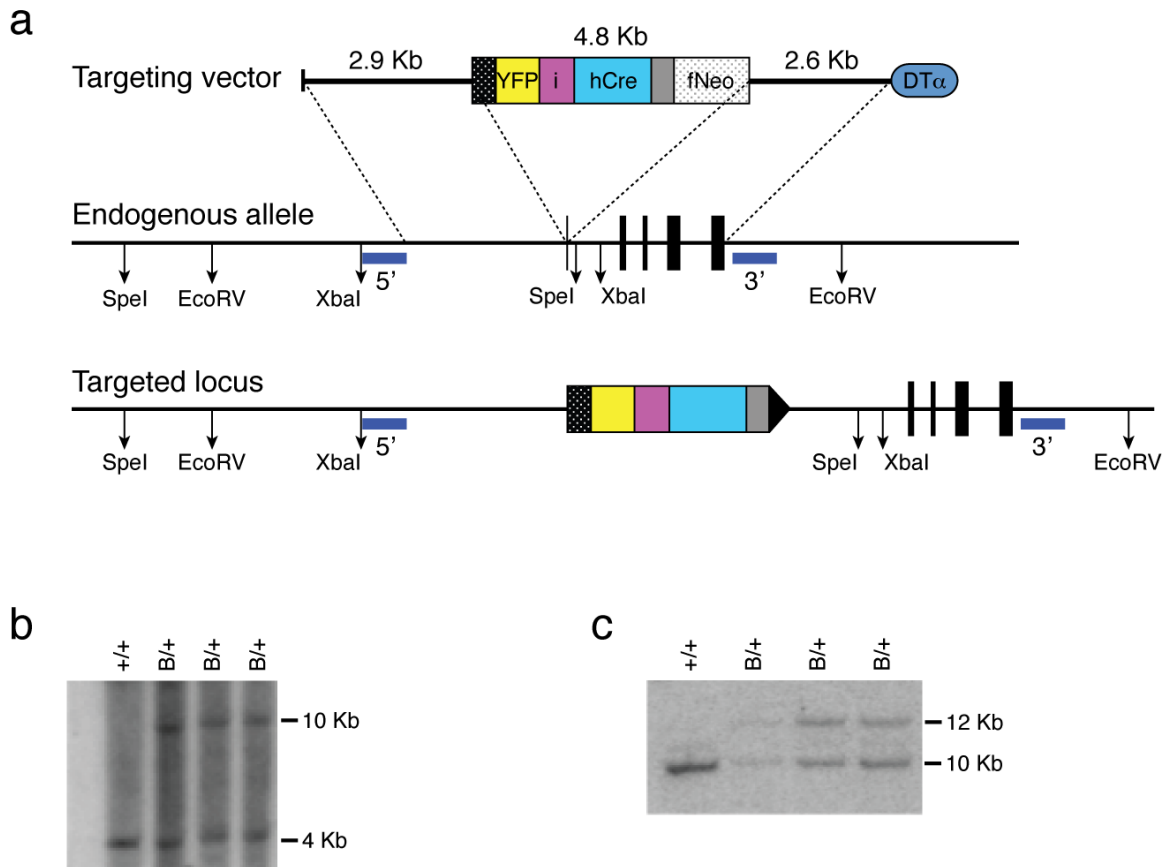
Supplementary Figure 2 Basophils, unlike CD4⁺ T cells, are not activated to secrete IL-4 in the spleen.

FACS analysis of IL-4 producing basophils and GFP⁺ CD4⁺ T cells from the spleens of *N. brasiliensis*-infected KN2 x 4get mice. Plots are representative of 3-5 independent experiments.



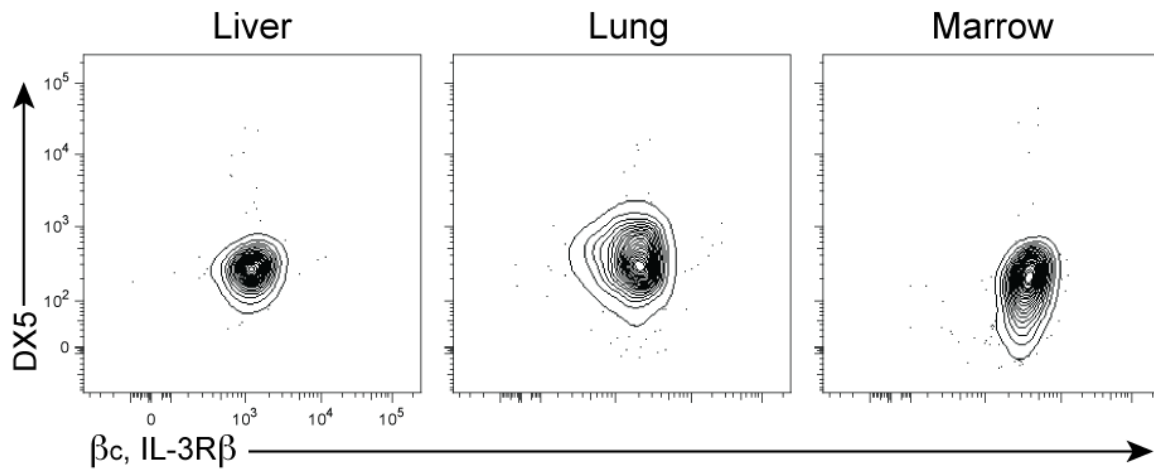
Supplementary Figure 3 Basophils are recruited to the small intestine during *N. brasiliensis* infection.

