

Basophils Orchestrate Chronic Allergic Dermatitis and Protective Immunity against Helminths

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SUMMARY

Basophils are associated with T helper 2 (Th2) cell-polarized immune responses such as allergic disorders or helminth infections. To directly address the role of basophils for type 2 immunity, we generated transgenic mice with constitutive and selective deletion of basophils. Differentiation and accumulation of Th2 cells, induction of eosinophilia, and increase in serum IgE or IgG1 induced by allergens or by infection with the helminth *Nippostrongylus brasiliensis* appeared to be basophil independent. Further, basophils were not required for passive IgE- or IgG1-mediated systemic anaphylaxis. However, basophils were essential for IgE-mediated chronic allergic dermatitis and for protection against secondary infection with *N. brasiliensis*. These results demonstrate that basophils play an important role for protective immunity against helminths and orchestrate chronic allergic inflammation, whereas primary Th2 cell responses can operate efficiently in the absence of this cell type.

INTRODUCTION

Type 2 immunity unfolds by concerted activation of innate and adaptive effector mechanisms in response to allergens or infection with helminth parasites. T helper 2 (Th2) cells are generally considered to be essential for orchestration of type 2 immune responses. However, increasing evidence suggests that cells of the innate immune system like mast cells, eosinophils, and basophils can at least in part contribute to protection against helminths and execute proinflammatory effector functions during allergic responses. Mast cells and basophils are functionally closely related since both cell types express the high-affinity receptor for IgE (FcεRI) and release a similar set of effector molecules including histamine, lipid mediators, and Th2-associated cytokines such as IL-4, IL-5, and IL-13. Despite their functional similarity, it remains unclear whether both lineages develop from a common precursor in vivo (Arinobu et al., 2009). Mast

cells leave the bone marrow as immature cells and undergo final differentiation to long-lived connective tissue mast cells (CTMCs) in the skin or to mucosal mast cells (MMC) in mucosal tissues. In contrast, basophils leave the bone marrow as mature cells with a lifespan of ~60 hr (Ohnmacht and Voehringer, 2009a). They can be transiently recruited into tissues in which they mediate their effector functions (Min et al., 2004; Voehringer et al., 2004). The role of basophils for type 2 immunity remains poorly defined, although considerable progress has been made over the past 3 years.

Karasuyama and colleagues demonstrated that antibody-mediated depletion of basophils from mice attenuates the late phase of chronic allergic dermatitis (Obata et al., 2007). They further showed that basophil-depleted mice are resistant to IgG-mediated passive anaphylaxis (Tsumimura et al., 2008). Others identified basophils as antigen-presenting cells for induction of Th2 cells in mice injected with IgE immune complexes, the proallergic protease papain or *Schistosoma mansoni* eggs (Perigou et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). In addition, basophils can rapidly release IL-4 and enhance memory Th2 cell responses in vivo (Khodoun et al., 2004). IL-4 and IL-6 from basophils have further been shown to promote the humoral memory response against *Streptococcus pneumoniae* (Denzel et al., 2008). Basophils further contribute to protective immunity against helminths (Voehringer, 2009).

Despite great advances in our understanding of in vivo functions of basophils over the past few years, one should bear in mind that currently used basophil-depleted mouse models are based on antibody-mediated depletion strategies using FcεRIα, CD200R3, or Thy1 as target molecules. To overcome potential bystander effects associated with antibody-mediated cell depletion like off-target binding, activation of complement, and stimulation of mast cells or macrophages, we sought to analyze type 2 immune responses in a newly developed genetically basophil-deficient mouse strain. To this end, we engineered bacterial artificial chromosome (BAC)-transgenic mice that constitutively lack more than 90% of basophils compared to control mice. Basophils were not required for in vivo priming of Th2 cells, induction of passive systemic anaphylaxis and allergen, or *N. brasiliensis*-induced type 2 immunity. However, basophils played an essential role for IgE-mediated chronic allergic inflammation (IgE-CAI) and protective immunity against secondary helminth infection.

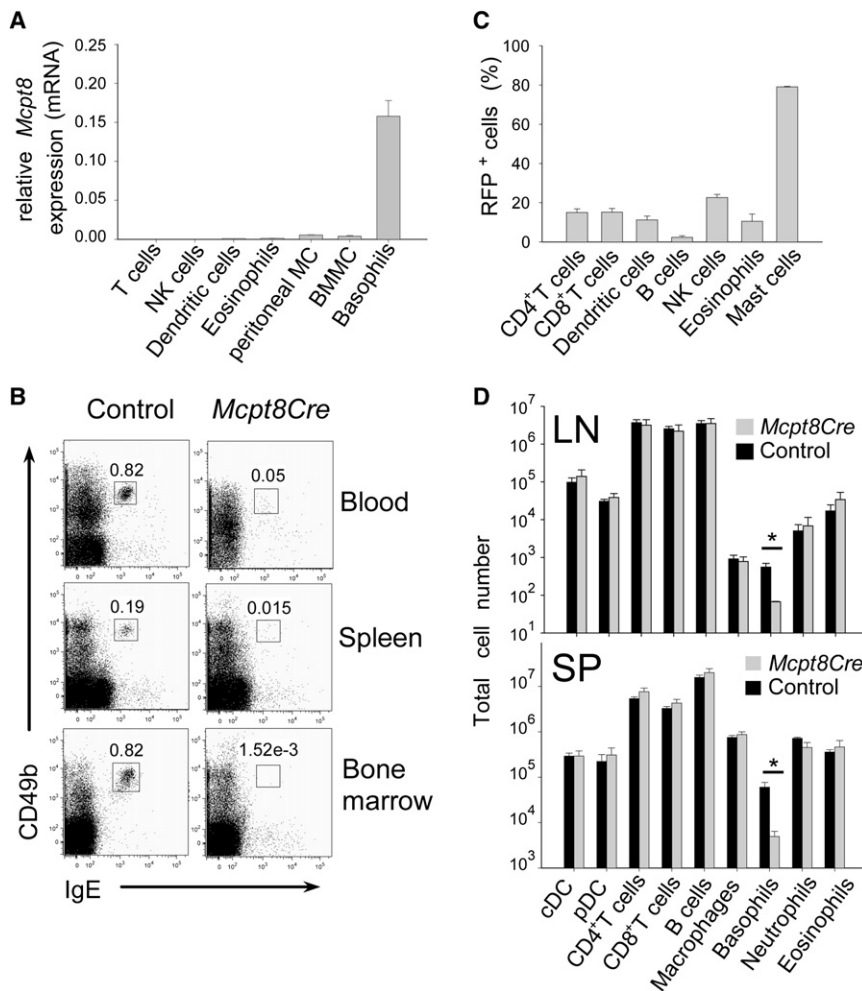


Figure 1. *Mcpt8Cre* Mice Are Constitutively Depleted of Basophils

(A) Relative expression of *Mcpt8* mRNA normalized to β -actin mRNA of indicated cell types analyzed by quantitative RT-PCR. Cells were isolated by sorting from bone marrow cultures (bone marrow-derived mast cells (BMMC) and basophils), the peritoneum (peritoneal MC), or the spleen (other cell types) of naive C57BL/6 mice.

(B) Blood, spleen, and bone marrow cells isolated from *Mcpt8Cre* or control mice were stained for CD49b and IgE to detect basophils. Numbers indicate the frequency of basophils among all leukocytes.

(C) *Mcpt8Cre* mice were crossed to *ROSAtdRFP* mice for identification of cells that had expressed *Mcpt8Cre* at some point during their development. Bars show the mean frequency + standard deviation (SD) of RFP-expressing cells among splenic CD4⁺ T cells, CD8⁺ T cells, DCs, B cells, NK cells, eosinophils, and peritoneal mast cells of four mice. (D) Bars show the mean + standard error (SE) of total number of indicated cell types in the spleen (SP) and mesenteric lymph nodes (LN) of four *Mcpt8Cre* mice (gray bars) and four negative littermates (control; black bars) from two independent experiments. **p* < 0.05.

ing from nonspecific recombination events of cryptic loxP sites in the mammalian genome. Cre toxicity has been described in various cell types and may occur when the expression of Cre is very high (Schmidt-Suppran and Rajewsky, 2007). To further determine the specificity of Cre expression in *Mcpt8Cre*

RESULTS

Selective Deletion of Basophils in *Mcpt8Cre* BAC Transgenic Mice

In order to address the role of individual genes in basophils for regulation of Th2 cell-associated immune responses, we constructed BAC-transgenic mice that express the Cre recombinase under control of regulatory elements for the mast cell protease 8 (*Mcpt8*) gene, which appears to be expressed in basophils but not in bone marrow-derived mast cells or ex vivo isolated mature T cells, natural killer (NK) cells, dendritic cells (DCs), or eosinophils (Ohnmacht and Voehringer, 2009a; Poorafshar et al., 2000) (Figure 1A and Figure S1 available online). Unexpectedly, more than 90% of basophils were spontaneously deleted in *Mcpt8Cre* mice as determined by double staining with anti-CD49b and anti-IgE (Figure 1B) or anti-CD49b and anti-CD200R3 in two independent founder lines, which contained five or seven copies of the BAC construct per genome (Figures S1 and S2). Basophilia could be induced in wild-type but not *Mcpt8Cre* mice by transfer of the IL-3-secreting cell line WEHI-3 (Figure S3). This demonstrates that basophil development in *Mcpt8Cre* mice could not be rescued by exogenous IL-3 and rather indicates that basophils are sensitive to Cre toxicity result-

mice and to trace cell lineages that might transiently express the *Mcpt8* gene during their development, we crossed them to *ROSAtdRFP* mice that contain a Cre-inducible conditional allele of the red fluorescent protein (RFP) in the ubiquitously expressed *ROSA26* locus (Luche et al., 2007). RFP was expressed in ~15% of peripheral T cells, NK cells, DCs, and eosinophils, suggesting that the *Mcpt8* gene was transiently expressed in a few precursor cells of these lineages, although this phenomenon was not observed in the B cell lineage (Figure 1C). The deletion of basophils in *Mcpt8Cre* mice did not affect other leukocyte populations in mesenteric lymph nodes and spleen (Figure 1D). Furthermore, *Mcpt8Cre* mice showed no defects in T cell development or basal T cell activation (Figure S4). Interestingly, ~80% of peritoneal mast cells were RFP positive in *Mcpt8Cre-ROSAtdRFP* double transgenic mice (Figure 1C), although *Mcpt8* mRNA was expressed at very low levels in these cells (Figure 1A), suggesting that *Mcpt8* was only transiently expressed in a yet to be defined precursor population of peritoneal mast cells. In contrast to basophils peritoneal and mucosal mast cells were not deleted in *Mcpt8Cre* mice (Figures 2A and 2B). *Mcpt8Cre* mice showed a normal response to induction of active systemic anaphylaxis (Figure 2C). In addition, passive systemic IgE-mediated anaphylaxis appeared normally in *Mcpt8Cre* mice

