

Th9 Cells Drive Host Immunity against Gastrointestinal Worm Infection

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SUMMARY

Type 2 inflammatory cytokines, including interleukin-4 (IL-4), IL-5, IL-9, and IL-13, drive the characteristic features of immunity against parasitic worms and allergens. Whether IL-9 serves an essential role in the initiation of host-protective responses is controversial, and the importance of IL-9- versus IL-4-producing CD4⁺ effector T cells in type 2 immunity is incompletely defined. Herein, we generated IL-9-deficient and IL-9-fluorescent reporter mice that demonstrated an essential role for this cytokine in the early type 2 immunity against *Nippostrongylus brasiliensis*. Whereas T helper 9 (Th9) cells and type 2 innate lymphoid cells (ILC2s) were major sources of infection-induced IL-9 production, the adoptive transfer of Th9 cells, but not Th2 cells, caused rapid worm expulsion, marked basophilia, and increased mast cell numbers in *Rag2*-deficient hosts. Taken together, our data show a critical and nonredundant role for Th9 cells and IL-9 in host-protective type 2 immunity against parasitic worm infection.

INTRODUCTION

Naive T cell differentiation into the CD4⁺ T helper 2 (Th2) cell subset is considered a central effector mechanism driving adaptive host protection against parasitic helminths and the pathophysiological sequelae of most allergic diseases (Palm et al., 2012). However, the understanding of type 2 inflammation has recently increased in complexity in that it also involves interleukin-9 (IL-9)-producing Th cell subsets (Th9 cells), type 2 innate lymphoid cells (ILC2s), and epithelial-cell-derived cytokines (thymic stromal lymphopoietin [TSLP], IL-25, and IL-33) (Allen and Maizels, 2011; Lloyd and Hessel, 2010; Paul and Zhu, 2010). Although type 2 inflammatory mechanisms function in both humans and mice to promote elevated IL-4, IL-5, IL-9, and IL-13 amounts and eosinophilia, mastocytosis, basophilia, goblet cell metaplasia, and immuno-

globulin E (IgE) production, the pathways responsible for initiating these responses remain controversial (Pulendran and Artis, 2012). Moreover, the functional redundancy among type 2 cytokines and effector mechanisms has generated considerable debate over which pathway, if any, serves an essential and nonredundant function.

IL-9 is a prototypical type 2 cytokine implicated in human and murine asthma, anaphylaxis, resistance to nematode infection, antiviral immunity, and tumorigenesis (Dodd et al., 2009; Fallon et al., 2000; Faulkner et al., 1997; Faulkner et al., 1998; Forbes et al., 2008; Gessner et al., 1993; Knoops et al., 2005; Lu et al., 2012; Osterfeld et al., 2010; Shimbara et al., 2000). IL-9 has been reported to be mainly produced by T cells but can also be produced by mast cells, eosinophils, and ILC2s (Gounni et al., 2000; Hültner et al., 2000; Schmitt et al., 1989; Uyttenhove et al., 1988; Wilhelm et al., 2011). IL-9 stimulates the growth, proliferation, and survival of T cells, enhances the production of IgE from B cells, promotes the proliferation and differentiation of mast cells and hematopoietic progenitors, and induces secretion of mucus and chemoattractant molecules by mucosal epithelial cells (Dong et al., 1999; Gounni et al., 2004; Petit-Frere et al., 1993; Uyttenhove et al., 1988; Van Snick et al., 1989). Recent identification of Th9 cells as a specialized Th cell subset secreting IL-9 in vitro has caused a reevaluation of the natural sources of this cytokine in vivo. Although previously considered to be a Th2-cell-derived cytokine, IL-9 is produced as an effector cytokine by Th9 cells (differentiated via transforming growth factor β [TGF- β] and IL-4), Th17 cells (differentiated with TGF- β and IL-6), and Foxp3⁺ T regulatory (Treg) cells (differentiated with TGF- β alone) (Dardalhon et al., 2008; Elyaman et al., 2009; Lu et al., 2006; Veldhoen et al., 2008). As such, whether Th9 cells serve a distinct biological role that is not shared by other T cell lineages is currently unknown.

Cytokines released from distinct Th cell lineages dramatically influence disease outcome, particularly in the context of parasitic helminth infections, which affect more than one billion people worldwide (Brooker, 2010). Previous studies directed toward elucidating the role of IL-9 in hookworm infection have suggested a dispensable role for IL-9 in promoting worm clearance (Townsend et al., 2000). However, other reports have supported a protective role for IL-9 during *Trichinella spiralis* and *Trichuris*

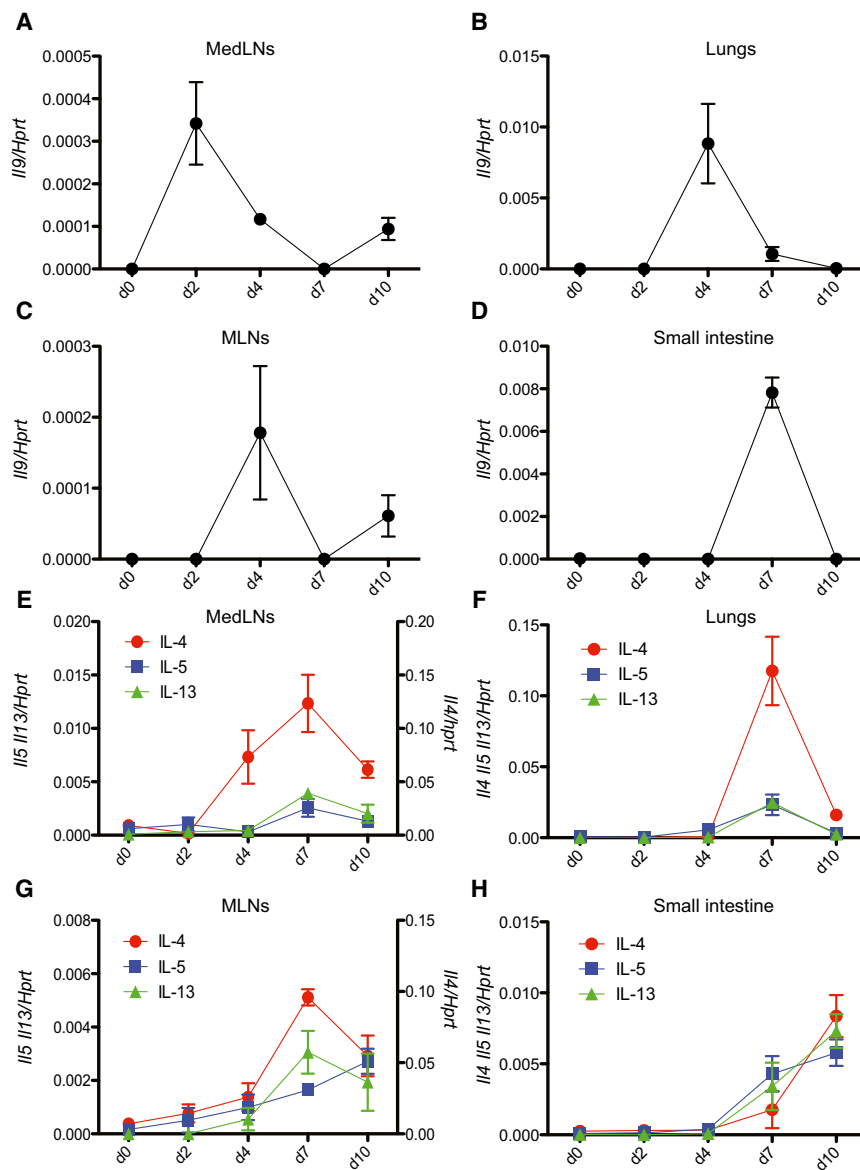


Figure 1. IL-9 Expression Precedes IL-4, IL-5, and IL-13 Expression during *N. brasiliensis* Infection

C57BL/6 mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae. MedLNs (A), lungs (B), MLNs (C), and the small intestine (D) were collected and homogenized at different days p.i. for assessment of *il9* mRNA expression by real-time RT-PCR. MedLNs (E), lungs (F), MLNs (G), and the small intestine (H) were analyzed for *il4*, *il5*, and *il13* mRNA expression. Data represent the mean $\pm$ SEM ratio of cytokine gene expression to *Hprt* expression as determined by the relative quantification method ($\Delta\Delta C_t$). The experiment was performed twice with similar results with two to three mice per day p.i. Statistically significant p values when comparing basal expression (d0) and at least one other time point for each gene were determined by one-way ANOVA.

were markedly more efficient than Th2 cells at driving basophilia, increased mast cell numbers, and rapid worm expulsion. Thus, our data show that IL-9 serves a critical role in the early stages of type 2 immunity and that its production from effector CD4⁺ T cells is alone sufficient for host protection against worm infection.

RESULTS

IL-9 Expression Precedes IL-4, IL-5, and IL-13 Expression In Vivo

Rapid induction of type 2 cytokines is critical for host immunity against most parasitic helminthes, but whether distinct cytokines are responsible for orchestrating host protection remains unclear. To gain a better understanding of type 2 cytokine regulation in vivo, we investigated the spatiotemporal expression patterns of IL-4, IL-5, IL-9, and IL-13 during *N. brasiliensis* infection. Upon infection, we found a strong induction of all type 2 cytokines, which are otherwise low or undetectable at steady state. As expected, the induction of these cytokines correlated with the presence of the parasite in the different target organs and associated lymphoid tissues; it peaked first in the lungs and later in the small intestine (Figure 1). Comparison of mRNA expression in wild-type (C57BL/6)-infected mice showed that IL-9 was the first among these cytokines induced by *N. brasiliensis* infection. IL-9 expression in mediastinal lymph nodes (medLNs) was detected as early as day 2 postinfection (p.i.) (Figure 1A) and peaked later around day 4 in the lungs (Figure 1B). In the mesenteric lymph nodes (MLNs), we observed the highest expression at day 4 p.i. (Figure 1C) and a subsequent strong increase by day 7 in the small intestine (Figure 1D). IL-9 expression was strictly transient, suggesting a very tight control in the expression

muris infections (Faulkner et al., 1997; Faulkner et al., 1998; Richard et al., 2000; Veldhoen et al., 2008). Thus, contradictory evidence has made it difficult to conclude the real contribution of IL-9 in the control of type 2 immune responses. In addition, definitive evidence identifying the cellular sources of IL-9 in infection models in vivo is still missing.

In this report, we investigated whether IL-9 is necessary and/or sufficient for host-protective type 2 immunity in vivo. In both mucosal tissues and secondary lymphoid organs, *Nippostrongylus brasiliensis* infection induced IL-9 expression, which preceded IL-4, IL-5, and IL-13 expression at these sites. IL-9 deficiency abrogated canonical type 2 cytokine production, basophilia, eosinophilia, mast cell amplification, and worm expulsion. Furthermore, we produced a strain of IL-9-fluorescent reporter mice and demonstrated that both CD4⁺ T cells and ILC2s were major sources of IL-9 secretion upon infection. When adoptively transferred into *Rag2*-deficient hosts, Th9 cells

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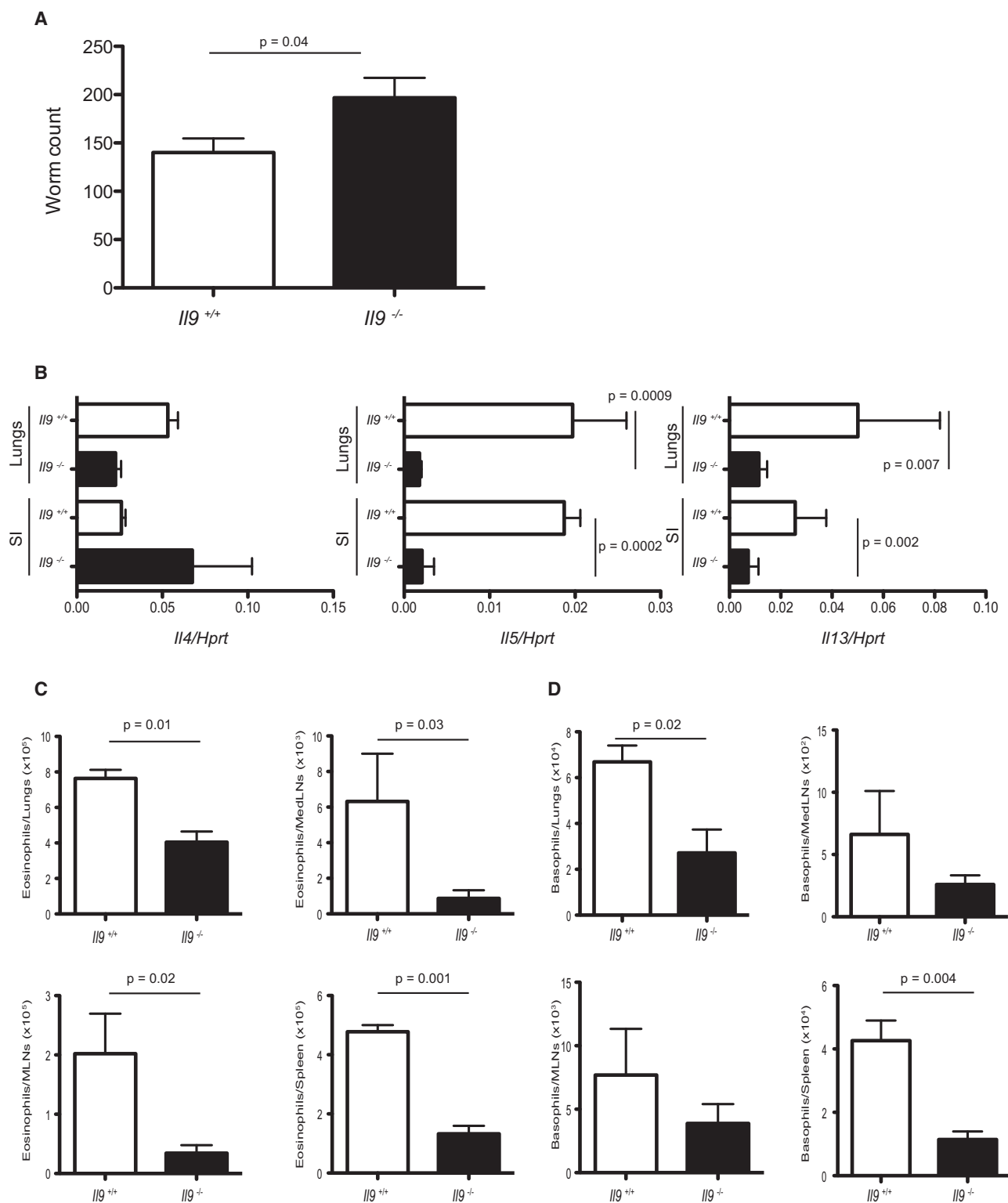


Figure 2. Impaired Worm Expulsion in the Absence of IL-9

(A) Infected wild-type *Il9*^{+/+} (white bars) and null *Il9*^{-/-} (black bars) mice were sacrificed at day 7 p.i. for obtaining intestinal worm counts. Data represent the mean $\pm$ SEM of six mice per group.