Basophils and eosinophils were isolated from the lamina propria of the small intestine from *N. brasiliensis*-infected KN2 x 4get mice. Data is presented as a percentage of total innate GFP⁺, CD4⁺ cells. Graphs represent the mean $\pm$ SEM, compiled from 4 independent experiments with a minimum of 7 total mice per timepoint.



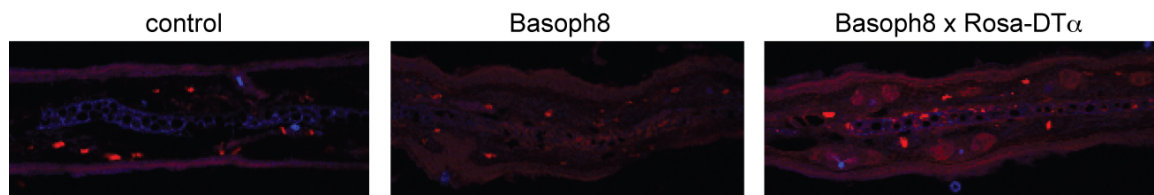
Supplementary Figure 4 Detailed targeting strategy used to generate the basophil reporter strain, Basoph8.

(a) The 4.8 Kb composite YFP reporter/hCre recombinase/floxed-Neo^r (fNeo) cassette is flanked by two homologous arms of the endogenous *Mcpt8* gene (2.9 Kb and 2.6 Kb respectively). Shown are restriction maps and exons (solid vertical bars) of the *mcpt8* locus before and after homologous recombination event in ES cells. External probes (both 5' and 3') used in genomic Southern are also depicted. (b and c) After selection against neomycin in culture, correctly targeted ES clones carrying one Basoph8 allele (B/+) and parental wild-type cells (+/+) were identified by Southern blot analyses using two restriction enzyme and external probe combinations. Genomic DNA was digested with either XbaI then probed with a 5' probe in b, or EcoRV and 3' probe in c. The wild-type allele yields a 4.4 Kb XbaI product and a 10 Kb EcoRV fragment which differs from the Basoph8 allele (9.2 Kb XbaI and 14 Kb EcoRV fragment).

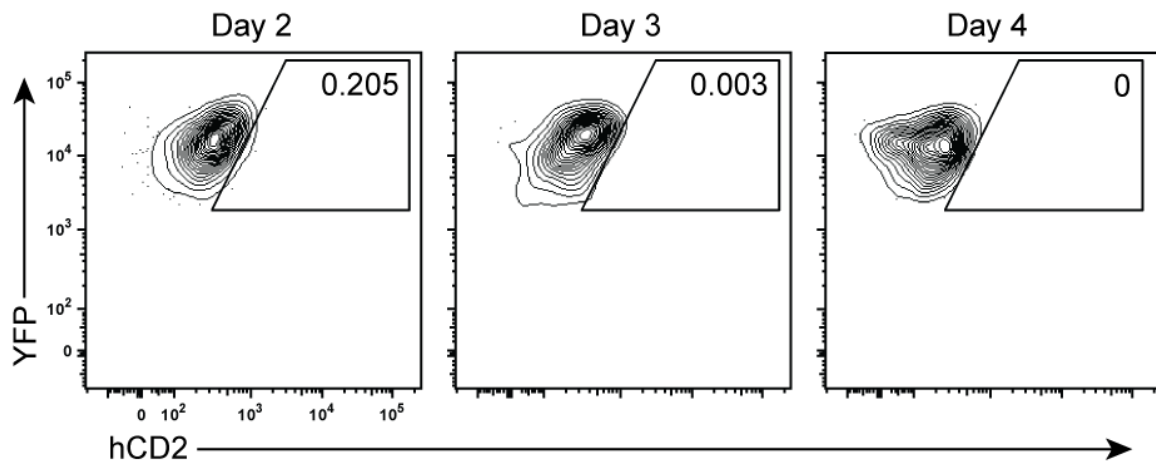


Supplementary Figure 5 Basophils exhibit variegated CD49b (DX5) expression in the bone marrow.

