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Ten Weeks of Infection with a Tissue-Invasive Helminth Protects against Local Immune Complex–Mediated Inflammation, but Not Cutaneous Type I Hypersensitivity, in Previously Sensitized Mice

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In this study, we evaluated the effect chronic helminth infection has on allergic disease in mice previously sensitized to OVA. Ten weeks of infection with *Litomosoides sigmodontis* reduced immunological markers of type I hypersensitivity, including OVA-specific IgE, basophil activation, and mast cell degranulation. Despite these reductions, there was no protection against immediate clinical hypersensitivity following intradermal OVA challenge. However, late-phase ear swelling, due to type III hypersensitivity, was significantly reduced in chronically infected animals. Levels of total IgG2a, OVA-specific IgG2a, and OVA-specific IgG1 were reduced in the setting of infection. These reductions were most likely due to increased Ab catabolism as ELISPOT assays demonstrated that infected animals do not have suppressed Ab production. Ear histology 24 h after challenge showed infected animals have reduced cellular infiltration in the ear, with significant decreases in numbers of neutrophils and macrophages. Consistent with this, infected animals had less neutrophil-specific chemokines CXCL-1 and CXCL-2 in the ear following challenge. Additionally, in vitro stimulation with immune complexes resulted in significantly less CXCL-1 and CXCL-2 production by eosinophils from chronically infected mice. Expression of Fc γ RI was also significantly reduced on eosinophils from infected animals. These data indicate that chronic filarial infection suppresses eosinophilic responses to Ab-mediated activation and has the potential to be used as a therapeutic for pre-existing hypersensitivity diseases. *The Journal of Immunology*, 2015, 195: 000–000.

Despite numerous epidemiologic and animal studies suggesting helminth infections are protective against allergy, the two prospective human clinical trials that have tested the efficacy of infection as a therapeutic have failed to show clinical benefit (1, 2). Lack of protection may be due to a variety of factors, including the possibility that helminth infections are better at preventing allergy than treating it. Interestingly, whereas >30 animal studies have demonstrated that helminth infection established prior to sensitization protects against the development of allergy, very few have investigated the use of helminths as therapeutics for pre-existing allergic disease [reviewed in (3)].

In this study, we sought to determine whether *Litomosoides sigmodontis*, a tissue-invasive filarial nematode that establishes chronic infection in immunocompetent BALB/c mice (4), protects against local hypersensitivity responses after sensitization has taken place. Similar to other helminths, *L. sigmodontis* induces

systemic immunomodulation (5–7), and a previous study demonstrated that *L. sigmodontis* can inhibit the development of allergic disease when infection is established prior to allergic sensitization (8). As we recently demonstrated that chronic *L. sigmodontis* infection suppresses the IgE-mediated activation of basophils (9), we hypothesized that infection may also protect against allergic disease in previously sensitized mice.

Our results demonstrate that whereas 10 wk of *L. sigmodontis* infection suppresses numerous immunologic markers of type I hypersensitivity, including allergen-specific IgE as well as basophil and mast cell degranulation in response to allergen exposure, it does not confer clinical benefit as measured by increases in local vascular permeability. Interestingly, though, we did find that infection protects the host from ear swelling due to type III (immune complex–mediated) hypersensitivity. This protection is associated with reduced neutrophil-specific chemokine production, fewer neutrophils trafficking to the site of immune complex deposition, reduced chemokine production by eosinophils after immune complex stimulation, and decreased Fc γ RI expression on eosinophils.

Materials and Methods

Animals

Four- to six-week-old female BALB/c mice (National Cancer Institute Mouse Repository, Frederick, MD), IgE-deficient mice (The Jackson Laboratory, Bar Harbor, ME), C57BL/6 mice (The Jackson Laboratory), mast cell–deficient W^{sh} mice (The Jackson Laboratory), eosinophil-deficient Δ dblGATA mice (The Jackson Laboratory), and Ab-deficient $J_H^{-/-}$ mice (Taconic, Hudson, NY) were housed at the Uniformed Services University Center for Laboratory Animal Medicine. All experiments were performed under protocols approved by the Uniformed Services University Institutional Animal Care and Use Committee.

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Abbreviations used in this article: CBC, complete blood count; LAA, local anaphylaxis assay.

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OVA sensitization

Mice were sensitized as previously described (10). In brief, mice received i.p. injections of 50 µg OVA (Sigma-Aldrich) adsorbed to 2 mg aluminum hydroxide (Pierce) in PBS on days 0, 7, and 14. Mock sensitization groups received i.p. injections of 2 mg aluminum hydroxide in PBS. Mice were given a 2- to 6-wk rest period before infection or OVA challenge.

L. sigmodontis infection

Infectious L3-stage larvae were isolated from the pleural cavity of infected jirds (*Meriones unguiculatus*, obtained from TRS Laboratory, Athens, GA), as previously described (11). Mice were infected by s.c. injection of 40 L3 larvae in RPMI 1640. Mock treatment groups were given a s.c. injection of RPMI 1640 (Mediatech). Worm counts, OVA challenge, and immunological assays were performed 10 wk postinfection.

Intradermal OVA challenge

The local anaphylaxis assay was performed as previously described (10). Mice were given an intradermal injection of 20 µg OVA in 10 µl PBS in one ear and 10 µl vehicle alone (PBS) in the other ear. Three minutes after challenge, 200 µl 0.5% Evans Blue dye (Sigma-Aldrich) was injected into the tail vein. Ten minutes later, animals were euthanized with CO₂ and ears were removed and placed in formamide (Sigma-Aldrich) overnight at 63°C. Extracted dye was measured at an absorbance of 620 nm. The OD of the vehicle-challenged ear was then subtracted from the OD of the OVA challenge ear for each animal.

For the ear thickness assay, a micrometer was used to measure baseline ear thickness prior to intradermal injection of 20 µg OVA or vehicle. Ear thickness was then measured at 1, 2, 12, 24, 48, 72, and 96 h post-challenge.

Basophil, CD4 cell, and platelet depletions

For basophil depletion, mice received an i.p. injection of 50 µg anti-CD200R3 clone Ba103 (Hycult Biotech) or rat IgG2b isotype control clone A95-1 (BD Biosciences) 24 h before challenge. For CD4⁺ T cell depletion, mice received an i.p. injection of 500 µg anti-CD4 clone GK1.5 (BioXcell) or rat IgG2b isotype control 24 h before challenge. Depletion of basophils and CD4⁺ T cells was confirmed via flow cytometry 4 d after challenge. For platelet depletion, mice received an i.p. injection of 4 µg anti-CD41 clone MWReg30 (provided by J. Semple, University of Toronto) or rat IgG1 isotype control clone R3-34 (BD Biosciences) 24 h before challenge. Platelet depletion was confirmed by complete blood count (CBC) 24 h after administration of the Ab.

Histology

Ten weeks postinfection, animals were challenged with PBS in one ear and 20 µg OVA in the other. Three and 24 h after challenge, animals were euthanized and ears were fixed in 10% formalin. H&E and toluidine blue staining was performed by Histoserv (Rockville, MD). Slides were digitized with a ×20 objective on a 2.0-RS NanoZoomer (Hamamatsu) using NDP.scan software version 2. Digitized slides were analyzed with NDP.view software version 1. Mast cell degranulation was assessed 24 h after challenge via toluidine blue stain. Based on the assumption that left and right ears contain a similar number of mast cells for each mouse, cells staining positive for toluidine blue for an entire ear section were enumerated. The number of toluidine-positive cells for the OVA challenge ear was then subtracted from the PBS challenge ear for each mouse to quantify the difference in mast cell degranulation as a result of OVA challenge. To assess ear pathology, a blinded pathologist (B.K.M.) scored H&E sections for each ear using four parameters (hemorrhage, edema, necrosis, and cellularity) on the basis of severity (0, absent; 1, mild; 2, moderate; or 3, severe) and focality (0, absent; 1, focal; 2, intermediate; and 3, diffuse) for a total maximum inflammation score of 24.

Immunohistochemistry

Ten weeks postinfection, animals were challenged with PBS in one ear and 20 µg OVA in the other. Three hours after challenge, animals were euthanized and ears were frozen with liquid nitrogen. Immunohistochemistry was performed by Histoserv. Ears were stained with anti-IgG FITC (Sigma-Aldrich) or DAPI, anti-C3 FITC (Immunology Consultants Laboratory), and anti-IgG Texas Red (Life Technologies). Confocal images of the entire ear were obtained with a ×10 objective using a Zeiss 710 microscope and Zen software. Prior to analysis, TIFF files were exported to Adobe Photoshop, and nonspecific staining by anti-C3 FITC along the outline of the ear was masked to ensure proper immune

complex identification (Supplemental Fig. 1A). Analysis of immune complexes was performed using the Puncta Analyzer plugin v2.0 (<https://github.com/physion/puncta-analyzer>) in ImageJ (imagej.nih.gov/ij/).