(B) At day 7 p.i., the lungs and small intestine (SI) of *Il9*^{+/+} (white bars) or *Il9*^{-/-} (black bars) infected mice were harvested and homogenized for assessment of *Il4*, *Il5*, and *Il13* mRNA expression. Data represent the mean $\pm$ SEM of cytokine gene expression to *Hprt* expression as determined by the $\Delta\Delta Ct$ method. Similar results were obtained in two independent experiments with six mice per group.

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of this cytokine in vivo, and was detected earlier than IL-4, IL-5, and IL-13 expression in all tissues examined (Figures 1E–1H). In the spleen, IL-9 expression also preceded that of IL-4, IL-5, and IL-13 (Figure S1, available online). Thus, IL-9 is expressed early upon helminth infection and could potentially act upstream of other type 2 cytokines in vivo.

IL-9 Is Necessary for IL-5 and IL-13 Induction, Eosinophilia, Basophilia, and Worm Clearance In Vivo

To investigate the role of IL-9 during the spontaneous development of type 2 immunity against *N. brasiliensis*, we generated IL-9-deficient mice on a BALB/c background through targeted deletion of exons 1–4. The targeting vector contained a neomycin (neo)-resistance cassette flanked by homology regions upstream of exon 1 and downstream of exon 4 of *Il9*, encoding IL-9 (Figure S2A). Genotyping of wild-type (+/+), heterozygous (+/-), and homozygous null (-/-) mice by PCR is shown in Figure S2B. IL-9-deficient mice were viable and fertile and did not show any gross phenotypic abnormalities. Analysis of the IL-9-deficient mice confirmed that *Il9* mRNA and IL-9 protein expression were undetectable in supernatants of sorted naive CD4⁺ T cells polarized under Th9-cell-culture conditions in vitro (Figure S2C and data not shown). During *N. brasiliensis* infection, IL-9 deficiency had a negative impact on worm clearance, shown by a significant increase in worm burden in *Il9*^{-/-} mice compared to wild-type mice at day 7 p.i. (Figure 2A). Because we found that IL-9 was expressed in the early phase of *N. brasiliensis* infection, we sought to determine whether other type 2 cytokines were affected in its absence. To this end, we analyzed type 2 cytokine induction in this infectious setting. We confirmed the absence of *Il9* mRNA in IL-9-deficient mice in vivo (Figure S2D). Compared to wild-type mice, IL-9-deficient mice showed severely impaired *Il5* and *Il13* mRNA expression in the lungs and small intestine at day 7 p.i. (Figure 2B). Expression of other type-2-related genes such as *Gata3*, *Il10*, and *Il9r* was not affected by IL-9 deletion (data not shown). Infection of IL-9-deficient mice with *N. brasiliensis* also resulted in a significant reduction in the cellularity of the spleen and medLNs at day 7 p.i. In contrast, total lymphoid infiltrates in the MLNs and lungs were not affected (Figure S2E). A detailed phenotypic analysis of the infiltrate present in the target organs of *Il9*^{-/-} mice and wild-type mice infected with *N. brasiliensis* showed a significant decrease in the absolute numbers of eosinophils (CD11b⁺Siglec F⁺) in all organs analyzed (Figure 2C). Unexpectedly, we also found a significant decrease in basophilia (CD49b⁺FcεR⁺c-Kit⁻) in the spleen and lungs of *Il9*^{-/-} mice (Figure 2D). Analysis of other cell subsets, including neutrophils (CD11b⁺Ly6G⁺) and ILC2s (lin⁻CD45⁺Thy1.2⁺Sca1⁺c-Kit⁺CD44⁺), showed similar numbers in affected tissues (Figures S2F and S2G and data not shown). Consistent with previous findings reporting a role for IL-9 in mast cell degranulation and goblet cell hyperplasia (Townsend et al., 2000), mast cell metalloproteinase 1 (Mcp1) secretion in serum and *mucin5b* mRNA expression in the small

intestine were reduced or absent in *Il9*^{-/-} infected mice (Figures S2H and S2I). Mast cell numbers were also significantly decreased in the lungs, MLNs, and spleen of *Il9*^{-/-} mice (Figure S2J). All together, our data support an important role for IL-9 in the potentiation and orchestration of anti-helminth responses in vivo.

Tracking of IL-9-Expressing Cells In Vivo with IL-9-Fluorescent Reporter Mice

To identify the cellular sources of IL-9 in vivo, we generated IL-9 reporter knockin mice designated INFER (Interleukin Nine Fluorescent Reporter) mice. Unlike the design in previous reports using fate reporter transgenic mice (Wilhelm et al., 2011), our design allows tracking of IL-9-expressing cells in real time. The targeting design consisted of the insertion of an internal ribosomal entry site (IRES) followed by the coding sequence for GFP (IRES-GFP) downstream of the stop codon of *Il9* (Figure 3A), thus allowing for reporter expression induced by the endogenous IL-9 promoter. The resulting mice were viable and fertile and showed no gross phenotypic defects.

Initial characterization of the INFER mice demonstrated that insertion of the IRES-GFP cassette into the *Il9* locus did not disrupt the endogenous expression of the gene or the protein. Kinetic analysis of *Il9* mRNA expression in vitro demonstrated that IL-9 peaked at day 3 and decreased by day 5 in sorted naive CD4⁺ T cells differentiated in Th9 cell cultures from INFER or wild-type mice. Measurement of IL-9 in the supernatants of these cultures by ELISA also demonstrated equivalent protein expression by day 5 (Figures S3A and S3B). We consistently observed a 24 hr delay between the peak of *Il9* mRNA expression and IL-9 protein secretion measured in supernatants of Th9 cell cultures in vitro. To validate IL-9 production and GFP expression, we sorted naive CD4⁺ T cells isolated from INFER mice and cultured them in vitro for 5 days in polarizing conditions suited to generate different effector Th cell subpopulations. Notably, GFP expression controlled by the endogenous IL-9 promoter occurred mostly under Th9-cell-polarizing conditions and only a low frequency of cells in Th2 and Th17 cell conditions (Figure 3B). We confirmed by ELISA the specific induction of IL-9 in Th9-cell-polarizing conditions and the respective cytokines in the different cultures to validate the corresponding induction of interferon-γ (IFN-γ), IL-4, and IL-17A in Th1, Th2, and Th17 cells, respectively (Figure 3C and data not shown). To further demonstrate that GFP expression in Th9 cell cultures of INFER naive CD4⁺ T cells corresponded to IL-9-producing cells, we sorted GFP⁺ and GFP⁻ cells from day 5 cultures and analyzed *Il9* mRNA and IL-9 cytokine expression. Both *Il9* mRNA and IL-9 protein levels were significantly higher in GFP⁺ cells than in GFP⁻ cells (Figure 3D and 3E). Moreover, intracellular staining performed in Th9 cell cultures with INFER naive T cells in vitro demonstrated that 80% of the cells expressing IL-9 also expressed GFP (Figure S3C). Thus, we generated a tool

(C) Total numbers of eosinophils (CD11b⁺Siglec F⁺) as determined by flow cytometry in the lungs, medLNs, MLNs, and spleen of *Il9*^{+/+} (white bars) and *Il9*^{-/-} (black bars) mice at day 7 p.i. Data represent the mean ± SEM of experiments with six mice per group.

(D) Total numbers of basophils (CD49b⁺FcεR⁺c-Kit⁻) as determined by flow cytometry in the lungs, medLNs, MLNs, and spleen of *Il9*^{+/+} (white bars) and *Il9*^{-/-} (black bars) mice at day 7 p.i. Data represent the mean ± SEM of experiments with six mice per group.

The p values determined by unpaired two-tailed Student's t test between groups are shown.

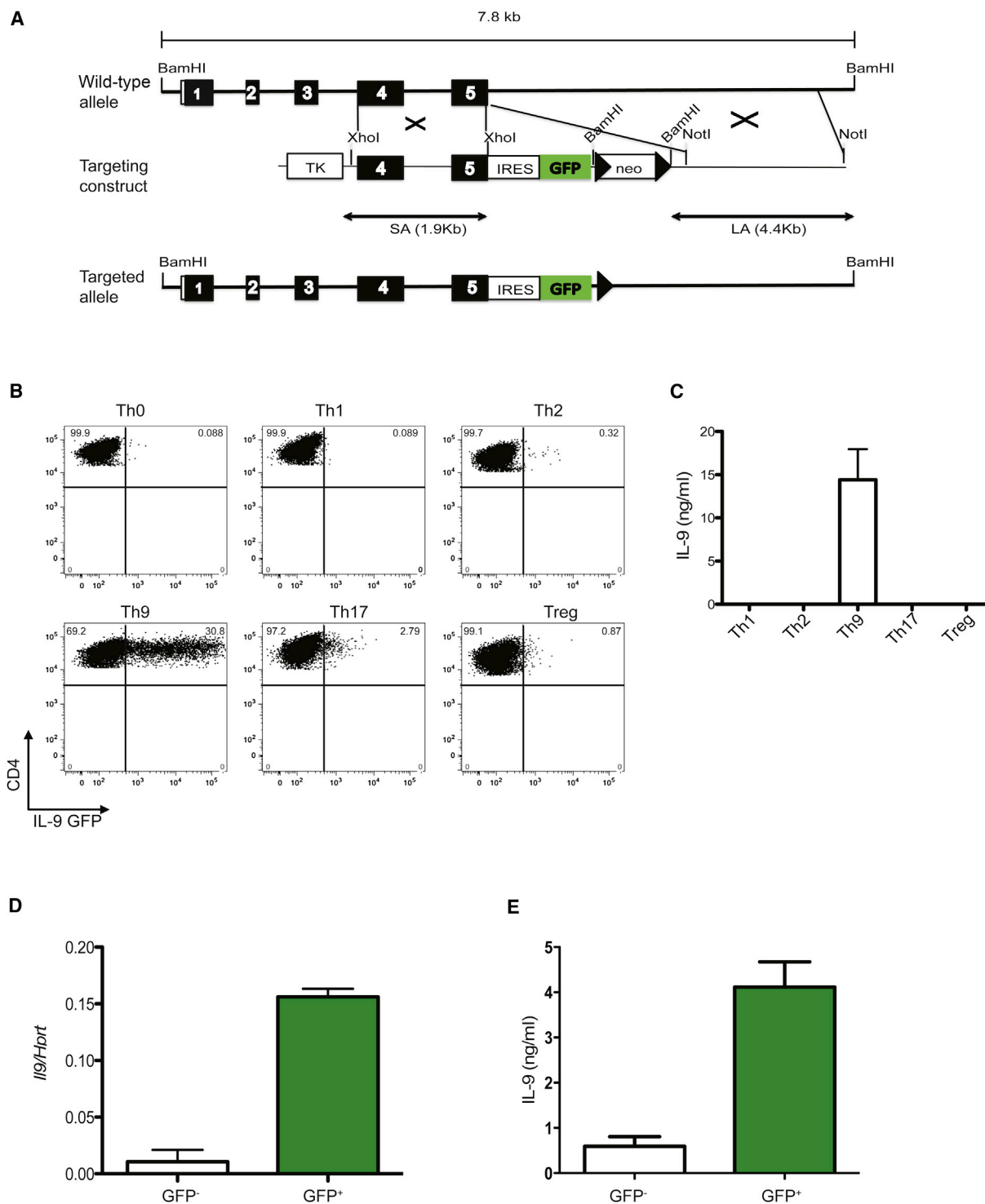


Figure 3. Generation of INFER Mice and Expression Analysis In Vitro

(A) A 7.8 kb genomic fragment of the *I9* locus was used for creating the targeting construct (middle), in which an IRES-GFP cassette followed by a loxP-flanked neo resistance gene was inserted downstream of the stop codon. Deletion of neo by Cre recombinase resulted in the final targeted allele (bottom). Filled boxes represent exons 1–5. loxP sites are shown as arrow heads. Restriction sites are depicted. TK stands for thymidine kinase.

(B) Sorted naive CD4⁺CD62L⁺CD25[−]CD44[−] T cells from INFER mice were cultured for 5 days in Th0-, Th1-, Th2-, Th9-, Th17-, or Treg-cell-polarizing conditions in vitro. Recovered cells were stained for surface CD4, and GFP expression was analyzed by flow cytometry. Numbers indicate the frequencies of CD4⁺GFP⁺ cells. The experiment was performed five times with similar results.

(C) Supernatants from cells isolated from cultures in (B) at day 5 were stimulated in vitro with anti-CD3 for 24 hr and analyzed by ELISA for IL-9 quantification. The mean $\pm$ SEM from one out of three independent experiments measured in triplicate is shown.

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that faithfully recapitulated active IL-9 expression and could be efficiently used for determining the source and function of this cytokine *in vivo*.

IL-9 Is Expressed in CD4⁺ T Cells and ILC2s *In Vivo*

To determine the source of IL-9 during helminth infection, we infected INFER mice with *N. brasiliensis* and analyzed GFP expression at days 0, 3, 5, 7, and 10 p.i. We did not find detectable expression of IL-9 (measured as GFP) under steady-state conditions in the spleen, lymph nodes, thymus, or bone marrow of INFER mice (data not shown). GFP expression was detected as early as day 3 p.i. in the medLNs, day 5 in the lungs, and later by day 7 in the MLNs of infected INFER mice (Figures 4A and 4B and Figures S4A and S4B). A detailed analysis demonstrated that the observed GFP expression corresponded to CD4⁺ T cells and ILC2s. Quantification of GFP⁺ cells in the lungs showed that CD4⁺GFP⁺ T cells increased from day 5 to day 7 p.i. However, there was a significant reduction by day 10 p.i., indicating transient expression of IL-9 in this cellular compartment. In contrast, GFP⁺ ILC2s in the lungs increased by day 5 and were maintained throughout the analysis (Figure 4C). In MLNs, we found a significant increase in CD4⁺ T cells and ILC2s expressing GFP by day 7 p.i.; this increase was further amplified by day 10 (Figure 4D). Importantly, the kinetics of IL-9 GFP expression closely correlated with worm trafficking and expulsion in most immunocompetent mouse strains. IL-9 GFP was not significantly expressed in eosinophils or mast cells during *N. brasiliensis* infection (data not shown). To definitively prove that GFP⁺ cells corresponded to IL-9-expressing cells *in vivo*, we sorted GFP⁺ and GFP⁻ CD4⁺ T cells or ILC2s from the lungs and MLNs at day 10 p.i. GFP⁺CD4⁺ T cells and GFP⁺ ILC2s expressed higher levels of IL-9 than did GFP⁻ cells (Figures S4C and S4D); hence, GFP expression efficiently reported IL-9-expressing cells *in vivo*. Thus, we used INFER mice to demonstrate that during *N. brasiliensis* infection, Th9 cells, together with ILC2s, develop and represent the cellular sources of IL-9 expression. The kinetic of IL-9 expression, as well as the original source, changes over time and depends on the presence of the relevant insult *in vivo*.

