

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

Sohee Kim, Melanie Prout, Hayley Ramshaw, Angel F. Lopez, Graham LeGros and Booki Min

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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-3Sohee Kim,* Melanie Prout,[†] Hayley Ramshaw,[‡] Angel F. Lopez,[‡] Graham LeGros,[†] and Booki Min*

Basophils are recognized as immune modulators through their ability to produce IL-4, a key cytokine required for Th2 immunity. It has also recently been reported that basophils are transiently recruited into the draining lymph node (LN) after allergen immunization and that the recruited basophils promote the differentiation of naive CD4 T cells into Th2 effector cells. Using IL-3^{-/-} and IL-3R β ^{-/-} mice, we report in this study that the IL-3/IL-3R system is absolutely required to recruit circulating basophils into the draining LN following helminth infection. Unexpectedly, the absence of IL-3 or of basophil LN recruitment played little role in helminth-induced Th2 immune responses. Moreover, basophil depletion in infected mice did not diminish the development of IL-4-producing CD4 T cells. Our results reveal a previously unknown role of IL-3 in recruiting basophils to the LN and demonstrate that basophils are not necessarily associated with the development of Th2 immunity during parasite infection. *The Journal of Immunology*, 2010, 184: 000–000.

Basophils, the least abundant granulocytes found in the circulation, have recently been proposed to be the most important innate source of IL-4 needed for the development of Th2 immunity (1). Such functions are primarily mediated by soluble factors (1–5), which are spontaneously expressed and readily released following activation, and by possible APC functions by which basophils present peptide Ag to naive T cells (6–8). However, for the regulatory roles to be effective, basophils need to be located in close proximity to the Ag-activated T cells in vivo. In support of this notion, it was demonstrated that after allergen immunization circulating basophils transiently enter the T cell zone of

the draining lymph node (LN), promoting Th2 differentiation (5). Therefore, basophil entry into the LN seems to be a critical step for the immune regulation by basophils to occur. However, it remains unclear what recruits circulating basophils into the draining LN during immune responses.

IL-3 has been demonstrated previously to play a key role in basophil development and maturation (9). In support of this finding, it was recently demonstrated that IL-3 induces basophil expansion by promoting granulocyte-monocyte progenitors and basophil-mast cell progenitors to differentiate into basophil lineage progenitors (10, 11). We previously reported that activated T cells are the major source of IL-3, which acts to enhance basophil generation in the bone marrow (BM) during parasite infection (12). Indeed, both basophil generation in the BM and basophil accumulation in the peripheral tissues are significantly impaired in mice deficient in IL-3 (12). However, the maintenance of basal basophil levels is not defective in IL-3^{-/-} mice (9), suggesting that IL-3 action on basophil generation is confined to immune responses.

In this study, we report that IL-3 plays an additional key role in recruiting circulating basophils into the lymphoid tissues. Similar to allergen immunization, circulating basophils were transiently recruited into the draining LN following parasite infection, and the recruitment was completely abolished in the absence of IL-3 or of IL-3 receptor (IL-3R). Paradoxically, wild type (WT) level Th2 immunity still developed in parasite-infected IL-3^{-/-} mice, suggesting that infection induced Th2 immune responses are independent of IL-3 and basophil LN recruitment. In support of this finding, basophil depletion did not abolish infection-induced development of Th2 CD4 T cells. These results suggest that basophil LN recruitment is not necessarily linked to Th2 immunity, suggesting that multiple mechanisms exist for fostering type 2 immune responses in vivo.

*Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195; [†]Malaghan Institute of Medical Research, Wellington, New Zealand; and [‡]Division of Human Immunology, Centre for Cancer Biology, Adelaide, South Australia, Australia

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Address correspondence and reprint requests to Booki Min, Department of Immunology/NB30, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195. E-mail address: minb@ccf.org

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Abbreviations used in this paper: BM, bone marrow; IL-3R, IL-3 receptor; LN, lymph node; medLN, mediastinal lymph node; mLN, mesenteric lymph node; WT, wild type.