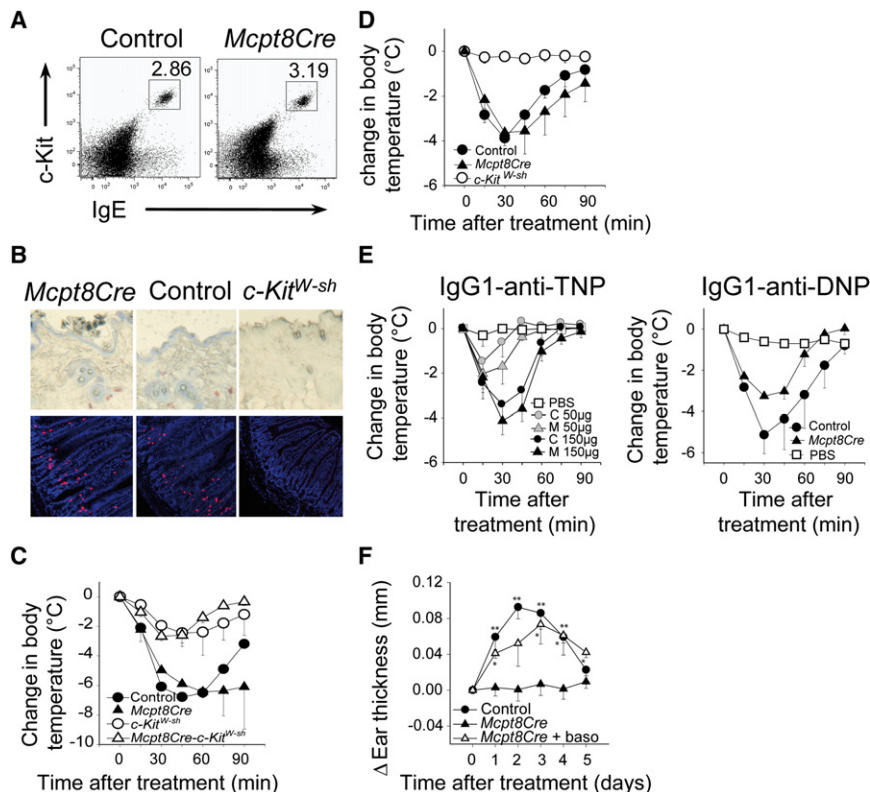


Figure 2. Basophils Are Required for Chronic Allergic Dermatitis but Not for Anaphylaxis

(A) Dot plots show the frequency of peritoneal mast cells (c-Kit⁺IgE⁺).

(B) As shown in the upper panel, Toluidin-blue staining in tissue sections from the dorsal skin of indicated mice. Mast cells (pink) are present in *Mcpt8Cre* and control mice but not in *c-Kit^{W-sh}* mice. The lower panel shows immunofluorescence staining for mMCP-1 (red) and DAPI (blue) of tissue sections from the small intestine at day 10 after primary *N. brasiliensis* infection to detect mucosal mast cells. Original magnification was 80 $\times$.

(C–E) Active (C), passive IgE-mediated (D), and passive IgG1-mediated (E) systemic anaphylaxis was induced in control (filled circles), *Mcpt8Cre* (filled triangle), *c-Kit^{W-sh}* (open circle), and *Mcpt8Cre-c-Kit^{W-sh}* (open triangle) mice and PBS-treated controls as described in the **Experimental Procedures**. Changes in rectal temperature after allergen challenge are shown. Data in (C) show the mean $\pm$ SD from two to three mice per group from one experiment. Data in (D) show the mean $\pm$ SE from five controls, three *Mcpt8Cre*, and three *c-Kit^{W-sh}* mice from three experiments. Data in (E) for anti-TNP anaphylaxis show the mean $\pm$ SE from three controls (C, circles) and three *Mcpt8Cre* mice (M, triangles) with 50 μ g (gray) or 150 μ g (black) mAb and two PBS-treated mice. Results for anti-DNP anaphylaxis are based

on three controls, three *Mcpt8Cre* mice and one PBS control. No significant differences were observed between *Mcpt8Cre* and control mice.

(F) IgE-mediated chronic allergic dermatitis. *Mcpt8Cre* mice (filled triangles), basophil-reconstituted *Mcpt8Cre* mice (open triangles), or control mice (circles) were sensitized i.v. with anti-TNP IgE and challenged in the left ear with TNP-OVA. The graph shows the difference in ear thickness between left and right ear. Data show the mean $\pm$ SE from seven *Mcpt8Cre* mice, three reconstituted *Mcpt8Cre* mice, and five controls from two experiments. * $p < 0.05$; ** $p < 0.01$ for control or reconstituted *Mcpt8Cre* mice versus non-reconstituted *Mcpt8Cre* mice.

(Figure 2D). We also tested two different IgG1 antibodies for their capacity to induce passive IgG1-mediated anaphylaxis and observed that both clones could induce anaphylaxis in *Mcpt8Cre* and control mice (Figure 2E). However, IgE-mediated chronic allergic inflammation of the skin (IgE-CAI) was abolished in basophil-ablated *Mcpt8Cre* mice (Figure 2F). Importantly, reconstitution of *Mcpt8Cre* mice with in vitro-generated basophils restored the IgE-CAI response, which demonstrates that basophils were required and sufficient to mediate the ear-swelling response (Figure 2F). This finding provides direct functional evidence for the loss of basophils in *Mcpt8Cre* mice and is consistent with a previous report that demonstrated that basophils play an essential role for IgE-CAI independently of mast cells and Th2 cells (Mukai et al., 2005). We conclude that the *Mcpt8Cre* transgenic construct causes selective deletion of basophils but not mast cells and that basophils are indeed essential for IgE-CAI, whereas they probably play no major role for active or passive systemic anaphylaxis.

Papain-Induced T Cell Proliferation and Th2 Cell Differentiation Depends on DCs and Not on Basophils

Recent reports by Sokol et al. demonstrated that injection of the protease papain into the footpad of mice results in transient recruitment of basophils into draining lymph nodes and Th2 cell differentiation. Papain-induced Th2 cell differentiation could be blocked by depletion of basophils with the Fc ϵ R1 α -specific

antibody MAR-1 (Sokol et al., 2008). Furthermore, DCs were found to play a minor role as APCs for Th2 cell polarization in vivo since selective expression of MHC-II on DCs was not sufficient for papain-induced Th2 cell polarization (Sokol et al., 2009). To determine whether these findings can be reproduced in another experimental setting, we directly compared the efficiency of papain-induced Th2 cell polarization in basophil-ablated or DC-ablated mice. To better visualize basophils and Th2 cells, we crossed *Mcpt8Cre* mice to *Il4eGFP* reporter mice (4get mice). As expected, basophils accumulated in draining lymph nodes of papain-injected 4get control mice but not in *Mcpt8Cre*-4get mice (Figure 3A). However, comparable frequencies and total numbers of Th2 cells were observed in both 4get and *Mcpt8Cre*-4get mice (Figure 3B). This demonstrates that papain can induce Th2 cells in the absence of basophils. To further address the requirement of basophils and DCs for papain-induced Th2 responses, we adoptively transferred OT-II cells into basophil-ablated *Mcpt8Cre*-4get mice, DC-ablated Δ DC mice or nontransgenic controls and immunized the mice with OVA-papain. Proliferation and Th2 cell differentiation was pronounced in control mice and *Mcpt8Cre*-4get mice. However, OT-II cells proliferated poorly and did not differentiate into Th2 cells in DC-ablated Δ DC mice (Figures 3C–3E). Importantly, Δ DC mice contained normal numbers of basophils and papain induced recruitment of basophils to draining lymph nodes was not impaired in these mice (Figures S2 and S5). The