IL-9 Derived from CD4⁺ T Cells Is Sufficient to Promote Host Protection against *N. brasiliensis*

Because ILC2s and CD4⁺ T cells produce IL-9 *in vivo*, we sought to determine the contribution of IL-9 produced by CD4⁺ T cells to host protection against *N. brasiliensis*. Thus, we transferred Th9 cells generated *in vitro* into IL-9-deficient hosts in the context of nematode infection. This experimental setting was devoid of IL-9 produced from the innate compartment (i.e., ILC2s). In IL-9-deficient hosts, IL-9 derived from CD4⁺ T cells was sufficient to promote a significant decrease in worm loads, equivalent to wild-type levels (Figure 5A). Moreover, IL-13 expression, which was significantly reduced in the absence of IL-9 (Figure 2B), increased to levels comparable to those of wild-type mice in the small intestine upon reintroduction of

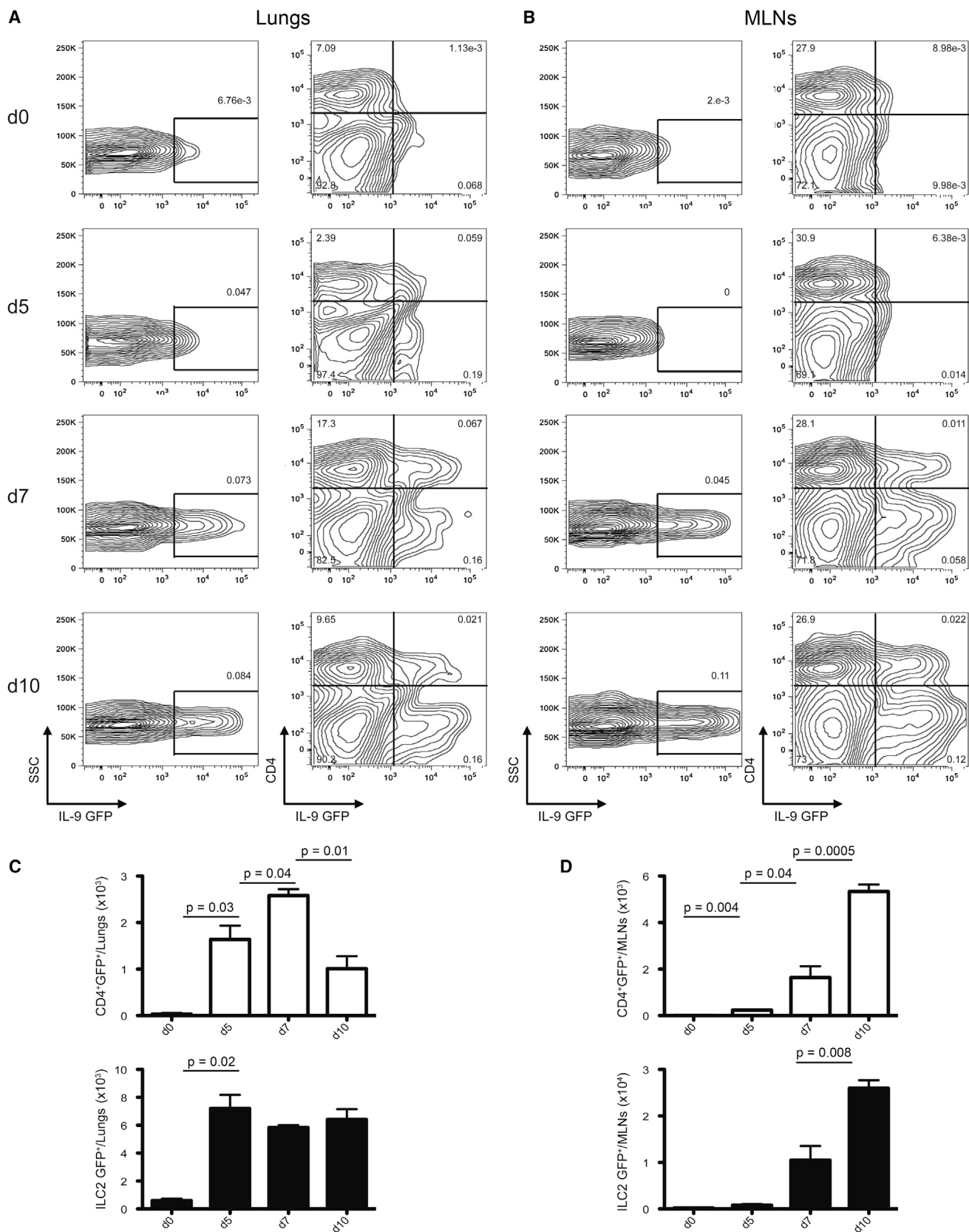
Th9 cells (Figure 5B). Likewise, IL-5 expression was partially restored in the small intestine (Figure 5B). Interestingly, there was no significant change in the levels of *Il13* and *Il5* mRNA between the lungs of *Il9*^{-/-} mice that received Th9 cells and those of *Il9*^{-/-} mice that did not receive Th9 cells (Figure 5B). A partial rescue was also observed in the numbers of eosinophils and basophils in the lungs, MLNs, and spleen of IL-9-deficient mice transferred with Th9 cells (Figures 5C and 5D). Thus, although ILC2-derived IL-9 could play a critical role in the promotion of optimal immune responses against nematodes, IL-9 derived from CD4⁺ T cells is sufficient to promote an effective anti-helminth response *in vivo*.

Th9 Cells Promote Basophilia and Increased Mast Cell Numbers, Leading to a Rapid Helminth Expulsion *In Vivo*

On the basis of our foregoing findings, we hypothesized that Th9 cells represent a protective subset of specialized effector Th cells *in vivo*. To characterize the function of Th9 cells, we sought to determine how the role of these cells compares to that of classical Th2 cells during *N. brasiliensis* infection. To this end, we adoptively transferred 2×10^5 purified effector Th9 or Th2 cells generated *in vitro* into immunodeficient *Rag2*^{-/-} recipient mice and infected them 24 hr later with *N. brasiliensis*. We analyzed the relevant tissues at days 5 and 7 p.i. To generate effector Th9 and Th2 cells, we sorted naive CD4⁺ T cells from INFER or IL-4 reporter 4get mice (Mohrs et al., 2001) and polarized them toward Th9 or Th2 cells, respectively, for 5 days. Sorted effector Th9 or Th2 cells (measured as GFP-expressing cells in these cultures) were used for the transfer experiments. Importantly, transfer of Th9 cells, but not Th2 cells, significantly decreased the worm burden in recipient mice starting at day 5 p.i., which was also observed by day 7 p.i. (Figure 6A). Interestingly, IL-9 expression on T cells promoted better engraftment of the transferred cells observed in MLNs by day 5 p.i., which was also recapitulated in the lungs and spleen by day 7 p.i. (Figure S5A). Despite increased eosinophilia observed in mice transferred with Th2 cells at day 7 p.i., we did not find important differences between groups in the numbers of this subset *in vivo* (Figure S5B), suggesting that the effect of Th9 cell transfer in worm expulsion was independent of eosinophils. At day 7 p.i., Th9 cells promoted significantly increased mast cell and basophil numbers in the lungs and spleen, but not MLNs, as well as a significant concomitant increase in ILC2s in the spleen, of *Rag2*^{-/-} recipient mice (data not shown) (Figures 6B and 6C). On the basis of microarray data, it has been suggested that ILC2s could directly respond to IL-9 (Price et al., 2010; Wilhelm et al., 2012); similarly, IL-9 has been shown to act as a mast cell growth factor (Hültner et al., 1990). However, there is no report so far for IL-9R expression on basophils. To determine whether basophils could represent a direct target for IL-9 *in vivo*, we isolated lung-infiltrating basophils from day 7 infected mice and analyzed *Il9r* mRNA expression. Indeed, basophils expressed *Il9r* mRNA, and this expression was lower than that in mast cells but higher than that in eosinophils isolated from the

(D) Sorted GFP⁻ or GFP⁺ cells from day 5 Th9 cell cultures with INFER naive CD4⁺ T cells were examined for IL-9 expression by real-time RT-PCR. Mean $\pm$ SEM normalized to *Hprt* is shown.

(E) GFP⁻ or GFP⁺ cells as described in (D) were stimulated with anti-CD3 for 24 hr. Supernatants were collected and analyzed by ELISA for IL-9 quantification. The mean $\pm$ SEM from one out of three independent experiments measured in triplicate is shown.



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same tissue (Figure S6). Hence, the effect of IL-9 on basophilia could be mediated directly in vivo.

Finally, to definitively prove that IL-9 was the effector cytokine responsible for the Th9-cell-induced responses in vivo, we performed transfer experiments in which we administered neutralizing antibodies against IL-9 during the course of the transfer experiment. IL-9 neutralization ablated the decrease in worm loads induced by Th9 cell transfers into *Rag2*^{-/-} recipient mice (Figure 7A). In addition, Th9-cell-induced mast cell and basophil amplification in the lungs and spleen was severely impaired in mice treated with anti-IL-9, but not in mice treated with isotype control (both groups of mice received effector Th9 cells) (Figures 7B and 7C). Thus, we demonstrated that IL-9 was strictly required for Th9 cells to promote the phenotype observed when transferred into immunocompromised hosts. All together, these data indicate that Th9 cells, through the expression of the effector cytokine IL-9, are more efficient than classical Th2 cells at orchestrating anti-helminth responses in vivo.

DISCUSSION

In this study, we identified a central and nonredundant role for IL-9 in driving host-protective type 2 immunity against hookworm infection. Using our generated IL-9-deficient mice, we found that IL-9 promotes IL-5 and IL-13 expression and increases basophilia, eosinophilia, and mast cell numbers in parasite-targeted tissues and ultimately leads to efficient worm expulsion. Generation of IL-9-fluorescent reporter mice allowed us to identify Th9 cells and ILC2s as natural sources of IL-9 in vivo. Both adaptive and innate IL-9-expressing subsets were rapidly induced within mucosal and lymphoid tissues upon *N. brasiliensis* infection. Furthermore, functional characterization of Th9 cells in adoptive transfer experiments demonstrated that Th9 cells are more efficient than Th2 cells at driving basophil and mast cell amplification and thus leading to worm expulsion. Our data also support IL-9 as a critical effector cytokine for Th9 cell function. As a whole, this study centers IL-9 as an important regulator driving mucosal type 2 immunity in vivo.

IL-9 has been reported as part of the type 2 cytokine effector panel; however, initial attempts to dissect its contribution in helminth infection did not demonstrate a significant role for this cytokine in worm clearance or type 2 cytokine induction in vivo (Townsend et al., 2000). Contradictory results regarding IL-9 function in the immune system have been described, perhaps mainly because of differences in the mouse strain analyzed and the disease model and experimental approaches employed to neutralize or amplify IL-9 expression in vivo (Faulkner et al., 1998; Richard et al., 2000). In this report, we demonstrated that IL-9 works as an initiator and/or amplifier of type 2 responses in vivo by activating innate subsets and promoting the expression of other type 2 cytokines, which further maintain an active response.

IL-9 was originally identified as a T-cell-derived cytokine (Schmitt et al., 1994; Schmitt et al., 1989). Although allergen-specific induction of IL-9 in peripheral-blood mononuclear cells of atopic patients and in murine models of allergy have been reported (Chang et al., 2010; Jones et al., 2012; Yao et al., 2011), there is no report of IL-9 expression in T cells in vivo without a requirement for ex vivo restimulation on isolated T cell populations. Studies have identified Th9 cells as a new Th cell subset responsible for IL-9 expression in vitro (Dardalhon et al., 2008; Veldhoen et al., 2008). Previous attempts to identify Th9 cells in vivo by using fate mapping of IL-9 reporter mice did not demonstrate IL-9-expressing T cells in a lung inflammation model, mainly because of incomplete reporter activity (Wilhelm et al., 2011). The generation of INFER mice in the present study enabled the tracking of active IL-9-expressing cells in real time. With this genetic tool, we were able to identify Th9 cells generated in vivo without the requirement of additional manipulation for their detection.