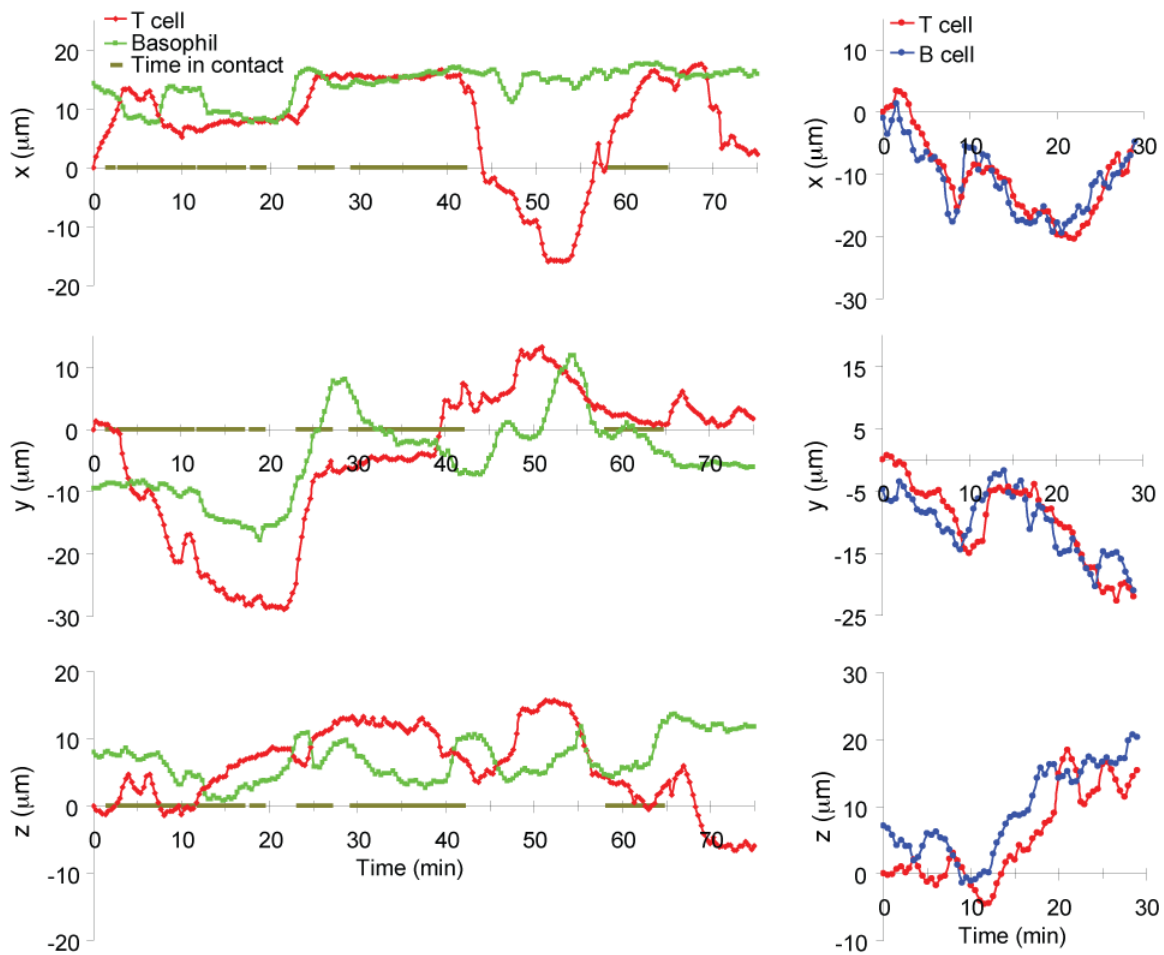
Flow cytometric analysis of CD49b (DX5) and β_c (CD131), IL3Rβ expression on total YFP⁺ basophils from *N. brasiliensis*-infected Basoph8 mice. Tissues were harvested from day 6 infected mice. Plots are representative of 3 individual experiments with 2-3 mice per experiment.



Supplementary Figure 6 Skin resident mast cell populations are intact in basophil deficient Basoph8 x Rosa-DT α mice. Ear samples were excised from wildtype, Basoph8 and Basoph8 x Rosa-DT α mice and sectioned (6 μ m). Naphthol AS-D chloroacetate staining revealed skin resident mast cells (red).