ELISAs and ELISPOT assays

ELISAs were performed on plasma samples from 10-wk infected mice. Total IgE (eBioscience), total IgG (eBioscience), total IgG2a (Kamiya Biomedical), OVA-specific IgE (MD Bioproducts), OVA-specific IgG1 (Cayman Chemical), and OVA-specific IgG2a (α Diagnostic) ELISAs were performed according to manufacturer instructions. *L. sigmodontis*-specific IgE ELISAs were performed by coating flat-bottom Immulon 4 plates (Thomas Scientific) with 20 µg/ml *L. sigmodontis* Ag overnight at 4°C. Plates were blocked with 5% BSA in PBS for 1 h. Prior to adding samples to the plate, IgG was adsorbed by incubating serum with GammaBind plus Sepharose (GE Healthcare) overnight at 4°C and diluted 1:8. Plates were then incubated with biotinylated rat anti-mouse IgE clone R35-118 (BD Biosciences), followed by 1:1000 dilution of alkaline phosphatase-conjugated streptavidin (BD Biosciences). Nitrophenyl phosphate disodium (Sigma-Aldrich) was used as substrate. Absorbance was detected with a Victor³ V microplate reader (PerkinElmer).

For ELISPOT assays, single-cell suspensions of splenocytes were prepared for each animal at 10 wk postinfection. Spleens were removed and homogenized through a 0.7-µm cell strainer (BD Biosciences), and RBCs were lysed with ACK lysing buffer (Quality Biological). Cells were frozen in RPMI 1640 containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES buffer, 30% FCS, and 10% DMSO. Total and OVA-specific IgG2a ELISPOTs (U-CyTech Biosciences) were performed according to manufacturer instructions. Spots were counted manually with a dissecting microscope.

CBCs

Ten weeks postinfection, whole blood was collected into EDTA tubes (BD Biosciences), and CBCs were obtained by BioReliance (Rockville, MD).

Obtaining cells from ear tissue

Tissue-processing methodology was adapted from Shannon et al. (12). Both ears were challenged by intradermal injection of 20 µg OVA. Twenty-four hours later, mice were euthanized and a terminal bleed was performed. Then animals were perfused with 5 ml PBS by intracardiac injection, after which ears were removed and rinsed with 70% ethanol. The dorsal and ventral dermal layers of both ears were then separated and placed in a 35 × 10-mm nontissue culture-treated petri dish (BD Biosciences) containing 3 ml digestion buffer (RPMI 1640, 25 mM HEPES [Mediatech], 1.5 g/L NaHCO₃, 100 U/ml DNase I [Roche], and 170 µg/ml Liberase [Roche]). Ear tissue was incubated at 37°C for 30 min and homogenized through a 0.7-µm cell strainer to create a single-cell suspension. A total of 4 ml PBS was used to rinse the strainer, and additional 3 ml PBS was added to bring the final sample volume to 10 ml. Cell counts were obtained with a Countess automated cell counter (Life Technologies) using trypan blue exclusion.

Basophil activation assay

Ten weeks postinfection, whole blood was collected into heparinized tubes (BD Biosciences). Samples were centrifuged at 600 × g for 10 min, plasma was removed, and remaining cells were washed with RPMI 1640. Washed cells from two animals were pooled for each sample. Samples were stimulated with RPMI 1640 and 40, 10, 2.5, and 0.625 µg/ml OVA for 1 h at 37°C and 5% CO₂. GolgiStop (BD Biosciences) was added, and cells were incubated for an additional 2 h at 37°C. Cells were washed twice with PBS, lysed, and fixed with a whole-blood lysing kit (Beckman Coulter). Basophil activation was assessed by flow cytometry.

Flow cytometry

To measure basophil activation, fixed cells were blocked with 1% PBS/BSA for 1 h at 4°C and then incubated with Perm/Wash buffer (BD Biosciences) for 15 min at 4°C. Cells were stained with anti-IgE FITC clone R35-72 (BD Biosciences), anti-CD4 PerCP clone RM4-5 (BD Biosciences), anti-B220 PerCP clone RA3-6B2 (BD Biosciences), and anti-IL-4 allophycocyanin clone 11B11 (BD Biosciences). Basophils were gated as CD4[−] B220⁺ IgE⁺, and activated basophils were gated as IL-4⁺. A total of 1 × 10⁵ events was analyzed per sample.

To determine cell types recruited to the ear following OVA challenge, single-cell suspensions of ear tissue were centrifuged at 290 × g for 5 min and resuspended in 500 µl 1% PBS/BSA for 30 min at 4°C. Cells were stained with anti-Ly6G allophycocyanin-eFluor780 clone RB6-8C5

(eBioscience), anti-F4/80 Pacific blue clone BM8 (eBioscience), anti-CD19 PE-Cy5 clone eBio1D3 (eBioscience), anti-CD11c BV421 clone N418 (BioLegend), anti-SiglecF PE-CF594 clone E50-2440 (BD Biosciences), and anti-CD45 allophycocyanin-Cy7 clone 30-F11 (BioLegend), and fixed in 4% paraformaldehyde. Eosinophils were gated as CD11c⁺CD45⁺SiglecF⁺, dendritic cells were gated as CD11c⁺, B cells were gated as CD19⁺, macrophages were gated as F4/80⁺, and neutrophils were gated as F4/80⁺Ly6G⁺. A total of 1.5×10^4 events was analyzed for mock-treated animals, 3×10^4 events for infected animals, 4×10^4 events for sensitized animals, and 4×10^4 events for sensitized plus infected animals.

To measure FcγR expression, aliquots of previously prepared frozen splenocytes were thawed and resuspended in 500 μl 1% PBS/BSA for 30 min at 4°C. Samples were stained with the aforementioned Abs used to determine cell types recruited to the ear. Additionally, samples were also stained with anti-CD64 PE clone x54-5/7.1 (BioLegend), anti-CD32 (Bio-ryb) conjugated to FITC (EasyLink FITC conjugation kit; Abcam), anti-CD16 clone EPR4333 (Abcam) conjugated to allophycocyanin (Lightning-Link allophycocyanin Ab labeling kit; Novus Biologicals), and anti-FcεRI PE clone MAR-1 (eBioscience). Cell types were gated as previously described. Each cell type was then assessed for FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcεRI positivity. A total of 1×10^6 events was analyzed per sample.

For all flow cytometric experiments, Abs were individually titrated using splenocytes from naive BALB/c mice, and OneComp eBeads (eBioscience) were used to calculate compensation for each flow cytometry run. Gates were established using the fluorescence-minus-one technique. Flow cytometric data were collected with a BD LSR-II flow cytometer (BD Biosciences) and analyzed with FlowJo software version 7 (Tree Star).

Chemokine production

Ten weeks postinfection, both ears were challenged with 20 μg OVA. Six hours later, mice were euthanized and ears were removed and cut into pieces ~1.5 cm in size. Ears were pooled for each mouse and added to Lysing Matrix D FastPrep tubes. One Complete Mini protease inhibitor mixture tablet (Roche) was dissolved in 10 ml PBS, and 500 μl was added to each tube. Samples were homogenized with a FastPrep-24 instrument

(MP Biomedicals) set at speed 5 for 20 s. Homogenization was repeated three times with a 30-s break between runs. Tubes were then centrifuged at $16,100 \times g$ for 10 min, and supernatants were collected and stored at -20°C. Samples were tested for the presence of chemokines with a Proteome Profiler Mouse Chemokine Array Kit (R&D Systems), according to manufacturer instructions. Membranes were developed for 10 min and digitized for analysis on ImageJ. Densitometry was performed using particle analysis, with measurements for each sample spot averaged and then normalized to membrane reference spots.

In vitro immune complex stimulation

Peritoneal macrophages were isolated by performing a peritoneal lavage with 10 ml HBSS. Cells were pelleted and resuspended in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES buffer. A total of 2.5×10^5 peritoneal cells in 500 μl RPMI 1640 was aliquoted to 48-well plates. Cells were incubated at 37°C for 2 h to allow macrophages to adhere. Nonadherent cells were removed by washing three times with 1 ml PBS. A total of 500 μl fresh RPMI 1640 media was added to each well.

A single-cell suspension of splenocytes was prepared by homogenizing spleens through a 0.7-μm cell strainer and removing contaminating RBCs by hypotonic lysis. Splenocytes were then used to generate an eosinophil-enriched cell suspension via negative selection. Splenocytes were incubated with CD45R (B220) and CD90 (Thy1.2) magnetic beads (MACS; Miltenyi Biotec) and applied to LD columns, as per manufacturer instructions. A total of 2×10^5 recovered cells in 500 μl RPMI 1640 was aliquoted to 48-well plates. A cytospin and DiffQuick stain was performed to assess the composition of recovered cells. Preparations typically resulted in 33% eosinophils, 57% polymorphonuclear cells, and 10% monocytes.