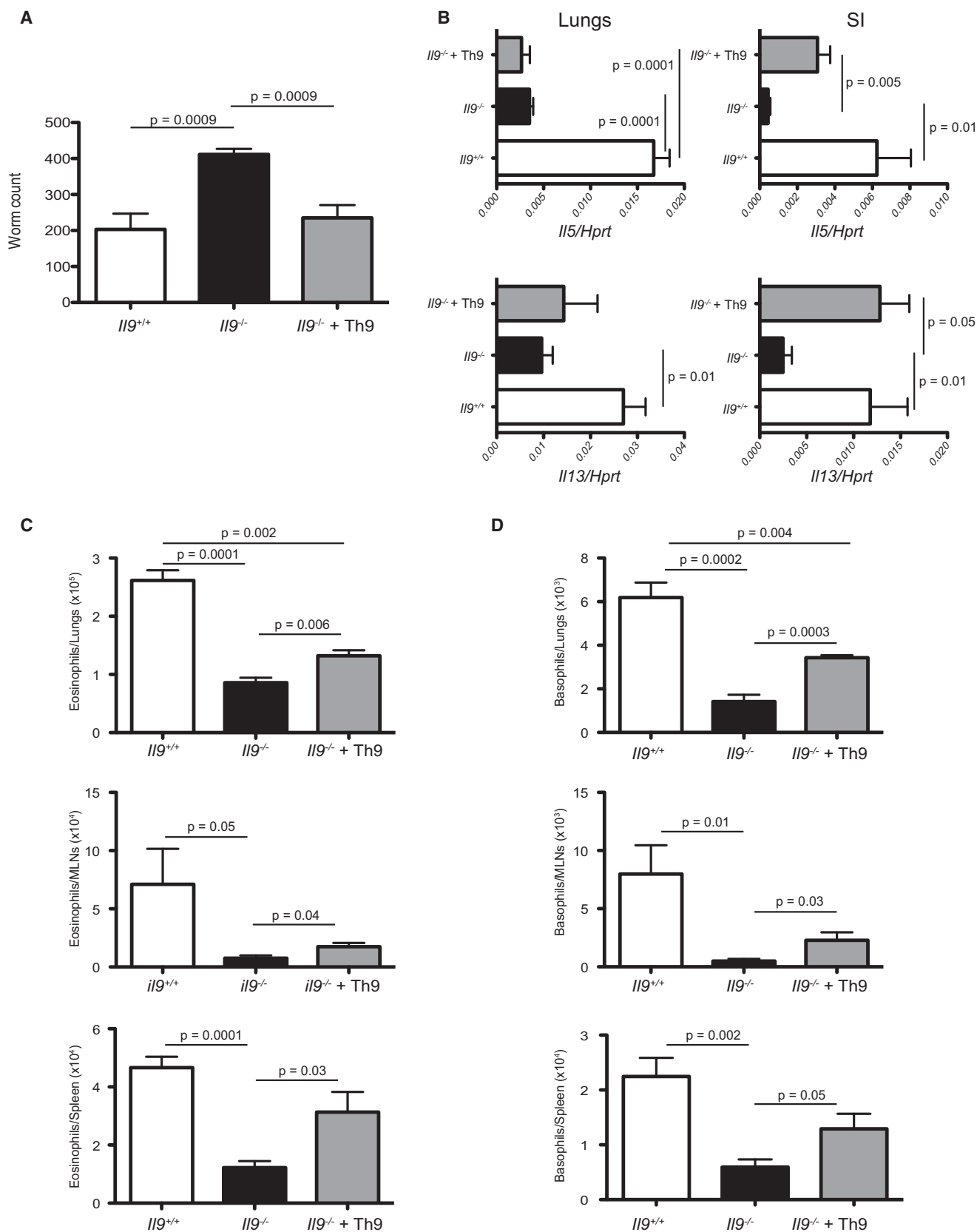
We also found that *N. brasiliensis* infection induced a second population of IL-9-expressing cells, identified as ILC2s. This innate subpopulation is an important source of IL-5 and IL-13 in vivo (Ikutani et al., 2012; Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010). IL-9-expressing ILC2s have also been observed in a papain-induced lung-inflammation model; however, this expression was transient and transitioned to IL-5 and/or IL-13 expression over time (Wilhelm et al., 2011). Our analysis of IL-9 expression in vivo confirmed that compared to other type 2 cytokines, this cytokine is expressed early during nematode infection (Faulkner et al., 1998); furthermore, IL-9 induction followed a strict temporal and compartmentalized regulation that might be important for avoiding detrimental effects of chronic exposure such as those observed in transgenic models (Temann et al., 1998). Even with these intrinsic limitations related to the nature of IL-9 induction, we have now demonstrated that both adaptive and innate sources of IL-9 are rapidly induced in response to helminth infection.

We further demonstrated that T-cell-derived IL-9 is sufficient to decrease worm loads and significantly increase IL-13 and IL-5 expression in the small intestine of IL-9-deficient mice. Moreover, IL-9 derived from CD4⁺ T cells induces significant increases in the levels of eosinophils and basophils in multiple organs. Interestingly, most of these parameters were only partially restored to wild-type levels upon Th9 adoptive transfer, suggesting that ILC2-derived IL-9 might be indispensable to amplify the immune response in infected mice. These results support the notion of a compartmentalized function for different sources of this cytokine in vivo, and the exact contribution of the innate IL-9⁺ compartment warrants further investigation. Nevertheless, the results presented here clearly demonstrate that IL-9 produced by T cells is sufficient to efficiently orchestrate anti-helminth responses in vivo.

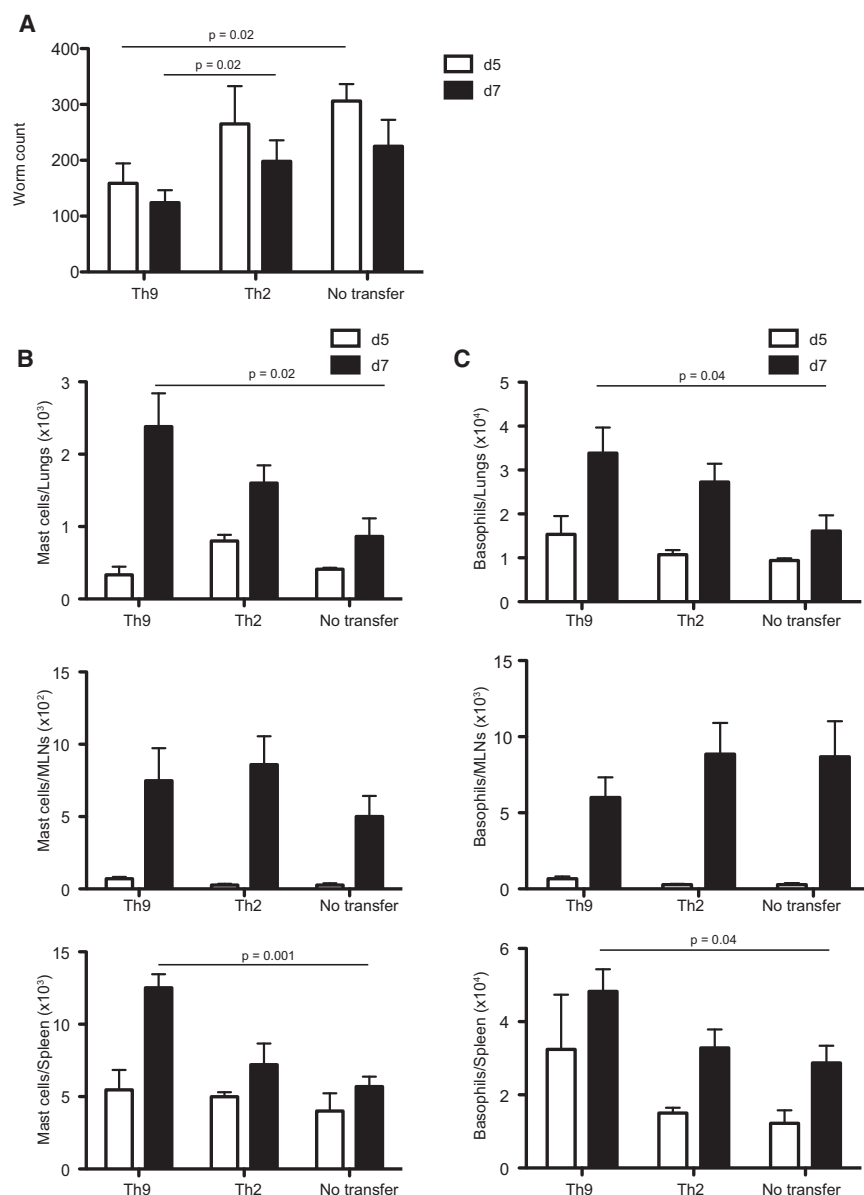
Because there has been recent controversy regarding the function of T cells expressing IL-9, we focused our study

Figure 4. IL-9 Is Expressed in CD4⁺ T Cells and ILC2s during *N. brasiliensis* Infection

INFER mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae. Lungs (A) and MLNs (B) were harvested and homogenized at different days p.i. for the assessment of IL-9 expression by flow cytometry. Numbers represent the frequencies of total GFP⁺ cells (left column) or GFP⁺ cells among CD4⁺ T cells (right column). Total numbers of CD4⁺ T cells (white bars) and ILC2s (black bars) expressing IL-9 (GFP⁺) in the lungs (C) or MLNs (D) of INFER infected mice as determined by flow cytometry are shown. Data represent the mean ± SEM from one out of three independent experiments with three to four mice per day p.i. The p values determined by unpaired two-tailed Student's t test between days are shown.



(legend on next page)



on whether Th9 cells represent a bona fide protective T cell subset in *N. brasiliensis* infection. Although growing evidence suggests that an IL-9-expressing T cell could account for diverse immune responses in vivo, functional characterization of purified Th9 cells has not been thoroughly addressed.

Increased basophilia in *Rag2*^{-/-} mice transferred with T cells during nematode infection has been previously reported (Min et al., 2004). We have now demonstrated that this characteristic phenotype is dependent on IL-9 expression by CD4⁺ T cells. Similarly, we have demonstrated that the phenotype observed

Figure 6. Adoptive Transfer of Th9 Cells, but Not Th2 Cells, Improves Worm Expulsion

(A) Sorted effector Th2 or Th9 cells (CD45.2⁺GFP⁺) from day 5 in vitro cultures with naive CD4⁺ T cells isolated from 4get or INFER mice were intravenously transferred into CD45.1⁺ *Rag2*^{-/-} recipient mice. After 24 hr, mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae. As a control, a third group of *Rag2*^{-/-} mice that did not receive T cells was also infected. Worm counts in the small intestine at days 5 (white bars) and 7 (black bars) p.i. are shown.

(B) Total mast cell numbers in the lungs, MLNs, and spleen of mice described in (A) as determined by flow cytometry.

(C) Total basophil numbers in the lungs, MLNs, and spleen of mice described in (A) as determined by flow cytometry.

Data represent the mean $\pm$ SEM. The experiment was repeated three times with similar results with four to five mice per group. Statistics were calculated by an unpaired two-tailed Student's t test. The p values between groups are shown.

Phenotypic characterization of Th9 cells in vivo has been done with heterogeneous populations of naive CD4⁺ T cells polarized toward Th9 cells in vitro (Dardalhon et al., 2008; Purwar et al., 2012; Staudt et al., 2010), where contaminating cells not expressing IL-9 are unavoidable. Our generated mouse model enabled us to study a pure population of IL-9-expressing T cells and its function in a physiological setting in vivo. With this approach, we have now demonstrated that Th9 cells provide better protection to *N. brasiliensis* infection than do classical Th2 cells, supporting a specialized role for this Th cell subset in vivo (Jones et al., 2012; Staudt et al., 2010).

Figure 5. T-Cell-Derived IL-9 Is Sufficient to Promote Worm Expulsion in IL-9-Deficient Hosts

(A) Sorted effector Th9 cells (GFP⁺) from day 5 in vitro cultures with naive CD4⁺ T cells isolated from INFER mice were intravenously transferred into *Il9*^{-/-} recipient mice (gray bars). After 24 hr, mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae. For comparison, *Il9*^{+/-} (white bars) and *Il9*^{-/-} (black bars) mice that did not receive Th9 cells were used. Worm counts in the small intestine at day 7 p.i. are shown.

(B) At day 7 p.i., the lungs and small intestine (SI) of mice described in (A) were harvested and homogenized for assessment of *Il5* and *Il13* mRNA expression. Data represent the mean $\pm$ SEM ratio of cytokine gene expression to *Hprt* expression as determined by the $\Delta\Delta$ Ct method. Similar results were obtained in three independent experiments with six mice per group.

(C) Total eosinophil numbers in the lungs, MLNs, and spleen of mice described in (A) as determined by flow cytometry.

(D) Total basophil numbers in the lungs, MLNs, and spleen of mice described in (A) as determined by flow cytometry.

Data represent the mean $\pm$ SEM. The experiment was repeated three times with similar results with five to six mice per group. Statistics were calculated by an unpaired two-tailed Student's t test. The p values between groups are shown.

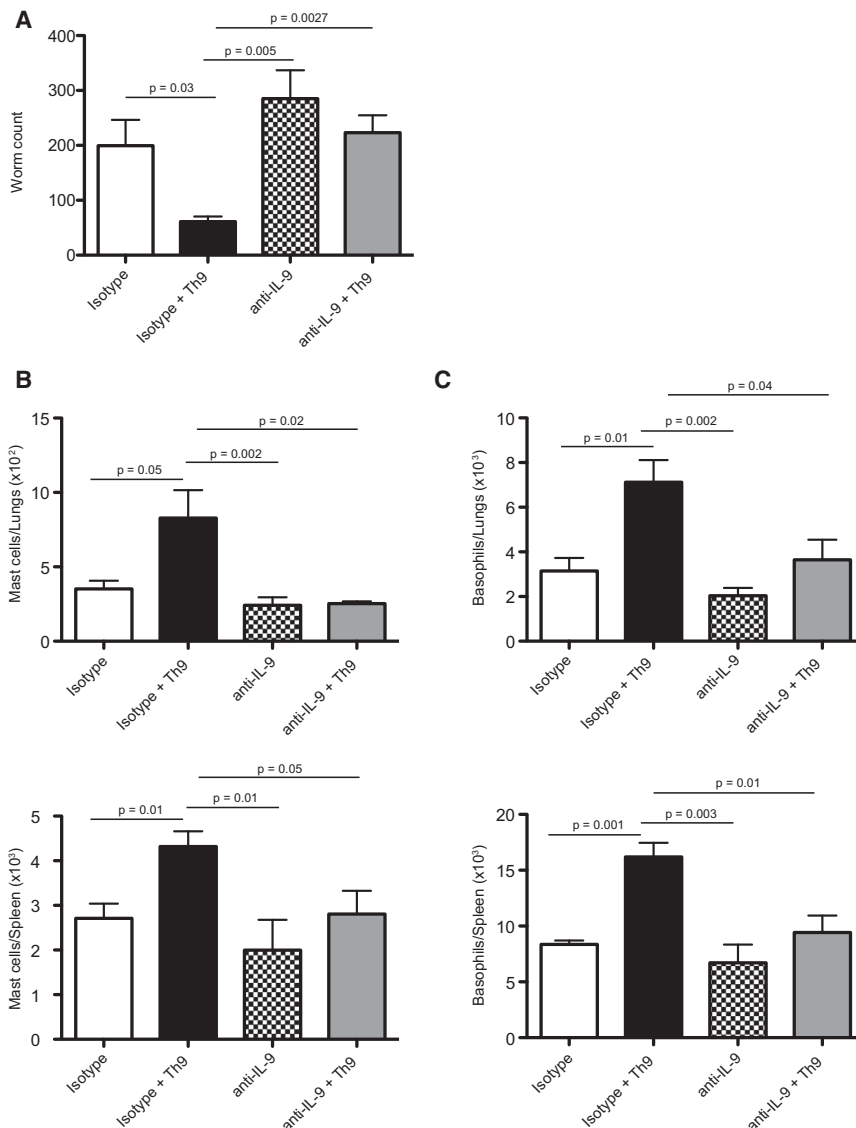


Figure 7. IL-9 Neutralization Abrogates the Th9-Cell-Induced Phenotype in *Rag2*^{-/-} Recipient Mice

Sorted effector Th9 cells (CD45.2⁺GFP⁺) from day 5 in vitro cultures with naive CD4⁺ T cells isolated from INFER mice were intravenously transferred into CD45.1⁺*Rag2*^{-/-} recipient mice treated with anti-IL-9 mAb (gray bars) or isotype control (black bars). After 24 hr, mice were subcutaneously infected with 625 L3 *N.brasiliensis* larvae. As additional controls, mice without transferred cells but treated with anti-IL-9 (checked bars) or isotype control (white bars) were used.

(A) Worm counts in the small intestine of mice described above at day 7 p.i.

(B) Total mast cell numbers in the lungs and spleen of mice described above as determined by flow cytometry.