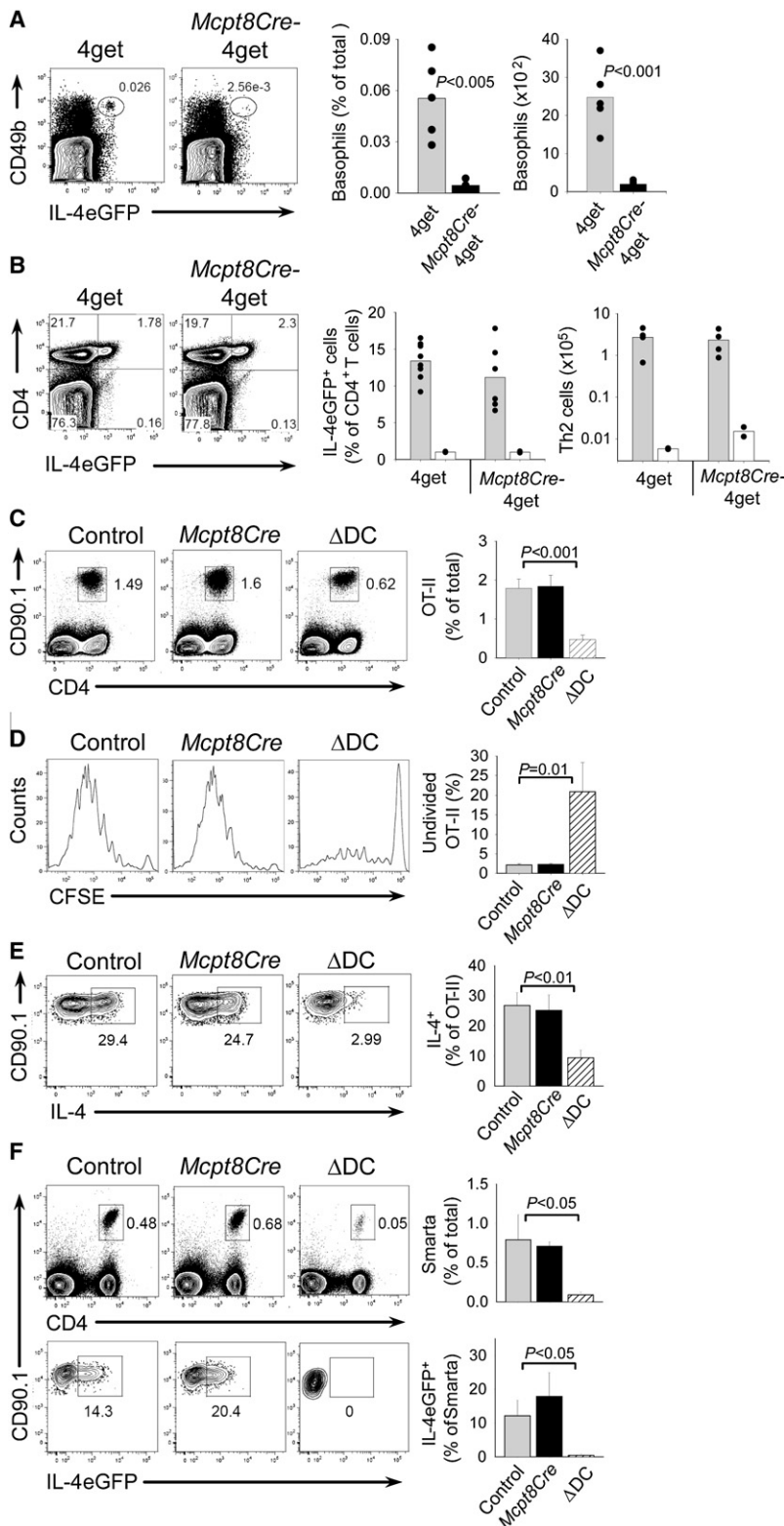


Figure 3. Papain-Induced T Cell Proliferation and Th2 Cell Differentiation Is Dependent on Dendritic Cells but Not Basophils

(A) Dot plots show the frequency of basophils (IL-4eGFP⁺CD49b⁺) in the popliteal lymph node 3 days after injection of papain into the foot of 4get or *Mcpt8Cre*-4get mice. Bars show the mean frequency and total number of basophils of five *Mcpt8Cre* mice (black bar) and five 4get mice (gray bar). Circles show values of individual mice.

(B) Dot plots show the frequency of Th2 cells (CD4⁺IL-4eGFP⁺) in the popliteal lymph node 4 days after papain administration. Bars show the mean frequency (left) or total number (right) of Th2 cells in the draining lymph node (gray bars) of eight or four *Mcpt8Cre*-4get mice and six or four 4get mice, respectively, from two experiments. Values from the contralateral lymph node from two mice per group are shown for comparison (white bars).

(C) Expansion of OVA-specific OT-II-CD90.1 cells in popliteal lymph nodes 4 days after transfer into control, *Mcpt8Cre* or DC-deficient Δ DC mice, and OVA-papain immunization. The dot plots show the frequency of transferred OT-II cells (CD4⁺CD90.1⁺) among total lymphocytes. The bars show the mean frequency +SE of transferred OT-II cells in 15 control mice, 12 *Mcpt8Cre* mice, and 5 Δ DC mice from 5 experiments.

(D) Histograms show the CFSE profile of OT-II cells 4 days after transfer and OVA-papain immunization in indicated recipient mice. Bars show the mean + SE of undivided OT-II cells in six control mice, six *Mcpt8Cre* mice and four Δ DC mice from three experiments.

(E) Dot plots show intracellular staining for IL-4 on gated OT-II cells (CD4⁺CD90.1⁺) on day 4 after OVA-papain immunization. Bars show the mean + SE of IL-4⁺ OT-II cells in 11 control mice, 9 *Mcpt8Cre* mice, and 8 Δ DC mice from 4 experiments.

(F) Expansion (upper panel) and Th2 cell polarization (lower panel) of transferred Smarta-4get-CD90.1 T cells on day 4 after LCMV-GP₆₁₋₈₀/papain immunization. Dot plots are gated on live cells (upper panel) or CD4⁺CD90.1⁺ cells (lower panel). Bars show the mean + SD of three mice per group from one experiment. p values were calculated by Student's t test.

for lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) peptide GP₆₁₋₈₀ (Figure 3F).

We conclude from these experiments that DCs are required to induce a Th2 cell response upon papain stimulation, whereas basophils play only a minor role. Given that our results are in apparent contrast to the report by Sokol et al. (2008), we thought that injection of the Fc ϵ R1 α -specific antibody (MAR-1) might cause bystander effects besides the depletion of basophils. We observed that in our hands, a single injection of 100 μ g MAR-1 induced efficient basophil depletion but also systemic anaphylaxis and increased serum concentrations of mast cell protease 1 (mMCP-1) in

basophil-independent Th2 cell differentiation was further confirmed in another system by transfer of purified Smarta-4get-CD90.1 T cells, which express a transgenic TCR specific

addition to 4-fold increased numbers of neutrophils in the spleen (Figure S6). These effects may in part explain the apparent difference between both models.

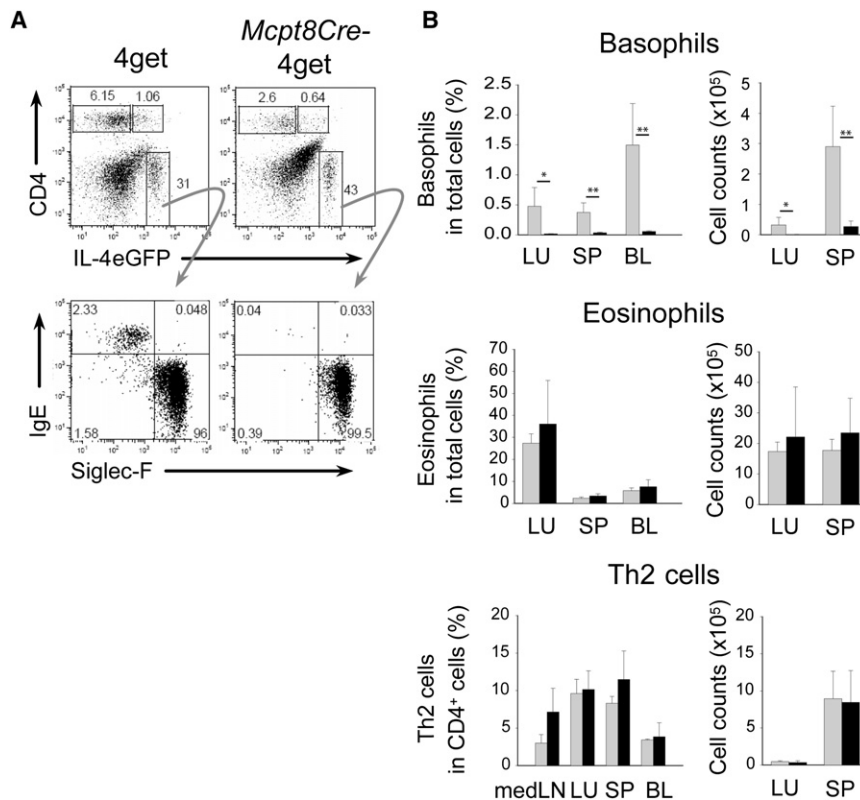


Figure 4. Basophils Do Not Contribute to OVA-Induced Airway Inflammation

(A) Detection of Th2 cells (CD4⁺IL-4eGFP⁺), eosinophils (CD4⁺IL-4eGFP⁺Siglec-F⁺IgE⁺), and basophils (CD4⁺IL-4eGFP⁺Siglec-F⁺IgE⁺) in total lung tissue 3 days after intranasal challenge of OVA-alum-sensitized mice. Numbers indicate the frequency of gated cells. The lower dot plots are gated on CD4⁺IL-4eGFP⁺ cells as indicated by arrows.

(B) Frequency (left) and absolute number (right) of basophils, eosinophils and Th2 cells in lung (LU), spleen (SP), blood (BL) and mediastinal lymph node (medLN) of 4get mice (gray bars) or *Mcpt8Cre-4get* mice (black bars). Bars show the mean + SD from five *Mcpt8Cre-4get* mice and three 4get mice. **p* < 0.05; ***p* < 0.01.