Rabbit anti-OVA Ab (Polysciences) was resuspended in ultra-pure H₂O and passed through a Mustang E endotoxin removal filter (Pall). OVA was resuspended in RPMI 1640 and also passed through a Mustang E filter. Immune complexes were generated by incubating 1 μg OVA with 100 μg rabbit anti-OVA Ab, with the total volume brought to 25 μl with RPMI 1640. OVA and anti-OVA Ab were incubated 37°C for 30 min, after which 25 μl immune complexes were added per well of 2×10^5 cells. As a control, additional cells were stimulated with 25 μl 1 μg OVA in RPMI

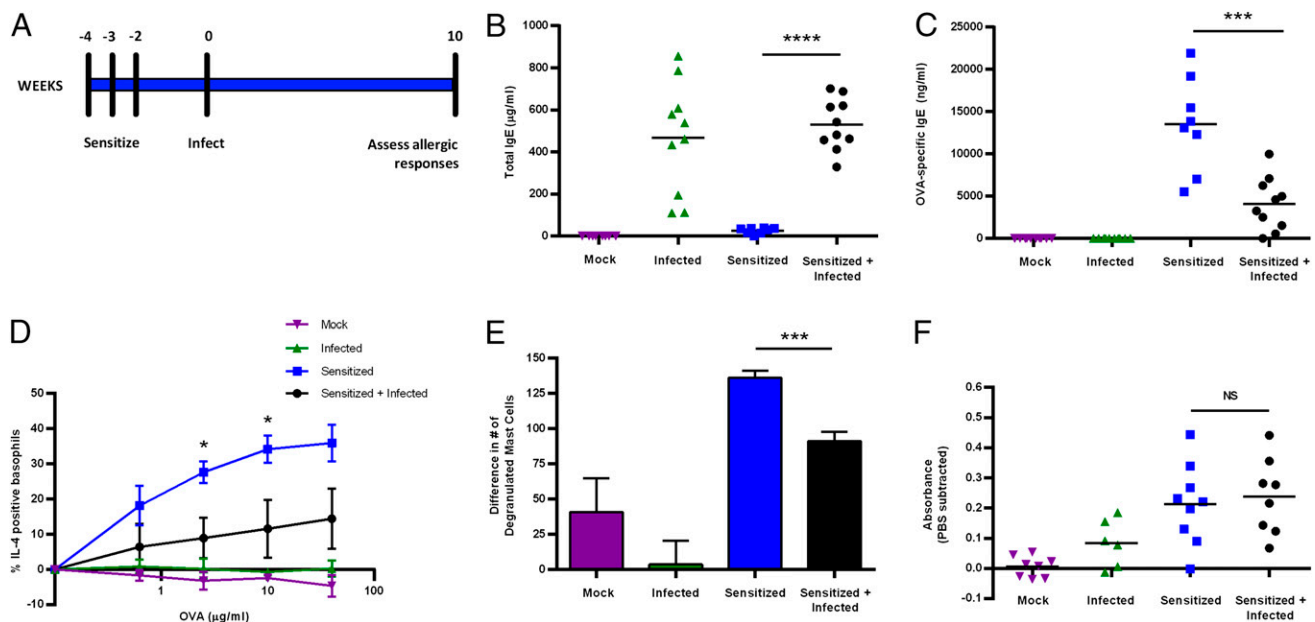


FIGURE 1. Chronic *L. sigmodontis* infection did not protect against clinical type I hypersensitivity. (A) Experimental design for the use of *L. sigmodontis* as a therapeutic agent for pre-existing allergic disease. Levels of circulating total IgE (B) and OVA-specific IgE (C) were assessed by ELISA at 10 wk postinfection. (D) Flow cytometric analysis of basophil activation in response to increasing concentrations of OVA stimulation. Basophils were gated as CD4⁺B220⁺IgE⁺, and activation was determined by intracellular IL-4 staining using fluorescence minus one controls. Values plotted represent percentage of IL-4⁺ basophils after OVA stimulation minus percentage of IL-4⁺ basophils after culture in media alone. Flow data are representative of two independent experiments with two animals pooled per sample and media levels subtracted. Sensitized and sensitized plus infected groups were compared at each concentration by the Mann-Whitney *U* test. (E) Differences in mast cell degranulation between PBS- and OVA-challenged ears. Nondegranulated mast cells were enumerated by toluidine blue stain of ear tissue 24 h after challenge. The total number of toluidine-positive staining cells for the OVA challenge ear was subtracted from the PBS challenge ear to quantify differences in mast cell degranulation. (F) LAA quantification of cutaneous type I hypersensitivity reactions in response to OVA challenge, with PBS values subtracted. Unless otherwise noted, data are representative of two independent experiments with four to five BALB/c mice per group. Error bars represent $\pm$ SEM.

p* < 0.05, **p* < 0.001, *****p* < 0.0001.

1640. Cells were stimulated with OVA or immune complexes at 37°C for 6 h, after which plates were centrifuged at $400 \times g$ for 10 min. Supernatants were collected and assayed with mouse KC and MIP-2 ELISAs (Ray Biotech), as per manufacturer instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 6 (GraphPad Software). Sensitized and sensitized plus infected groups were analyzed using the Mann–Whitney *U* test. The *p* values <0.05 were considered significant. Unless otherwise noted, data are representative of two individual experiments with four to five animals per group.

Results

Chronic *L. sigmodontis* infection does not protect against cutaneous type I hypersensitivity

To determine whether chronic helminth infection protects against type I, IgE-mediated hypersensitivity, BALB/c mice were sensitized to OVA for 3 wk, given a rest period, infected with the rodent filarial parasite *L. sigmodontis* for 10 wk, and then assessed for local anaphylaxis after intradermal injection of OVA (Fig. 1A). *L. sigmodontis* is a chronic tissue-invasive nematode in which adult worms reside in the pleural cavity and microfilariae circulate in the blood. In all experiments, sensitization did not affect worm burdens, which were assessed at the study end point (data not shown).

OVA-sensitized animals had elevated total and OVA-specific IgE levels compared with mock animals (Fig. 1B, 1C), confirming that OVA sensitization successfully elicited a type 2 immune response. As expected, all infected animals had high levels of total IgE

(Fig. 1B), and levels of *L. sigmodontis*-specific IgE levels were the same for infected and sensitized plus infected animals (data not shown). Despite having higher levels of total IgE than sensitized mice, sensitized plus infected animals had significantly lower levels of OVA-specific IgE compared with sensitized animals (Fig. 1C).

Basophils and mast cells are two major allergy effector cells that release histamine and other proinflammatory mediators in response to IgE cross-linking. To determine whether basophils were suppressed in response to allergen, whole blood was stimulated with increasing concentrations of OVA and basophil activation was assessed by measurement of IL-4 production by intracellular flow cytometry. Basophils, identified as $CD4^+B220^-IgE^+$ cells, had reduced IL-4 production in sensitized plus infected animals compared with sensitized animals (Fig. 1D).

To evaluate whether mast cell function was reduced in the setting of chronic *L. sigmodontis* infection, mast cell degranulation was measured by enumerating total and degranulated mast cells in ear tissue 24 h after intradermal challenge with OVA or PBS. The number of mast cells in the OVA challenge ear was then subtracted from the PBS challenge ear. As seen in Fig. 1E, chronically infected animals had significantly fewer degranulated mast cells after OVA challenge than sensitized animals.

Given that sensitized plus infected animals had lower levels of OVA-specific IgE, decreased IL-4 production by basophils, and reduced mast cell degranulation following OVA challenge, we next tested whether infection protected against clinical allergic responses using a local anaphylaxis assay (LAA). The LAA monitors changes in vascular permeability by quantifying dye extravasation