(C) Total basophil numbers in the lungs and spleen of mice described above as determined by flow cytometry.

Data represent the mean $\pm$ SEM. The experiment was repeated three times with similar results with four to five mice per group. Statistics were calculated by an unpaired two-tailed Student's t test. The p values between groups are shown.

derived IL-9 could potentially account for the increased ILC2 numbers observed in *Rag2*^{-/-} recipients transferred with Th9 cells.

In conclusion, our work indicates that IL-9 from both innate and adaptive sources orchestrates and amplifies type 2 immunity. Importantly, Th9 cells, but not Th2 cells, promote effective anti-helminth responses in vivo, supporting the fact that Th9 cells have specialized functions that are strictly dependent on IL-9 and that are not shared with those of other Th cell subsets. Future studies monitoring

in Th9 cell recipient mice on mast cell amplification is also dependent on IL-9, supporting the concept that IL-9 is a potent mast cell growth factor (Camberis et al., 2003; Hültner et al., 1990; Jones et al., 2012). In the future, the use of novel murine models for analyzing the specific role of basophils in vivo will be important for determining the contribution of IL-9 production by Th9 cells to basophil expansion and activation during nematode infection (Ohnmacht et al., 2010; Wada et al., 2010). Similarly, the development of new experimental tools will be critical for dissecting the effects of IL-9 on other cell types, such as ILC2s and nonhematopoietic cells, such as mucosal epithelial cells (Gounni et al., 2004). Our present findings regarding ILC2 amplification induced by Th9 transfer are particularly relevant given that ILC2s and T cells seem to work in concert to potentiate and induce robust *N. brasiliensis* elimination (Neill et al., 2010). An interesting observation is that *Il9r* is one of the genes most highly expressed on the ILC2 subpopulation (Price et al., 2010). On the basis of this observation, we propose that T-cell-

IL-9 expression with INFER mice will facilitate the identification of the relevant sources and functions of IL-9 in vivo not only in different type 2 immune responses, such as asthma, but also in other IL-9-driven inflammatory disorders.

EXPERIMENTAL PROCEDURES

Mice

For the generation of IL-9-deficient mice, a genomic region containing exons 1–4 of *Il9* was deleted and substituted for the neo resistance gene. The targeting construct contained 1.2 and 2.8 kb homology arms flanking the neo cassette. Downstream of the 3' homology arm, the thymidine kinase gene was inserted for additional screening (Figure S2). The linearized vector was electroporated into BALB/c embryonic stem cells. G418-resistant, gancyclovir-sensitive clones were screened and injected into C57BL/6 blastocysts. Chimeric males in which germline transmission was successful were bred to BALB/c mice for more than ten generations. Screening of *Il9*^{-/-} mice with primers 5'-CAGGAT GATTGTACCACACCG-3' and 5'-CTCATTTGCTTGGATGTGAGC-3' or 5'-TC GCCTCTTGACGAGTTCT-3' and 5'-CCAACAGGTGGAGAGAAGACA-3' amplified a 250 bp wild-type allele or a 480 bp targeted allele, respectively.

INFER mice were generated with a standard cloning strategy. In brief, the targeting construct was generated by standard subcloning techniques using appropriate restriction enzymes and the parental vectors pEasyfloX and pMCSV2.2. The targeting construct contained two homology arms of 1.9 and 4.4 kb. The IRES-GFP was introduced immediately after the stop codon for avoiding disruption of *cis*-regulatory elements in the 3' UTR and the polyadenylation signal. In addition, the construct contained a neo gene flanked by loxP sites and driven by a promoter that allowed for positive selection in both bacterial and mammalian cells (Figure 3). The targeting construct was electroporated in JM8 embryonic stem cells derived from the purebred C57BL/6 background, and individual clones were tested for successful homologous recombination by PCR. Embryonic stem cells carrying the targeted allele were injected into BALB/c blastocysts, which were then implanted in CD1 pseudopregnant foster mothers. Male chimeras were bred with the Ella Cre germline deleter (C57BL/6) strain (Jackson Laboratories) for removal of the neo cassette and screening for germline-transmitted offspring. Mice bearing the targeted allele were screened by PCR. Primers for the wild-type allele, 5'-GGGACGTTCCAGAAGACAGA-3' and 5'-CCAAAGCAACTGTGAAGCA-3', amplified a 179 bp product. Primers for targeted allele, 5'-GGGACGTTCCAGAAGACAGA-3' and 5'-GGAAAGACCCCTAGGAATGC-3', amplified a 228 bp product. For transfer experiments into *IL9*^{-/-} mice, donor cells from INFER mice backcrossed at least eight generations (N8) into BALB/c background were used.

C57BL/6 and BALB/c mice were purchased from the National Cancer Institute, and *Rag2*^{-/-} mice on the C57BL/6 background (Taconic) and IL-4 reporter 4get mice (Mohr et al., 2001) (Jackson Laboratories) were backcrossed for more than ten generations to the C57BL/6 background in house.

All mouse experiments were approved by the Institutional Animal Care and Use Committee of Yale University.

Helminth Infection

Individual mice were inoculated subcutaneously with 625 viable third-stage *N. brasiliensis* larvae. Animals were sacrificed at different time points p.i., and harvested tissues were snap frozen for mRNA analysis or processed for flow cytometry staining. Worm burdens were determined as described (Camberis et al., 2003), and serum was collected.

Lymphocyte Preparation and Flow Cytometry

Spleen, medLNs, and MLNs were removed from euthanized mice, placed into RPMI media supplemented with 5% fetal calf serum (FCS), and passed through a cell strainer (70 μ m). For isolation of lung infiltrates, cells were collected after 1 hr digestion in RPMI media supplemented with 5% FCS, 1 mg/ml Collagenase D (Roche), and 10 μ g/ml DNase I (Invitrogen) at 37°C. Homogenates were passed through a cell strainer, and infiltrates were separated with a 27.5% Optiprep gradient (Axis-Shield) by centrifugation at 2,750 rpm for 20 min. Cells were removed from the interface, and ammonium-chloride-potassium lysing buffer was used. Cells were acquired on an LRS II Flow Cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

For intracellular IL-9 staining, day 5 Th9 cell cultures were restimulated for 4 hr with phorbol 12-myristate 13-acetate (20 ng/ml) and ionomycin (1 μ g/ml) in the presence of brefeldin A (1 μ g/ml).

Antibodies

All antibodies for flow cytometry analysis were from eBioscience. For the lin⁻ cocktail, biotin-coupled antibodies against CD4, CD8, CD11b, CD11c, CD19, NK1.1, B220, Gr-1, Ter119, TCR $\gamma\delta$, and TCR β were used. Fluorescent-coupled streptavidin or antibodies against CD4, CD8, CD11b, CD25, CD44, CD45, CD45.1, CD45.2, CD49b, CD62L, Thy1.2, Sca-1, c-kit, Ly6G, Fc ϵ R, and Siglec-F were used. For intracellular staining, anti-IL-9-PE (BD) and anti-GFP (Invitrogen) were used. For ELISA, purified and biotinylated antibodies (BD Biosciences) against IFN- γ , IL-4, IL-9, and IL-17 were used.

For neutralization experiments, anti-murine IL-9 (9C1) and an isotype control (C1.18.4) from BioXcell were used.

In Vitro T Cell Differentiation

CD4⁺ T cells from the spleen and axillary and inguinal lymph nodes of C57BL/6, INFER, or 4get mice were enriched by magnetic-activated cell sorting using

anti-CD4 beads (Milteny Biotech). Then, naive CD4⁺ T cells (CD25⁻CD62L⁺CD44⁻) were purified by flow cytometry sorting and cultured in Clicks medium (Sigma-Aldrich) supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and β -mercaptoethanol (40 nM) in the presence of plate-bound anti-CD3 (2C11, 2 μ g/ml for Th2 and Th9 cells, 5 μ g/ml for Th1 and Treg cells, 10 μ g/ml for Th17 cells) and anti-CD28 (37N51, 10 μ g/ml for Th2 and Th9 cells, 2 μ g/ml for Th1 and Treg cells, 2 μ g/ml for Th17 cells with soluble antibody). A total of 0.75×10^6 cells/ml were cultured for 5 days under Th1-, Th2-, Th9-, Th17-, or Treg-cell-polarizing conditions. Cytokines for effector differentiation were as follows: Th1 cells: IL-12 (10 ng/ml PeproTech), anti-IL-4 (10 μ g/ml, 11B11), and IL-2 (20 U/ml, PeproTech); Th2 cells: IL-4 (20 ng/ml, PeproTech), anti-IFN- γ (10 μ g/ml, XMG1.2), and IL-2 (20 U/ml); Th9 cells: IL-4 (10 ng/ml), TGF- β (1 ng/ml, R&D), IL-1 β (10 ng/ml, PeproTech), IL-25 (20 ng/ml, R&D), TSLP (50 ng/ml, PeproTech), and anti-IFN- γ (10 μ g/ml); Th17 cells: TGF- β (0.5 ng/ml), IL-6 (20 ng/ml), IL-23 (50 ng/ml), anti-IL-4 (10 μ g/ml), anti-IFN- γ (10 μ g/ml), and anti-CD28 (5 μ g/ml); and Treg cells: TGF- β (2 ng/ml).

Adoptive-Transfer Experiments

Naive CD4⁺ T cells (CD25⁻CD62L⁺CD44⁻) from 4get or INFER mice were cultured in Th2- or Th9-cell-skewing conditions, respectively. On day 5, in-vitro-generated effector cells were sorted by flow cytometry on the basis of GFP expression. A total of 2×10^5 IL-4 GFP⁺ Th2 cells or IL-9 GFP⁺ Th9 cells were transferred intravenously into CD45.1 *Rag2*^{-/-} recipient mice. CD45.2 expression on transferred cells was used for identifying donor cells. One day after transfer, mice were infected with *N. brasiliensis* as described above and euthanized for analysis 5–7 days p.i.

Transfer experiments with neutralizing antibodies against IL-9 were performed as described above with Th9 cells. Mice were intravenously (first two doses) or intraperitoneally injected with 200 μ g/dose of antibody every other day starting at day -2. Mice were euthanized for analysis at day 7 p.i.

For transfer experiments into *IL9*^{-/-} mice, 5×10^5 sorted Th9 cells (GFP⁺) from day 5 cultures were used. One day later, transferred mice were infected with *N. brasiliensis* as described above and euthanized for analysis at day 7 p.i.

Real-Time RT-PCR

RNA from cells or organs was extracted with Trizol reagent (Invitrogen) and reverse transcribed with Superscript II (Invitrogen) and oligo dT according to the manufacturer's protocol. cDNA was semiquantitated with commercially available TaqMan primer and probe sets with the use of the ABI-PRISM 7500 Sequence Detection System (Applied Biosystems). Expression was calculated with the $\Delta\Delta C_T$ method normalized to *Hprt* expression.

Cytokine and mMCPT-1 Measurements

Cells from day 5 Th cell cultures in vitro were harvested and directly recultured or cultured after sorting based on GFP expression in anti-CD3 (10 μ g/ml) plate-bound culture dishes. Cells were resuspended at 1×10^6 to 2×10^6 cells/ml in Click's media, and supernatants were collected 24 hr later for determining cytokine concentration by ELISA. For mMCPT-1 quantification, serum from mice infected with *N. brasiliensis* was collected at day 7 p.i. and analyzed according to the manufacturer's protocol (eBioscience).

Statistical Analysis

Statistical analyses were calculated in Prism 5.0 (Graphpad Software) with the use of a two-tailed Student's t test or one-way ANOVA; $p \leq 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2013.07.020>.