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Materials and Methods

Mice

BALB/c and BALB/c Rag2^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). BALB/c IL-3^{-/-} (9) were provided by Dr. Chris Lantz (James Madison University, Harrisonburg, VA). IL-3Rβ^{-/-} mice, deficient in both β_c and β_{IL-3} on a BALB/c background, were generated, screened, and bred as described (13) and maintained in the animal facility of the Lerner Research Institute (Cleveland, OH). G4 knock-in mice expressing GFP under the IL-4 promoter were previously described (14). All experimental procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee.

Parasite infection

Mice were infected s.c. with 500 L3 *Nippostrongylus brasiliensis* larvae as previously reported (15). Basophil recruitment and T cell cytokine production were examined as described below. In indicated experiments, 40 μg MAR-1 or hamster IgG was injected i.v. into mice (prior to infection and day 4 postinfection).

Flow cytometry

LNs and liver cells were examined for basophils. Liver cells were prepared from animals perfused with PBS as previously described (15). Cells were stained with anti-FcγR (clone 93) and anti-CD45 (30-F11). In some experiments, basophils were identified as FcεRIα/CD49b-expressing cells using anti-FcεRIα (MAR1) and anti-CD49b (HMA2). To measure T cell cytokine production, harvested cells were stimulated with 10 ng/ml PMA plus 1 μM ionomycin (Calbiochem, San Diego, CA) for 4 h; 2 μM monensin (Calbiochem) was added to the culture during the last 2 h of culture. Cells were harvested and immediately fixed in 4% paraformaldehyde. Fixed cells were subsequently permeabilized in PBS/0.1% saponin/0.1% BSA buffer, and incubated with anti-CD4 (RM4-5), anti-IL-3 (MP2-8F8), anti-IL-4 (11B11), and anti-IL-13 (eBio13A). All Abs were purchased from eBioscience (San Diego, CA). Samples were acquired using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed using FlowJo (Treestar, Ashland, OR).

BM reconstitution

BM cells collected from the tibia and femur of the donor animals were transferred i.v. into the lethally irradiated (1100 rad) recipients (~10 × 10⁶ cells per recipient); 1 mg gentamycin was injected i.p. into the recipients at days 0 and 2 of BM transfer. Successful BM cell reconstitution was confirmed by FACS analysis before the experiments. Typically, reconstituted mice were used between 6 and 8 wk after BM transfer.

Data analysis

Statistical significance was determined by the Student *t* test using the Prism (GraphPad Software, La Jolla, CA), and *p* < 0.05 indicated a significant difference.

Results and Discussion

IL-3-dependent basophil LN recruitment following parasite infection

Infection with the intestinal nematode *Nippostrongylus brasiliensis* induces robust type 2 immune responses (16), although the mechanisms underlying *N. brasiliensis* that induce Th2 immunity remain elusive. The infection also enhances basophil generation in the BM and subsequent accumulation in the peripheral tissues, including liver, lung, and spleen (15, 17). It was recently reported that circulating basophils are transiently recruited into the draining LNs after s.c. allergen immunization, and these recruited basophils play key roles in the development of allergen-specific type 2 immune responses by primarily producing IL-4 (5). We therefore examined whether such basophil recruitment occurs during *N. brasiliensis* infection and, if so, whether it acts to promote *N. brasiliensis*-induced Th2 immunity in vivo. Our initial attempts to detect basophil accumulation in the draining LNs failed when measured at the peak of the responses (15); however, in this study we examined whether basophil accumulation occurs early during infection as seen during allergen

immunization (5, 8). Draining mediastinal LNs (medLNs) were examined for the presence of basophils at 3, 4, and 10 d postinfection. Basophils were identified as FcγR^{high}CD45^{int} cells as previously reported (1, 2). As seen in allergen and *Schistosoma* egg-induced immune responses (5, 8), basophils were indeed recruited into the medLNs at 3 and 4 d postinfection (222 ± 108 basophils in naive animals and 3738 ± 1796 basophils in *N. brasiliensis*-infected animals at day 4 postinfection; Fig. 1A). The recruitment was transient; thus, almost no basophils remained in the medLNs 10 d postinfection (Fig. 1A). Interestingly, basophils were also recruited into the mesenteric LN (mLN) later during infection (i.e., 10 d postinfection; Fig. 1A). Because the medLNs and mLNs are the major sites of immune responses during early and late infection, respectively (18), these results suggest that the major Ag-draining LNs are the key sites for basophil recruitment.