Basophils Are Not Required for Orchestration of Acute OVA-Alum-Induced Allergic Inflammation of the Lung

Basophils, eosinophils and Th2 cells are known to accumulate during allergic inflammatory responses in the lung. Since basophils produce large amounts of pro-inflammatory cytokines they might be involved in orchestration of the inflammatory immune response in the lung. To address this possibility, we immunized normal 4get or basophil-ablated *Mcpt8Cre-4get* mice with OVA-alum and challenged the mice 2 weeks later by intranasal application of OVA. The inflammatory infiltrate of the lung was analyzed 3 days later by flow cytometry. Basophils in lung, spleen, and blood were reduced by more than 90% in *Mcpt8Cre-4get* mice as compared to wild-type 4get mice, whereas eosinophils and Th2 cells were present at equal levels in these organs in both mice (Figures 4A and 4B).

The Humoral Immune Response to OVA-Alum Is Not Impaired in Basophil-Deficient Mice

It has been shown that mice depleted of basophils by anti-FcεRIα treatment develop poor humoral immune responses (Denzel et al., 2008). The authors could further demonstrate by in vitro experiments that IL-4 and IL-6 from basophils instruct T cells to provide efficient help for B cell activation. To address whether humoral immunity was impaired in our model, we first analyzed the concentration of different immunoglobulin isotypes in the serum of naive mice and observed no difference between *Mcpt8Cre* or control mice (Figure 5A). Next, we immunized *Mcpt8Cre* or control mice intraperitoneally (i.p.) with OVA-alum, challenged the mice 14 days later by injection of OVA,

and determined antigen-specific antibodies in the serum at different days after challenge by ELISA. The alum adjuvant is well known to promote Th2 cell-polarized immune responses in mice. Therefore, we expected increased concentrations of OVA-specific IgE and IgG1 antibodies given that both isotypes are induced by IL-4 or IL-13. The kinetics of OVA-specific IgE and IgG1 in the serum were comparable between *Mcpt8Cre* and control mice, which demonstrates that efficient humoral immune responses can develop in the absence of basophils (Figure 5B).

The Primary Immune Response against *N. brasiliensis* Appears to Be Normal in Basophil-Deficient Mice

Infection of mice with the helminth *N. brasiliensis* induces a strong type 2 immune response characterized by high serum IgE concentrations and pronounced accumulation of basophils, eosinophils, and Th2 cells in the lung and small intestine. Here, we determined the accumulation of these cells in the lung, mediastinal lymph nodes, mesenteric lymph nodes, and blood of 4get and *Mcpt8Cre-4get* mice at different time points during infection (Figures 6A–6C and Figure S7). *N. brasiliensis* infection induced accumulation of basophils in lung, blood, and lymph nodes in 4get but not in *Mcpt8Cre-4get* mice. Importantly, the numbers of eosinophils and Th2 cells were not significantly different at any time in these organs in both mouse strains. We further observed no difference in early neutrophil recruitment to draining lymph nodes between *Mcpt8Cre* and wild-type mice when L3 larvae were injected into the ear as described by Pesce et al. (2008) (Figure S8). Serum IgE was also comparable and both mice had eliminated all adult worms from the small intestine by day 10 after infection (Figures 6D and 6E). This demonstrates that basophils are dispensable for orchestration of a primary immune response to *N. brasiliensis*.

The Protective Memory Response against *N. brasiliensis* Is Impaired in Basophil-Deficient Mice

Basophils express activating receptors for IgE and IgG on the cell surface and can be sensitized with parasite-specific

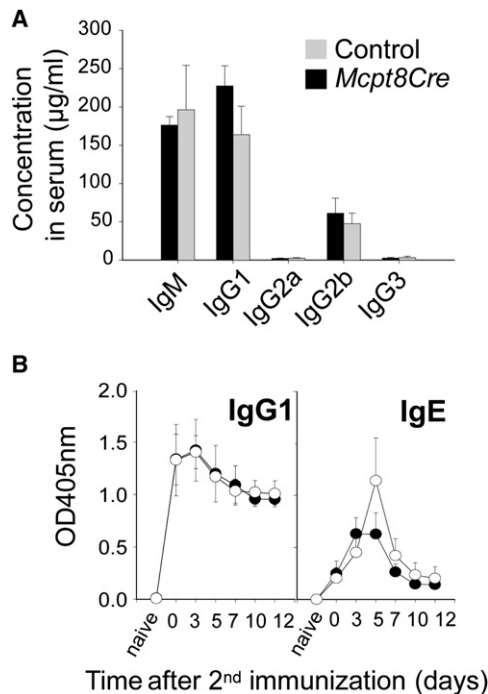


Figure 5. Humoral Immune Response to OVA-Alum

(A) Serum concentration of different immunoglobulin isotypes in naive *Mcpt8Cre*-4get mice (black bars) and 4get control mice (gray bars). Bars show the mean + SD of four mice per group from two experiments. (B) The graphs show OVA-specific antibodies in serum of *Mcpt8Cre*-4get mice (open symbols) or 4get control mice (filled symbols) before immunization (naive) and at indicated time points after secondary OVA administration. Data show the mean + SD from four *Mcpt8Cre*-4get mice and three 4get mice.

antibodies that are generated during the first infection with *N. brasiliensis* (Ohnmacht and Voehringer, 2009a). Because basophils can rapidly produce large amounts of IL-4, they might enhance expansion or de novo polarization of Th2 cells, promote eosinophilia, and contribute to efficient worm expulsion during secondary infection with *N. brasiliensis*. Interestingly, protection against secondary infection with *N. brasiliensis* operates independently of memory CD4⁺ T cells (Katona et al., 1988). Worm expulsion requires IL-4 or IL-13, which can be produced by basophils in large quantities. Therefore, basophils could be involved in protective immunity against this parasite. To directly address this possibility, we infected 4get and *Mcpt8Cre*-4get mice twice with *N. brasiliensis* at a 4 week interval. Effector cell recruitment, the amount of IgE, and worm expulsion were measured on day 5 after the second infection. This time point was chosen because it takes ~4 days before the larvae appear in the small intestine, if they are not eliminated before they reach this organ. Further, it has been shown that a few worms can be detected at this time point but not later during secondary infection of wild-type mice (Harris et al., 1999). Basophil-ablated *Mcpt8Cre*-4get mice showed no defect in Th2 cell and eosinophil recruitment to the lung (Figure 7A). Th2 cells were also increased to similar extent in the lung draining mediastinal lymph nodes in both *Mcpt8Cre*-4get and 4get control mice, whereas eosinophils were reduced in *Mcpt8Cre*-4get mice, suggesting

that basophils contribute to recruitment or survival of eosinophils in this tissue (Figure 7A). However, Th2 cells and eosinophils did not accumulate in the mesenteric lymph nodes, suggesting that the immune response is mainly localized to the lung. Serum IgE (Figure 7B) and mucosal mastocytosis (Figure 7C) were also comparable between both groups of mice. However, *Mcpt8Cre*-4get mice showed impaired worm expulsion, which demonstrates that basophils contribute to protective immunity during the first 4 days after secondary infection before the larvae reach the intestines (Figure 7D).

DISCUSSION

We have studied the role of basophils for immune responses against allergens and parasites with newly developed genetically ablated basophil-deficient mice. Basophils were essential for induction of IgE-mediated chronic allergic inflammation of the skin (IgE-CAI). This finding confirms a previous report that demonstrated that transient depletion of basophils with the anti-CD200R3 mAb Ba103 results in protection from IgE-CAI (Obata et al., 2007). Although basophils constitute only a small minority of infiltrating cells of the skin during IgE-CAI they are required for orchestration of the inflammatory response including tissue swelling and recruitment of eosinophils and neutrophils (Mukai et al., 2005). Pronounced basophil recruitment to the dermis has been observed already 40 years ago during induction of cutaneous basophil hypersensitivity (CBH) in guinea pigs that were sensitized with protein antigens in incomplete Freund's adjuvant (Dvorak et al., 1970). This indicates that basophils might contribute to dermatitis in other inflammatory settings. Future experiments will hopefully lead to identification of basophil-derived factors that mediate inflammation of the skin. The constitutive lack of basophils in *Mcpt8Cre* mice should allow us to reconstitute these mice with basophils from different gene-targeted mice to further dissect the mechanism(s) by which basophils mediate chronic allergic inflammation. IL-4 and IL-13 are potential candidates given that recruitment of eosinophils into tissues is at least in part mediated by the eotaxin-family of chemokines that are induced by IL-4 and/or IL-13 (Zimmermann et al., 2003). It seems plausible that basophils are not only required for allergic inflammation of the skin but might also contribute to chronic allergic inflammation of other tissues including lung and intestines. Indeed, infiltrating basophils have been detected in cases of chronic asthma (Macfarlane et al., 2000; Nouri-Aria et al., 2001). Passive systemic anaphylaxis (PSA) can be induced in mice by a classical pathway that depends on IgE-mediated mast cell activation and release of histamine or by two alternative pathways that operate by IgG1-mediated release of platelet-activating factor (PAF) from basophils or macrophages (Strait et al., 2002; Tsujimura et al., 2008). The relative contribution of basophils and macrophages for the alternative pathway remains unclear (Mukai et al., 2009). Our experiments with two different clones of IgG1 antibodies demonstrate that IgG1-mediated PSA appeared to be normal in basophil-ablated *Mcpt8Cre* mice, suggesting that the alternative PSA pathway is mainly mediated by activated macrophages.