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REFERENCES

- Allen, J.E., and Maizels, R.M. (2011). Diversity and dialogue in immunity to helminths. *Nat. Rev. Immunol.* **11**, 375–388.
- Brooker, S. (2010). Estimating the global distribution and disease burden of intestinal nematode infections: adding up the numbers—a review. *Int. J. Parasitol.* **40**, 1137–1144.
- Camberis, M., Le Gros, G., and Urban, J., Jr. (2003). Animal model of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Curr. Protoc. Immunol. Chapter 19*, 12.
- Chang, H.C., Sehra, S., Goswami, R., Yao, W., Yu, Q., Stritesky, G.L., Jabeen, R., McKinley, C., Ahji, A.N., Han, L., et al. (2010). The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat. Immunol.* **11**, 527–534.
- Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R.A., Mitsdoerffer, M., Strom, T.B., Elyaman, W., Ho, I.C., et al. (2008). IL-4 inhibits TGF- β -induced Foxp3+ T cells and, together with TGF- β , generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat. Immunol.* **9**, 1347–1355.
- Dodd, J.S., Lum, E., Goulding, J., Muir, R., Van Snick, J., and Openshaw, P.J. (2009). IL-9 regulates pathology during primary and memory responses to respiratory syncytial virus infection. *J. Immunol.* **183**, 7006–7013.
- Dong, Q., Louahed, J., Vink, A., Sullivan, C.D., Messler, C.J., Zhou, Y., Haczku, A., Huaux, F., Arras, M., Holroyd, K.J., et al. (1999). IL-9 induces chemokine expression in lung epithelial cells and baseline airway eosinophilia in transgenic mice. *Eur. J. Immunol.* **29**, 2130–2139.
- Elyaman, W., Bradshaw, E.M., Uyttenhove, C., Dardalhon, V., Awasthi, A., Imitola, J., Bettelli, E., Oukka, M., van Snick, J., Renauld, J.C., et al. (2009). IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc. Natl. Acad. Sci. USA* **106**, 12885–12890.
- Fallon, P.G., Smith, P., Richardson, E.J., Jones, F.J., Faulkner, H.C., Van Snick, J., Renauld, J.C., Grecis, R.K., and Dunne, D.W. (2000). Expression of interleukin-9 leads to Th2 cytokine-dominated responses and fatal enteropathy in mice with chronic *Schistosoma mansoni* infections. *Infect. Immun.* **68**, 6005–6011.
- Faulkner, H., Humphreys, N., Renauld, J.C., Van Snick, J., and Grecis, R. (1997). Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *Eur. J. Immunol.* **27**, 2536–2540.
- Faulkner, H., Renauld, J.C., Van Snick, J., and Grecis, R.K. (1998). Interleukin-9 enhances resistance to the intestinal nematode *Trichuris muris*. *Infect. Immun.* **66**, 3832–3840.
- Forbes, E.E., Groschwitz, K., Abonia, J.P., Brandt, E.B., Cohen, E., Blanchard, C., Ahrens, R., Seidu, L., McKenzie, A., Strait, R., et al. (2008). IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J. Exp. Med.* **205**, 897–913.
- Gessner, A., Blum, H., and Rölinghoff, M. (1993). Differential regulation of IL-9 expression after infection with *Leishmania major* in susceptible and resistant mice. *Immunobiology* **189**, 419–435.
- Gounni, A.S., Nutku, E., Koussih, L., Aris, F., Louahed, J., Levitt, R.C., Nicolaides, N.C., and Hamid, Q. (2000). IL-9 expression by human eosinophils: regulation by IL-1 β and TNF- α . *J. Allergy Clin. Immunol.* **106**, 460–466.
- Gounni, A.S., Hamid, Q., Rahman, S.M., Hoeck, J., Yang, J., and Shan, L. (2004). IL-9-mediated induction of eotaxin1/CCL11 in human airway smooth muscle cells. *J. Immunol.* **173**, 2771–2779.
- Hültner, L., Druetz, C., Moeller, J., Uyttenhove, C., Schmitt, E., Rüde, E., Dörmer, P., and Van Snick, J. (1990). Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). *Eur. J. Immunol.* **20**, 1413–1416.
- Hültner, L., Kölsch, S., Stassen, M., Kaspers, U., Kremer, J.P., Mailhammer, R., Moeller, J., Broszeit, H., and Schmitt, E. (2000). In activated mast cells, IL-1 up-regulates the production of several Th2-related cytokines including IL-9. *J. Immunol.* **164**, 5556–5563.
- Ikutani, M., Yanagibashi, T., Ogasawara, M., Tsuneyama, K., Yamamoto, S., Hattori, Y., Kuro, T., Itakura, A., Nagai, Y., Takaki, S., and Takatsu, K. (2012). Identification of innate IL-5-producing cells and their role in lung eosinophil regulation and antitumor immunity. *J. Immunol.* **188**, 703–713.
- Jones, C.P., Gregory, L.G., Causton, B., Campbell, G.A., and Lloyd, C.M. (2012). Activin A and TGF- β promote T(H)9 cell-mediated pulmonary allergic pathology. *J. Allergy Clin. Immunol.* **129**, 1000–1010, e3.
- Knoops, L., Louahed, J., Van Snick, J., and Renauld, J.C. (2005). IL-9 promotes but is not necessary for systemic anaphylaxis. *J. Immunol.* **175**, 335–341.
- Lloyd, C.M., and Hessel, E.M. (2010). Functions of T cells in asthma: more than just T(H)2 cells. *Nat. Rev. Immunol.* **10**, 838–848.
- Lu, L.F., Lind, E.F., Gondek, D.C., Bennett, K.A., Gleeson, M.W., Pino-Lagos, K., Scott, Z.A., Coyle, A.J., Reed, J.L., Van Snick, J., et al. (2006). Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* **442**, 997–1002.
- Lu, Y., Hong, S., Li, H., Park, J., Hong, B., Wang, L., Zheng, Y., Liu, Z., Xu, J., He, J., et al. (2012). Th9 cells promote antitumor immune responses in vivo. *J. Clin. Invest.* **122**, 4160–4171.
- Min, B., Prout, M., Hu-Li, J., Zhu, J., Jankovic, D., Morgan, E.S., Urban, J.F., Jr., Dvorak, A.M., Finkelman, F.D., LeGros, G., and Paul, W.E. (2004). Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* **200**, 507–517.
- Mohrs, M., Shinkai, K., Mohrs, K., and Locksley, R.M. (2001). Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter. *Immunity* **15**, 303–311.
- Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J., Ohtani, M., Fujii, H., and Koyasu, S. (2010). Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* **463**, 540–544.
- Neill, D.R., Wong, S.H., Bellosi, A., Flynn, R.J., Daly, M., Langford, T.K., Bucks, C., Kane, C.M., Fallon, P.G., Pannell, R., et al. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367–1370.
- Ohnmacht, C., Schwartz, C., Panzer, M., Schiedewitz, I., Naumann, R., and Voehringer, D. (2010). Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* **33**, 364–374.
- Osterfeld, H., Ahrens, R., Strait, R., Finkelman, F.D., Renauld, J.C., and Hogan, S.P. (2010). Differential roles for the IL-9/IL-9 receptor α -chain pathway in systemic and oral antigen-induced anaphylaxis. *J. Allergy Clin. Immunol.* **125**, 469–476, e2.
- Palm, N.W., Rosenstein, R.K., and Medzhitov, R. (2012). Allergic host defences. *Nature* **484**, 465–472.
- Paul, W.E., and Zhu, J. (2010). How are T(H)2-type immune responses initiated and amplified? *Nat. Rev. Immunol.* **10**, 225–235.
- Petit-Frere, C., Dugas, B., Braquet, P., and Mencia-Huerta, J.M. (1993). Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine B lymphocytes. *Immunology* **79**, 146–151.
- Price, A.E., Liang, H.E., Sullivan, B.M., Reinhardt, R.L., Eisle, C.J., Erle, D.J., and Locksley, R.M. (2010). Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci. USA* **107**, 11489–11494.
- Pulendran, B., and Artis, D. (2012). New paradigms in type 2 immunity. *Science* **337**, 431–435.
- Purwar, R., Schlapbach, C., Xiao, S., Kang, H.S., Elyaman, W., Jiang, X., Jetten, A.M., Khoury, S.J., Fuhlbrigge, R.C., Kuchroo, V.K., et al. (2012). Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nat. Med.* **18**, 1248–1253.
- Richard, M., Grecis, R.K., Humphreys, N.E., Renauld, J.C., and Van Snick, J. (2000). Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in *Trichuris muris*-infected mice. *Proc. Natl. Acad. Sci. USA* **97**, 767–772.

- Saenz, S.A., Siracusa, M.C., Perrigoue, J.G., Spencer, S.P., Urban, J.F., Jr., Tocker, J.E., Budelsky, A.L., Kleinschek, M.A., Kastelein, R.A., Kambayashi, T., et al. (2010). IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464, 1362–1366.
- Schmitt, E., Van Brandwijk, R., Van Snick, J., Siebold, B., and Rüde, E. (1989). TCGF III/P40 is produced by naive murine CD4+ T cells but is not a general T cell growth factor. *Eur. J. Immunol.* 19, 2167–2170.
- Schmitt, E., Germann, T., Goedert, S., Hoehn, P., Huels, C., Koelsch, S., Kühn, R., Müller, W., Palm, N., and Rüde, E. (1994). IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J. Immunol.* 153, 3989–3996.
- Shimbara, A., Christodoulopoulos, P., Soussi-Gounni, A., Olivenstein, R., Nakamura, Y., Levitt, R.C., Nicolaides, N.C., Holroyd, K.J., Tsicopoulos, A., Lafitte, J.J., et al. (2000). IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J. Allergy Clin. Immunol.* 105, 108–115.
- Staudt, V., Bothur, E., Klein, M., Lingnau, K., Reuter, S., Grebe, N., Gerlitzki, B., Hoffmann, M., Ulges, A., Taube, C., et al. (2010). Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* 33, 192–202.
- Temann, U.A., Geba, G.P., Rankin, J.A., and Flavell, R.A. (1998). Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J. Exp. Med.* 188, 1307–1320.
- Townsend, J.M., Fallon, G.P., Matthews, J.D., Smith, P., Jolin, E.H., and McKenzie, N.A. (2000). IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity* 13, 573–583.
- Uyttenhove, C., Simpson, R.J., and Van Snick, J. (1988). Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. *Proc. Natl. Acad. Sci. USA* 85, 6934–6938.
- Van Snick, J., Goethals, A., Renauld, J.C., Van Roost, E., Uyttenhove, C., Rubira, M.R., Moritz, R.L., and Simpson, R.J. (1989). Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40). *J. Exp. Med.* 169, 363–368.
- Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorp, A., Buer, J., Martin, B., Wilhelm, C., and Stockinger, B. (2008). Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* 9, 1341–1346.
- Wada, T., Ishiwata, K., Koseki, H., Ishikura, T., Ugajin, T., Ohnuma, N., Obata, K., Ishikawa, R., Yoshikawa, S., Mukai, K., et al. (2010). Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J. Clin. Invest.* 120, 2867–2875.
- Wilhelm, C., Hirota, K., Stieglitz, B., Van Snick, J., Tolaini, M., Lahl, K., Sparwasser, T., Helmby, H., and Stockinger, B. (2011). An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat. Immunol.* 12, 1071–1077.
- Wilhelm, C., Turner, J.E., Van Snick, J., and Stockinger, B. (2012). The many lives of IL-9: a question of survival? *Nat. Immunol.* 13, 637–641.
- Yao, W., Tepper, R.S., and Kaplan, M.H. (2011). Predisposition to the development of IL-9-secreting T cells in atopic infants. *J. Allergy Clin. Immunol.* 128, 1357–1360, e5.

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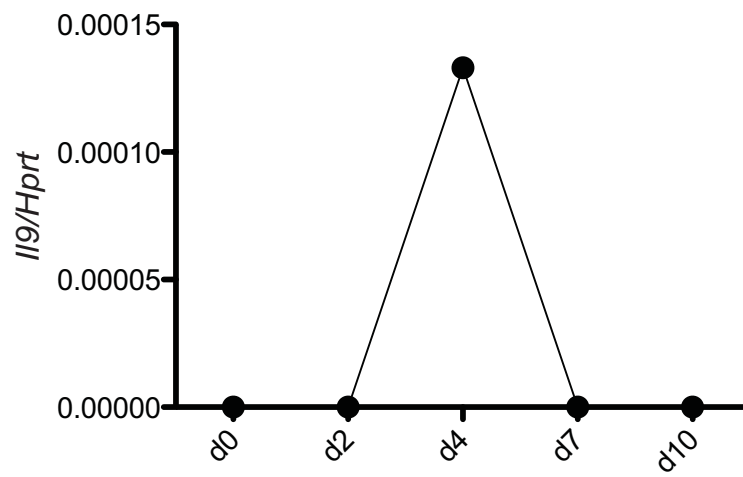
Supplemental Information

Th9 Cells Drive Host Immunity

against Gastrointestinal Worm Infection

Paula Licona-Limón, Jorge Henao-Mejia, Angela U. Temann, Nicola Gagliani, Ileana Licona-Limón, Harumichi Ishigame, Liming Hao, De'Broski R. Herbert, and Richard A. Flavell

A



B

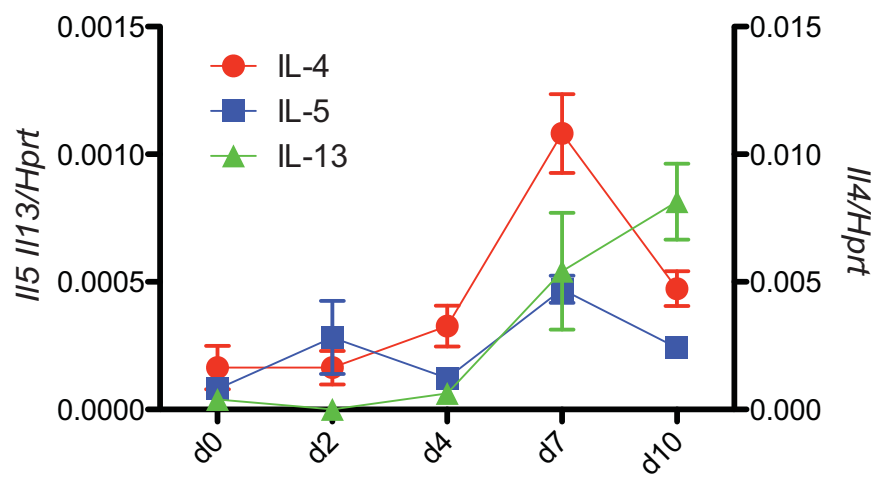


Figure S1. IL-9 expression precedes IL-4, IL-5 and IL-13 in spleen of *N. brasiliensis* infected mice

(A) C57BL/6 mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae. Spleen was collected and homogenized at different days p.i. for assessment of *Il9* mRNA expression by real time RT-PCR. The experiment was performed two times with similar results with 2-3 mice per day p.i. Statistically significant p values were determined by one-way ANOVA when comparing basal expression (d0) with at least one other time point for each gene.

(B) Same samples as above analyzed for *Il4*, *Il5* and *Il13* mRNA expression.

Data represent the mean $\pm$ SEM ratio of cytokine gene to *Hprt* expression as determined by the relative quantification method ($\Delta\Delta Ct$). The experiment was performed two times with similar results with 2-3 mice per day p.i. Statistically significant p values were determined by one-way ANOVA when comparing basal expression (d0) with at least one other time point for each gene.