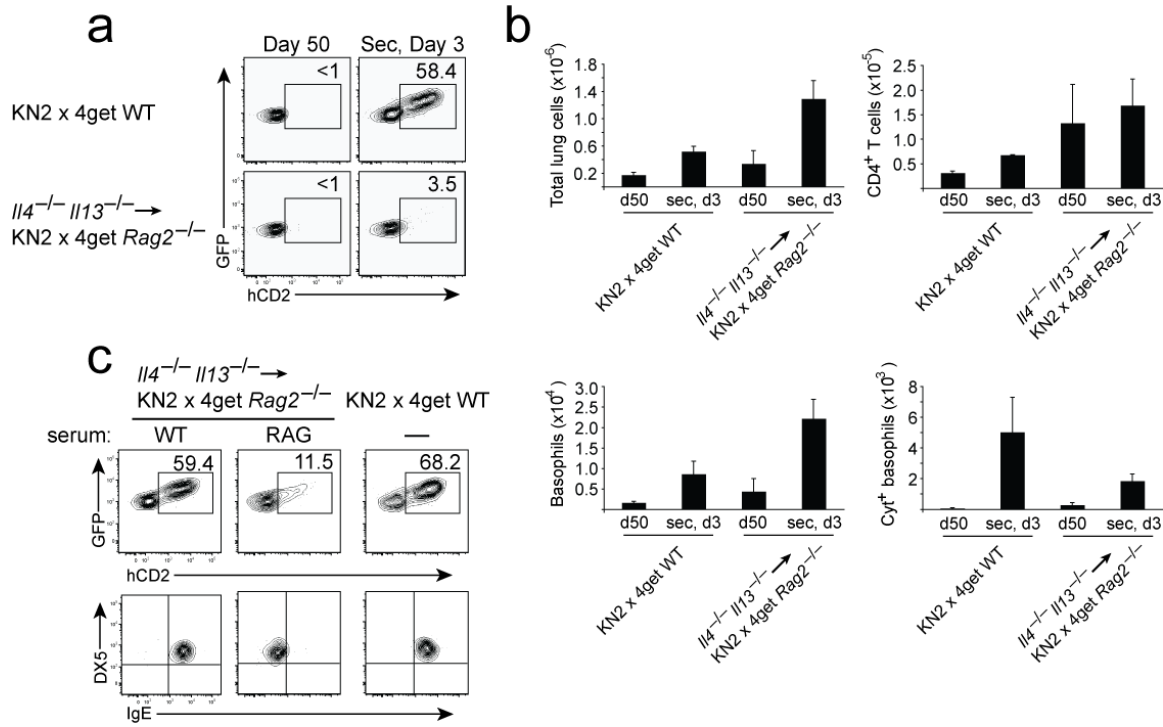


Supplementary Figure 7 Basophils do not express IL-4 in the draining lymph node. Plots represent hCD2 expression by basophils from the draining popliteal node of Basoph8 x KN2 mice immunized with *S. mansoni* eggs. The plots are representative of data presented in **Fig. 4b, c, and d**.



Supplementary Figure 8 CD4⁺ T cells closely follow the movements of B cells but not basophils.

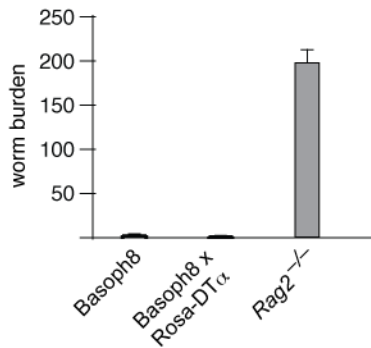
Cell centroids are tracked over time for movements in *xyz* compared to a reference of 0,0,0 as the starting position of the T cell. The left panels show a T cell in contact with a basophil in the lung (corresponding to **Fig. 5a,d** and **Supplementary Video 4**), whereas the right panels show a T cell in contact with a B cell in a lymph node (corresponding to **Fig. 5a,d**). On the left panels, the T cell and basophil were only in contact during portions of the time-lapse recording; periods in which the cells were touching are shown by a brown line on the time scale. The T cell and B cell tracked on the right panel were in contact throughout the entire 30-minute time-lapse recording.



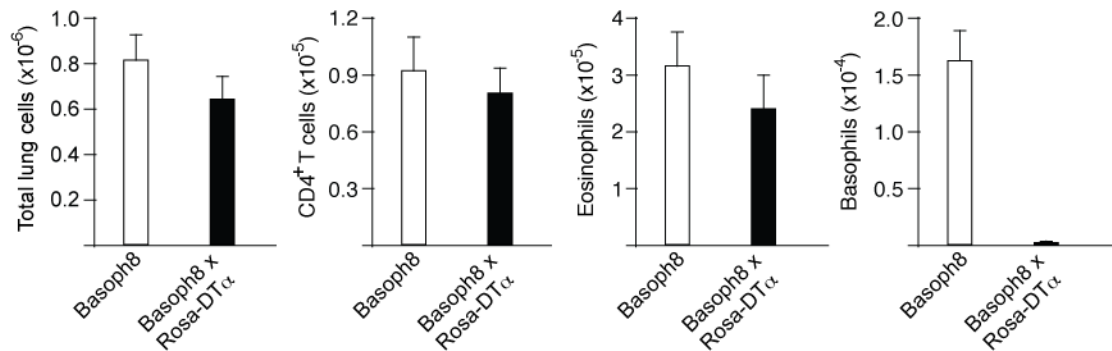
Supplementary Figure 9 Secondary *N. brasiliensis* infection results in Ig-dependent IL-4 production by basophils.