Three recent studies questioned the role of DCs and rather proposed an essential role for basophils as antigen-presenting

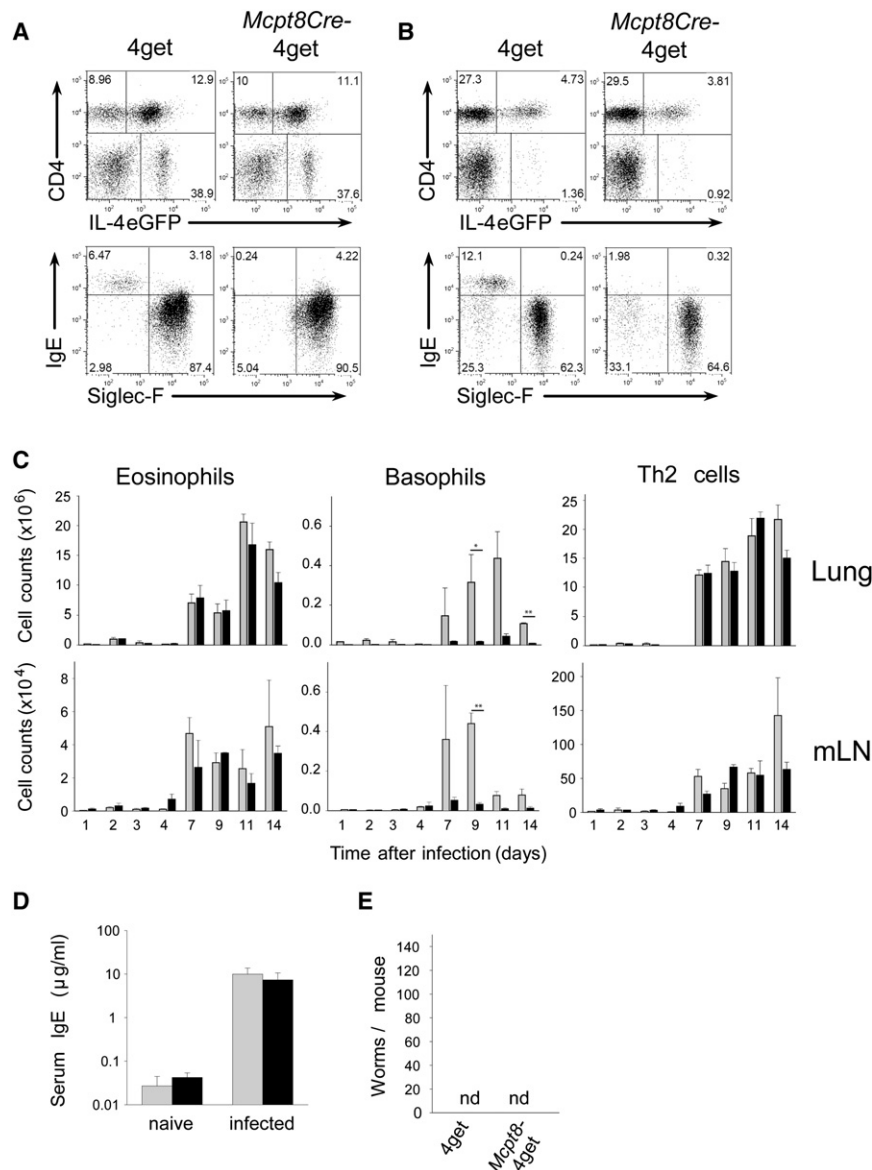


Figure 6. Basophils Are Not Required for Th2 Cell Differentiation and Eosinophilia after Primary *N. brasiliensis* Infection

(A and B) Detection of Th2 cells (CD4⁺IL-4eGFP⁺), eosinophils (CD4⁺IL-4eGFP⁺Siglec-F⁺IgE⁺) and basophils (CD4⁺IL-4eGFP⁺Siglec-F⁺IgE⁺) in total lung tissue (A) and mesenteric lymph nodes (B) of day 9 *N. brasiliensis* infected 4get and *Mcpt8Cre*/4get mice. Numbers indicate the frequency of gated cells. The lower dot plots are gated on CD4⁺IL-4eGFP⁺ cells as indicated in the upper dot plots.

(C) Number of eosinophils, basophils, and Th2 cells in lung and mesenteric lymph nodes (mLNs) of 4get (gray bars) and *Mcpt8Cre*-4get mice (black bars) at indicated time points after *N. brasiliensis* infection. Bars show the mean + SD of two to six mice per time point and mouse strain. *p < 0.05; **p < 0.01.

(D) Serum IgE concentrations of 4get mice (gray bars) and *Mcpt8Cre*-4get mice (black bars) that had been infected 10 days before with *N. brasiliensis* (infected) or not (naive). Bars show the mean + SD from three individual mice per group.

(E) No adult worms were detected in the small intestine of 4get or *Mcpt8Cre*-4get mice 10 days after infection with *N. brasiliensis*. nd, not detected.

cells during induction of Th2 cells by papain, IgE immune complexes, or helminth parasites (Perrigou et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009).

Sokol et al. described a nonredundant role for basophils as Th2 cell-inducing APCs after OVA-papain immunization because transfer of basophils into MHC-II deficient mice was sufficient for Th2 cell polarization and MAR-1-mediated basophil-depletion abolished the response (Sokol et al., 2009). Th2 cell polarization and basophil recruitment to lymph nodes appeared normal in *CD11c-diphtheria toxin receptor (DTR)* bone marrow chimeras in which DCs had been depleted by diphtheria toxin (DT), whereas the response was abolished in mice with exclusive expression of MHC-II on DCs. Tang et al. proposed a model in which DCs and basophils cooperate to induce a Th2 cell response against papain (Tang et al., 2010). In contrast to the previous study, they observed a reduced Th2 cell response and impaired basophil recruitment after papain injection in

after immunization with OVA-papain, OVA-alum, or infection with *N. brasiliensis*. Interestingly, Urban et al. reported that injection of papain into the footpad induces transient IL-4 expression in the draining popliteal lymph node already 1 hr after injection (Urban et al., 1992). Because basophils are not yet recruited at that time point, it appears unlikely that they are the initial source for IL-4 after papain injection.

The DC- or basophil-ablated mice in our study show a high efficiency of constitutive deletion and they did not undergo any further manipulation such as irradiation, or injection of DT, or cell-depleting antibodies before immunization or infection. This is an apparent and important difference to the other models and might provide a possible explanation for the contradictory results. Antibody-mediated depletion of basophils may cause bystander effects including depletion of FcεRIα expressing DCs, activation of mast cells and macrophages, formation of immune complexes, and activation of the complement system.

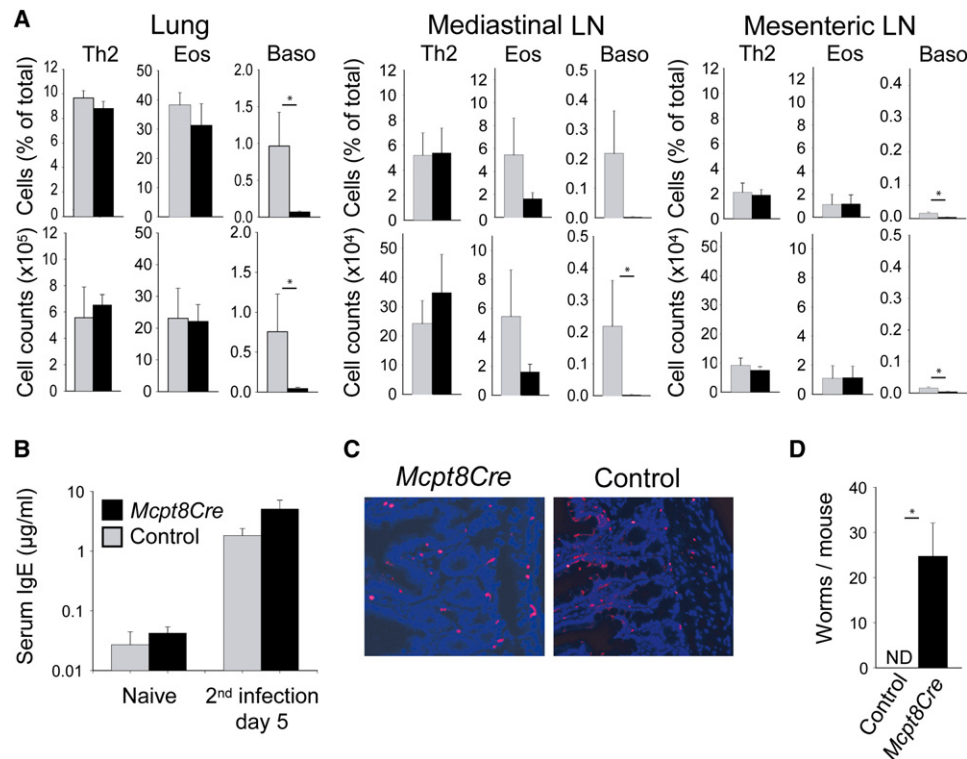


Figure 7. Basophils Mediate Protective Immunity against Secondary Infection with *N. brasiliensis*.

(A) Frequency (upper panel) and total number (lower panel) of Th2 cells, eosinophils, and basophils in the lung, mediastinal, and mesenteric lymph nodes at day 5 after secondary infection with *N. brasiliensis*. The graphs show the mean + SE from six *Mcpt8Cre*-4get mice (black bars) and three 4get mice (gray bars) from two experiments. **p* < 0.05.

(B) Serum concentrations of IgE at day 5 after secondary infection in comparison to naive mice. The graph shows the mean + SD from three *Mcpt8Cre*-4get mice (black bars) and three 4get mice (gray bars).

(C) Immunofluorescence staining for mMCP-1 (red) to detect mucosal mast cells in the small intestine at day 5 after secondary infection.

(D) Number of worms in the small intestine at day 5 after secondary infection. The graph shows the mean + SE from nine *Mcpt8Cre*-4get mice (black bar) and six 4get mice from three experiments (nd, not detected). **p* < 0.05.

Indeed, we observed that in our hands MAR-1 injection caused systemic anaphylaxis, increased serum concentrations of mMCP-1, and increased numbers of neutrophils in the spleen. There is good evidence that DCs act as Th2 cell-inducing APCs and orchestrate type 2 immune responses against allergens and *N. brasiliensis*, although DCs do not produce IL-4 (Lambrecht and Hammad, 2009; Ohnmacht et al., 2009). In fact, T cell-derived IL-4 is sufficient for Th2 cell polarization during helminth infection as demonstrated by adoptive transfers of TCR-transgenic or polyclonal CD4 T cells into IL-4-and-IL-13-deficient recipient mice (Liu et al., 2005; Voehringer et al., 2006).