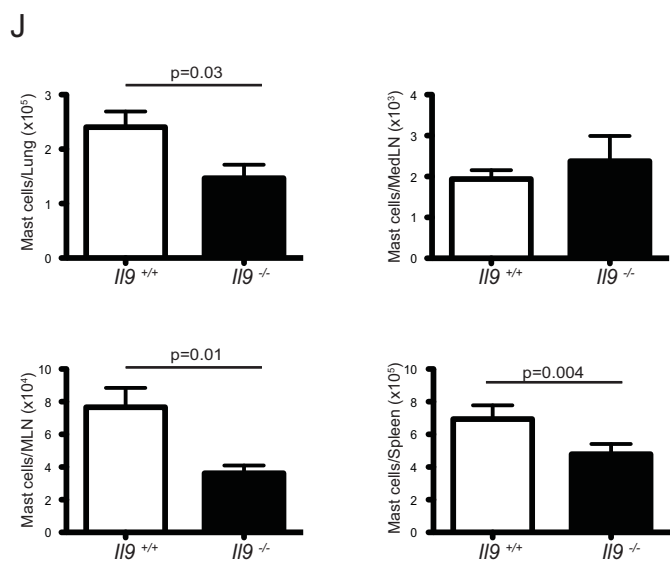
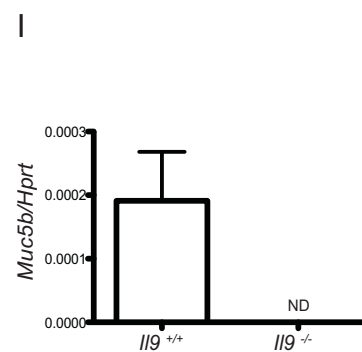
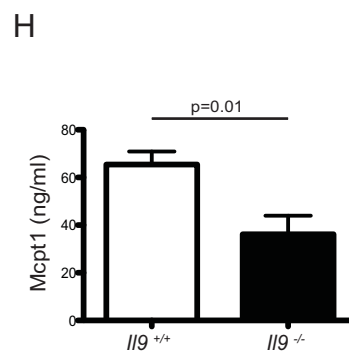
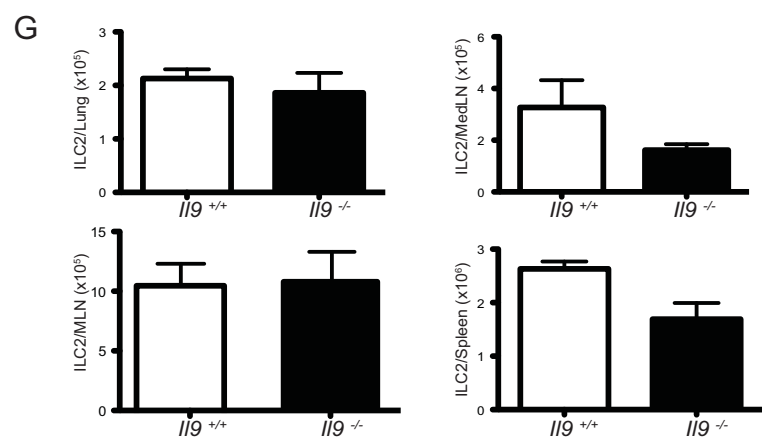
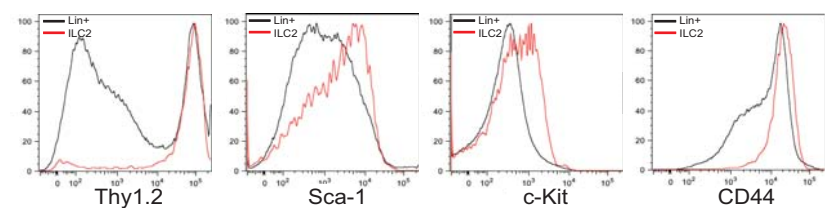
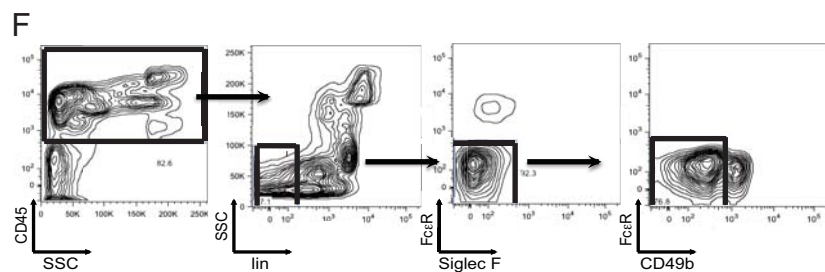
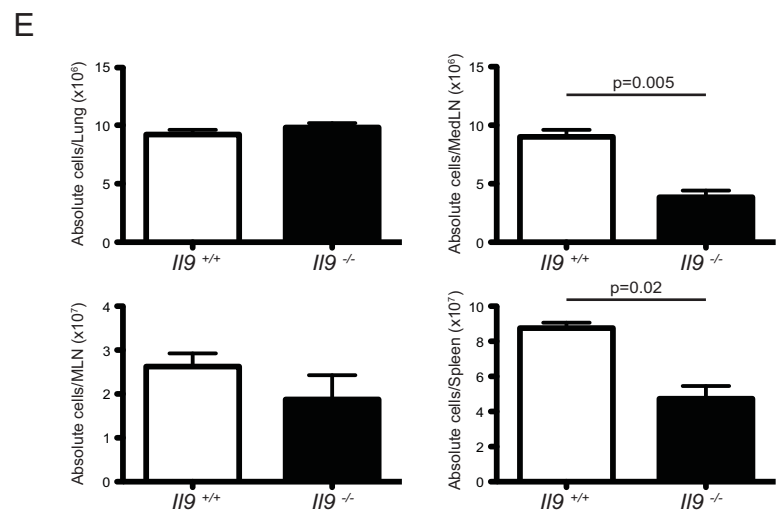
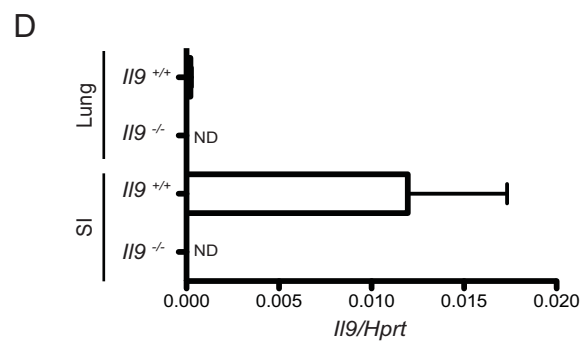
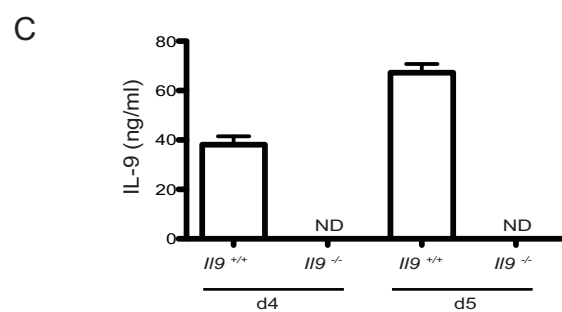
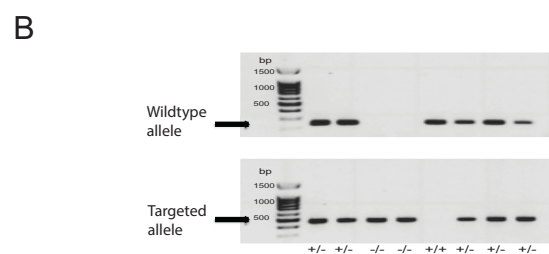
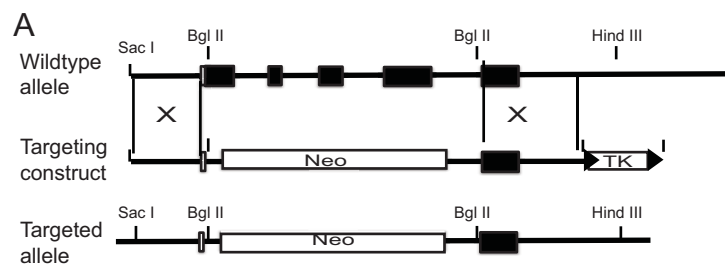


Figure S2. Generation and characterization of IL-9 deficient mice

(A) Schematic representation of IL-9 wild-type allele, targeting construct and targeted allele. The targeting construct (middle) had a neomycin resistance gene substituting exons 1-4, followed by a loxp-flanked thymidine kinase (TK) downstream of exon 5. Deletion of TK by Cre recombinase resulted in the targeted allele (bottom). Filled boxes represent exons 1-5. Loxp sites are shown as arrow heads. Restriction sites are depicted.

(B) Genotyping of IL-9 deficient mice showing PCR amplification of a 250 bp and 480 bp product corresponding to the IL-9 wild-type (+/+) and targeted (-/-) allele, respectively.

(C) Sorted naïve CD4⁺ T cells from *Il9*^{+/+} or *Il9*^{-/-} mice were polarized *in vitro* in Th9 cultures. IL-9 secretion was determined by ELISA at day 4 and day 5. Data represents mean +/- SEM. The experiment was repeated three times with similar results. ND; not detected.

(D) *Il9*^{+/+} or *Il9*^{-/-} mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae. Lung and small intestine was collected and homogenized at day 7 p.i. for assessment of *Il9* mRNA expression by real time RT-PCR. Data represent the mean +/- SEM expression of the cytokine gene to *Hprt* using the $\Delta\Delta C_t$ method.

(E) Total numbers of cells in lung, MedLN, MLN and spleen of *Il9*^{+/+} or *Il9*^{-/-} mice at day 7 p.i.

(F) Gating strategy for analysis of ILC2 cells by flow cytometry in *N. brasiliensis* infected mice. Example shown is lung at day 7 p.i. Numbers represent percentage of boxed cells. The lower panels are representative flow cytometry analysis for surface

expression of Thy1.2, Sca-1, c-kit and CD44 in ILC2 cells (red line) and lin^+ cells (black line) from lung infected mice.

(G) Total numbers of ILC2 cells as determined by flow cytometry in lung, medLN, MLN and spleen of $\text{Il9}^{+/+}$ or $\text{Il9}^{-/-}$ mice at day 7 p.i.

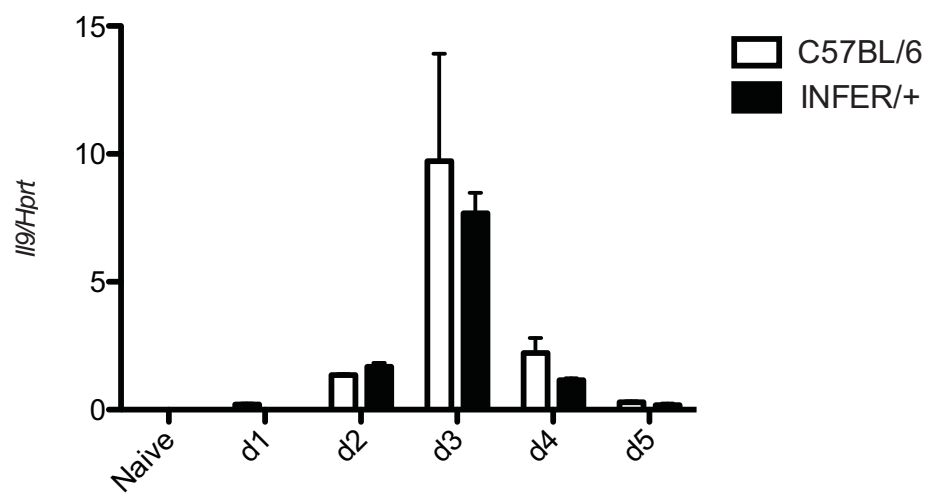
(H) Serum concentration of Mcpt1 as determined by ELISA in $\text{Il9}^{+/+}$ (white bars) or $\text{Il9}^{-/-}$ (black bars) mice at day 7 p.i.

(I) Small intestine from $\text{Il9}^{+/+}$ (white bars) or $\text{Il9}^{-/-}$ (black bars) mice was collected and homogenized at day 7 p.i for assessment of *Muc5b* mRNA expression by real time RT-PCR. Data represent the mean $\pm$ SEM expression of mucin to *Hprt* using the $\Delta\Delta\text{Ct}$ method.

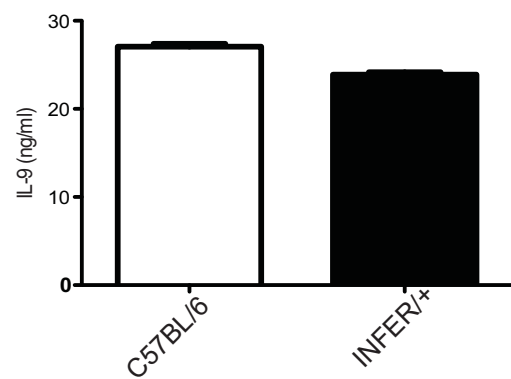
(J) Total mast cell (FceRI^+ c-Kit^+) numbers in lung, medLN, MLN and spleen of $\text{Il9}^{+/+}$ (white bars) or $\text{Il9}^{-/-}$ (black bars) at day 7 p.i. Mean $\pm$ SEM of experiments with 6 mice per group.

Density plots and histograms are representative results of six mice per group analyzed. Data are represented as mean $\pm$ SEM. All the experiments were performed twice with similar results. $n = 6$ mice per group. p value determined by unpaired two-tailed Student's t test is shown. ND; not detected.

A



B



C

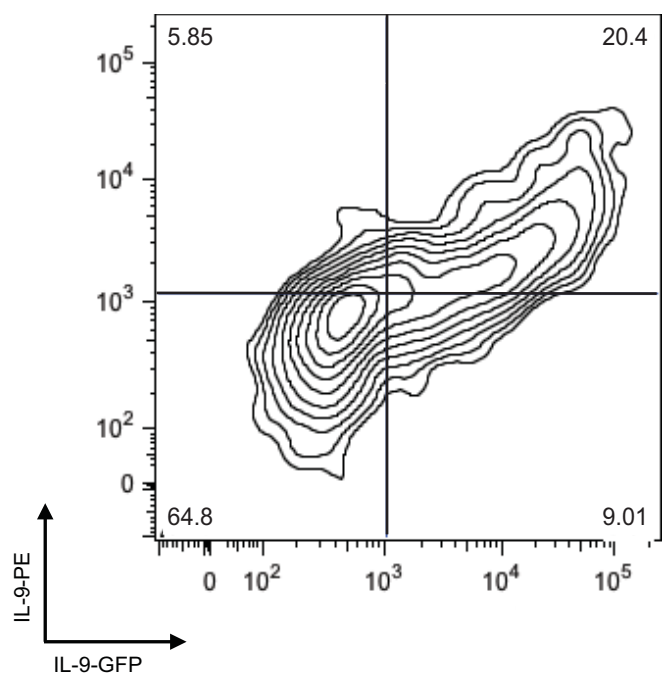


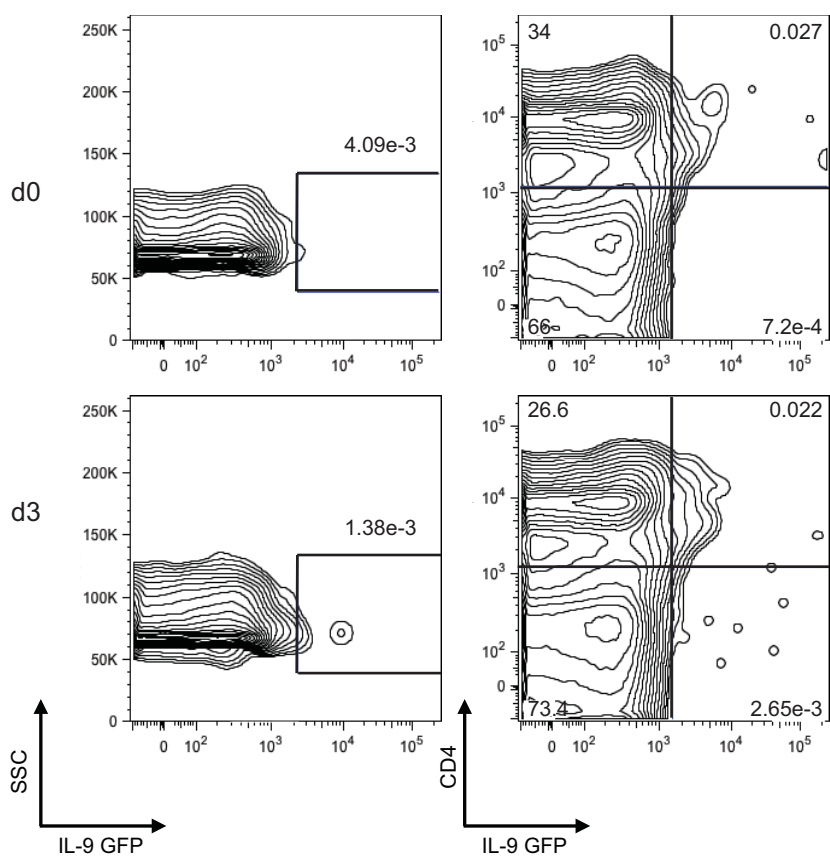
Figure S3. Validation of INFER mice *in vitro*

(A) Sorted naïve CD4⁺CD62L⁺CD25⁻CD44⁻ T cells from wild-type C57BL/6 or INFER/+ mice were cultured in Th9 conditions for 5 days. *Il9* mRNA expression was assessed in samples collected daily by quantitative RT-PCR using the $\Delta\Delta C_t$ method. Mean $\pm$ SEM normalized to *Hprt* from one out of three independent experiments is shown.

