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Basophils Amplify Type 2 Immune Responses, but Do Not Serve a Protective Role, during Chronic Infection of Mice with the Filarial Nematode *Litomosoides sigmodontis*

Marina N. Torrero,* Marc P. Hübner,* David Larson,* Hajime Karasuyama,[†] and Edward Mitre*

Chronic helminth infections induce a type 2 immune response characterized by eosinophilia, high levels of IgE, and increased T cell production of type 2 cytokines. Because basophils have been shown to be substantial contributors of IL-4 in helminth infections, and because basophils are capable of inducing Th2 differentiation of CD4⁺ T cells and IgE isotype switching in B cells, we hypothesized that basophils function to amplify type 2 immune responses in chronic helminth infection. To test this, we evaluated basophil function using the *Litomosoides sigmodontis* filaria model of chronic helminth infection in BALB/c mice. Time-course studies showed that eosinophilia, parasite Ag-specific CD4⁺ T cell production of IL-4 and IL-5 and basophil activation and IL-4 production in response to parasite Ag all peak late (6–8 wk) in the course of *L. sigmodontis* infection, after parasite-specific IgE has become detectable. Mixed-gender and single-sex worm implantation experiments demonstrated that the relatively late peak of these responses was not dependent on the appearance of circulating microfilariae, but may be due to initial low levels of parasite Ag load and/or habitation of the developing worms in the pleural space. Depletion of basophils throughout the course of *L. sigmodontis* infection caused significant decreases in total and parasite-specific IgE, eosinophilia, and parasite Ag-driven CD4⁺ T cell proliferation and IL-4 production, but did not alter total worm numbers. These results demonstrate that basophils amplify type 2 immune responses, but do not serve a protective role, in chronic infection of mice with the filarial nematode *L. sigmodontis*. *The Journal of Immunology*, 2010, 185: 000–000.

In contrast to most other pathogens, helminths induce a type 2 immune response characterized by eosinophilia, elevated serum levels of Ag-specific and polyclonal IgE, and increases in T cell production of IL-4, IL-5, and IL-13. This type 2 response is likely helpful in controlling helminth infections as deficiencies of IL-4, IL-4R signaling, and IgE are associated with increased parasite burdens in a number of animal models (1–7).

Basophils, which typically comprise >0.5% of all circulating leukocytes, have become increasingly recognized as playing a central role in the development of type 2 immune responses in allergic and helminth diseases (8–12). Of all PBMCs, only basophils have the ability to release large amounts of IL-4 early in response to appropriate stimuli, releasing preformed IL-4 within 5–10 min of activation (13). Studies we and others have conducted suggest that basophils produce substantially more IL-4 on a per-cell basis than other cell types in response to antigenic challenge (14–16). In addition to IL-4, basophils also synthesize and release

thymic stromal lymphopoietin, which can also promote type 2 immune responses (17, 18).

The concept that basophils can drive type 2 immune responses is supported by in vitro studies that have demonstrated that basophils, which express CD40L as well as release IL-4, can induce B cells to switch to IgE isotype (19, 20) and can cause naive T cells to differentiate to a type 2 phenotype (21). Consistent with these findings, in vivo depletion of basophils has been shown to decrease numbers of Th2 cells 4 d after a Th2-inducing allergen challenge (22), IL-4 mRNA expression from colon tissue 21 d postinfection (p.i.) of mice with the gastrointestinal helminth *Trichuris muris* (23), tissue eosinophilia in a cutaneous anaphylaxis model (24), and circulating and tissue eosinophilia as well as mRNA expression of IL-4, IL-5, and IL-13 in mice sensitized with serum from *Nippostrongylus brasiliensis*-infected mice and then challenged with that parasite (25).

In this study, we sought to determine the physiologic importance basophils may have in the development of type 2 responses in a model of chronic helminth infection. To achieve this, we evaluated the function of basophils over a 10-wk time period in mice infected with *Litomosoides sigmodontis*, a mouse model of filariasis. In this model, infective-stage L3 larvae injected into the s.c. tissue migrate to the pleural cavity during the first week p.i., develop into adult stage worms by 4 wk p.i., and begin to release L1 stage microfilariae into the bloodstream by 7 wk p.i. As in filarial infections of humans, infection of mice with *L. sigmodontis* results in a predominant type 2 immune response with high levels of circulating eosinophils and IgE Abs as well as production of type 2 cytokines by CD4⁺ T cells.

Our findings demonstrate that both type 2 immune responses and basophil activation in response to *L. sigmodontis* become maximal late (6–8 wk p.i.) in the course of *L. sigmodontis* infection after

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Abbreviations used in this paper: Depleted, *Litomosoides sigmodontis*-infected mice depleted of basophils with Ba103; Iso, *Litomosoides sigmodontis*-infected mice treated with isotype control Ab; LsAg, *Litomosoides sigmodontis* Ag; p.i., postinfection; U, uninfected.

specific IgE has become detectable. Worm implantation experiments demonstrated that the relatively late peak of these responses was not dependent on the appearance of circulating microfilariae. Finally, basophil depletion clearly shows that basophils play a major role in amplifying type 2 immune responses toward *L. sigmodontis* as eosinophilia, parasite Ag-specific IgE levels, CD4⁺ T cell proliferation in response to parasite Ag, and parasite Ag-driven IL-4 production from CD4⁺ T cells were all significantly diminished in *L. sigmodontis*-infected mice depleted of basophils for an 8-wk time period.

Materials and Methods

Mice and infection

Female BALB/c mice (National Cancer Institute Mouse Repository, Frederick, MD) were maintained at the Uniformed Services University animal facility. Experiments were performed with mice between 5 and 8 wk of age under a protocol approved by the Uniformed Services University Institutional Animal Care and Use Committee (Bethesda, MD).

Infectious-stage L3 larvae from *Litomosoides sigmodontis* were isolated by lavage from the pleural cavity of 4-d infected jirds (*Meriones unguiculatus*, obtained from TRS Laboratory, Athens, GA) as previously described (26). Adult worms were obtained by physical extraction from the pleural cavity of killed mice that had been infected for 8 wk. Mice were infected by s.c. injection of 40 L3 larvae or by i.p. surgical implantation of five adult worms. For surgical implantation of adult worms, mice were anesthetized with a mixture of ketamine, acetopromazine, and xylazine as described before (27). At study end points, mice were euthanized with carbon dioxide and whole blood obtained by cardiac puncture and collected in heparinized microfuge tubes (Sarstedt, Nümbrecht, Germany).

Basophil depletion

For in vivo basophil depletion, Ba103, a rat monoclonal IgG2b Ab that recognizes CD200R3 was obtained as described before (24) and injected weekly into mice i.p. starting 2 d preinfection or injected once at 8 wk p.i. Control mice in basophil depletion experiments were given i.p. injections of rat IgG2b isotype control Ab (BD Biosciences, San Jose, CA).

Flow cytometric basophil activation assays

Whole blood was diluted by half with RPMI 1640 (Cellgro, Mediatech, Herndon, VA) and stimulated with *L. sigmodontis* Ag (LsAg) at 20 µg/ml for 2 h at 37°C in 5% CO₂. When intracellular IL-4 was measured along with CD200R, monensin (BD GolgiStop protein transport inhibitor, BD Biosciences) was added after 1 h of incubation at 2 mM final concentration, and the tubes were incubated for 2 more h at 37°C in 5% CO₂. Cells were washed twice with 2 ml PBS (Mediatech) and centrifuged at 500 × *g* for 5 min. Supernatants were aspirated and RBCs lysed using a whole blood lysing reagent kit (Beckman Coulter, Fullerton, CA). Immuno-Lyse was diluted 1/25 in PBS, and 1 ml working solution was added to each tube and incubated 1 min at room temperature. Leukocytes were immediately fixed with 250 µl fixative solution (9.25% formaldehyde and 3.75% methanol), washed twice with 2 ml PBS, and centrifuged at 500 × *g* for 5 min. Supernatants were aspirated and nonspecific binding sites blocked by resuspending cells in 100 µl 1% BSA/PBS and incubating at 4°C for 1 h. Cells were stained with anti-IgE FITC, anti-CD4 PerCP, anti-B220 PerCP, and anti-CD200R-PE for 30 min at 4°C, washed twice with 2 ml PBS, and centrifuged at 500 × *g* for 5 min. In studies in which intracellular IL-4 was also evaluated, cells were stained in a two-step manner. After surface staining and two washes, cells were permeabilized with BD Perm/Wash buffer (BD Biosciences), resuspended in 1% BSA/PBS, stained with anti-IL-4 allophycocyanin for 30 min at 4°C, and washed twice. Cells were resuspended in 200 µl PBS and analyzed using a BD LSR II Optical Bench flow cytometer and FACSDiVa 6.1 software (BD Biosciences).