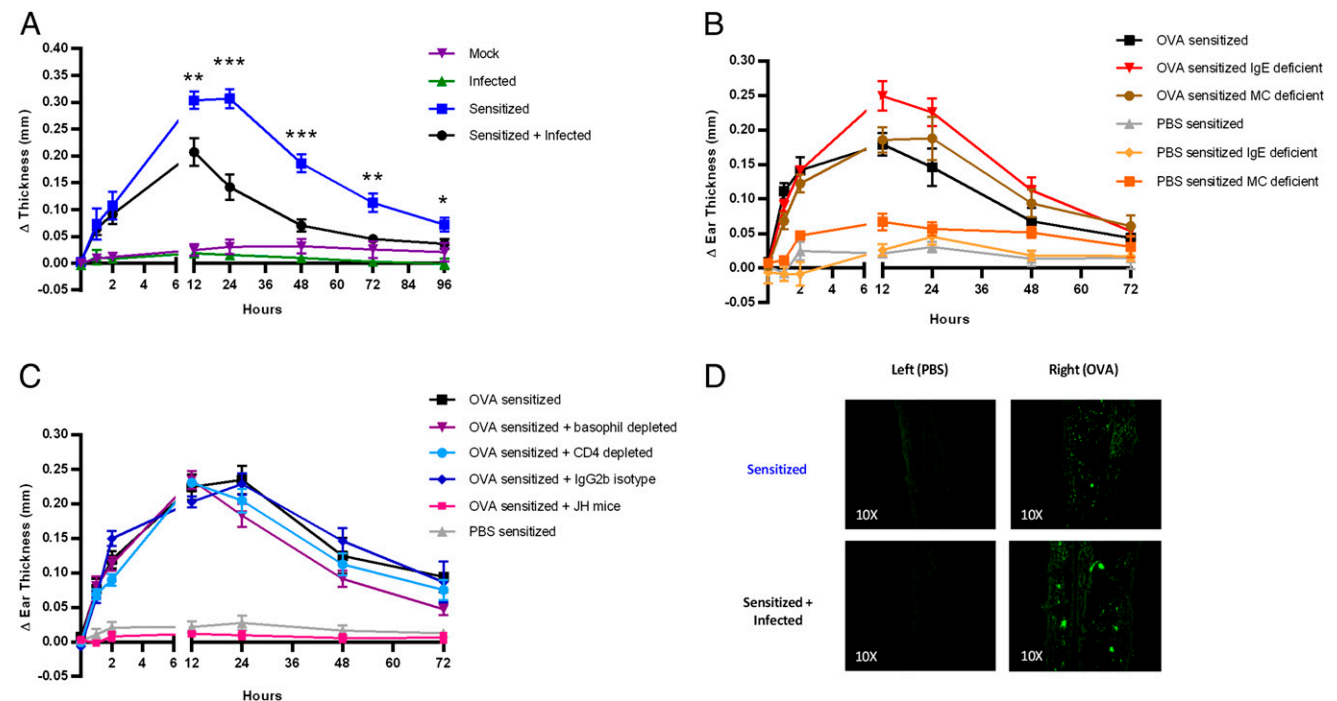


FIGURE 2. Immune complex-mediated ear swelling was significantly reduced in sensitized plus infected animals. (A) Ear swelling was measured by micrometer for 4 d following OVA and PBS challenge at 10 wk postinfection. Data points for sensitized and sensitized plus infected groups were compared at each time point by the Mann–Whitney *U* test. Data are representative of two independent experiments composed of four to five BALB/c mice per group. (B) Ear swelling for uninfected OVA- and PBS-sensitized mice on C57BL/6 background, including IgE-deficient, mast cell-deficient (*W^{sh}*), and C57BL/6 control mice. Data are representative of two independent experiments composed of three animals per group. (C) Ear swelling for uninfected OVA- and PBS-sensitized mice on BALB/c background, including Ab-deficient *J^H* and BALB/c control mice. Basophils and CD4 cells were depleted from BALB/c mice by administration of anti-CD200R and anti-CD4, respectively, 24 h prior to challenge. Depletion was confirmed 72 h postchallenge by flow cytometry. Data are representative of two independent experiments composed of three animals per group. Δ Thickness represents differences in ear thickness between PBS- and OVA-challenged ears (OVA-PBS) for each animal. (D) Visualization of immune complexes in OVA-sensitized BALB/c mice by immunohistochemistry 3 h after PBS or OVA challenge by staining with anti-mouse IgG-FITC. Error bars represent $\pm$ SEM. **p* < 0.05 , ***p* < 0.01 , ****p* < 0.001 .

in the tissue following allergen challenge (10). Sensitized and sensitized plus infected animals did not show any difference in dye extravasation (Fig. 1F), indicating that 10 wk of infection with *L. sigmodontis* does not protect against the symptoms of type I hypersensitivity in previously sensitized mice. These results suggest that, although *L. sigmodontis* suppresses several immunological drivers of type I hypersensitivity (allergen-specific IgE, basophil activation, and mast cell degranulation), the extent to which it does so is not sufficient to appreciably alter clinical responses that occur after local allergen challenge.

Chronic L. sigmodontis infection protects against late-phase ear swelling due to type III hypersensitivity

We next assessed the effect chronic helminth infection had on late-phase inflammation after intradermal allergen exposure. Animals were challenged with 20 μ g OVA in the right ear and PBS in the left ear, and ear thickness was serially measured over a period of 4 d using a micrometer.

Unsensitized animals (infected and mock groups) did not respond to OVA challenge and had negligible ear swelling throughout the time course (Fig. 2A). During the early time points, 1 and 2 h after challenge, sensitized and sensitized plus infected animals exhibited the same degree of swelling, confirming results from the LAA that infection does not suppress immediate allergic responses. However, at all of the late-phase time points (12–96 h postchallenge), sensitized plus infected animals had significantly less ear swelling than sensitized animals (Fig. 2A).

To determine whether late-phase ear swelling was due to type I hypersensitivity, we performed the ear thickness assay on mast cell-deficient (W^{sh} , C57BL/6 background), IgE-deficient (C57BL/6 background), and C57BL/6 control mice. When OVA-sensitized mice from each strain were challenged with OVA, they exhibited increases in ear thickness comparable to sensitized wild-type mice (Fig. 2B). These data indicate that neither IgE nor mast cells are required for late-phase swelling.

Because previous studies have implicated basophils as important mediators of late-phase swelling in type I hypersensitivity (13), we next evaluated whether basophils were contributing to the late-phase swelling observed in our allergy model. To deplete basophils, BALB/c mice were given 50 μ g anti-CD200R3 24 h prior to OVA challenge. Flow cytometry demonstrated that this approach resulted in >85% basophil depletion for the duration of the monitoring period (data not shown). As seen in Fig. 2C, basophil depletion did not reduce late-phase ear swelling in response to intradermal OVA challenge, indicating that basophils were not important for swelling in our allergy model. Similar results were found for OVA-sensitized BALB/c mice that were depleted of CD4 T cells, indicating that late-phase swelling was not due to type IV hypersensitivity (Fig. 2C). Finally, platelet depletion by i.p. administration of anti-CD41 24 h prior to challenge also had no effect on ear swelling (Supplemental Fig. 2).

It was only when Ab-deficient J_H mice (BALB/c background) were used that we observed complete abrogation of the late-phase response (Fig. 2C). This result suggests that late-phase

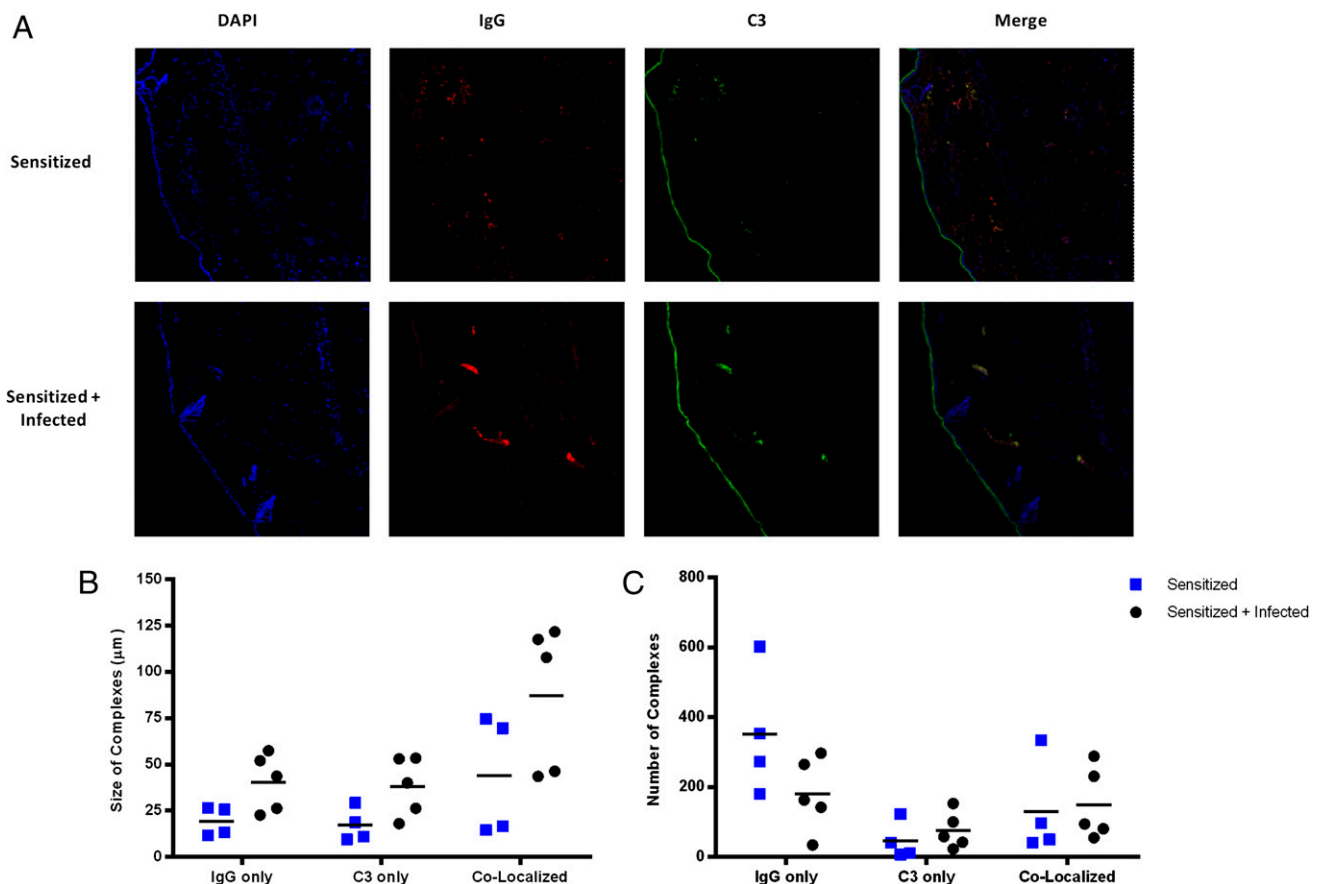


FIGURE 3. Chronic *L. sigmodontis* infection does not alter immune complex morphology. Immunohistochemistry was performed on ears 3 h after OVA challenge. Ear sections were stained with DAPI, anti-mouse IgG-Texas Red, and anti-mouse C3-FITC to visualize immune complexes. Data are from two independent experiments with two to three BALB/c mice per group. **(A)** Representative images taken with a Zeiss 710 confocal microscope at $\times 10$ with 50% zoom to show detail. For each animal, the entire ear was imaged and analyzed using the Puncta Analyzer plugin on ImageJ. **(B)** Average size of IgG only, C3 only, and colocalized complexes. **(C)** Number of IgG only, C3 only, and colocalized complexes.

ear swelling is due to immune complex-mediated type III hypersensitivity.