(a) IL-4 expression by lung basophils on day 3 following a secondary *N. brasiliensis* infection. (b) Total cell numbers from infected lungs in a, harvested at the indicated timepoints. Results represent the mean $\pm$ SEM of 4-5 mice per group compiled from two independent experiments. (c) Basophil IL-4 production in the lung at day 3 after secondary *N. brasiliensis* infection. KN2 x 4get x $Rag2^{-/-}$ reconstituted with $Il4^{-/-} Il13^{-/-}$ CD4⁺ T cells received 2 injections of serum from either *N. brasiliensis*-infected wildtype or $Rag2^{-/-}$ mice. Surface bound IgE on basophils was used to assess the efficiency of serum transfers and immunoglobulin loading. Data are representative of 2 independent experiments with 2-3 mice per group.

a

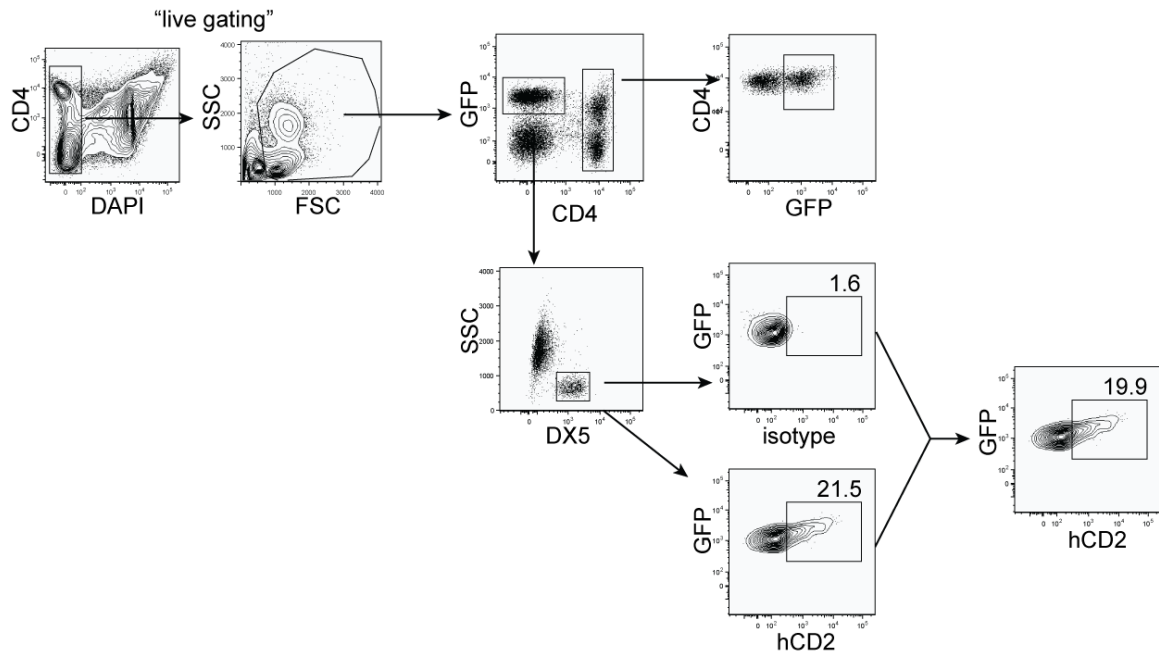


b



Supplementary Figure 10 Intact response against secondary *N. brasiliensis* infection in basophil deficient Basoph8 x Rosa-DT α mice.

(a) Parasite burden in the small intestine 5 days after secondary *N. brasiliensis* infection. Basophil reporter, Basoph8, and basophil deficient, Basoph8 x Rosa-DT α , mice were re-infected 30-40 days after the initial *N. brasiliensis* infection. Worm burden in Rag2^{-/-} mice at day 5 after a primary infection was used as a control. (b) Analysis of lung inflammation in secondary-infected mice. Total leukocytes, eosinophils, basophils, and CD4⁺ T cells were assessed by FACS analysis. Graphs represent the mean $\pm$ SEM from 2 independent experiments, a total 8 mice per group.



Supplementary Figure 11 Gating strategy for isolating and assessing IL-4 production by eosinophils, basophils and CD4⁺ T cells from *N. brasiliensis* infected KN2 x 4get mice.

Live cells were gated based on DAPI-negative staining and FSC/SSC characteristics. Eosinophils were identified as GFP⁺, CD4⁻, DX5⁻, SSC^{hi} cells. Basophils were identified as GFP⁺, CD4⁻, DX5⁺ (or β_c (CD131), IL3R β in some experiments), SSC^{lo} cells. Human CD2 expression on CD4⁺ T cells is presented for the GFP⁺ subpopulation to normalize with constitutive GFP⁺ eosinophils and basophils. Cytokine-positive populations are presented as the percent hCD2⁺ minus isotype control staining for each subset.