For all flow cytometry experiments, Abs were individually titrated before use and compensations assessed using BD compBeads (BD Biosciences) bound to the Abs being used for that experiment. For basophil activation assays, cutoff gates for CD200R-PE and IL-4-allophycocyanin positivity were established using the fluorescence-minus-one technique (28). Anti-IgE FITC (R35-72), anti-CD4 PerCP (RM4-5), anti-B220 PerCP (RA3-6B2), anti-Siglec-F PE (E50-2440), anti-CD49b allophycocyanin (HMα2), and anti-IL-4 allophycocyanin (11B11) were purchased from BD Pharmingen (San Diego, CA) and anti-CD200R-PE (OX-110) from AbD Serotec (Kidlington, Oxford, U.K.).

LsAg

Soluble LsAg was made from adult male and female *L. sigmodontis* worms harvested from the pleural space of infected jirds and frozen at -20°C. Worms were thawed, washed with PBS, refrozen at -20°C, and lyophilized overnight. Lyophilized worms were resuspended in PBS and mechanically disrupted by stirring with a magnetic bar at 4°C overnight. Postcentrifugation at 750 × *g* for 10 min at 4°C with no brake, the supernatant was collected. The pellet was stirred again overnight, centrifuged at 1200 × *g* for 10 min at 4°C, and the supernatant again collected. The two supernatants were combined and centrifuged for a final time at 12,000 × *g* for 30 min at 4°C. This final supernatant was passed through a 0.22-µm filter (Millex-GV; Millipore, Bedford, MA) and total protein concentration measured using a BCA protein assay kit (Pierce, Rockford, IL). The final soluble Ag product, LsAg, contains Ags from both male and female adult worms as well as from microfilariae that are present in the uteri of adult female worms. Although there are no L3 worms used in the production of LsAg, Ab and cellular immune responses induced by L3 stage worms are reactive to LsAg (29).

Worm recovery and cell counting

L4 and adult worms were recovered and enumerated by dissection of the pleural cavity. For enumeration of microfilariae, 30 µl aliquots of whole blood were incubated in ACK lysing buffer for 10 min, centrifuged, and then resuspended in 30 µl PBS and visualized unstained using integrated modulation contrast.

For the time-course study, basophil and eosinophil numbers were analyzed using a Bayer Advia 120 differential leukocyte counter. For worm implantation experiments, basophil and eosinophil numbers were obtained by manual differential count of blood smears stained by May Grünwald/Giemsa. For basophil depletion studies, eosinophil numbers were determined by surface staining blood samples with anti-CD11b FITC (BD Pharmingen) and anti-CCR3-PE (R&D Systems, Minneapolis, MN) and identifying eosinophils by flow cytometric analysis as CD11b^{du}CCR3⁺. In a subset of animals, eosinophils were also identified as anti-Siglec-F PE⁺ and anti-IgE FITC⁻.

Polyclonal and specific IgE ELISA

Blood was collected from *L. sigmodontis*-infected female BALB/c mice at various time points by retro-orbital bleed or cardiac puncture and analyzed for polyclonal IgE and LsAg-specific IgE by colorimetric sandwich ELISA. Flat-bottom Immulon 4 plates (Thomas Scientific, Swedesboro, NJ) were coated overnight at 4°C with 10 µg/ml anti-mouse IgE (clone R35-72) for polyclonal IgE or with 20 µg/ml LsAg for parasite-specific IgE. Blocking was performed by 1 h incubation of plates with 5% BSA in PBS. Prior to testing, IgG was adsorbed from serum samples by incubation of serum with GammaBind G Sepharose (Amersham Biosciences, Uppsala, Sweden) overnight at 4°C. Serum samples were then diluted 1:5 and 1:50 for polyclonal IgE measurements. Plates were washed and incubated with biotinylated rat anti-mouse IgE (clone R35-118) in PBS. Following washing, 1/1000 dilution of alkaline phosphatase-conjugated streptavidin (BD Pharmingen) was added, and plates were incubated for 1 h at 37°C. Nitrophenyl phosphate disodium (Sigma-Aldrich, St. Louis, MO) was used as substrate. Purified mouse IgE was used as standard for total IgE (BD Biosciences). For specific IgE measurements, samples from control and experimental groups were analyzed as duplicates on the same plate to allow accurate comparison between groups by OD. Absorbance was detected at 405 nm using a PerkinElmer Victor³ V microplate reader (PerkinElmer, Waltham, MA).

Cytokine quantification and proliferation assays

Splenocytes were resuspended in ACK Lysing buffer (Quality Biological, Gaithersburg, MD) to allow RBC lysis. Cells were washed and then resuspended in Iscove's DMEM (Mediatech) supplemented with 10% FCS (Valley Biomedical, Winchester, VA), 1% L-glutamine (Mediatech), 1% insulin-transferrin-selenium medium (Invitrogen, Carlsbad, CA), and 80 µg/ml gentamicin (Quality Biological). CD4⁺ cells and CD11c⁺ cells were isolated from splenocytes by magnetic cell sorting (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. CD4⁺ cells were plated at 2 × 10⁶ cells/ml with 10% dendritic cells isolated from uninfected mice. Cells were stimulated with 20 µg/ml LsAg or 5 µg/ml anti-CD3 (eBioscience, San Diego, CA) and cultured at 37°C, 5% CO₂. Supernatants were collected from CD4⁺ cell cultures incubated for 3 d and assayed for mouse Th1/Th2 6-Plex (BioSource International, Camarillo, CA) as specified by the manufacturer. IL-4 was quantified by ELISA (eBioscience) according to the manufacturer's instruction. CD4⁺ cell proliferation was quantified by chemiluminescence after 3 d cultures using

a BrdU incorporation immunoassay (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instruction.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 4 statistics software (GraphPad Software, San Diego, CA). Differences between multiple groups for time-course studies were tested for significance using the Kruskal-Wallis test, followed by Dunn's post hoc multiple comparisons. Comparisons between two groups were performed by Student *t* test followed by Welch's correction for variance. *p* values <0.05 were considered significant. Data are expressed as mean value $\pm$ SEM. All data are representative of at least two independent experiments except for the adult worm implantation experiments, which were only conducted once.

Results

Type 2 immune responses become prominent 6–8 wk p.i. with *L. sigmodontis*

In the *L. sigmodontis* model, L3 larvae injected into the s.c. tissue migrate to the pleural cavity by days 4–6 p.i., when subsequent development takes place. Larvae molt to the L4 stage between days 8 and 12 p.i., become adults by days 25–30 p.i., and are able to produce microfilariae that circulate in the blood by day 50 p.i. As in filarial infections of humans, infection of mice with *L. sigmodontis* results in a predominant type 2 immune response characterized by high levels of circulating eosinophils and IgE Abs as well as production of type 2 cytokines by T cells. To better understand the development of type 2 immune responses in chronic filariasis, we conducted an observational time-course study of these markers of type 2 immunity. Mice were euthanized at various time points over the course of a 10-wk infection, and numbers of worms

residing in the thoracic cavity, numbers of microfilariae in the blood, circulating eosinophil and basophil counts, polyclonal and LsAg-specific IgE Ab concentrations, and IL-4, IL-5, and IFN- γ concentrations produced by splenic CD4⁺ T cells after in vitro stimulation with LsAg were measured.