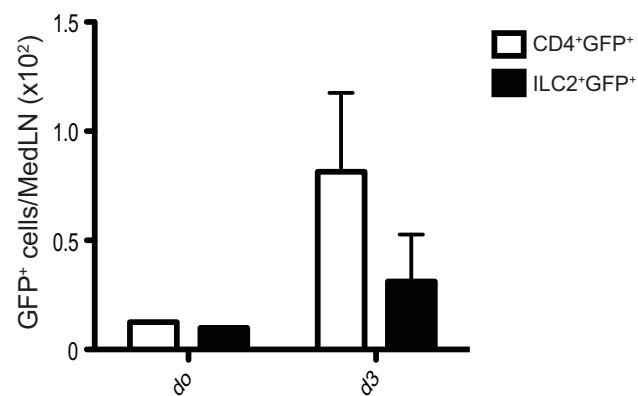
(B) Supernatants from cultures in (A) at day 5 were collected and analyzed by ELISA for IL-9 quantification. Mean $\pm$ SEM from one out of three independent experiments measured in triplicate is shown.

(C) Intracellular staining for IL-9 and GFP expression analyzed by flow cytometry in Th9 cultures from INFER/+ naïve T cells at day 5.

A

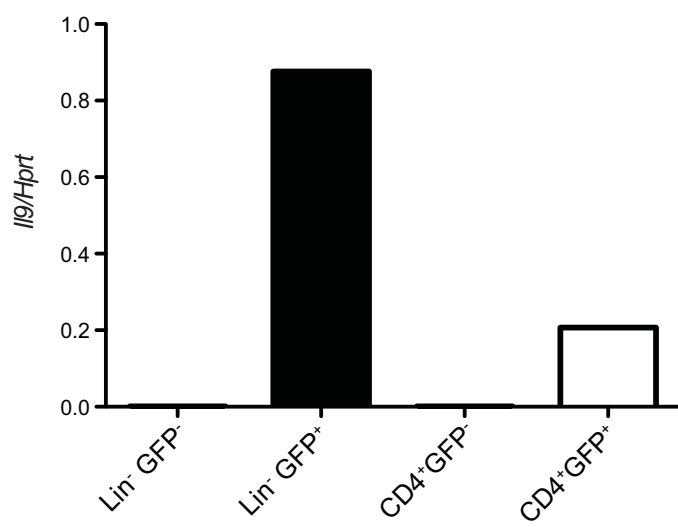


B



C

Lung



D

MLN

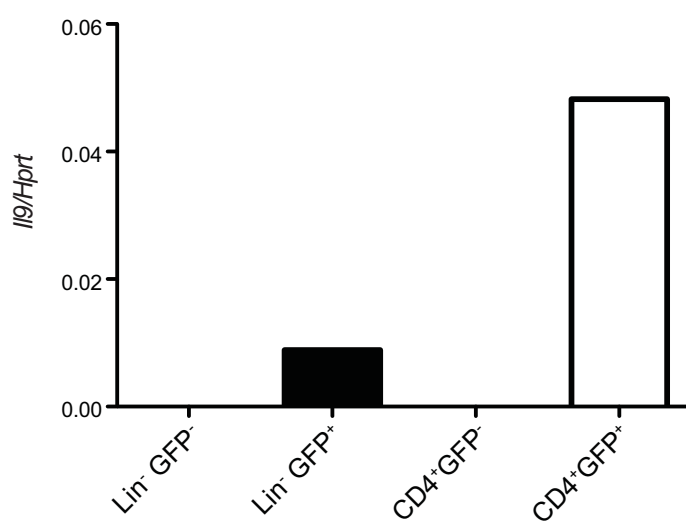


Figure S4. IL-9 is expressed in GFP⁺ cells *in vivo*

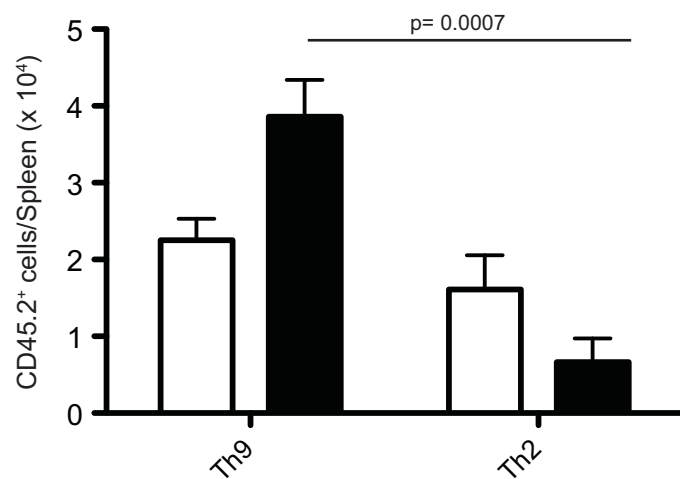
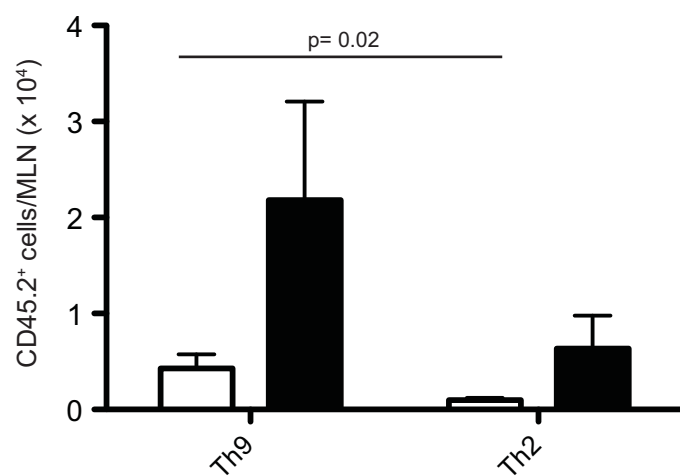
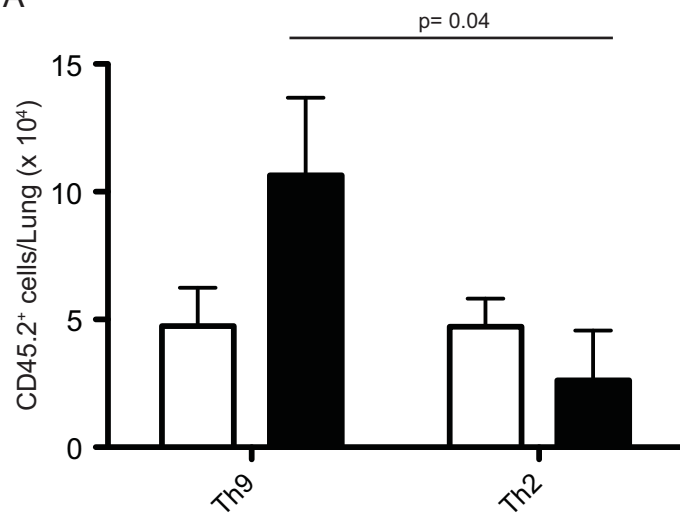
INFER mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae.

(A) MedLN were harvested and homogenized at days 0 and 3 p.i for assessment of IL-9 expression by flow cytometry. Numbers represent the frequencies of total GFP⁺ cells (left column) or GFP⁺ among CD4⁺ T cells (right column).

(B) Total numbers of CD4⁺ T cells (white bars) and ILC2 cells (black bars) expressing IL-9 (GFP⁺) in medLN as determined by flow cytometry at days 0 and 3 p.i. Data are represented as mean $\pm$ SEM from one out of three independent experiments with 3-4 mice per day p.i.

(C-D) CD4⁺ GFP⁻, CD4⁺ GFP⁺, ILC2 GFP⁻ and ILC2 GFP⁺ cells, isolated from lung (C) or MLN (D) of INFER infected mice at day 10 p.i. were assessed for *Il9* mRNA expression by quantitative RT-PCR. Data represent ratio of IL-9 to *Hprt* expression by the relative quantification method ($\Delta\Delta C_t$). The experiment was repeated three times with similar results using 10 pulled INFER mice.

A



B

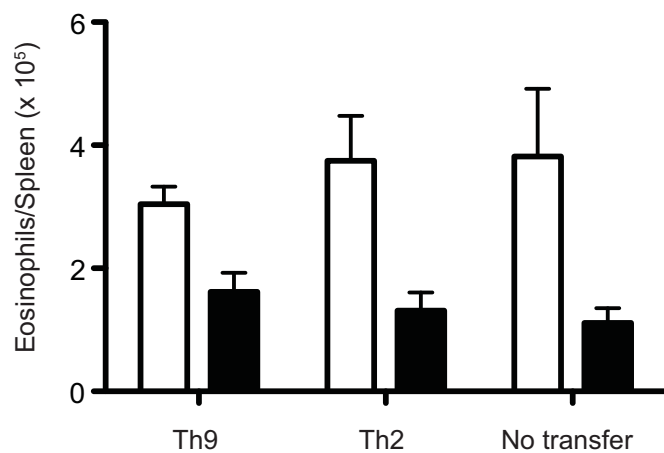
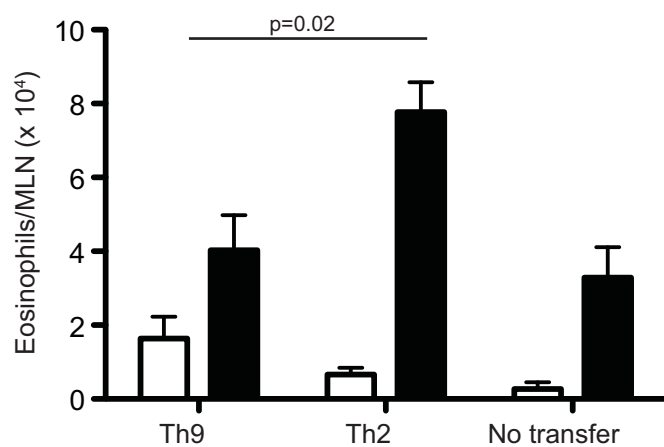
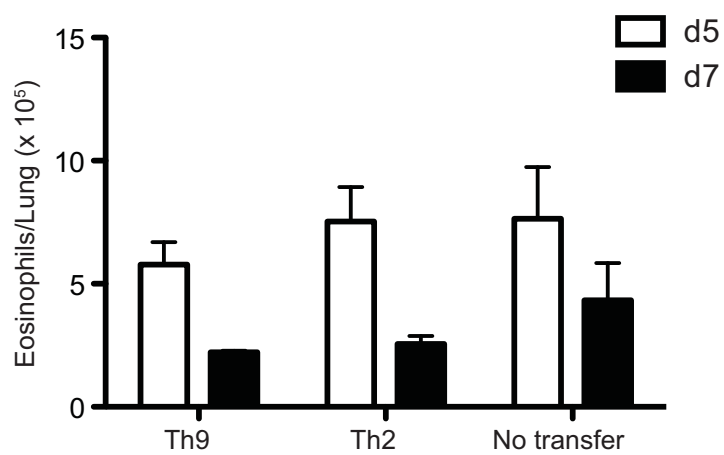


Figure S5. Donor cells and eosinophil quantification in $Rag2^{-/-}$ mice adoptively transferred with Th2 or Th9 cells

(A) Sorted effector Th2 or Th9 (2×10^5) cells ($CD45.2^+GFP^+$) from day 5 *in vitro* cultures with naïve $CD4^+$ T cells isolated from 4get or INFER mice were intravenously transferred into $CD45.1^+ Rag2^{-/-}$ recipient mice. 24 hrs later, mice were subcutaneously infected with 625 L3 *N. brasiliensis* larvae. Total numbers of transferred Th9 or Th2 cells recovered from lung, MLN, and spleen at day 5 (white bars) and day 7 (black bars) p.i. were determined by flow cytometry (gated on $CD45.2^+$).

(B) Total numbers of eosinophils in lung, MLN and spleen of $Rag2^{-/-}$ mice adoptively transferred with effector Th2 or Th9 (2×10^5) cells ($CD45.2^+GFP^+$) at day 5 (white bars) and day 7 (black bars) p.i. As a control a third group of $Rag2^{-/-}$ mice that did not received T cells was also infected.

Data are represented as mean $\pm$ SEM. The experiment was repeated three times with similar results. n= 4-5 mice per group. Statistics were calculated by unpaired two-tailed Student's t test. The p values between groups are shown.

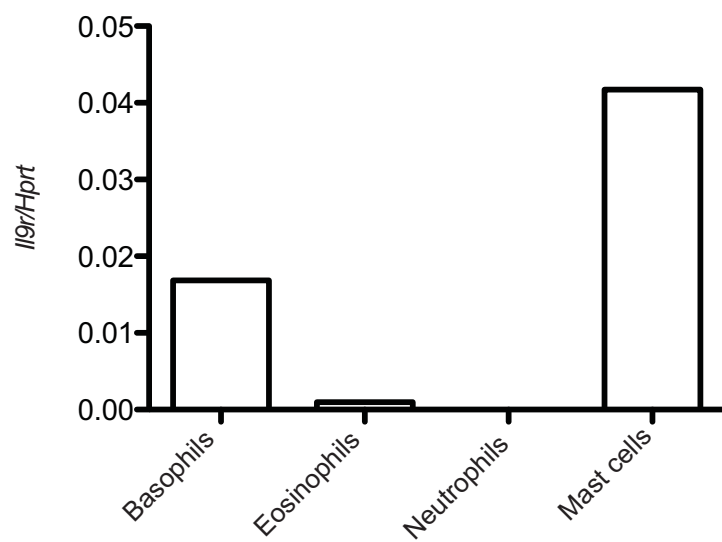


Figure S6. IL-9R expression in sorted populations from *N. brasiliensis* infected lung

Sorted eosinophils (CD11b⁺ SiglecF⁺), basophils (CD49b⁺ FceR⁺ c-kit⁻), neutrophils (Ly6G⁺CD11b⁺) and mast cells (FceR⁺ c-Kit⁺) from *N. brasiliensis* infected lung at day 7 p.i. were assessed for *Il9r* mRNA expression by real-time RT-PCR. Data represent ratio of *Il9r* to *Hprt* mRNA expression by the relative quantification method ($\Delta\Delta Ct$). The experiment was repeated twice with similar results using 5 pulled wildtype C57BL/6 mice.