Basophils can induce important effector functions for protective immunity against helminths given that they produce large amounts of IL-4 and IL-13, which are required for expulsion of most gastrointestinal helminths (Voehringer, 2009). We could show that basophils contribute to worm expulsion during primary infection with the hookworm *N. brasiliensis* in the absence of IL-4 and/or IL-13 producing T cells, suggesting that IL-4 and/or IL-13 from basophils can be sufficient for worm expulsion (Ohnmacht and Voehringer, 2009a). Perrigoue et al. demonstrated that depletion of basophils by MAR-1 treatment results in lower IL-4 concentrations, reduced goblet cell hyperplasia, and impaired worm expulsion after infection with the helminth

Trichuris muris, which resides in the caecum (Perrigoue et al., 2009). We and others could further demonstrate that antibody-mediated basophil-depletion rendered mice susceptible to secondary infection with *N. brasiliensis*, whereas Th2 cells and mast cells were not required for protective immunity (Katona et al., 1988; Ohnmacht and Voehringer, 2010). In the present study, we have extended these findings by using constitutively basophil-ablated *Mcpt8Cre* mice that showed efficient worm expulsion during primary infection while they were susceptible to secondary infection. The protective role for basophils during reinfection could be explained by the presence of parasite-specific antibodies produced by long-lived plasma cells that keep basophils sensitized so that large amounts of IL-4 and IL-13 can be released after secondary encounter of parasite-derived antigens. Basophils are probably less important during primary infection with *N. brasiliensis* given that parasite-specific antibodies are generated relatively late after infection. Another study has demonstrated that parasite-specific antibodies are required for protective memory against reinfection with the murine hookworm *Heligmosomoides polygyrus*, although it remains unclear whether basophils are also involved in this system (McCoy et al., 2008). Human hookworm infections are a major socioeconomic problem with an estimated 600 million

infected people worldwide. Vaccines are not available yet and a better understanding of protective immunity against these pathogens is urgently needed because individuals are frequently reinfected with the same pathogens in endemic regions (Hotez et al., 2003).

Basophils have gained much interest in the recent past since new roles for these rare cells have been defined with murine models in which basophils were depleted by monoclonal antibodies. Here, we used newly developed basophil-ablated mice to address the contribution of basophils for type 2 immunity. Although we could confirm that basophils are required for IgE-CAI and protective immunity against *N. brasiliensis*, we found no evidence that basophils are required for Th2 cell polarization induced by allergens or helminth infection, OVA-IgG1-mediated PSA or humoral immunity to OVA-alum or *N. brasiliensis*. Future studies with constitutively basophil-ablated *Mcpt8Cre* mice will focus on dissecting the effector mechanism(s) used by basophils to orchestrate chronic allergic inflammation and protective immunity against helminths.

EXPERIMENTAL PROCEDURES

Mice

Mcpt8Cre BAC-transgenic mice were generated by pronuclear injection of fertilized C57BL/6 oocytes with a modified bacterial artificial chromosome (BAC) where the coding sequence of Cre-recombinase was inserted behind the start codon of the *mcpt8* gene by homologous recombination in bacteria. In brief, BAC clone RP23-16G10 (C57BL/6 library, ResGen Invitrogen) was transfected together with a targeting vector containing a Cre-loxP-neo-loxP cassette flanked by two short (500 bp) homologous sequences into the bacterial strain EL250 (Liu et al., 2003). Recombination was induced by a temperature shift followed by deletion of the neo-cassette by growth in arabinose-containing medium (Figure S1). Dendritic cell-ablated Δ DC mice have been described (Ohnmacht et al., 2009). In brief, these mice were generated by crossing *CD11cCre* mice (Caton et al., 2007) to mice that express the diphtheria toxin A behind a loxP flanked Stop cassette in the *ROSA26* locus (Voehringer et al., 2008). 4get mice contain an IRES-eGFP cassette behind the stop codon of *Irf4*, serve as sensitive IL-4 reporter mice, and were provided by R.M. Locksley (Mohrs et al., 2001). Mast cell-deficient *c-Kit^{W-sh}* mice (Lyon and Glenister, 1982) were obtained from D. Lee (Brigham & Women's Hospital, Boston, MA). *ROSAtdRFP* reporter mice (Lucas et al., 2007) were obtained from H. Fehling (University Clinics Ulm, Germany). OT-II TCR-tg mice (specific for OVA₃₂₃₋₃₃₉ peptide) have been described (Barnden et al., 1998) and were obtained from the Jackson Lab. Smarta TCR-tg mice (specific for LCMV-GP₆₁₋₈₀ peptide) were kindly provided by A. Oxenius (ETH Zurich, Switzerland) (Oxenius et al., 1998). All mice had been backcrossed to C57BL/6 background and housed in accordance with institutional guidelines. Animal experiments were approved by the federal government of Bavaria. Mice were used at 6–12 weeks of age.

Nippostrongylus brasiliensis Infection

Third-stage larvae (L3) of *N. brasiliensis* were washed in sterile 0.9% saline (37°C) and injected subcutaneous (s.c.) (500 organisms) into mice. Mice were provided antibiotic-containing water (2 g/l neomycin sulfate, 100 mg/l polymyxin B sulfate; Sigma-Aldrich, St. Louis, MO) for the first 5 days after infection.

Ovalbumin-Induced Airway Inflammation and Induction of OVA-Specific Humoral Immune Responses

For inducing airway inflammation, mice were first primed by injecting a mixture of 50 μ g ovalbumin (OVA; Sigma-Aldrich) and 200 μ l Imject Alum (Pierce) intraperitoneally (i.p.) at day 0 and 7. Mice were challenged at day 14 and 15 by intranasal administration of 500 μ g OVA diluted in 50 μ l PBS. Analysis was performed at day 18 after priming.

For inducing a humoral immune response, mice were primed by i.p. injection of 200 μ l Imject Alum together with 50 μ g OVA. Four weeks later, mice were

challenged by i.p. injection of 500 μ g OVA in 300 μ l PBS. Serum was analyzed at indicated time points after challenge by OVA-specific ELISA.

Anaphylaxis and Allergic Dermatitis Experiments

For passive anaphylaxis, mice were sensitized by injecting intravenously 50 μ g TNP-specific IgE (clone IgELb4), 50 μ g or 150 μ g TNP-specific IgG1 (clone 1B7.11; ATCC TIB-191), or 400 μ g DNP-specific IgG1 (clone U-7.6; kindly provided by Z. Eshhar, Weizmann Institute of Science, Rehovot, Israel) at 24 hr (IgE) or 3 hr (IgG1) before challenge, respectively. Systemic anaphylaxis was then induced by injection of 50 μ g TNP₁₄-BSA or 50 μ g DNP₉-BSA (Biosearch Technologies), respectively, diluted in 300 μ l PBS. For active systemic anaphylaxis, mice were sensitized with 50 μ g OVA in 200 μ l alum i.p. and challenged 14 days later with 20 μ g OVA in 300 μ l PBS i.v.. Body temperature was measured at indicated time points after challenge with an electronic thermometer (Qtemp 200, Greisinger GmbH).

Allergic dermatitis was induced by i.v. injection of 33 μ g TNP-specific IgE (IgELb4) in 300 μ l PBS 1 day before injection of 10 μ g TNP-OVA in 10 μ l PBS into the left ear and 10 μ g OVA in 10 μ l PBS into the right ear. Ear thickness was measured every day and the difference between both ears was calculated. For reconstitution experiments, mice were transferred 1 day before antibody injection with 2.5×10^6 basophils derived from bone marrow cultures of mast cell deficient *c-Kit^{W-sh}* mice. In brief, bone marrow cells were cultured for 1 week in medium containing 1 ng/ml IL-3 (Immunotools) resulting in 40% mature basophils (CD49b⁺Fc ϵ RI α ⁺CD200R1⁺CD200R3⁺c-Kit⁺) and 40% of a potential basophil precursor population that lacks CD49b expression but shares expression of the other markers with basophils (Ohnmacht and Voehringer, 2009a). Cultures were not further purified before the transfer to prevent activation and loss of basophils.

Papain Treatment and Stimulation of TCR-tg Cells In Vivo

A total of 50 μ g papain (Lot# D00053300, Calbiochem, Merck KG) was diluted in 50 μ l PBS and injected into the rear footpad of mice as previously described by Sokol et al. (2008). Basophil recruitment was assessed on day 3 and Th2 cell polarization on day 4 after papain injection by flow cytometry. For induction of antigen-specific T cell responses untouched TCR-tg CD4⁺ T cells specific for LCMV-GP₆₁₋₈₀ (Smarta-4get-CD90.1 mice) or OVA₃₂₃₋₃₃₉ (OT-II-CD90.1 mice) were first isolated by magnetic bead technology (MACS, Miltenyi Biotech). Purified TCR-tg cells were then either labeled with carboxyfluorescein succinimidyl ester (CFSE) or injected directly (10^6 cells / mouse) i.v. and then the mice were injected with 50 μ g papain containing 30 μ g GP₆₁₋₈₀ or 50 μ g OVA₃₂₃₋₃₃₉ into the footpad. On day 4 the draining popliteal lymph nodes were excised and analyzed for T cell proliferation and Th2 cell differentiation.