As seen in Fig. 1A, the numbers of parasites recovered from the pleural cavity remained fairly constant over the time periods studied. Postinjection of 40 infective-stage L3 larvae, a maximum mean of 23 worms were recovered at 6 wk p.i., and a minimum mean of 18 worms were recovered at both 4 and 10 wk p.i. As expected, microfilariae appeared in blood at time points later than 6 wk p.i. (Fig. 1B). Polyclonal IgE Abs were detected in plasma as early as 2 wk p.i., became extremely elevated by 6 wk p.i., and remained very high up to 10 wk p.i. (Fig. 1C). Testing for parasite Ag-specific responses was conducted using LsAg. LsAg is a crude homogenate soluble Ag mixture that contains Ag from adult male worms, adult female worms, and microfilariae and is cross-reactive with immune responses induced by challenge with L3 stage worms (29). LsAg-specific IgE concentrations were not detected until 4 wk p.i., when they were present at concentrations just above the sensitivity of the ELISA and then continued increasing up to 10 wk p.i. (Fig. 1C).

Basophil numbers did not appreciably increase early in the course of infection, but clearly increased late in infection from a baseline of 119 cells/ μ l in uninfected mice to a peak of 403 cells/ μ l at 8 wk p.i. (Fig. 1D). Circulating eosinophils increased early, from a baseline mean of 424 cells/ μ l in uninfected mice to a mean of 613 cells/ μ l by 4 wk p.i., and then rose to a peak mean of 1520 cells/ μ l at 8 wk p.i. (Fig. 1E). IL-4, IL-5, and IFN- γ pro-

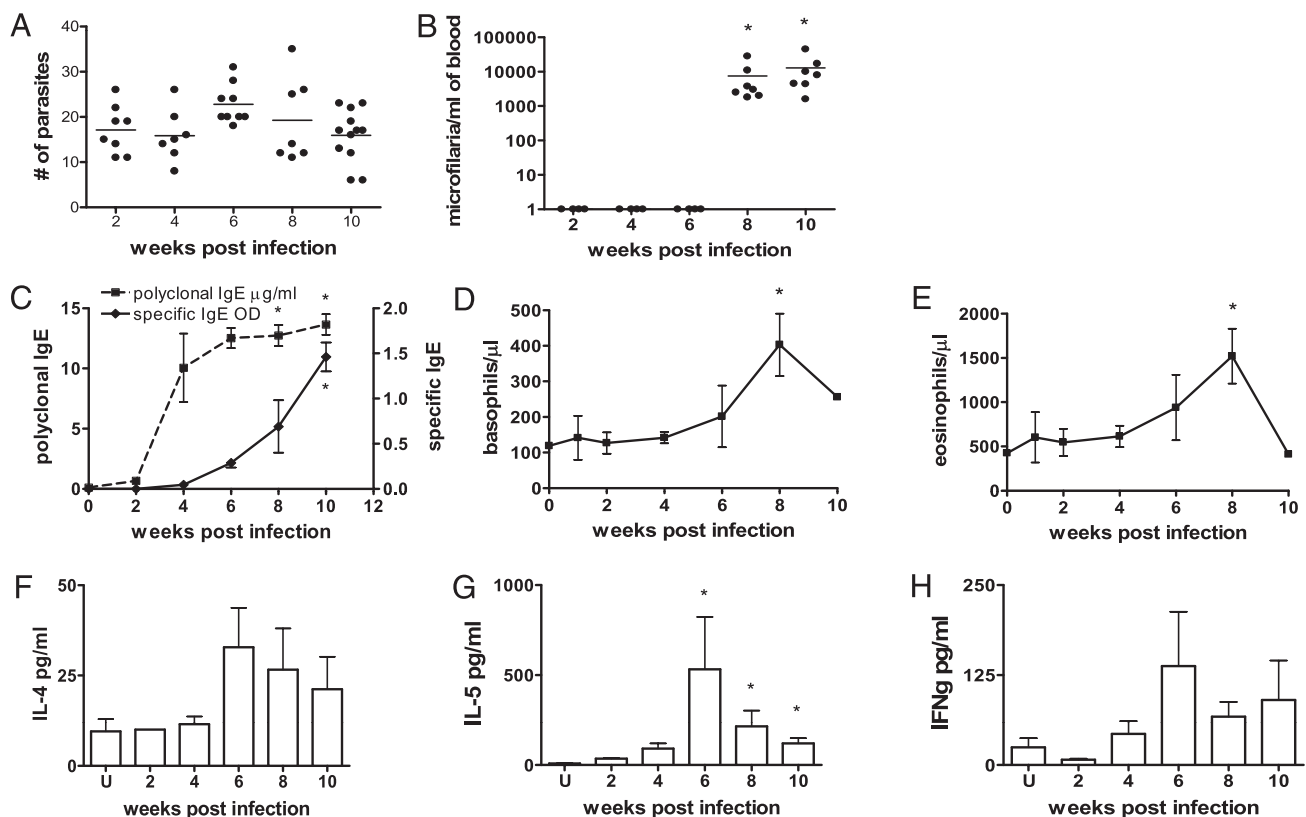


FIGURE 1. Time course of the immune response to *L. sigmodontis*. Mice were infected by s.c. injection of 40 *L. sigmodontis* L3 larvae and then euthanized at different time points. *A*, Numbers of parasites recovered from the thoracic cavity. *B*, Numbers of circulating microfilariae. *C*, Levels of polyclonal and LsAg-specific IgE measured in plasma. *D*, Numbers of circulating basophils. *E*, Numbers of circulating eosinophils. Concentrations of IL-4 (*F*), IL-5 (*G*), and IFN- γ (*H*) in supernatants of LsAg-stimulated splenic CD4⁺ cells cultured for 3 d postsubtraction of media control. Data shown are from one of two experiments using 5–10 mice per group. Error bars represent $\pm$ SEM. Significant differences between groups were analyzed by the Kruskal-Wallis test followed by Dunn's post hoc multiple comparisons. **p* < 0.05 compared with uninfected time point. U, uninfected mice.

duction from CD4⁺ T cells stimulated with LsAg peaked at 6 wk p.i. and subsequently decreased (Fig. 1F–H).

In aggregate, these findings demonstrate that type 2 immune responses peak late in the course of infection. Maximal production of the type 2 cytokines IL-4 and IL-5 occurs 6 wk p.i., and maximal eosinophil and basophil counts occur at 8 wk p.i. These peaks occur after parasite-specific IgE first becomes present in the circulation and are coincident with the development of circulating microfilariae. Interestingly, although both polyclonal and LsAg-specific IgE levels progressively increased throughout the course of infection, eosinophilia, basophilia, and LsAg-driven cytokine production from T cells all waned late in the course of infection despite the continued presence of both adult worms and circulating microfilariae.

Substantial basophil activation in response to parasite Ag does not occur until several weeks into the course of infection

To determine when basophils from *L. sigmodontis*-infected mice become activated in response to parasite Ag, basophil expression of intracellular IL-4 and surface CD200R were measured by flow cytometry after in vitro culture of whole blood cells with parasite Ag for 3 h at different time points of infection.

Basophils were identified as CD4⁺B220⁺IgE⁺ cells by flow cytometry (Supplemental Fig. 1A, 1B), a strategy that identifies a highly pure basophil population in the blood (30). Additional staining on a subset of blood samples confirmed that CD4⁺B220⁺IgE⁺ cells in this study were almost entirely basophils, as these cells routinely stained >98% negative for c-Kit (Supplemental Fig. 1C) and >96% positive for CD49b (Supplemental Fig. 1D).

The percentages of basophils that stained positive for surface CD200R and intracellular IL-4, as well as the mean fluorescence intensity staining of these markers, routinely increased when blood from infected mice was incubated with LsAg or anti-IgE as compared with media (representative FACS plots in Supplemental Fig. 1E). As seen in Fig. 2A, basophils from *L. sigmodontis*-infected mice started producing IL-4 in response to LsAg at 4–6 wk p.i. The maximum frequency of basophils staining positive for IL-4 in response to LsAg occurred at 8 wk p.i. (mean 25% IL-4⁺ basophils) followed by a sharp decrease at 10 wk p.i. (mean 8% IL-4⁺ basophils). Analyses of basophil surface expression of CD200R, which has been demonstrated to function as an activation marker for murine basophils in response to both IgE-mediated and IgE-independent stimuli (30), paralleled the findings of IL-4 expression. The percentages of basophils expressing CD200R in response to LsAg increased slightly at time points of 4–6 wk p.i., became maximal at 8 wk p.i., and then decreased at 10 wk p.i. (Fig. 2B). To confirm the flow cytometric evidence that basophils from 8 wk infected mice become activated upon stimulation with LsAg, IL-4 was measured from supernatants of blood cells after 2 h in vitro stimulation with media alone, LsAg, or anti-IgE. As seen in Fig. 2C, LsAg as well as anti-IgE stimulation of blood from 8 wk infected mice markedly increased supernatant IL-4 concentrations.