Supplementary Methods

Gut preparation

The small intestines from *N. brasiliensis*-infected mice were collected, flushed with PBS and Hanks' balanced salt solution (HBSS), cut longitudinally and incubated for 13 min in Sputolysin/HBSS solution (Caldon Biotech) at room temperature. The tissue was cut into 5 mm pieces, washed and filtered in HBSS before digestion. Tissue fragments were placed in 20 ml collagenase VIII solution (200 U/ml collagenase VIII, 5 U/ml Dnase I in RPMI/10% FCS) and incubated at 37°C with gentle stirring. After 35 min, digested material was filtered and the remaining fragments were subjected to additional collagenase digestion. After 5 rounds of tissue digestion, all of the samples were pooled, overlayed onto a 40/90% Percoll gradient and centrifuged at 2200 RPM for 20 min prior to analysis.

Mast cell isolation and naphthol chloroacetate AS-D staining

Ear tissue from euthanized mice was split into dorsal and ventral halves, and then minced. Tissue was resuspended in PBS with Liberase (2U/ml), CI or TL, (Roche) and incubated at 37°C for 35 minutes on an orbital shaker. Collagenase activity was quenched with PBS supplemented with 2% fetal calf serum (FCS) and the cells were analyzed by flow cytometry. Naphthol AS-D chloroacetate staining (Sigma, St. Louis, MO) was performed per manufacturer's instructions on paraformaldehyde-fixed 6 µm sections.

Serum transfer

Serum was collected from *N. brasiliensis*-infected BALB/c and *Rag2*^{-/-} mice between days 10-20 post-infection. KN2 x 4get *Rag2*^{-/-} mice, reconstituted with *Il4*^{-/-} *Il13*^{-/-} *CD4*⁺ T cells, were infected with *N. brasiliensis*. Mice were re-infected 30 days after the primary infection and collected serum was administered intravenously (300 µl), one day prior and two days following secondary infection. Basophil IL-4 production was assessed by hCD2 expression 3 days after secondary infection.

Enzyme-linked immunosorbent assay

Serum IgE concentrations were quantified as described¹. Cytokines from culture supernatants were detected using the following combinations of capture and biotinylated detection antibodies, respectively: IL-3 (MP28F8 and BAF403, R&D Systems); IL-4 (11B11 and BVD6-24G2, Pharmingen); IL-5 (TRFK5 and TRFK4, Pharmingen); IL-13 (38213 and BAF413, R&D Systems).

Cell purification and transfer for imaging

Spleens and/or the inguinal, axillary, brachial and facial lymph nodes were collected from donor mice and cells were dissociated by mechanical disruption on 40 µm cell strainers (BD Falcon) in ice-cold DMEM medium (Invitrogen) containing 4.5 g/L of glucose, 2% fetal bovine serum (FBS, Invitrogen), antibiotics (50 IU/mL of penicillin and 50 µg/mL of streptomycin; Invitrogen), 2 mM L-glutamine (Invitrogen) and 10 mM HEPES (Invitrogen). Cells were centrifuged at 350g at 4°C for seven minutes. 10 µl of DNase I (10 mg/mL, Roche) were added to each cell pellet and cells were gently resuspended in 0.5-1.0 mL of the DMEM media described above for B cell purification, or in D-PBS

without calcium and magnesium (Invitrogen), containing 0.1% BSA (Roche), 2mM EDTA (Invitrogen) for T cell purification. Cells were counted on a Coulter Counter Z2.

For B cell purification, cells were labeled for 25-60 minutes with 10 μ L of biotinylated rat anti-mouse CD43 (clone S7, BD Biosciences) and 10 μ L of biotinylated hamster anti-mouse CD11c (clone N418, Biolegend) per 10^8 cells. Cells were then washed with a 10-fold volume of room-temperature ACK Lysing Buffer (Quality Biological) to lyse erythrocytes, centrifuged and resuspended as described above, and then transferred to 2 mL microcentrifuge tubes. Dynabeads MyOne Streptavidin T1 were washed once in the same media and then 250 μ L were added per 10^8 cells and the tubes were placed on a rotator/agitator for 30 minutes at 4°C. Tubes were placed in a DynaMag-2 magnet (Invitrogen) for two minutes and the negative fraction was collected, centrifuged at 350g at room temperature for five minutes, resuspended in PBS and counted. B cell purity was typically greater than 98%.

CD4⁺ T cells were purified with the Dynabeads FlowComp Mouse CD4 kit (Invitrogen) according to the manufacturer's instructions, with the following modifications: 1) prior to adding the CD4 antibody, the cells were incubated at 4°C for 5 minutes with 5 μ g/ml rat anti-CD16/32 (TruStain fcX, clone 93, Biolegend) and 112 μ g/ml rat gamma globulin (Jackson ImmunoResearch) to minimize Fc-mediated binding, and 2) $\frac{1}{2}$ the recommended amount of antibody, beads and release buffer were utilized. CD4⁺ T cell purity was typically greater than 95%.