To assess for this, we performed immunohistochemistry on ears from sensitized and sensitized plus infected animals 3 h after OVA challenge. Anti-mouse IgG conjugated to FITC was then used to visualize immune complex deposition in the ear tissue via confocal microscopy (Fig. 2D). Immune complexes were readily visible in both groups, confirming that late-phase ear swelling in our model is due to type III hypersensitivity. Indeed, the time course of the swelling observed in our model is most consistent with that of immune complex-mediated inflammation.

Infection does not affect immune complex size, number, or complement activation

To evaluate whether infection induced changes in immune complex morphology, we performed immunohistochemistry for IgG and C3 and visualized immune complexes via confocal microscopy. Both sensitized and sensitized plus infected animals showed IgG, C3, and colocalized immune complex staining (Fig. 3A). There were no significant differences between groups with regard to immune complex size or number (Fig. 3B, 3C). These results suggest that the mechanism by which infection protects against ear swelling due to type III hypersensitivity most likely occurs downstream of immune complex formation.

*Chronic *L. sigmodontis* infection reduces levels of allergen-specific IgG*

Total IgG, total IgG2a, OVA-specific IgG2a, and OVA-specific IgG1 Ab levels were measured by ELISA at 10 wk postinfection. Sensitized plus infected animals had elevated levels of total IgG (Fig. 4A), but a significant reduction in total IgG2a (Fig. 4B), the predominant IgG subclass that participates in immune complex formation. Furthermore, OVA-specific IgG2a levels were also significantly lower in sensitized plus infected animals compared with sensitized animals (Fig. 4C). The reduction in OVA-specific IgG was not subclass dependent, as OVA-specific IgG1, an anti-inflammatory IgG subclass associated with tolerance (14),

was also lower in sensitized plus infected animals compared with the sensitized group (Fig. 4D).

To ascertain whether the reduction in OVA-specific IgG2a was due to a defect in Ab production, ELISPOT assays were performed. Sensitized plus infected animals had higher numbers of total IgG2a (Fig. 4E) and similar numbers of OVA-specific IgG2a (Fig. 4F)-producing cells as sensitized animals. These data indicate that Ab production is not compromised during chronic infection, suggesting that the reduction in OVA-specific Ab levels in infected animals may be due to increased Ab catabolism.

Infection significantly reduces pathology at 24 h postchallenge

Ear pathology was assessed by H&E stain at 3 h postchallenge (Fig. 5A), the time point at which immune complexes were visualized by immunohistochemistry, and 24 h postchallenge (Fig. 5C), the time point of maximal ear swelling. A blinded pathologist scored the tissue sections based on severity and focality of edema, hemorrhage, necrosis, and cellularity. Scores for OVA-challenged ears were comparable between sensitized plus infected and sensitized animals at the 3-h time point (Fig. 5B). At 24 h postchallenge, ears of sensitized animals exhibited marked cellular infiltration. As seen in Fig. 5C, cellular infiltration was also present in sensitized plus infected animals, but to a much lower degree. Sensitized plus infected animals had a significant reduction in pathology score at 24 h postchallenge (Fig. 5D), confirming the protective effect observed when performing the ear thickness assay.

Infection reduces neutrophil and macrophage recruitment following OVA challenge

After observing reduced swelling and cellularity in sensitized plus infected animals, we quantified the specific cell types recruited to the ear tissue after allergen exposure. A single-cell suspension of ear tissue was prepared 24 h after OVA challenge. Cells were enumerated on a hemacytometer and then stained for flow cytometry to determine the cell types present. The number of live cells in the ear tissue was significantly reduced in sensitized plus infected animals compared with sensitized animals (Fig. 6A);

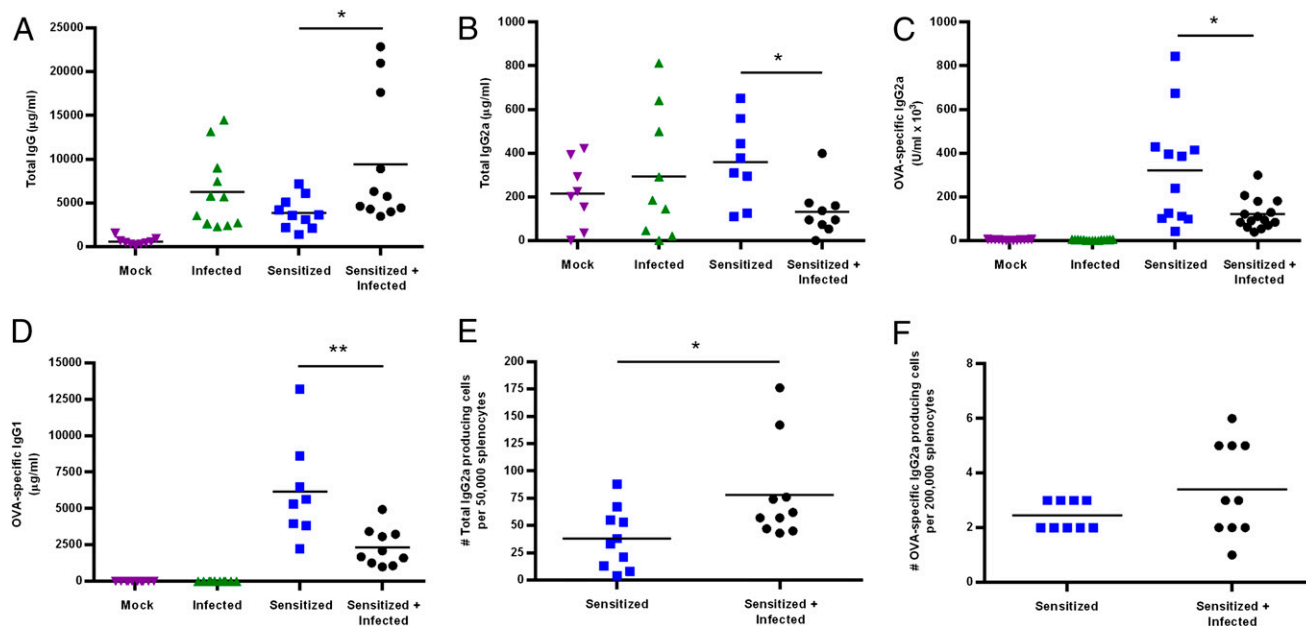


FIGURE 4. Chronically infected animals had reduced levels of allergen-specific Abs. Levels of circulating total IgG (A), total IgG2a (B), OVA-specific IgG2a (C), and OVA-specific IgG1 (D) were detected by ELISA at 10 wk postinfection. ELISPOT assays for total IgG2a (E) and OVA-specific IgG2a (F) were performed on live, frozen splenocytes that were isolated from animals at the 10-wk time point. Data are representative of two independent experiments with four to five BALB/c mice per group. Statistical differences between sensitized and sensitized plus infected groups were determined by the Mann-Whitney *U* test. **p* < 0.05, ***p* < 0.01.