To demonstrate that basophils are the principle source of IL-4 in 2-h blood cultures stimulated with LsAg, IL-4 release in response to LsAg was evaluated in uninfected mice and in BALB/c mice infected with *L. sigmodontis* for 8 wk and then treated with basophil-depleting Ab (Ba103) or isotype Ab 24 h prior to in vitro stimulation. Flow cytometric analysis demonstrated that Ba103 treatment resulted in >95% depletion of basophils 24 h post-injection (data not shown). As seen in Fig. 2D, no substantial IL-4 release was observed from whole blood of mice depleted of basophils, demonstrating that basophils are indeed the primary source of IL-4 in response to LsAg in 2-h blood cultures and substantiating

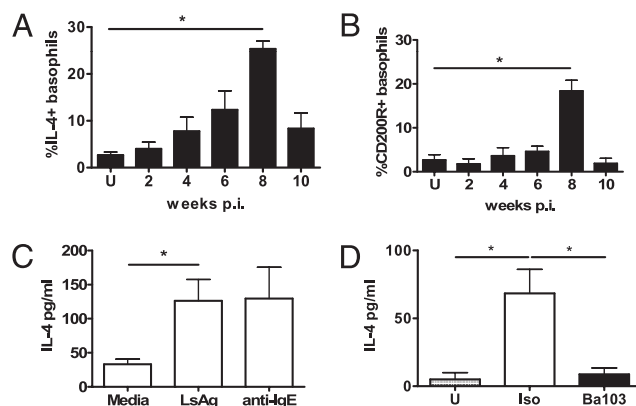


FIGURE 2. Parasite-specific basophil activation peaks 8 wk p.i. with *L. sigmodontis*. Percentages of basophils positive for IL-4 (A) or CD200R (B) postsubtraction of media controls from in vitro cultures of blood stimulated with LsAg for 3 h. C, IL-4 released from in vitro cultures of blood stimulated with media alone, LsAg, or anti-IgE for 2 h measured at 8 wk p.i. by ELISA. D, IL-4 released from in vitro cultures of blood stimulated with LsAg for 2 h measured at 8 wk p.i. by ELISA from uninfected control mice, 8 wk infected mice treated with isotype Ab (iso), and 8 wk infected mice depleted of basophils by i.p. injection of Ba103 Ab 24 h prior to blood draw. Data shown are from one of two experiments using five mice per group. Error bars represent $\pm$ SEM. Significant differences between groups for A and B were analyzed by the Kruskal-Wallis test followed by Dunn's post hoc multiple comparisons. Significant differences between groups for C and D were analyzed by Student *t* test followed by Welch's correction for variance. **p* < 0.05. U, uninfected mice.

a prior study that showed that basophils are the only circulating cell capable of rapid release of IL-4 (13).

These results demonstrate that basophils become activated and release IL-4 over the course of filaria infection. Of note, substantial basophil activation was only observed at time points after 4 wk p.i. when LsAg-specific IgE is already present (Fig. 1C), consistent with the notion that cross-linking of specific Abs may be the primary means of basophil activation in helminth infection. Also, it is interesting to note that even though parasite-specific IgE levels are very high at 10 wk p.i., basophil responsiveness to LsAg at this late time point is substantially attenuated.

Microfilariae are not required to induce late type 2 immune responses in mice infected with L. sigmodontis

Because type 2 responses became maximal close to the time circulating microfilariae appear in the bloodstream (Fig. 1), we conducted single-sex worm implantation experiments to determine if microfilariae are the main drivers of the type 2 response in chronic filariasis. Implantation of 8-w-old female microfilaria-producing worms into the peritoneum of BALB/c mice resulted in circulating microfilaremia throughout the course of the experiment in all tested mice, whereas no microfilaremia ever occurred post-implantation of adult male worms (data not shown). Eight weeks postimplantation, single-sex worm infections showed no differences in parasite Ag-specific IgE levels (Fig. 3A), eosinophilia (Fig. 3B), or basophilia (Fig. 3C). Similarly, CD4⁺ T cells from female-infected (microfilaremic) mice and male-infected (amicrofilaremic) mice demonstrated no significant differences in IL-4 production after in vitro stimulation with parasite Ag (Fig. 3D). IL-5 levels trended higher in male worm-infected mice (Fig. 3E), and IFN- γ levels trended higher in female worm-infected mice (Fig. 3F), though neither of these differences was statistically significant.

Finally, basophil activation in response to parasite Ag was equivalent in the two groups, with similar percentages of basophils

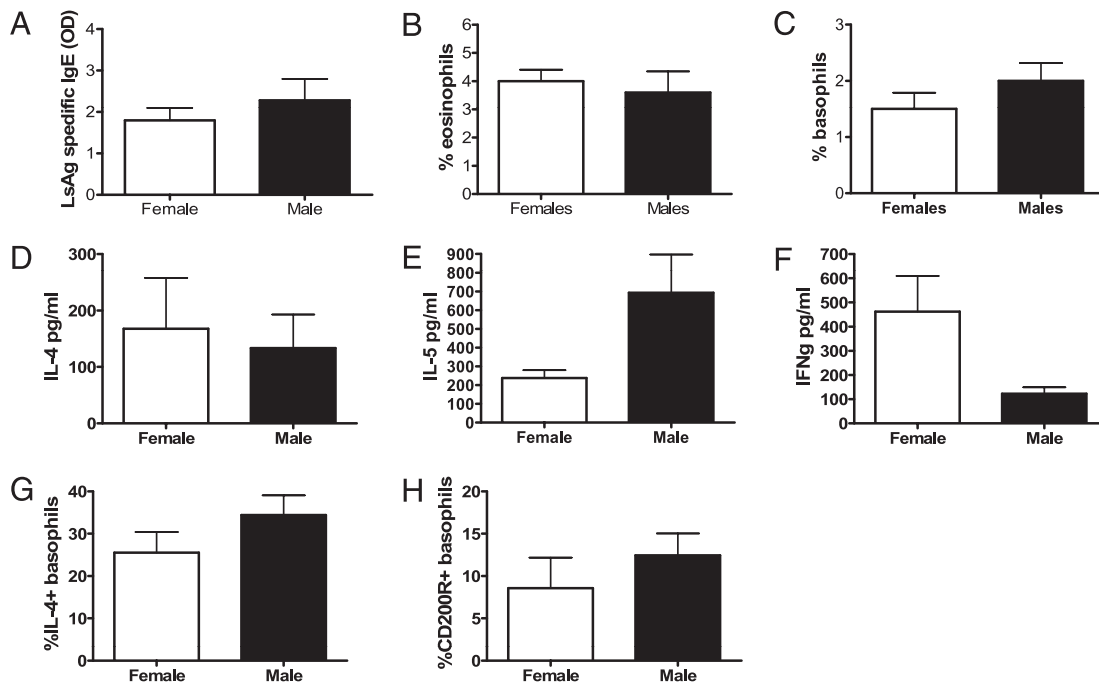


FIGURE 3. Immune responses 8 wk after i.p. implantation with either five male or five microfilaria-producing female adult *L. sigmodontis* worms. *A*, LsAg-specific IgE levels measured in plasma. Circulating levels of eosinophils (*B*) and basophils (*C*). Concentrations of IL-4 (*D*), IL-5 (*E*), and IFN- γ (*F*) in supernatants of LsAg-stimulated splenic CD4⁺ T cells cultured for 3 d postsubtraction of media control. Percentages of basophils positive for intracellular IL-4 (*G*) or surface CD200R (*H*) postsubtraction of media controls from in vitro cultures of blood stimulated with LsAg for 3 h. Five mice per group. Error bars represent $\pm$ SEM. Significant differences between groups were analyzed by Student *t* test followed by Welch's correction for variance. $p < 0.05$.

expressing IL-4 (Fig. 3*G*) and CD200R (Fig. 3*H*) after in vitro incubation with LsAg. These single-sex implantation experiments demonstrate that microfilariae are not required for the development of type 2 immune responses late in the course of chronic filariasis.