The frequency of HEL-binding B cells was determined by flow cytometry as described². The frequency of TCR-transgenic T cells was determined by flow cytometry² with the following antibodies: rat anti-mouse V α 2 TCR (clone B20.1, PerCP-Cy5.5,

Biolegend), mouse anti-mouse V β 5.1,5.2 TCR (clone MR9-4, FITC, BD Biosciences), rat anti-mouse CD4 (clone GK1.5, APC-H7, BD Biosciences) and hamster anti-mouse CD3 (clone 145-2C11, Alexa 647, Biolegend) or rat anti-mouse CD3 (clone 17A2, Pacific Blue, Biolegend). Cells were analyzed on an LSRII (Becton Dickinson) and data were analyzed using FlowJo software (Treestar, Inc.).

Cells were adoptively transferred into the retro-orbital plexus of anesthetized Basoph8 mice 12-24 hours prior to immunization. Variable numbers of OT-II CD4⁺ T cells (5,000-300,000) and Hy10 B cells (10,000-100,000) were transferred, as high numbers were unexpectedly found to inhibit basophil recruitment to draining lymph nodes, whereas too low numbers were difficult to visualize at early timepoints. OT-II (subline 426-6) human CD2-DsRed mice were typically used as donors; in this subline on average about 75% of CD4⁺ T cells expressed the transgenic V α 2 V β 5 TCR and only these cells expanded upon immunization. However, for imaging at early timepoints in which the cells had not substantially expanded, we transferred cells from OT-II (subline 425-2) *Rag1*^{-/-} mice in which approximately 99% of the CD4⁺ T cells express the transgenic TCR. We also found unexpectedly that the human CD2-DsRed transgene was downregulated specifically around day 2 after immunization, and thus CMTMR labeling² gave brighter fluorescence at this timepoint.

Preparation of lung slices and lymph node explants for imaging

Lung slices were prepared for imaging by a modification of established methods (E. Thornton and M. Krummel, manuscript in preparation)³. Mice were euthanized with an overdose of isoflurane and the vena cava was severed. Lungs were deflated by

perforating the diaphragm and were exposed by a thoracotomy. A 20G x 1” SurFlash catheter (Terumo) was inserted into the trachea and stabilized with a 2/0 suture (Fine Science Tools). Lungs were inflated with a syringe with 0.8-1.2 mL of 2% low-melt agarose (Type VII, Sigma-Aldrich) that had been maintained liquid at 41°C. The large airways were cleared of agarose by flushing an additional 0.2 mL of air. The agarose was solidified by pouring 4°C PBS (UCSF Cell Culture Facility or Invitrogen) over the lungs. The lungs were then excised and placed into a 50 mL conical tube filled with cold PBS. Within 30 minutes, lung lobes were adhered with Vetbond (3M) to specimen mounting blocks (Leica Microsystems) and approximately 600 µm thick sections were cut in a bath of cool (approximately 5-10°C) PBS on a vibratome (Model G, Oxford Laboratories) with stainless steel, PTFE-treated vibratome injector blades (Ted Pella). Lung sections were transferred to a 12-well plate (BD Falcon) and maintained in PBS at room temperature until imaging.

Intact lymph nodes were explanted, cleaned to remove excess fat under a Stereo Discovery V8 microscope (Carl Zeiss MicroImaging), and mounted with a thin layer of Vetbond (3M) on plastic unbreakable coverslips (Fisher Scientific). Until imaging, lymph nodes were kept at room temperature in microcentrifuge tubes filled with RPMI media with only L-glutamine added.

Samples were imaged in a JG-23W/HP Ultra Quiet Imaging Chamber (Warner Instruments) attached to a PM-1 chamber platform and Series 20 stage adapter. RPMI (Invitrogen) with only L-glutamine added was bubbled with 95% O₂/5% CO₂ in a 39°C water bath and then pumped with a mini-variable flow pump (VWR International) at a rate of approximately 1.5 mL per minute, through an in-line heater (Warner Instruments),

into the imaging chamber. The temperature of the sample was carefully maintained at 35-37°C with a dual-channel temperature controller (Warner Instruments). In order to secure the lymph node explants to the bottom of the imaging chamber, a small amount of silicone vacuum grease (Beckman) was applied to the bottom of the plastic coverslip onto which the lymph node had been glued. Lung slices were secured with a stainless steel slice hold-down anchor with 1.5 mm thread spacing (Warner Instruments).