We recently reported that the IL-3 produced by activated CD4 T cells plays a key role in inducing basophil generation in the BM and the subsequent accumulation in the peripheral tissues (12). To test whether IL-3 also plays a role in basophil LN recruitment, groups of WT and IL-3^{-/-} mice were infected with *N. brasiliensis*, and the medLN was examined for the presence of recruited basophils. To our surprise, basophils failed to enter the medLN in the absence of IL-3 (Fig. 1A). Similarly, recruitment of basophils to the mLN at 10 d postinfection was also abolished in *N. brasiliensis*-infected IL-3^{-/-} mice (Fig. 1A). Because IL-3 is mainly produced by activated T cells (12), this result suggests that basophil LN recruitment requires IL-3 produced by CD4 T cells (17). Indeed, basophil mLN recruitment was observed in the mLNs of *N. brasiliensis*-infected Rag2^{-/-} mice that received WT CD4 T cells, but not in mice that received IL-3^{-/-} CD4 T cells (Fig. 1B). Notably, infection-induced basophil generation in the BM only becomes detectable after 7 d of infection (12); therefore, basophils recruited into the medLN are likely from the preexisting pools in the circulation. Because the basal maintenance of basophils without infection is independent of IL-3 (9), the lack of basophil recruitment in IL-3^{-/-} mice is not due to defects in infection- or IL-3-mediated basophil generation. These results suggest that basophils are recruited into Ag-draining lymphoid tissues and that the recruitment appears to be dependent on T cell activation and IL-3 production.

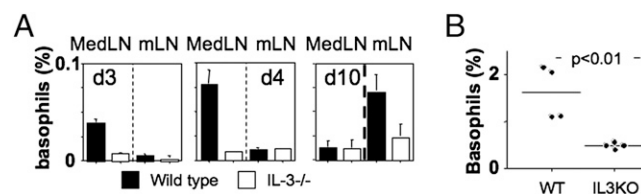


FIGURE 1. Basophil LN recruitment requires IL-3. *A*, Groups (*n* = 4) of WT and IL-3^{-/-} mice were infected with *N. brasiliensis* and sacrificed 3, 4, and 10 d postinfection. MedLN and mLN were examined for basophils (FcγR^{high}CD45^{intermediate}) by FACS. Data represent the mean ± SD of individually tested mice. *B*, WT and IL-3^{-/-} CD4 T cells were transferred (5 × 10⁶ per recipient) into Rag2^{-/-} mice. The recipients were infected with *N. brasiliensis* 14 d after the transfer. Basophil mLN recruitment was determined by FACS analysis 10 d postinfection. Each symbol represents an individual mouse.