Implantation of adult worms into the peritoneal cavity hastens the onset of basophil activation in response to LsAg

L3 larvae of *L. sigmodontis* are much smaller than adult worms (850 μ m versus 4–8 cm in length) and within 3 to 4 d of infection migrate to the pleural space, where they develop over several weeks to adult worms. To determine whether the late onset of basophil activation in response to parasite Ag was related to either a low initial parasite Ag burden or to habitation of the adult worms in the pleural space, three female and two male adult worms were surgically implanted into the peritoneum of BALB/c mice and IgE levels and basophil responses to LsAg assessed at varying time points.

As with initial infection using L3 larvae, total and LsAg-specific IgE levels peaked relatively late (6–8 wk) in the course of infection (Fig. 4*A*, 4*B*). Interestingly, onset and peak basophil IL-4 production in response to LsAg occurred much earlier post-implantation of adult worms than had been observed after s.c. injection of L3 larvae (day 7 p.i. for onset and peak in worm-implanted mice, Fig. 4*C*, versus 14–28 d p.i. for onset and 56 d p.i. for peak in L3-infected mice, Fig. 2*A*). Similarly, the time point at which basophil activation in response to LsAg decreased occurred earlier in implanted mice (56d p.i., Fig. 4*C*) than in L3-infected mice (70d p.i., Fig. 2*A*, 2*B*). These results suggest that the late onset of basophil activation observed postinfection with L3 larvae may be due to relatively low initial parasite Ag burden, sequestration of worms and worm Ags in the pleural space, or a combination of these factors. Of note, as observed with the L3 infections, onset of basophil activation in response to LsAg correlated with the detection of parasite-specific IgE, as levels of LsAg-

specific IgE increased to just above the level of detection at time points of 7 and 14 d p.i. (Fig. 4*B*).

*Basophil depletion suppresses type 2 immune responses during *L. sigmodontis* infection*

To study the role basophils may play in the immune response to *L. sigmodontis*, we injected *L. sigmodontis*-infected mice weekly with Ba103, a mAb against CD200R3 that specifically depletes circulating basophils (24), and evaluated immune responses at 8 wk p.i., as this was the time point at which most type 2 immune responses were found to be maximal (Fig. 1). Fifty micrograms Ba103 administered i.p. every week was sufficient to clear >95% of circulating basophils in all mice throughout the course of infection (Supplemental Fig. 2*A*). Importantly, and as reported previously (24), Ba103 injections did not alter peritoneal mast cell numbers even though mast cells can also express CD200R3 (Supplemental Fig. 2*B*).

Immunological studies clearly demonstrate that basophil depletion results in attenuated type 2 immune responses 8 wk p.i. with *L. sigmodontis*. First, polyclonal and parasite-specific IgE Ab production were significantly reduced in infected mice depleted of basophils compared with isotype-treated *L. sigmodontis*-infected mice (Fig. 5*A*, 5*B*). As expected, uninfected mice developed no detectable specific IgE and significantly lower levels of polyclonal IgE than either infected group (Fig. 5*A*, 5*B*). Similarly, whereas all *L. sigmodontis*-infected mice developed some degree of eosinophilia in comparison with uninfected mice, *L. sigmodontis*-infected mice depleted of basophils mounted significantly less eosinophilia than nondepleted infected mice (Fig. 5*C*, 5*D*). This decrease in eosinophilia was confirmed by peripheral smears and, in a subset of animals, by flow cytometric identification of eosinophils as Siglec-F⁺ and IgE⁺ (data not shown).

With respect to cellular immune responses, measurement of cytokines from LsAg-stimulated CD4⁺ T cells demonstrated sig-

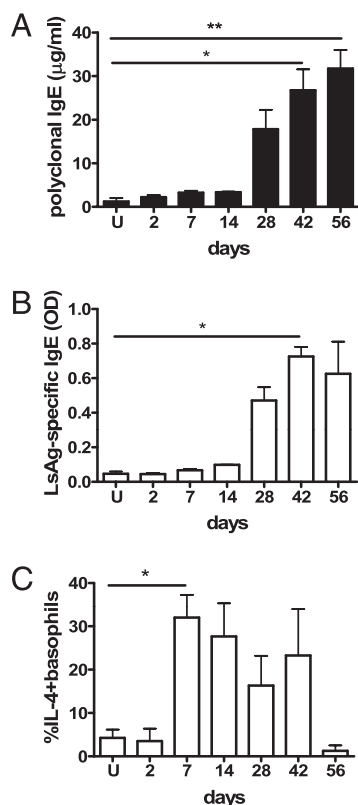


FIGURE 4. Time course of IgE levels and basophil activation in response to LsAg after i.p. implantation of both male (three) and female (two) adult *L. sigmodontis* worms. Levels of polyclonal (A) and LsAg-specific IgE (B) in plasma. C, Percentages of basophils positive for IL-4 postsubtraction of media controls from in vitro cultures of blood stimulated with LsAg for 3 h. Five mice per group. Error bars represent $\pm$ SEM. Significant differences between groups were analyzed by the Kruskal-Wallis test followed by Dunn's posthoc multiple comparisons. * $p < 0.05$; ** $p < 0.01$. U, uninfected.

nificantly less IL-4 production from CD4⁺ T cells of basophil-depleted mice compared with control group-infected mice (Fig. 6A). IL-5 and IFN- γ production from LsAg-stimulated CD4⁺ T cells were also less in basophil-depleted mice than control mice, but these differences were not statistically significant (Fig. 6B, 6C). Additionally, CD4⁺ T cells of basophil-depleted mice exhibited significantly less proliferation in response to parasite-Ag than control mice infected with *L. sigmodontis* (Fig. 6D). This was not due to an intrinsic difference in the capability of CD4⁺ T cells to proliferate, because CD4⁺ T cells from basophil-depleted and control mice mounted equivalent proliferative responses after anti-CD3 stimulation (Fig. 6E). Uninfected mice did not exhibit any measurable cytokine release (Fig. 6A–C) or proliferation in response to LsAg (data not shown). Finally, depletion of basophils with Ba103 did not alter the numbers of adult worms recovered from the pleural cavity 8 wk p.i. (Fig. 6F) or levels of circulating microfilaremia (data not shown).

In summary, basophil depletion with Ba103 resulted in an impaired type 2 immune response during chronic *L. sigmodontis* infection. Basophil depletion did not completely abrogate the initiation of type 2 responses because, unlike uninfected mice, *L. sigmodontis*-infected mice depleted of basophils were still able to develop measurable levels of parasite-specific IgE and CD4⁺ T cell production of IL-4 and IL-5 in response to parasite Ag. Basophil-depleted *L. sigmodontis*-infected mice also developed significantly greater eosinophilia than uninfected mice. However, when compared with basophil-competent *L. sigmodontis*-infected mice, basophil-depleted *L. sig-*

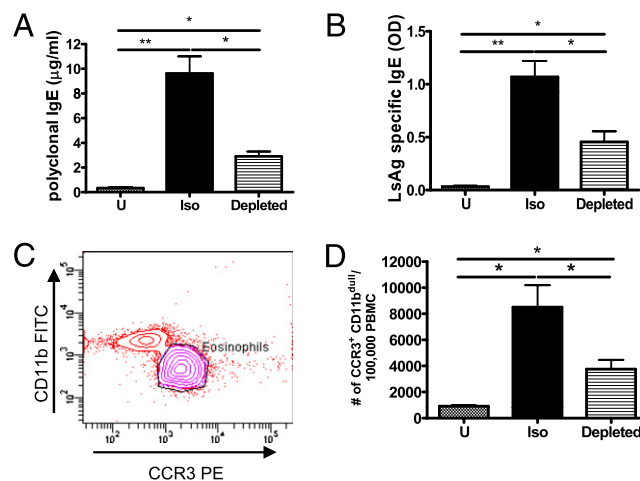


FIGURE 5. IgE and circulating eosinophil levels in uninfected mice, *L. sigmodontis*-infected mice treated with isotype control Ab (Iso), and *L. sigmodontis*-infected mice depleted of basophils with Ba103 (Depleted). Mice were infected by s.c. injection of 40 *L. sigmodontis* L3 larvae, given weekly i.p. injections of isotype control Ab or Ba103 Ab to deplete basophils, and euthanized 8 wk p.i. Polyclonal IgE (A) and LsAg-specific IgE (B) levels measured in plasma. C, Eosinophils identified by flow cytometry as CD11b^{dim} CCR3⁺. D, Levels of circulating eosinophils. Data shown are from one of two experiments using five mice per group. Error bars represent $\pm$ SEM. Significant differences between groups were analyzed by Student *t* test followed by Welch's correction for variance. * $p < 0.05$; ** $p < 0.01$. U, uninfected mice.

modontis-infected mice developed significantly lower levels of polyclonal IgE, LsAg-specific IgE, eosinophilia, and LsAg-driven CD4⁺ T cell IL-4 production and proliferation.