Flow Cytometry

Lungs were perfused with 10 ml PBS before single cell suspensions were performed by mechanical dispersion followed by incubation with CD16/32 blocking antibody (2.4G2) at room temperature. Cell suspensions were stained with diluted antibody mixtures on ice. The following antibodies were purchased from eBioscience unless otherwise stated: PE-labeled anti-CD3 (145-2C11), biotinylated and PerCP-Cy5.5-labeled anti-CD4 (RM4-5), FITC-labeled anti-CD8 (5H10; Invitrogen Caltag), PE-labeled anti-CD11b (M1/70), biotinylated and PE-labeled anti-CD11c (N418), PE-labeled anti-CD44 (IM-7), FITC- and Alexa Fluor 647-labeled anti-CD45R (RA3-6B2), APC-labeled anti-CD49b (HM α 2, Biolegend), FITC-labeled anti-CD62L (MEL-14; Invitrogen Caltag), PE-labeled anti-CD90.1 (HIS51), PE-labeled anti-CD117 (B8; Invitrogen Caltag), biotinylated and eFluor 450-labeled anti-Gr-1 (RB6-8C5), biotinylated and PE-labeled anti-IgE (23G3; Southern-Biotech), APC-labeled anti-MHC-II (M5/114.15.2), biotinylated and APC-labeled anti-NK1.1 (PK136), AlexaFluor 647-labeled anti-PDCA-1 (ebio927), and PE-labeled anti-Siglec-F (E50-2440; BD Pharmingen). FITC-, PE- or APC-labeled streptavidin (SouthernBiotech) were used for visualization of biotinylated antibodies. CFSE staining was performed as previously described (Weston and Parish, 1990). Intracellular cytokine staining was performed with APC-labeled anti-IL-4 (11B11) after cells had been stimulated for 4 hr with 1 μ g/ml ionomycin and 40 ng/ml PMA with Brefeldin A added at 5 μ g/ml for the last 2 hr. Data were acquired on a FACS Calibur or FACS Canto II instrument (Becton Dickinson) and analyzed with FlowJo software (TreeStar).

ELISA

IgE and mMCP-1 concentrations were determined as described (Ohnmacht and Voehringer, 2010). Basal immunoglobulin concentrations in the serum of naive mice were determined with the SBA clonotyping system with alkaline phosphatase and the mouse immunoglobulin panel (SouthernBiotech). For detection of OVA-specific antibodies, ELISA plates were coated with 50 µg/ml OVA overnight at 4°C. After blocking with 3% bovine serum albumin and incubation with a 1:10 dilution of serum samples, OVA-specific antibodies were determined by incubation with anti-IgG1 linked to alkaline phosphatase (SouthernBiotech) or biotinylated anti-IgE and streptavidin-coupled alkaline phosphatase.

Quantitative RT-PCR

T cells (CD3⁺NK1.1[−]), NK cells (NK1.1⁺CD3[−]), dendritic cells (CD11c⁺MHC II⁺), and eosinophils (Siglec-F⁺SSC^{hi}) were sorted from spleens of C57BL/6 mice with a FACS Aria instrument (BD) (purity >95%). Mast cells were sorted from bone marrow cultures or the peritoneum. Basophils were sorted from bone marrow cultures. RNA was isolated from sorted cells with an RNA isolation kit (RNeasy, QIAGEN). Quantitative RT-PCR was performed on a LightCycler 2.0 (Roche) with primers for *Mcp18* (fwd: 5'-CAGTCAACGCTGA AGGAG-3', rev: 5'-TGGATGGAGTCGTGTAG-3') and *β-actin* (fwd: 5'-ATGGA TGACGATATCGCT-3', rev: 5'-TGAGGTAGTCTGTCAGGT-3').

Histology

Cryosections of dorsal skin tissue that had been fixed in 4% paraformaldehyde were stained with acidified toluidine blue. For immunofluorescent staining, cryosections from the small intestine were blocked with TNB-buffer (TSA amplification kit, Perkin Elmer) supplemented with 1% rat serum, 1% mouse serum and CD16/32-specific antibody. Mouse mast cell protease-1 (mMCP-1) was detected with a horseradish-peroxidase coupled anti-mMCP-1 (MS-RM8; Moredun Scientific Limited). For visualization, sections were first incubated with biotin-tyramide (TSA amplification kit, Perkin Elmer) and then with streptavidin-Cy3.

Statistics

Student's *t* test was performed with Sigmaplot 6.0 (Systat Software). *p* values of equal or less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and can be found with this article online at doi:10.1016/j.immuni.2010.08.011.

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Supplemental Information

Basophils Orchestrate Chronic Allergic Dermatitis and Protective Immunity against Helminths

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Figure S1. Generation of *Mcpt8Cre* mice.

(A) Localization of the *Mcpt8* gene within the BAC clone used to generate *Mcpt8Cre* mice. (B) *Mcpt8Cre* mice were generated by inserting the coding sequence of the Cre-recombinase directly behind the start codon of the coding region of the *mcpt8* gene. We used a targeting vector containing a Cre-loxP-neo-loxP cassette flanked by two short homologous sequences which we electroporated into the bacterial strain EL250 together with a modified bacterial artificial chromosome (BAC). After homologous recombination, the neo-cassette was deleted by growth in arabinose-containing medium and the BAC clone was injected into the pro-nucleus of fertilized C57BL/6 oocytes. (C) The copy number of the integrated BAC in two different founder lines was determined by quantitative PCR for *Mcpt8* (primer1: 5'-ggtagctcttcagggtctgaccag-3', primer2: 5'-gacacagaggcatcctactg-3') and normalized to PCR-products of the *ROSA26* locus (primer1: 5'-aaagtcgctctgagttgttat-3', primer2: 5'-ggagcgggagaaatggatatg-3') using the Dynamo SYBR green capillary Kit (Finnzymes, Espoo, Finland) and LightCycler instrument (Roche, Basel, Switzerland). Since two copies of the *Mcpt8* gene are present in the genome of unmanipulated mice we can calculate that the BAC-construct integrated with 5 copies in line#1 and 7 copies in line#2. Bars show the mean + SD from 3 mice per group.

Figure S2. Identification of basophils by flow cytometry in different mouse strains.

(A) Dot plots show staining for CD49b vs CD200R3 of total live cells from blood, spleen and bone marrow of indicated naïve mice. CD49b⁺CD200R3⁺ cells are basophils. (B) Naïve control mice, two different founder lines of *Mcpt8Cre* mice and dendritic cell depleted Δ DC mice were stained with anti-CD49b and anti-IgE to detect basophils in indicated organs. The figure is related to figure 1.

Figure S3. IL-3-induced basophilia is not observed in *Mcpt8Cre* mice.

Mice were injected i.p. with 10^7 cells of the IL-3 producing cell line WEHI-3 (ATCC #TIB-68) and basophil levels in the blood were analysed by flow cytometry at indicated timepoints by staining for IgE and CD49b.

Figure S4. *Mcpt8Cre* mice have a normal T cell compartment.

(A) Dot plots show the frequency of SP CD4 and CD8 T cells in thymus (upper row) and spleen (lower row) of naïve control (left) and *Mcpt8Cre* (right) mice. (B) Splenocytes were stained for CD4, CD8, CD44 and CD62L to detect naïve (CD44^{lo}CD62L^{hi}) and activated (CD44^{hi}CD62L^{lo}) subpopulations within the CD4 (upper row) and the CD8 (lower row) compartment. The figure is related to figure 1.

Figure S5. Δ DC mice have no defect in basophil recruitment after papain immunization.

(A) Dot plots show the frequency of basophils in draining popliteal lymph nodes on day 3 after papain immunization. (B) Bars show the mean frequency of basophils in

the lymph nodes and circles show results from individual mice. The figure is related to figure 3.

Figure S6. Effects of MAR-1 administration.

Mice were injected i.v. with 100 µg anti-FcεRIα (MAR-1, eBioscience) or PBS. **(A)** Dot plots show the frequency of basophils in the blood on day 1 after injection of MAR-1 or PBS. **(B)** Change in body temperature at indicated timepoints after MAR-1 or PBS injection. 6 mice per group were analyzed. ** $P < 0.01$. **(C)** Serum levels of mMCP-1 at 24h after MAR-1 or PBS injection. Bars show the mean + SE from 10 mice per group. **(D)** Total number of neutrophils (Gr-1^{hi} CD11b⁺ cells) in the spleen at 24h after MAR-1 or PBS injection. Bars show the mean + SD from 3 mice per group. P values were calculated by Student's t-test.

Figure S7. Effector cells in mediastinal lymph node and blood.

4get (grey bars) and *Mcpt8Cre*-4get (black bars) mice were infected with *N. brasiliensis* and analyzed at indicated time points by flow cytometry. The absolute numbers of eosinophils, basophils and Th2 cells in mediastinal lymph nodes (medLN; upper panels) or their frequency among total leukocytes in the blood (lower panels) are depicted. Bars show the mean number or frequency + SD of 2-4 mice per time point and mouse strain. ** $P < 0.01$. The figure is related to figure 6.

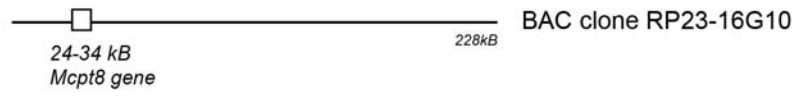
Figure S8. Neutrophils in draining lymph nodes after *N. brasiliensis* infection.

Control and *Mcpt8Cre* mice were infected with 500 L3 larvae of *N. brasiliensis* intracutaneously into the ear and neutrophils were detected 18h later in the draining

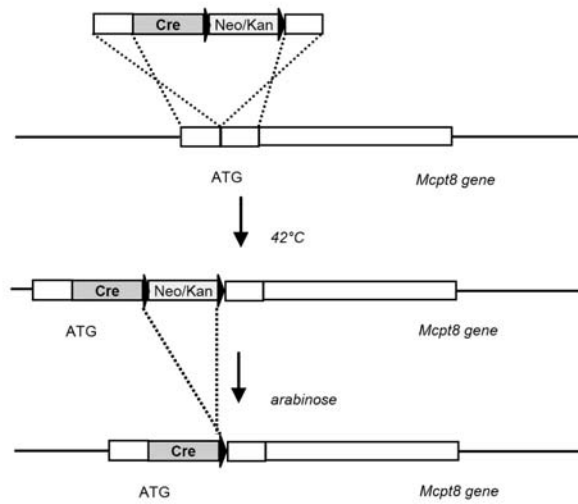
cervical lymph nodes by staining for Gr-1 and CD11b. Dot plots are representative of two mice per group. The figure is related to figure 6.