Dependence on IL-3R for basophil recruitment

The mechanism by which IL-3 mediates basophil recruitment to the LN is unclear. It was previously reported that IL-3 can induce chemokine and adhesion molecule expression on endothelial cells, enhancing transendothelial migration of human basophils in vitro (19, 20). To directly examine the IL-3 target cells involved in basophil recruitment, we generated BM chimeras using IL-3R $\beta^{-/-}$ mice deficient in both IL-3R β_c and IL-3R β_{IL-3} (13). WT BM cells were transferred into lethally irradiated IL-3R $\beta^{-/-}$ recipients, in which only recipient-derived cells including endothelial cells are IL-3R $\beta^{-/-}$. Alternatively, IL-3R $\beta^{-/-}$ BM cells were transferred into lethally irradiated WT recipients, in which BM derived cells are deficient in IL-3R β but endothelial cells express the receptor. Successful reconstitution was confirmed by measuring IL-3R β_c (CD131) expression of blood cells (data not shown). Groups of reconstituted mice were infected with *N. brasiliensis*, and basophil recruitment into the medLN was examined 4 d postinfection (Fig. 2A). Basophil recruitment was found in *N. brasiliensis*-infected WT BM $\rightarrow$ IL-3R $\beta^{-/-}$ but not in the IL-3R $\beta^{-/-}$ BM $\rightarrow$ WT group, strongly suggesting that IL-3R β expression on the BM-derived cells is necessary for the recruitment (Fig. 2A) and that the IL-3 target cells are of BM origin. Of note, basophil levels in the blood of these BM chimeras were similar prior to infection; therefore, the lack of basophil recruitment to the LN is not a defect of basophil development (Fig. 2B). *N. brasiliensis* infection-induced basophil accumulation in the liver occurred in WT BM $\rightarrow$ IL-3R $\beta^{-/-}$ mice compared with uninfected mice; however, the accumulation was only marginally induced in IL-3R $\beta^{-/-}$ BM $\rightarrow$ WT recipients (Fig. 2C). WT BM $\rightarrow$ WT recipients showed substantial basophil accumulation in the liver, whereas such accumulation was not found in IL-3R $\beta^{-/-}$ BM $\rightarrow$ IL-3R $\beta^{-/-}$ mice (Supplemental Fig. 1). These data

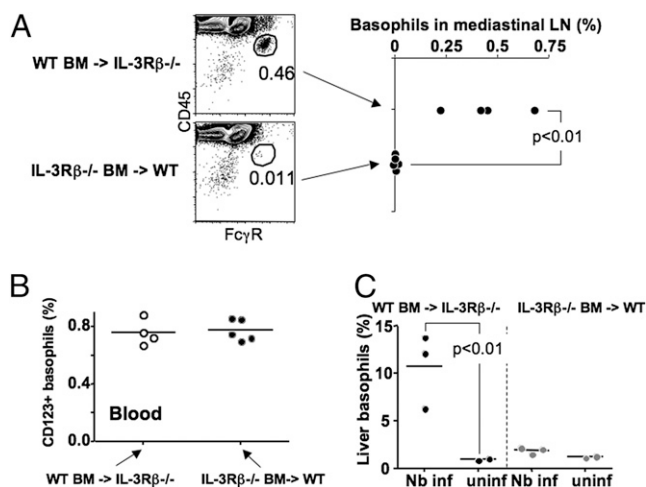


FIGURE 2. IL-3 acts on BM-derived cells to mediate basophil recruitment. WT and IL-3R $\beta^{-/-}$ BM cells were transferred into lethally irradiated IL-3R $\beta^{-/-}$ and WT recipients, respectively. Reconstitution was confirmed by measuring IL-3R β expression. *A*, Mice were infected with *N. brasiliensis*, and medLN was examined for basophils 4 d postinfection. *B*, Blood basophil levels of reconstituted mice were determined prior to *N. brasiliensis* infection. *C*, Mice were infected with *N. brasiliensis*, and liver basophils were examined 10 d postinfection. Uninfected control mice are shown as controls. Each symbol represents an individual mouse.

strongly suggest that the IL-3 target cells involved in basophil LN recruitment are not endothelial cells in vivo.

IL-3 $^{-/-}$ but not IL-3R $\beta^{-/-}$ mice develop *N. brasiliensis*-specific Th2 immunity