Discussion

The importance of basophils in the development of type 2 immune responses has become increasingly recognized over the past several years (8–12). Through the release of IL-4 and upregulation of CD40L, basophils are capable of inducing IgE isotype switching in B cells (19, 20) and Th2 differentiation of CD4⁺ T cells (21). Even though they are the least common circulating leukocytes, basophils have been shown to be substantial contributors of IL-4, the principal cytokine driving type 2 immunity, in both human and animal studies of helminth infections (14–16, 31–33). In humans, the numbers of basophils that release IL-4 in response to parasite Ag are equivalent to the number of CD4⁺ T cells releasing IL-4 (15). Further, basophils from filaria-infected patients require less Ag for activation and release more IL-4 on a per-cell basis than CD4⁺ T cells (15). By using the *L. sigmodontis* murine model of filariasis, this study demonstrates that basophils play a major role in the amplification of type 2 immune responses during chronic helminth infection.

Time-course evaluations revealed that type 2 immune responses become maximal fairly late in the course of murine filarial infection. Specifically, CD4⁺ T cell production of the type 2 cytokines IL-4 and IL-5 peaked at 6 wk p.i., and eosinophilia and basophilia peaked at 8 wk p.i. Specific IgE did not become detectable until 4 wk p.i. and then continued to increase throughout the time period studied (10 wk).

The late type 2 immune responses we observed are consistent with other studies evaluating chronic helminth infections. In *L. sigmodontis* infection, maximal parasite Ag-driven IL-4 and IL-5 production from splenocytes was previously found to occur at 10–12 wk p.i. (34), and maximal IL-4 and IL-5 cytokine transcripts at 4 to 5 wk p.i. (35). Similarly, eosinophil levels were greater at 30 d p.i.

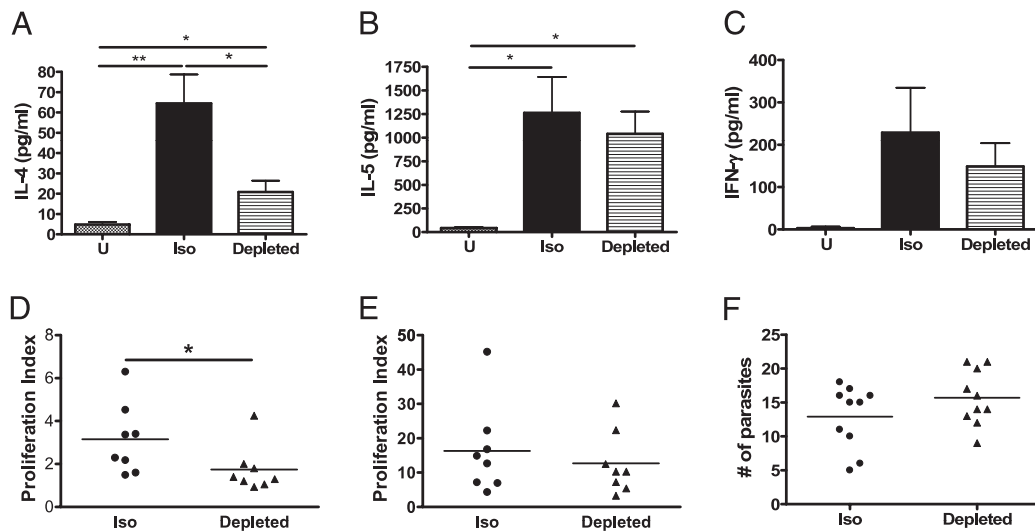


FIGURE 6. CD4⁺ T cell responses and adult worm burdens in uninfected mice, *L. sigmodontis*-infected mice treated with isotype control Ab, and *L. sigmodontis*-infected mice depleted of basophils with Ba103. Mice were infected by s.c. injection of 40 *L. sigmodontis* L3 larvae, given weekly i.p. injections of isotype control Ab or Ba103 Ab to deplete basophils, and euthanized 8 wk p.i. Concentrations of IL-4 (A), IL-5 (B), and IFN-γ (C) in supernatants of LsAg-stimulated splenic CD4⁺ T cells cultured for 3 d postsubstitution of media control. Splenic CD4⁺ T cell proliferation in response to LsAg (D) and anti-CD3 stimulation (E). F, Numbers of adult worms recovered from the thoracic cavity at study endpoint. Data shown are from one of two experiments using 5–10 mice per group. Error bars represent $\pm$ SEM. Significant differences between groups were analyzed by Student *t* test followed by Welch's correction for variance. **p* < 0.05; ***p* < 0.01. Depleted, *L. sigmodontis*-infected mice depleted of basophils with Ba103; Iso, *L. sigmodontis*-infected mice treated with isotype control Ab; U, uninfected mice

in *L. sigmodontis*-infected mice than at either 10 d or 80 d p.i. (36), and eosinophil levels in pigs infected with *Trichuris suis* peaked at 5 wk p.i. (37). Guinea pigs infected with *Fascioloides magna* demonstrated maximal eosinophil levels at 7–10 wk p.i. and maximal basophil levels at 10 wk p.i. (38). With regard to basophil activation, the late onset basophil activation we observed is consistent with a recent human study in which basophils of individuals experimentally infected with hookworms did not exhibit an activated phenotype until 4–6 wk p.i. (39)

It is important to note that the late type 2 immune responses seen in *L. sigmodontis* infection and other models of chronic helminth infection differ from that observed in rodents infected with *Nippostrongylus brasiliensis*, an acute helminth infection model in which intestinal parasites are rapidly expelled within ~2 wk of infection. Daily examination of blood smears in rats infected with *N. brasiliensis* demonstrated peak eosinophil and basophil levels 12–14 d p.i. (40). In this study, we observed that surgical implantation of adult *L. sigmodontis* worms in the peritoneum of mice moved up the time point at which basophils become activated in response to LsAg, suggesting that the late onset of basophil activation observed in *L. sigmodontis* infection may be due to insufficient Ag load or to Ag sequestered in the pleural space. Thus, differences in the time course of basophil activation and other type 2 immune responses observed in various helminth infections may be related in part to issues of Ag load and exposure to the immune system.

The finding that basophils from uninfected mice and from mice infected for 2 wk after L3 injection and for 2 d after adult worm implantation do not become substantially activated in response to parasite Ag, as measured by both IL-4 expression and CD200R upregulation by flow cytometry, demonstrates that filarial Ags do not activate basophils in a nonspecific manner. Although a number of helminth-derived molecules can directly activate basophils (41–43), the results of this study are consistent with prior studies we have conducted with human cells that showed that crude soluble extracts of whole filarial worms do not activate basophils of uninfected individuals (15). Given that basophil activation in L3 infection and adult worm implantation experiments did not occur

until parasite-specific IgE became detectable in the serum, it is likely that basophil activation in our model is primarily dependent on sensitization of basophils with specific Ab.

Because several type 2 responses peaked at time points when microfilariae were circulating in the blood, we examined type 2 immune responses in the setting of single-sex infections. We found that circulating IgE levels and concentrations of IL-4 and IL-5 released by CD4⁺ T cells in response to parasite Ag were equivalent 8 wk postimplantation of either adult female worms, in which microfilaremia developed, or male worms, in which no microfilaremia developed. Additionally, basophilia, eosinophilia, and percentages of activated basophils in response to parasite Ag were similar whether female or male adult worms were implanted. Thus, the late peak in type 2 immune responses was not attributable to the development of circulating microfilariae.