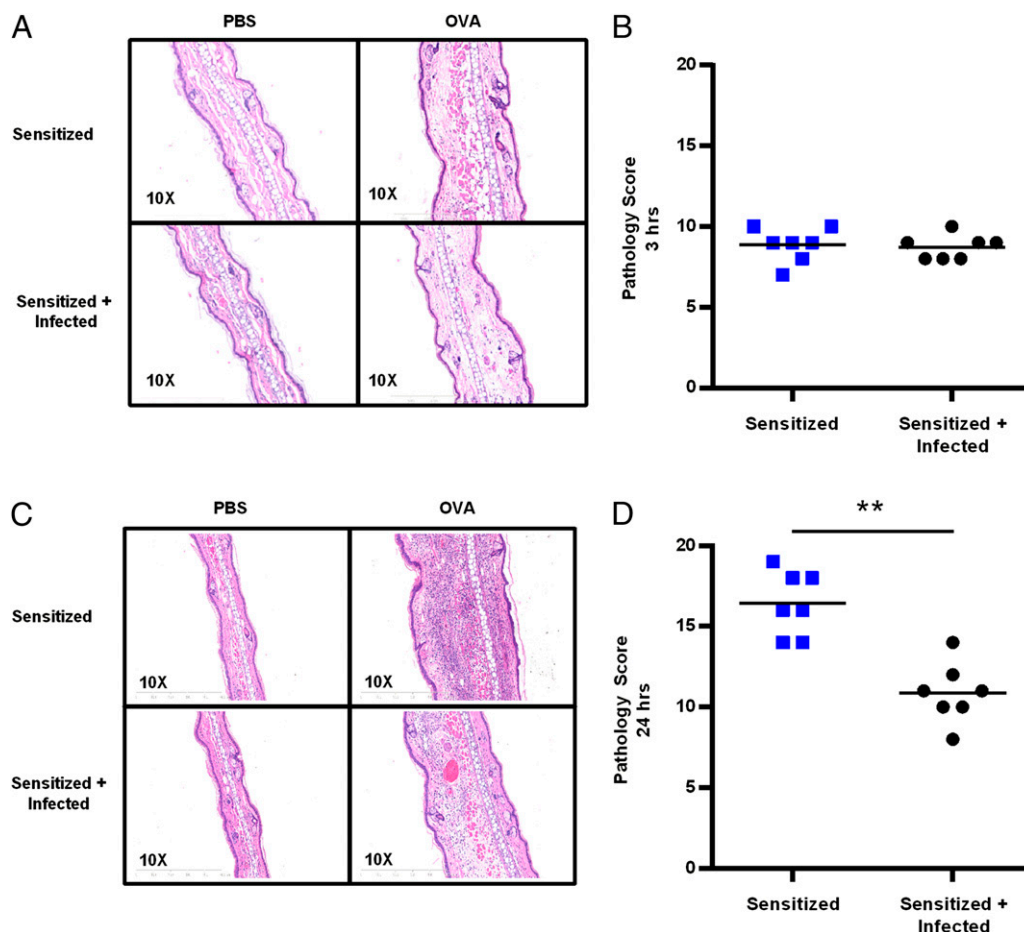


FIGURE 5. Sensitized plus infected animals exhibited reduced ear pathology 24 h postchallenge. **(A)** H&E stain of ear tissue at 3 h postchallenge, the time point at which immune complexes were detected by immunohistochemistry. **(B)** Pathology score for 3-h time point. Four parameters were measured (hemorrhaging, edema, necrosis, and cellularity) on the basis of severity (0, absent; 1, mild; 2, moderate; or 3, severe) and focality (0, absent; 1, focal; 2, intermediate; and 3, diffuse) for a total maximum score of 24. **(C)** H&E stain of ear tissue 24 h postchallenge, the time point at which there was the greatest difference in ear swelling between groups. **(D)** Pathology score for 24-h time point. Data are representative of two independent experiments with three to four BALB/c mice per group. Statistical differences between groups were analyzed by the Mann–Whitney *U* test. ***p* < 0.01.

however, there was no difference in the number or percentage of dead cells between groups (data not shown).

Neutrophils are the primary cell type that participates in immune complex-mediated inflammation, and the severity of type III hypersensitivity reactions can be correlated to the number of neutrophils present (15). We found a significant reduction in the number of neutrophils recruited in sensitized plus infected animals following OVA challenge (Fig. 6B). Macrophages were also present in lower numbers in infected animals (Fig. 6C). There was no difference in the numbers of eosinophils (Fig. 6D) or dendritic cells (Fig. 6E) between sensitized and sensitized plus infected groups. B cell numbers were below the limit of detection for sensitized and sensitized plus infected mice (Fig. 6F).

To ensure that differences in cellular infiltration were not due to reduced availability of circulating WBCs, we performed a CBC analysis on animals 10 wk postinfection. Sensitized plus infected animals had equal or higher numbers of circulation lymphocytes and granulocytes, including neutrophils and monocytes (data not shown). These data indicate that helminth infection suppresses neutrophil and monocyte recruitment to immune complexes.

Infection suppresses neutrophil-specific chemokine production by eosinophils

To investigate the underlying mechanism driving impaired cell recruitment in sensitized plus infected animals, we used a chemokine

profiler array to measure relative concentrations of chemokines present in the ear 6 h after OVA challenge. Of the 25 chemokines assayed by the array, 10 were present at detectable levels in the ear tissue 6 h after challenge. Of these, only neutrophil-specific chemokines CXCL-1 (KC) and CXCL-2 (MIP-2) were significantly reduced in sensitized plus infected animals (Fig. 7A). These data correlate with the reduced neutrophil recruitment observed in Fig. 6B. The remaining chemokines, including monocyte-specific chemokines CCL-2, CCL-6, and CCL-12, tended to be only slightly lower in sensitized plus infected animals compared with sensitized animals. Although other time points may have elicited a more robust difference between groups, the slight reduction in multiple monocyte-specific chemokines in infected animals may have had a cumulative effect on macrophage recruitment following OVA challenge (Fig. 6C).

Two cell types that release CXCL-1 and CXCL-2 in response to immune complex activation are macrophages and eosinophils (15, 16). To test the effect chronic helminth infection has on chemokine production by these cells, we next stimulated enriched populations of macrophages and eosinophils from sensitized and sensitized plus infected mice with immune complexes in vitro. Immune complexes were generated by combining polyclonal rabbit anti-OVA IgG Ab with OVA at a ratio of 100:1. This resulted in the formation of large, precipitating immune complexes after a 30-min incubation at 37°C.

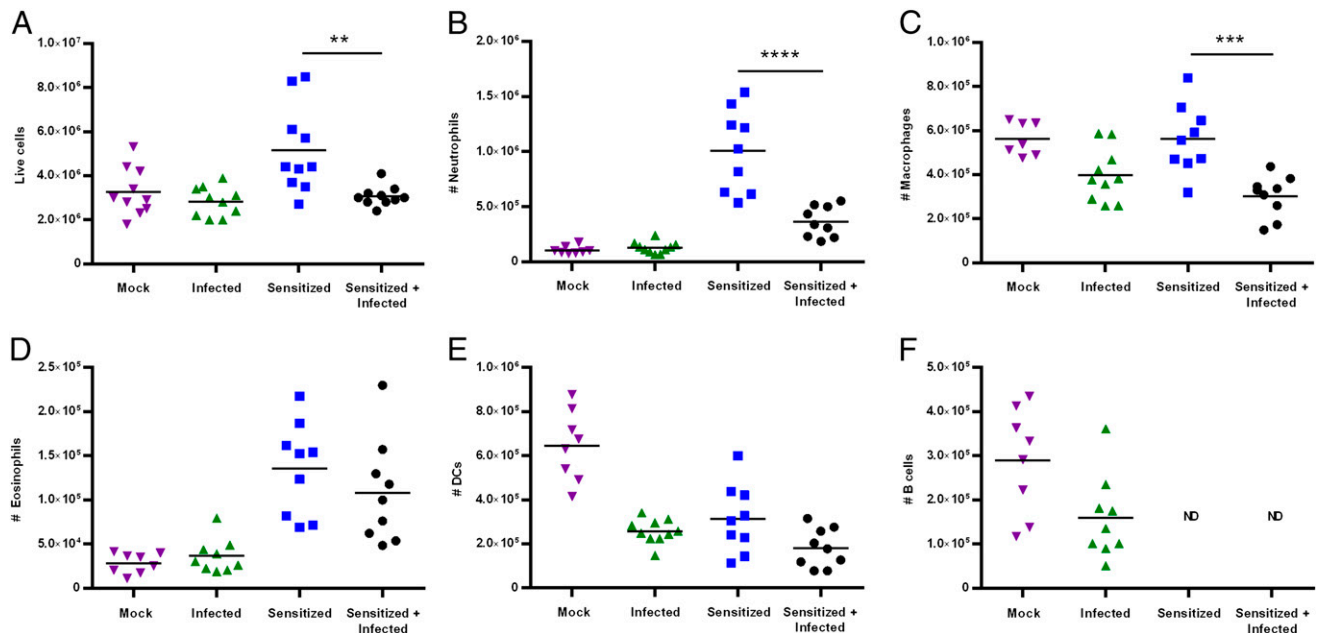


FIGURE 6. Sensitized plus infected animals showed impaired neutrophil and macrophage recruitment to the ear following allergen challenge. (A) Twenty-four hours after OVA challenge, a single-cell suspension ear tissue was generated and live cell counts were obtained by hemacytometer. Flow cytometry was then performed on the cell suspensions to determine the number of (B) neutrophils (F4/80⁺Ly6G⁺), (C) macrophages (F4/80⁺), (D) eosinophils (CD11c⁺CD45⁺SiglecF⁺), (E) dendritic cells (CD11c⁺), and (F) B cells (CD19⁺) present. Gates were determined using fluorescence minus one controls. Data are representative of two independent experiments with four to five BALB/c mice per group. Statistical differences between groups were analyzed by the Mann–Whitney *U* test. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

As seen in Fig. 7B and 7C, CXCL-1 and CXCL-2 production by macrophages was greater in sensitized plus infected animals than sensitized animals, although the difference was not statistically significant. In contrast, eosinophil production of CXCL-1 (Fig. 7D) and CXCL-2 (Fig. 7E) was markedly reduced in chronically infected mice compared with sensitized mice (*p* values < 0.05 and < 0.01, respectively). The suppression of CXCL-2 production by eosinophils in sensitized plus infected animals was more robust than suppression of CXCL-1 production, consistent with the data obtained by the chemokine array (Fig. 7A). These results suggest that the suppression of neutrophil-specific chemokine production in chronically infected mice may be due to suppression of eosinophil function.