Two-photon microscopy and data analysis

As the Basoph8 YFP fluorescence was near the threshold for detection on a standard photomultiplier tube-based non-descanned detector (NDD), we collected YFP fluorescence in a more sensitive GaAsP detector (Carl Zeiss MicroImaging) situated directly above the objective. The GaAsP was custom outfitted with a dichroic that passed through orange-red wavelengths to the standard NDD housing, where DsRed or CMTMR were collected through an ET605/70 emission filter (Chroma). CFP, which emits at both blue and green wavelengths, could also be detected with the GaAsP. In order to spectrally separate CFP from YFP, we took advantage of the rapid tuning capability of the Chameleon laser to change excitation wavelengths in an approach similar to the “multi-tracking” technique in confocal microscopy. An excitation of 1015 nm was observed to give optimal YFP and DsRed/CMTMR signal, yet did not excite CFP. In contrast, excitation wavelengths from 850-910 nm excited CFP and gave diminished YFP fluorescence. 890 nm was found to give the brightest CFP signal, whereas 850 nm gave the least YFP excitation. The Multi-Time module in ZEN 2009 was set up to collect each z-stack at one wavelength, then to switch wavelengths and adjust beam intensity, and

finally repeat the z -stack collection at the second wavelength. The overall process was repeated every 30 s and the images from each wavelength collection, shifted by approximately 10-15 seconds, were combined. The red channel at 850 nm or 890 nm exhibited higher autofluorescence and low DsRed signal compared to 1015 nm, and thus this channel was collected as well to assist with semi-automated tracking as described below. Thus, we generated four channel images from two detectors with little spectral overlap by alternating laser excitation wavelengths.

Contact times were determined by a semi-automated measurement task in Volocity 5.2.1 or 5.4.2 (PerkinElmer). Cells in each channel were identified as objects by SD intensity and size thresholds. Noise was removed from the objects and touching objects were separated as needed. In some cases, we reduced the contribution of objects identified from autofluorescent material by subtracting the objects identified in one channel from another channel, as autofluorescent cells typically fluoresced in multiple channels whereas the cells of interest primarily fluoresced in a single channel. In order to identify cells in contact, a set of objects from one channel was subjected to one round of dilation, and then we selected objects in the other channel that were touching these objects. This procedure resulted in the identification of objects in one channel that were within one voxel of objects in the other channel, which were then tracked over time. Each track was manually visualized in 3-D and verified, and tracks were joined if cells remained in contact but there were gaps in automated tracking of the conjugate. The track times were then compiled to make contact time scatter graphs in GraphPad Prism and histograms in Microsoft Excel 2003. Centroid distances between the objects were

calculated in Microsoft Excel 2003 with a custom macro written in Microsoft Visual Basic for Applications and were plotted in GraphPad Prism.

Supplementary References

1. Voehringer, D., Shinkai, K. & Locksley, R.M. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* **20**, 267-277 (2004).
2. Allen, C.D., Okada, T., Tang, H.L. & Cyster, J.G. Imaging of germinal center selection events during affinity maturation. *Science* **315**, 528-531 (2007).
3. Delmotte, P. & Sanderson, M.J. Ciliary beat frequency is maintained at a maximal rate in the small airways of mouse lung slices. *Am J Respir Cell Mol Biol* **35**, 110-117 (2006).

Supplementary Video Legends

Supplementary Video 1 Basophils and antigen-specific CD4⁺ T cells do not interact in draining lymph nodes after primary immunization with *S. mansoni* eggs and OVA. A time-lapse sequence of 24 µm z-projection images from an inguinal lymph node explant is shown approximately three days after subcutaneous immunization. Elapsed time is indicated as hh:mm:ss.

Supplementary Video 2 Basophils and antigen-specific CD4⁺ T cells do not interact in draining lymph nodes after primary immunization with papain and OVA. A time-lapse sequence of 32 µm z-projection images from an inguinal lymph node explant is shown approximately 2.25 days after subcutaneous immunization. Elapsed time is indicated as hh:mm:ss.

Supplementary Video 3 Antigen-specific CD4⁺ T cells interact with cognate antigen-specific B cells, but not basophils, in draining lymph nodes after primary immunization. A time-lapse sequence of 33 µm z-projection images from an inguinal lymph node explant is shown approximately 3.7 days after subcutaneous immunization with a mixture of papain and DEL-OVA. Elapsed time is indicated as hh:mm:ss.

Supplementary Video 4 Basophils and antigen-specific CD4⁺ T cells engage in multiple serial interactions in the lung after primary *N. brasiliensis* infection. A time-lapse sequence of 29 µm z-projection images from a lung slice is shown approximately 7.5 days after *N. brasiliensis* infection, with intranasal OVA administered on days 1 and 6. Elapsed time is indicated as hh:mm:ss. The video corresponds to the still images shown in **Fig. 5a** and the characteristics of the serial encounters are quantified in **Fig. 5d,e** and **Supplementary Fig. 8**.

Supplementary Video 5 Basophils and polyclonal T cells engage in multiple serial interactions in the lung after primary *N. brasiliensis* infection. A time-lapse sequence of 26 µm z-projection images from a lung slice is shown approximately 7.25 days after *N. brasiliensis* infection of a Basoph8 mouse carrying a hCD2-dsRed transgene. This transgene is highly expressed in T cells and weakly expressed in subsets of dendritic cells, macrophages, NK cells, and other undefined cell types. In this image sequence, a single green basophil and bright red T cell are shown interacting in the center, with a weakly fluorescent macrophage off to the right side. Elapsed time is indicated as hh:mm:ss.