The finding that, in the absence of either IL-3 or of IL-3R β , basophils fail to be recruited to the draining LN prompted us to test whether recruited basophils contribute to the T cell immunity in vivo. T cell differentiation was examined in *N. brasiliensis*-infected WT, IL-3 $^{-/-}$, and IL-3R $\beta^{-/-}$ mice. We found that IL-3 $^{-/-}$ mice mounted WT level Th2 responses; CD4 T cells from the medLN and the liver of *N. brasiliensis*-infected IL-3 $^{-/-}$ mice expressed comparable levels of IL-4 and IL-13 (Fig. 3A, 3B, 3D, 3E). In addition, no difference was found in CD4 T cell cytokine production between WT and IL-3 $^{-/-}$ mice when measured 4 d postinfection (Supplemental Fig. 2). By contrast, CD4 T cells from *N. brasiliensis*-infected IL-3R $\beta^{-/-}$ mice failed to develop Th2 type T cell responses. The failure of IL-3R $\beta^{-/-}$ mice to mount Th2 immunity could be partly due to defective T cell activation, because of defective IL-3 production in IL-3R $\beta^{-/-}$ CD4 T cells (Fig. 3C, 3F). Indeed, the frequency of activated phenotype (CD44^{hi}CD62L^{lo}) CD4 T cells in *N. brasiliensis*-infected IL-3R $\beta^{-/-}$ mice was lower than that in WT mice (15.5 ± 1.1 for IL-3R $\beta^{-/-}$ and 30.5 ± 3.8 for WT mice). The frequency of activated phenotype CD4 T cells in naive mice was slightly higher in WT ($\sim 14\%$) than in IL-3R $\beta^{-/-}$ ($\sim 9\%$) mice (data not shown). Of note, the development of Th1 phenotype CD4 T cells is relatively minor during *N. brasiliensis* infection; thus, no major differences in IFN γ + CD4 T cells in *N. brasiliensis*-infected WT and IL-3R $\beta^{-/-}$ mice were noticed (Supplemental Fig. 3). The exact reason for a defective Th2 response in IL-3R $\beta^{-/-}$ mice remains to be examined. T cells may require IL-5 or GM-CSF to become Th2 cells; alternatively, these mice are biased to respond inappropriately. Indeed, it was recently reported that the cells from these mice exhibit defects in activation and recruitment to sites of challenge (13), which may be a direct consequence of being unable to recognize GM-CSF, IL-5, or IL-3, or an effect secondary to limited numbers of dendritic cells known to require GM-CSF or IL-3 for their full development and function. Serum IgE concentration measured at the peak of the responses was found similar to T cell responses:

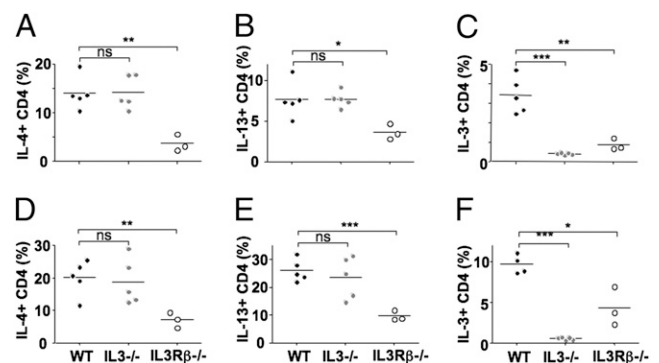


FIGURE 3. *N. brasiliensis* infection-induced Th2 responses are unaffected in IL-3 $^{-/-}$ mice. Groups of WT, IL-3 $^{-/-}$, and IL-3R $\beta^{-/-}$ mice were infected with *N. brasiliensis* and sacrificed 10 d postinfection. MedLN (*A–C*) and liver (*D–F*) cells were stimulated with PMA plus ionomycin and CD4 T cell cytokine production; IL-4 (*A* and *D*), IL-13 (*B* and *E*), and IL-3 (*C* and *F*) were determined by FACS analysis. Each symbol represents an individual mouse. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