Two lines of evidence from this study suggest basophils are responsible for amplifying the type 2 immune responses in *L. sigmodontis* infection. First, we found that the late amplification of type 2 cytokine production and eosinophilia, which occurred at 6 and 8 wk p.i., respectively, occurred after the 4 wk time point at which basophils first become substantially activated and release IL-4 in response to parasite Ag. To our knowledge, this is the first time-course study of basophil activation in a chronic helminth infection model, and the observation that basophil activation begins prior to peaks in other type 2 responses is consistent with the concept that basophils amplify type 2 immunity.

The second, and more direct, line of evidence that suggests basophils are important for the amplification of type 2 immune responses comes from the basophil depletion experiments. Depletion of basophils throughout chronic *L. sigmodontis* infection resulted in significant decreases in parasite-specific IgE levels, eosinophilia, and parasite Ag-driven CD4⁺ T cell proliferation and IL-4 production. Although basophil depletion experiments have not previously been conducted on a prolonged helminth infection model, our results are consistent with results from two other experiments that have evaluated the effects of basophil depletion on type 2 responses during helminth infection. Depletion of basophils

with Ab to Thy 1.2 in 4get/rag^{-/-} mice in which basophils had been sensitized with serum from *N. brasiliensis*-infected wild-type mice resulted in decreased circulating and tissue eosinophilia as well as mRNA expression of IL-4, IL-5, and IL-13 10 d after challenge infection with *N. brasiliensis* (25). Similarly, depletion of basophils with the MAR-1 Ab to FcεR1 substantially decreased IL-4 mRNA expression from colon tissue 21 d p.i. of mice with the gastrointestinal helminth *Trichuris muris* (23).

Importantly, our study demonstrates that basophils boost IgE production in chronic helminth infection. Prior in vitro studies have clearly shown that basophils, which express CD40L as well as release IL-4, have the ability to induce B cells to switch to IgE isotype (19, 20). As highlighted in a recent review, however, whether basophils are actually important for IgE production during helminth infection has not been previously evaluated in an in vivo model (12). Because basophil depletion resulted in significant decreases in both total and parasite-specific IgE levels in the circulation of mice chronically infected with *L. sigmodontis*, our results suggest that basophils play a physiologically important role in driving IgE production. Although it is likely that IL-4 is the principal molecule responsible for the type 2 amplifying effects of basophil, release of thymic stromal lymphopoietin, which also skews CD4⁺ T cells toward a Th2 phenotype, may also be important. Also, although we believe it is probable that basophils are directly involved in the amplification of many aspects of type 2 inflammation, it is possible that some effects basophils have on type 2 inflammation are indirect. For example, basophils may principally function to increase numbers of Th2 cells, which then cause further increases in eosinophilia and IgE production.

With regards to protective immunity, we found no increased susceptibility for *L. sigmodontis* infection in mice depleted of basophils. This finding is in contrast to those of other studies in which the presence of basophils correlates with decreased susceptibility to helminth infection. Mice depleted of basophils exhibited increased intestinal carriage of *T. muris* (23) and IL-3-deficient mice, which maintain physiologic baseline levels of basophils but do not increase basophil numbers in response to helminth infection, displayed decreased clearing of *Strongyloides venezuelensis* (44) compared with control infected mice. It is possible that basophils are important effectors of immunity against some, but not all helminths, as the same authors who noted a correlation between eosinophilia and lower worm burdens with *S. venezuelensis* did not see any appreciable differences in parasite burdens between IL-3-deficient and IL-3-competent mice post-infection with *N. brasiliensis* (44). The disparate findings we and others report on the role of basophils in protective immunity demonstrate that immunological correlates of protection for one helminth infection cannot be generalized to all helminths.

Because RAG mice reconstituted with IL-4/IL-13-deficient T cells exhibit decreased *N. brasiliensis* expulsion postdepletion of basophils, it is possible that for some helminth infections the protective effects provided by basophils are redundant, and perhaps not additive, with those of intact CD4⁺ T cells (25). It is important to note that in our study, we observed that basophil depletion resulted in diminution, but not elimination, of Th2 responses. Because a complete deficiency of IL-4 or IL-5 results in, respectively, increased *L. sigmodontis* microfilaria and adult worm burdens at 60 d p.i. (45), and because we did not see any differences in worm burdens at 56 d p.i. despite decreased type 2 immune responses, our study suggests that the basophil contribution to type 2 immunity does not enhance immune protection against primary filaria infection.

Given that IgE-mediated activation of basophils in response to parasite Ag persists for years posttreatment of filarial infection in

humans (46), future studies will evaluate whether basophils play a role in protective immunity against secondary helminth infections after animals have been previously exposed or vaccinated.

Interestingly, our time-course study showed a substantial decrease in basophil activation in response to parasite Ag at the 10-wk time point compared with the 8-wk time point despite the continued presence of adult worms, microfilariae, and parasite-specific IgE. Chronic helminth infections are known to induce immunoregulatory networks that downregulate immune reactivity. Mechanisms include upregulation of regulatory T cells, induction of alternatively activated macrophages, increased production of suppressor cytokines such as IL-10 and TGF-β, and direct immunomodulatory effects of helminth Ags (47–49). Downregulation of CD4⁺ T cell immune reactivity to parasite Ag has been well demonstrated late in the course of *L. sigmodontis* infection (50), consistent with the decreases in parasite Ag-driven CD4⁺ T cell cytokine production we observed at the 10-wk time point, and has been shown to be due in part to active suppression by regulatory T cells (50). The decreased basophil reactivity we observed in response to parasite Ag 10 wk p.i. suggests that basophils may also undergo some type of active suppression in chronic helminth infection. Such a phenomenon could be one of the reasons chronic helminth infections are often associated with decreased allergic disease. The effects chronic *L. sigmodontis* infection may have on basophil releasability will be evaluated in greater detail in future studies.

Finally, it is important to note that recently three independent groups have demonstrated that basophils, which express MHC class II, are able to act as APCs and, when they do so, induce the development of Th2 CD4⁺ T cells (18–20). Whereas these studies show that basophils have the ability to initiate type 2 immune responses, the extent to which basophils actually do so during helminth infections remains uncertain. The results of this study suggest that basophils are not necessary for the induction of type 2 immune responses toward helminth infection because eosinophilia, parasite-specific IgE, and parasite Ag-driven IL-4 production from CD4⁺ T cells all developed in *L. sigmodontis*-infected mice depleted of basophils, albeit at lower levels than in basophil-competent mice. However, we cannot definitively conclude that basophils are dispensable for the initiation of type 2 immune responses because Ab depletion of basophils routinely eliminated >95%, but not all, circulating basophils. As such, whether basophils are the principal cells responsible for the initiation of type 2 immune responses in helminth infections will likely not be determined with certainty until the development of basophil-deficient mice.

In summary, the results of this study demonstrate that basophils amplify type 2 immune responses in chronic helminth infection. Activation of basophils in response to parasite Ag did not occur until 2–4 wk into the course of infection, peaked at 8 wk p.i., and coincided with a late amplification in type 2 immune responses. Single-sex worm implantation experiments revealed that late type 2 immune responses were not dependent on the development of circulating microfilariae and mixed-gender worm implantation suggested that delayed onset of basophil activation may be due in part to initial low parasite Ag load or sequestering of Ag in the pleural space. Direct evidence for the functional role of basophils in amplifying type 2 immunity came from basophil depletion experiments, which found that depletion of basophils throughout chronic infection causes decreased levels of circulating eosinophils, decreased production of parasite specific IgE, decreased CD4⁺ T cell proliferation in response to parasite Ag, and decreased parasite Ag-driven IL-4 production from CD4⁺ T cells in chronic helminth infection. Finally, basophil depletion did not alter parasite burden, suggesting basophils do not play a significant role in protective immunity during primary filarial infection.

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Disclosures

The authors have no financial conflicts of interest.

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Figure 1 Supplemental

Representative flow cytometry dot plots of basophil activation in an *L. sigmodontis*-infected mouse as assessed by intracellular IL-4 and surface CD200R expression. A-B, Gating strategy to identify basophils by flow cytometry. A. Initial large gate encompassing lymphocyte region and lower portion of granulocyte region on forward and side scatter plot. B, Basophils gated as CD4⁻B220⁻IgE⁺ cells in blood. Percentages of CD4⁻B220⁻IgE⁺ cells that stain negative for c-kit (C) and positive for CD49b (D). E, Percentages of basophils positive for intracellular IL-4 (upper panel) or surface CD200R (lower panel) from in vitro cultures of blood stimulated with Media control, anti-IgE, or LsAg for 3 hours. FMO=Fluorescence minus one, MFI=Mean Fluorescence Intensity of IL-4 or CD200R staining for all cells in the basophil gate.