Although eosinophils are known to produce chemokines, the role that eosinophils play during type III hypersensitivity responses has yet to be defined. To determine whether eosinophils were required for type III hypersensitivity, we sensitized Δ dblGATA (eosinophil-deficient) mice to OVA, challenged the ears with OVA and PBS, and monitored ear thickness over time. As seen in Fig. 7F, eosinophil-deficient animals exhibit reduced ear swelling compared with BALB/c control animals. This indicates eosinophils are important for eliciting immune responses to immune complex deposition.

Eosinophil expression of FcγRI is suppressed in chronically infected animals

To understand why eosinophils from chronically infected animals produced lower levels of neutrophil-specific chemokines, we measured the surface expression of activating FcγRI and FcγRIII and inhibitory FcγRII on multiple cell types by flow cytometry.

Sensitized and infected animals showed a significant reduction in the percentage of eosinophils expressing FcγRI compared with sensitized animals (Fig. 8A). There was no difference in the percentage of eosinophils expressing the other activating receptor FcγRIII, or the inhibitory receptor FcγRII. Furthermore, there was no difference between groups with regard to the percentage of

macrophages (Fig. 8B), neutrophils (Fig. 8C), dendritic cells (Fig. 8D), or B cells (Fig. 8E) expressing FcγRI, FcγRII, or FcγRIII. The only appreciable difference in receptor expression levels on the surface of any cell type was a reduction in FcγRI on eosinophils, as indicated by mean fluorescence intensity values (Supplemental Fig. 3). We have also observed that eosinophils from sensitized plus infected animals have decreased expression of FcεRI (data not shown), indicating that filariae may be exerting global suppression of eosinophil function by decreasing important Ab receptors on the cell surface. Notably, the absence of neutrophils expressing FcγRI or FcγRIII (Fig. 8C) signifies that neutrophils are not likely to be major contributors to the chemokine production observed from the eosinophil-enriched cell fraction (Fig. 7).

Discussion

In this study, we found that chronic helminth infection with the filarial parasite *L. sigmodontis* protects against type III, but not type I, hypersensitivity in a murine ear challenge model. Protection was associated with reduced neutrophil influx into the ear, decreased local levels of the CXCL-1 and CXCL-2 neutrophil chemokines, and diminished production of these chemokines by eosinophils in response to immune complex stimulation.

In our first experiment, we evaluated whether chronic *L. sigmodontis* infection protects against type I hypersensitivity in mice previously sensitized against OVA. Even though 10 wk of infection resulted in lower OVA-specific IgE levels, reduced basophil activation in response to OVA, and decreased numbers of degranulated tissue mast cells after intradermal OVA challenge, no clinical protection against immediate local anaphylaxis was observed using an Evans Blue assay to measure changes in vascular permeability. Future work will address whether infection can protect against systemic anaphylaxis. Both local and systemic anaphylaxis are type I hypersensitivity responses; however, local anaphylaxis is primarily due to mast cell activation, whereas

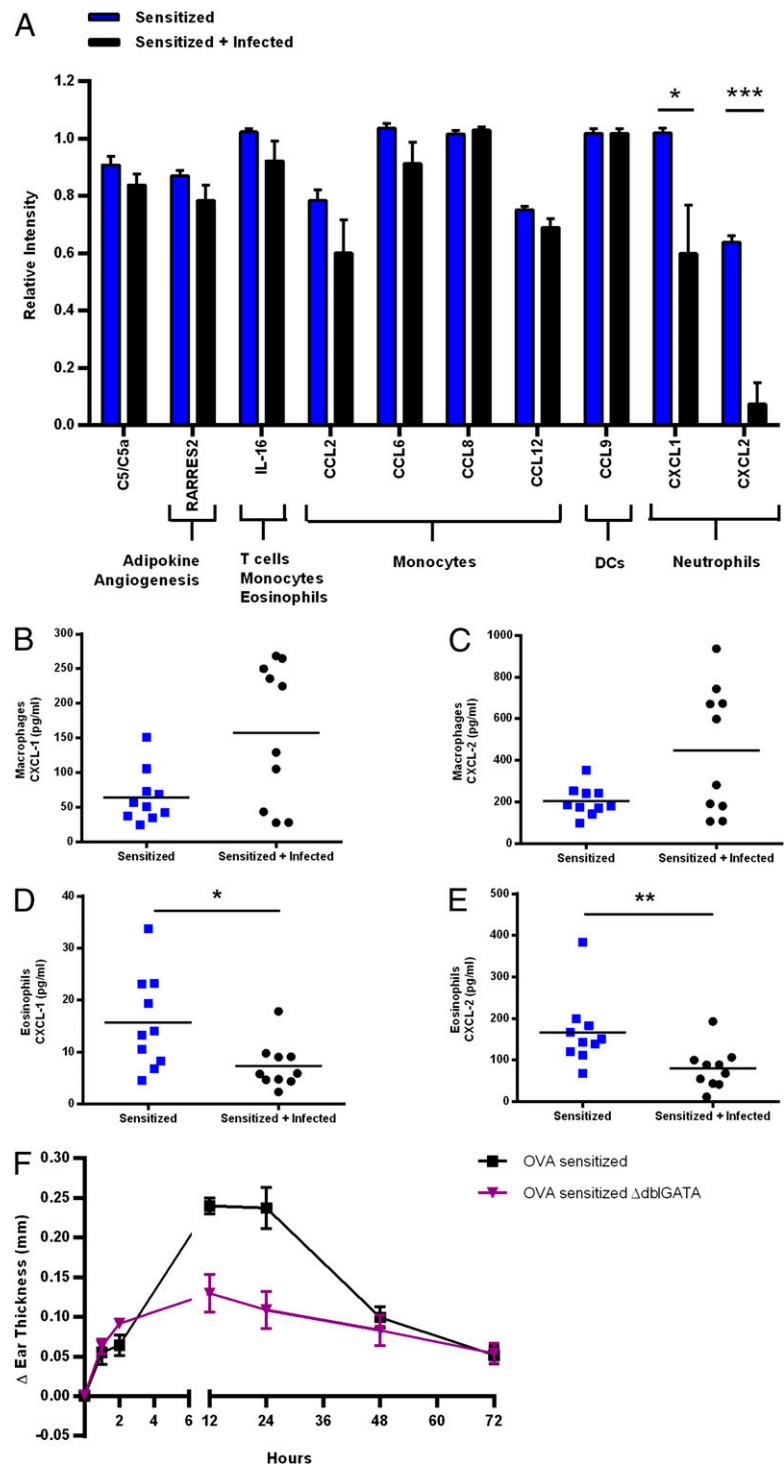


FIGURE 7. Neutrophil-specific chemokine production by eosinophils was reduced in sensitized plus infected animals. **(A)** Chemokine array performed on ear tissue 6 h after OVA challenge. Both ears were challenged with OVA and pooled for each animal. Tissues were homogenized with FastPrep lysing matrix D beads in the presence of protease inhibitors, and supernatants were assayed for the presence of 25 distinct chemokines. To determine the cell types responsible for neutrophil-specific chemokine production, macrophages were isolated from the peritoneal cavity and an eosinophil-enriched cell fraction was derived from splenocytes. Cells were stimulated with immune complexes for 6 h in vitro, and supernatants were harvested to assess macrophage production of CXCL-1 (**B**) and CXCL-2 (**C**), and eosinophil production of CXCL-1 (**D**) and CXCL-2 (**E**) by ELISA. Data are representative of two independent experiments with four to five BALB/c mice per group. **(F)** Ear swelling for uninfected OVA-sensitized eosinophil-deficient $\Delta dbiGATA$ (BALB/c background) and control BALB/c mice. Data are representative of two independent experiments composed of three animals per group. Error bars represent $\pm$ SEM. Significant differences between groups were analyzed by the Mann-Whitney *U* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

systemic anaphylaxis is due to the activity of basophils and/or IgG-mediated activation of inflammatory macrophages (17).

Although numerous studies have shown helminths protect against allergy when given prior to sensitization, the few animal studies that have infected after sensitization have produced mixed results (18–21). Given that *L. sigmodontis* was shown to be beneficial when given prior to allergic sensitization (8), it is likely that helminth infections are more effective at blunting sensitization than preventing symptoms after sensitization has occurred. Thus, these results support the conclusion that helminth infections may not readily protect against immediate hypersensitivity reactions in previously sensitized individuals. Indeed, to date, the only two

clinical studies that have prospectively tested whether helminth infections can be given to protect against allergic disease have been negative (1, 2).