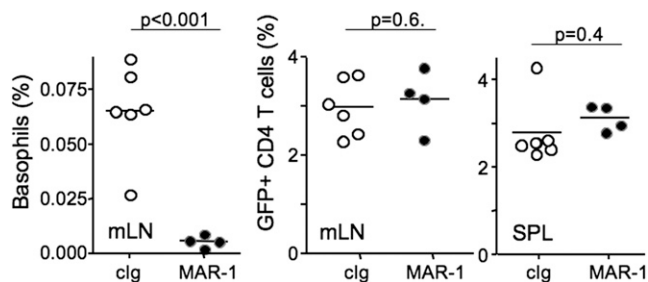


FIGURE 4. Basophil-independent Th2 differentiation in *N. brasiliensis*-infected mice. Groups of G4 mice received 40 μ g MAR-1 i.v. or control Ab before infection. Following *N. brasiliensis* infection, mice were treated with Ab on day 4 postinfection. The basophil levels in mLN as well as GFP expression of splenic and mLN CD4 T cells were determined 10 d post-infection. Each symbol represents an individual mouse.

915 $\pm$ 62 μ g/ml in WT mice, 675 $\pm$ 216 μ g/ml in IL-3^{-/-} mice, and 104 $\pm$ 12 μ g/ml in IL-3R β ^{-/-} mice.

To confirm that basophils are dispensable for *N. brasiliensis*-induced Th2 immunity, we injected GFP/IL-4 (G4) knock-in mice (14) with basophil-depleting Ab, MAR-1 into *N. brasiliensis*-infected mice, and examined the development of GFP (IL-4)-expressing CD4 T cells. As shown in Fig. 4, MAR-1 Ab injection efficiently depleted basophils in the mLN. However, CD4 T cell IL-4 production (GFP expression) was not reduced by the absence of basophils (Fig. 4), further supporting the finding that basophils are dispensable for the development of Th2 immunity following *N. brasiliensis* infection.

The mechanisms underlying the development of in vivo Th2 immune responses remain unclear. IL-4 has been considered the master regulator that promotes Th2 differentiation via activation of STAT6 and GATA3 (21), although there have been several reports showing that Th2 immunity can develop independently of IL-4 under certain circumstances (22, 23). Our study of IL-3^{-/-} mice further provides evidence that basophils are dispensable during *N. brasiliensis*-induced Th2 immunity. This finding is consistent with previous studies showing that non-CD4 T cell-derived IL-4 is not necessary for Th2 differentiation in vivo (5, 22, 24, 25). As recently been reported, the nature of Ag may determine the mechanism of Th2 immune responses (26). For example, parasite-associated Ag may bypass the requirement for IL-4, and further the requirement of basophils, to generate in vivo Th2 immune responses. Further investigation will be necessary to examine this possibility.

In vitro stimulation of endothelial cells with IL-3 has been demonstrated to induce selective transmigration of human basophils (20, 27, 28). However, our data show that in vivo the cellular target of IL-3 is of BM origin. Further investigation will be required to identify these targets. Chemokines including CCL11 and CCL2 were shown to mediate basophil migration (20, 29). Adhesion molecules such as β 2 integrin, P-selectin, and CD49d have been demonstrated to induce basophil rolling and adhesion (27, 28). Whether these mechanisms operate during IL-3-dependent basophil recruitment to the LN will be an important area of investigation.

The roles of IL-3 in basophil biology seem to be manifold (9, 10, 12, 30) and now include the recruitment of basophils into the draining LN. Given that IL-3 is mainly produced by activated T cells, these results imply the requirement of adaptive immunity for the basophil responses to develop (12, 17). Un-

raveling the cellular mechanisms will prove useful in developing therapeutic approaches to inhibit basophil recruitment into effector sites. Moreover, our data also demonstrate that Th2 immune responses may develop in a basophil-independent manner and that cautious analysis of each Th2 immune response will be necessary.