Figure S1

A



B



C

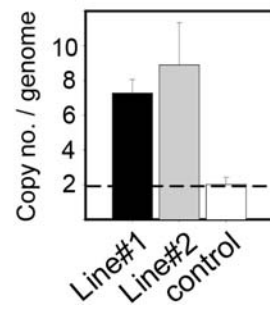


Figure S2

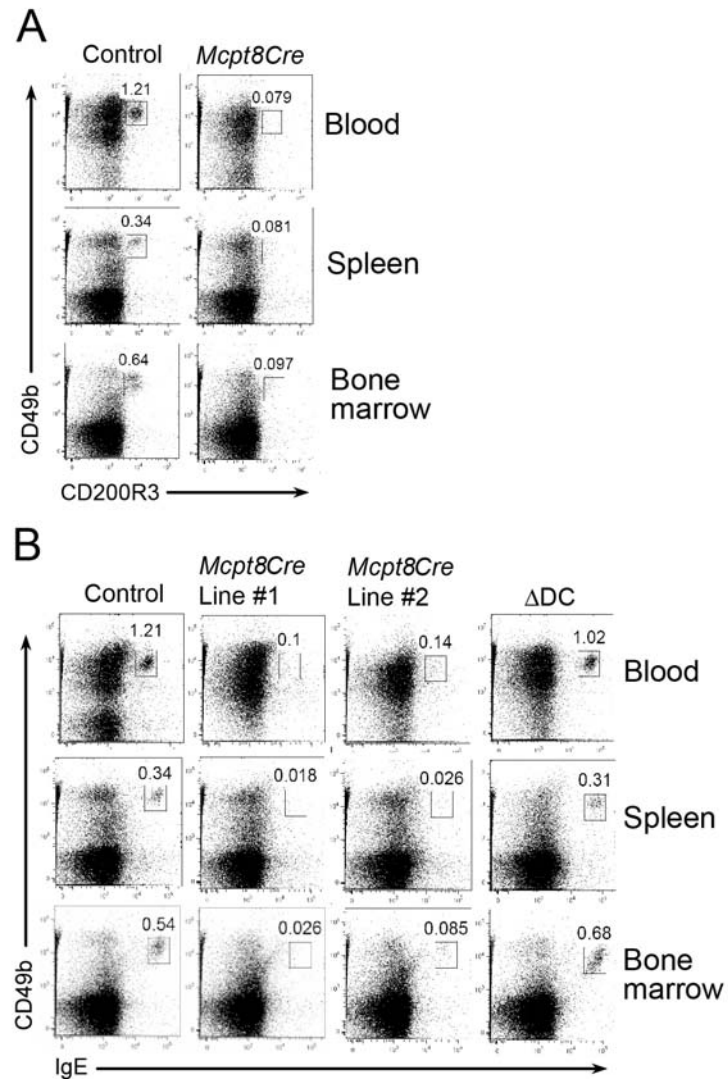


Figure S3

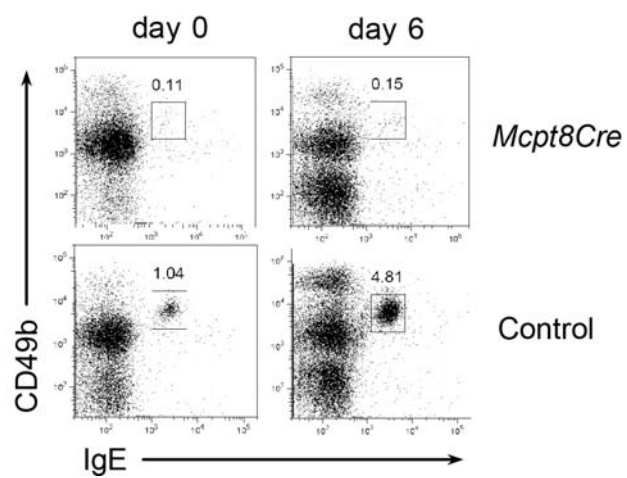


Figure S4

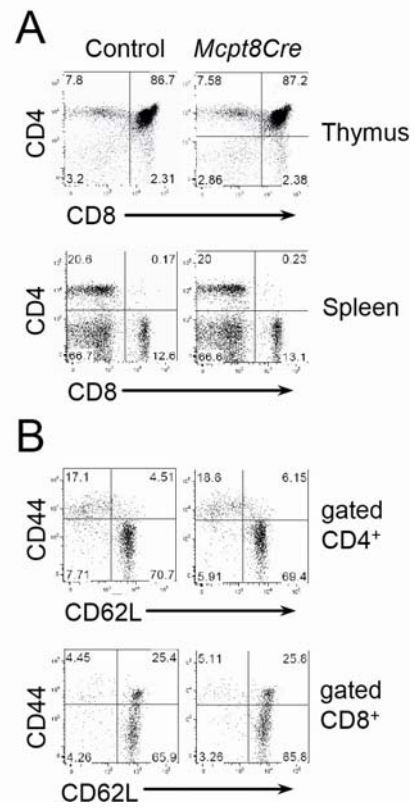


Figure S5

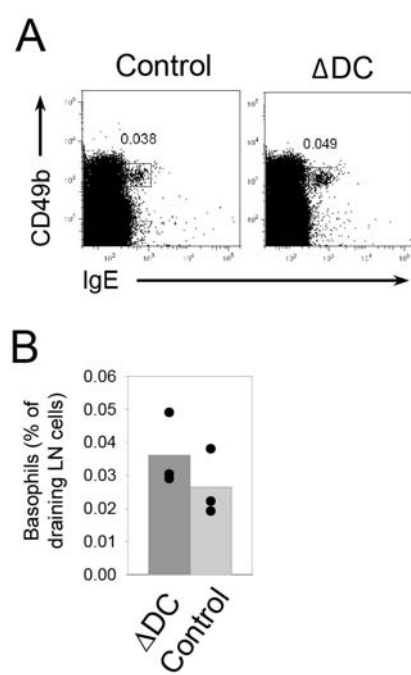


Figure S6

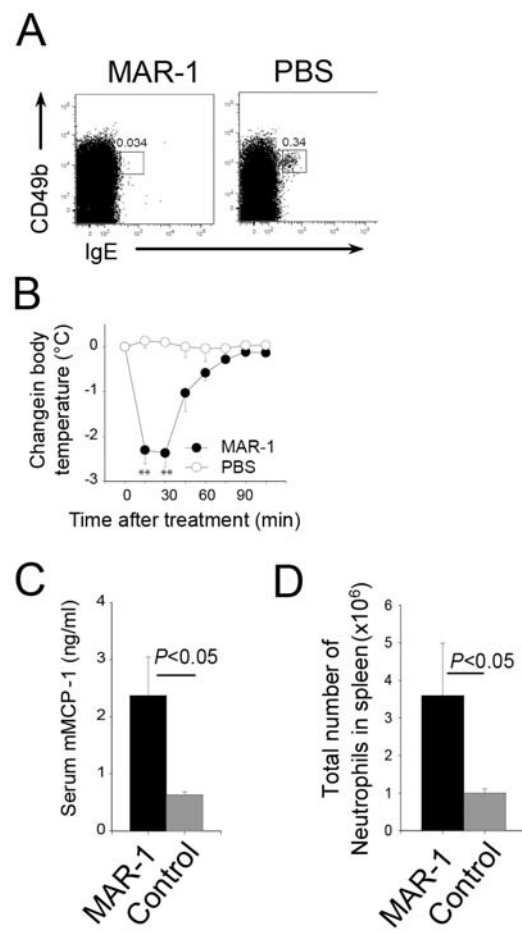


Figure S7

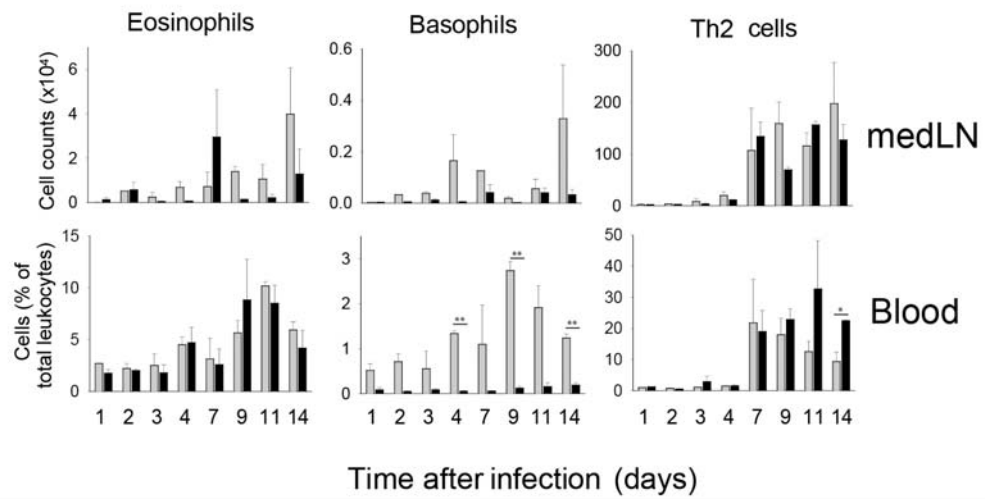


Figure S8