Figure 2 Supplemental

Weekly Ba103 treatment results in depletion of basophils, but not peritoneal mast cells, throughout the course of *L. sigmodontis* infection. Mice were injected with isotype control antibody (upper panels) or Ba103 antibody every week and euthanized at 8 weeks p.i. Basophils were gated as CD4⁻B220⁻IgE⁺ cells (A) and peritoneal mast cells as IgE⁺ c-Kit⁺ (B). Blood samples from 2, 4 and 6 weeks p.i. were drawn just prior to the next injection of Ba103 (or isotype control) antibody.

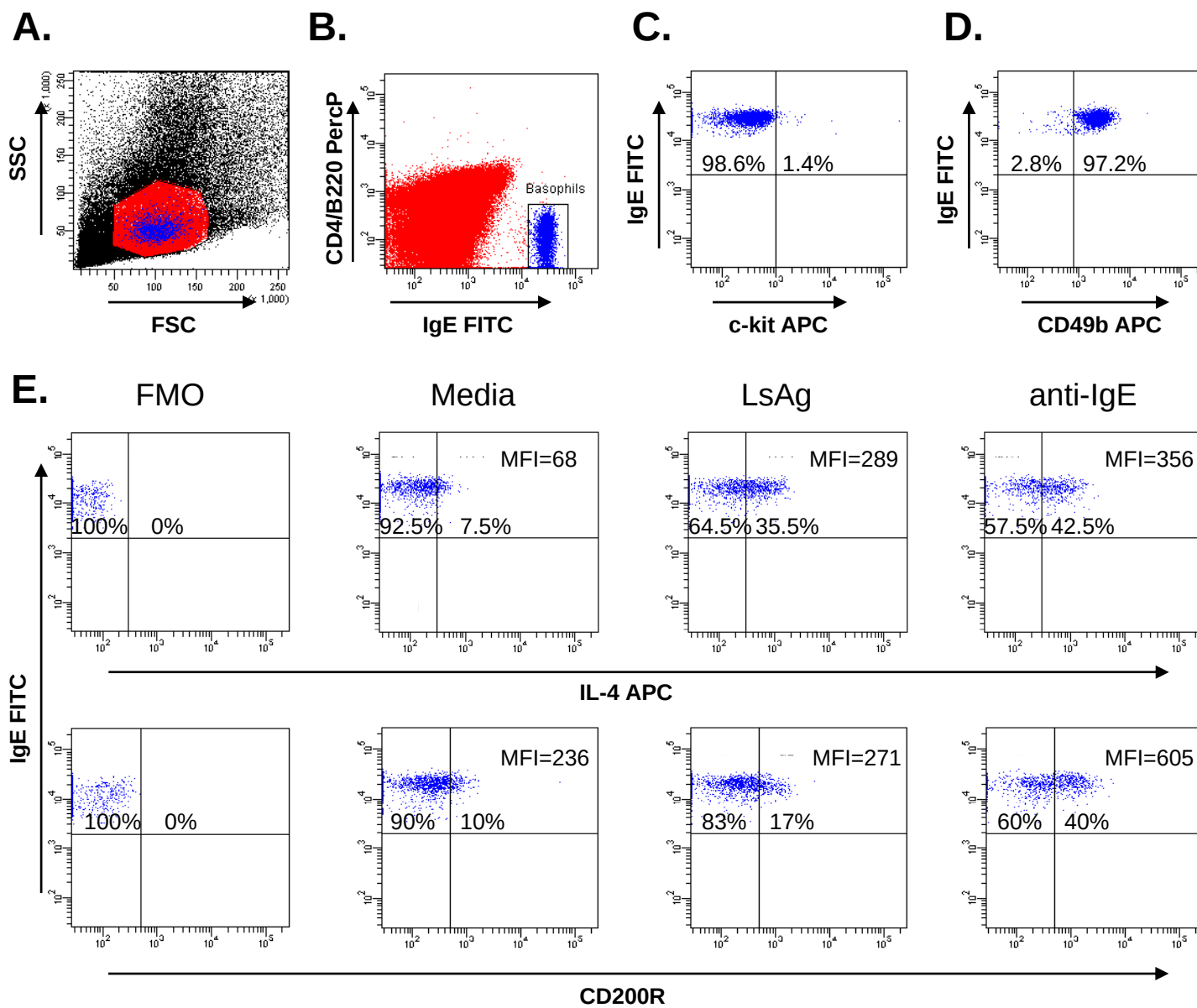


FIGURE 1 Suppl.

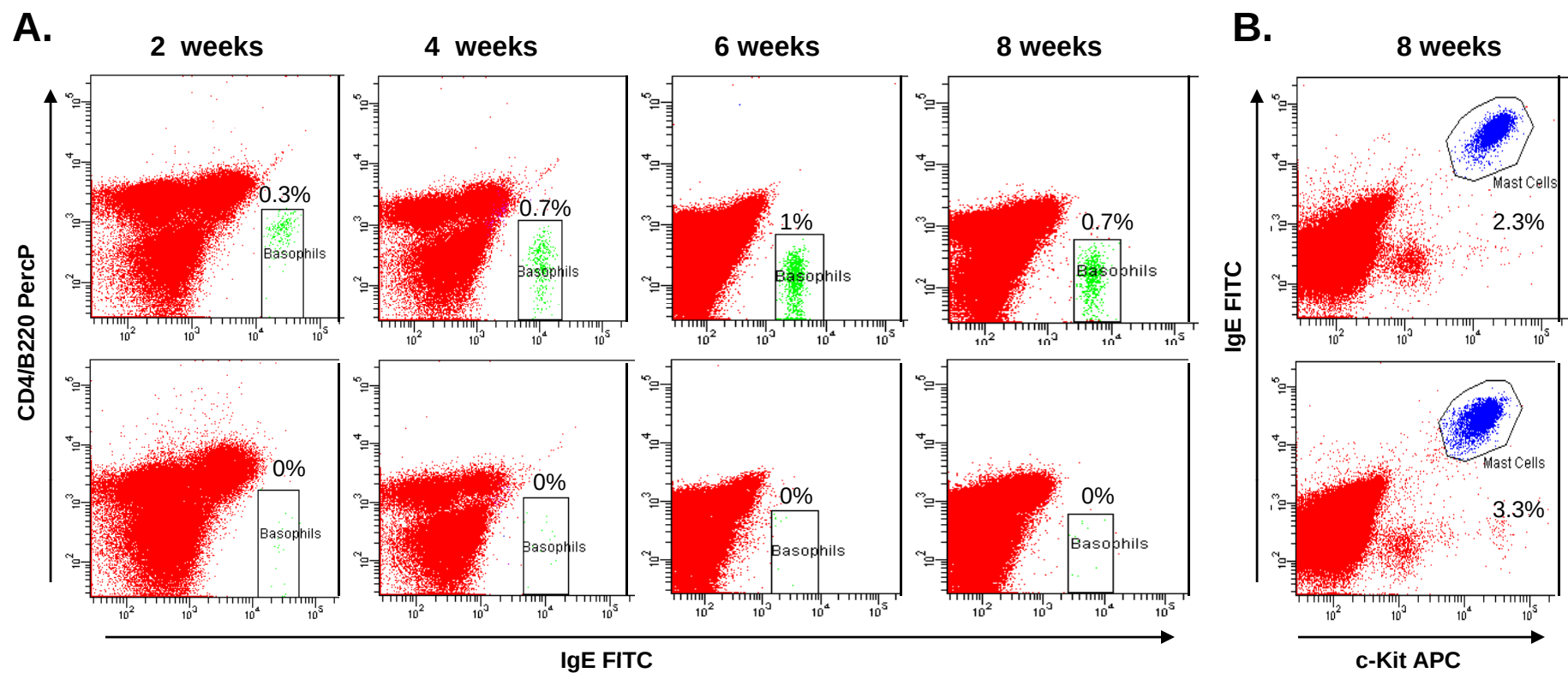


FIGURE 2 Suppl.