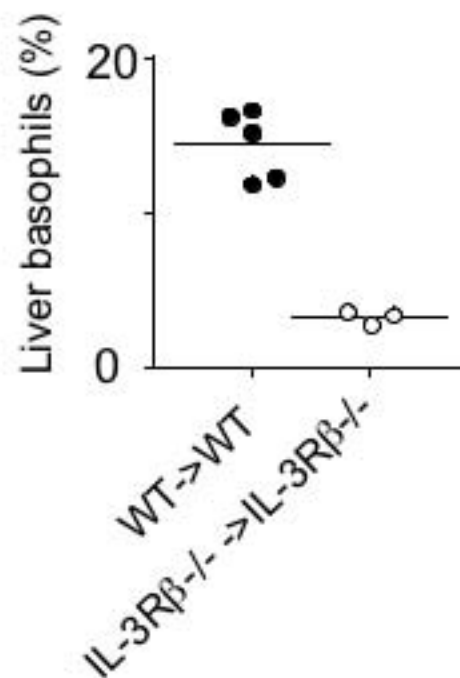
Disclosures

The authors have no financial conflicts of interest.

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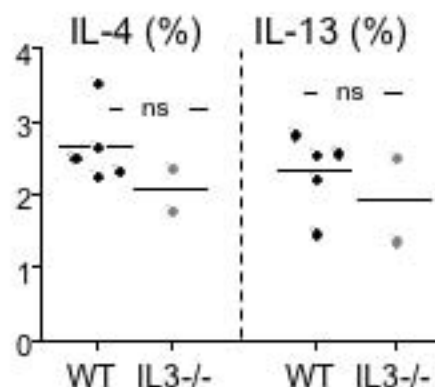
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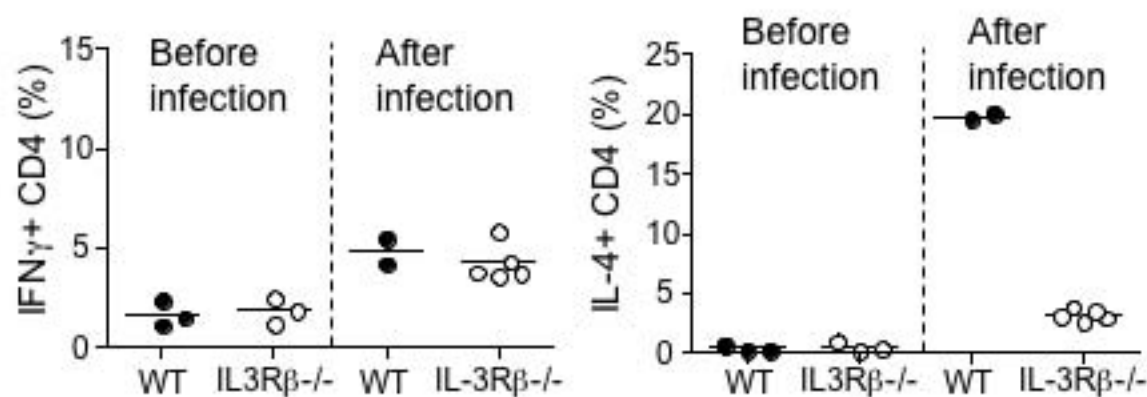
Supplementary Fig 1. Basophil accumulation in the liver of BM control mice.

WT and IL-3R β ^{-/-} BM cells were transferred into lethally irradiated WT and IL-3R β ^{-/-} recipients, respectively. After 6 weeks of reconstitution, the mice were infected with Nb. Basophil accumulation in the liver was determined by FACS analysis on day 10 post infection. Each symbol represents individual mouse.



Supplementary Fig 2. CD4 T cell Th2 cytokine expression in Nb infected WT and IL-3-/- mice.

Groups of WT and IL-3-/- mice were infected with Nb. Draining medLN cells were collected 4 days post infection, ex vivo stimulated with PMA/ionomycin, and stained for intracellular IL-4 and IL-13. Shown are the frequencies of cytokine+ CD4 T cells. Each symbol represents individual mouse. ns, not significant.



Supplementary Fig 3. CD4 T cell cytokine expression in Nb infected WT and IL-3R β ^{-/-} mice.

Groups of WT and IL-3R β ^{-/-} mice were infected with Nb. mLN cells were collected 10 days post infection, ex vivo stimulated with PMA/ionomycin, and stained for intracellular IL-4 and IFN γ . Shown are the frequencies of cytokine+ CD4 T cells. Each symbol represents individual mouse.