That said, the results of our experiment do not completely rule out the possibility that helminth infections can have beneficial effects on individuals with established immediate hypersensitivities. Indeed, the major immunologic correlates of type I hypersensitivity (allergen-specific IgE as well as basophil activation and mast cell degranulation in response to allergen) were decreased in the setting of *L. sigmodontis* infection. We speculate that these decreases did not translate into clinical protection because either they were not of sufficient magnitude for the dose of

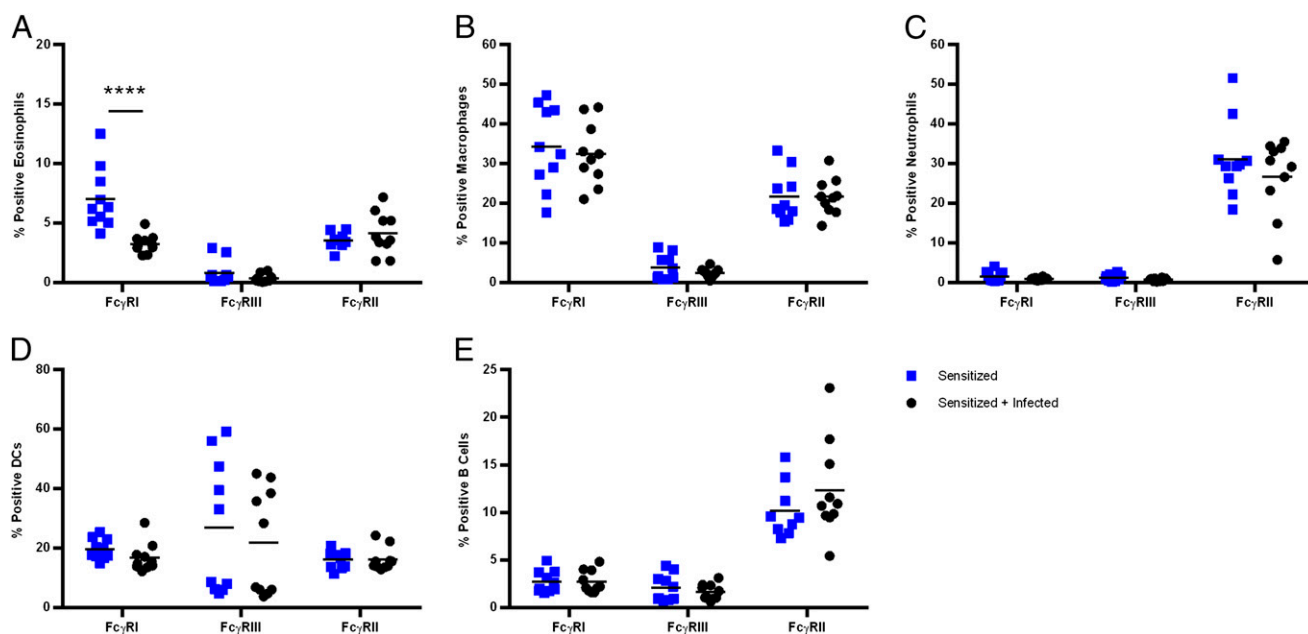


FIGURE 8. Infection results in decreased percentage of eosinophils expressing FcγRI. Flow cytometry was used to assess the percentage of cells expressing activating FcγRI and FcγRIII and inhibitory FcγRII. Single-cell suspensions of live splenocytes were first gated as (A) eosinophils (CD11c⁺CD45⁺SiglecF⁺), (B) macrophages (F4/80⁺), (C) neutrophils (F4/80⁺Ly6G⁺), (D) dendritic cells (CD11c⁺), or (E) B cells (CD19⁺). Each cell type was then gated on the basis of FcγRI (CD64⁺), FcγRIII (CD16⁺), and FcγRII (CD32⁺) positivity using fluorescence minus one (FMO) controls. Data are representative of two independent experiments with four to five BALB/c mice per group. Error bars represent ± SEM. Significant differences between groups were analyzed by the Mann–Whitney *U* test. *****p* < 0.0001.

allergen given, or infection did not occur for a long enough period of time. With regard to magnitude of change, the decrease in OVA-specific IgE may not have been great enough to decrease mast cell sensitivity to IgE cross-linking. In terms of duration, a 10-wk infection may not be long enough to substantially alter the repertoire of IgE Abs on the surface of tissue-resident mast cells. Because these are long-lived cells with slow turnover at tissue sites (22), it is possible that decreases in circulating levels of allergen-specific IgE may take months to result in substantial reductions in mast cell sensitivity to allergen. Testing whether a longer exposure to helminths can provide clinical protection against immediate hypersensitivity will be the focus of future studies.

Interestingly, although we did not observe clinical protection against type I hypersensitivity, we did see significant protection against late ear swelling due to type III hypersensitivity. Type III hypersensitivity is driven by immune complex deposition and is a major pathogenic mechanism for diseases such as systemic lupus erythematosus (23), serum sickness (24), and poststreptococcal glomerulonephritis (25). To our knowledge, this is the first study to specifically demonstrate that helminth infection can protect against immune complex-mediated hypersensitivity.

Whereas immune complexes were visualized at 3 h postchallenge and showed no difference in size or number between groups, a reduction in pathology and cellular infiltration was observed at 24 h postchallenge (Fig. 5). This difference in pathology was associated with fewer neutrophils and macrophage trafficking to the site of allergen challenge (Fig. 6).

To determine why sensitized plus infected animals had fewer cells recruited to the ear, we monitored chemokine production after OVA challenge and *in vitro* immune complex stimulation. We observed a reduction in the neutrophil attractant chemokines CXCL-1 and CXCL-2 and noted that eosinophils were specifically impaired in their ability to secrete these chemokines (Fig. 7). Conversely, macrophage production of chemokines was not suppressed, and in some animals was even exacerbated. Although

macrophages are considered to be the primary cell type responsible for neutrophil-specific chemokine production, eosinophils have the capacity to produce large volumes of these chemokines in response to stimulation (26). Confirming this, eosinophil-deficient *AdblGATA* mice exhibit attenuated inflammation following OVA challenge. To our knowledge, this is the first time eosinophils have been shown to be important contributors to immune complex-mediated inflammation, most likely through the production of neutrophil-specific chemokines.

In addition to neutrophils, the number of macrophages present in the ear tissue was significantly suppressed in chronically infected animals (Fig. 6), although this was not reflected by a substantial suppression of monocyte-specific chemokines produced 6 h postchallenge (Fig. 7). Although assessing chemokine production at other time points may reveal more distinct differences, reduced monocyte recruitment may have been due to an impaired ability of monocytes to respond to chemokines, rather than reduced chemokine production. Indeed, macrophages and neutrophils from mice infected with *Echinococcus multilocularis* lost their ability to migrate in response to stimulation with worm Ag or endotoxin-activated mouse serum (27). Interestingly, this inhibitory effect was observed for chronic, but not acute, infection. Furthermore, *Taeniaestatin*, a protease inhibitor isolated from *Taenia taeniaeformis*, prevented neutrophil chemotaxis in response to C5a (28).

The activation of cells by immune complexes involves ligation of activating (FcγRI and FcγRIII) and inhibitory (FcγRII) receptors. The ratio of activating and inhibitory receptor binding plays an integral role in determining whether cellular responses will be proinflammatory or anti-inflammatory. Whereas the number of circulating eosinophils was not decreased in sensitized plus infected mice, the percentage of eosinophils expressing FcγRI was reduced by 50% compared with sensitized animals (Fig. 8). Furthermore, the amount of FcγRI expressed on the surface of eosinophils was also reduced, as indicated by mean fluorescence intensity data (Supplemental Fig. 3). This suggests that chronic

L. sigmodontis infection lowers the propensity for eosinophils to express FcγRI, which in turn reduces the ability of this cell type to produce chemokines upon immune complex stimulation (Supplemental Fig. 4).

In addition to establishing systemic immunoregulatory networks, helminths release immune-modulatory factors in the form of excretory–secretory products. Because only eosinophils displayed a marked difference in FcγRI expression, it is possible that *L. sigmodontis* worms release factors that directly suppress eosinophils. Previous studies have already shown that worm products are associated with altered eosinophil function and reduced chemotaxis (29–31). The selective suppression of FcγRI expression on eosinophils during the course of infection would be advantageous to the parasite, as eosinophils are known to mediate worm clearance through Ab-dependent cell-mediated cytotoxicity (32).

The fact that eosinophils were the only cell type to display decreased FcγRI expression and chemokine production suggests that we may be able to develop medications that specifically suppress eosinophil function. Because eosinophils exhibited enhanced immunoregulation during infection, worm-mediated therapies may be particularly well suited for eosinophil-driven diseases.

Interestingly, even though they did not play a mechanistic role in protecting mice against the allergic responses evaluated in this study, both OVA-specific IgG and IgE levels were lower in the setting of chronic *L. sigmodontis* infection. The reduction in allergen-specific Ab levels is an interesting phenomenon, as other groups have reported similar results when infection is given prior to sensitization (8, 33, 34). In contrast to reductions in Abs specific to environmental or self-Ags, levels of *L. sigmodontis*-specific and nonspecific Abs rise throughout the course of infection (35). Indeed, our ELISA and ELISPOT data (Fig. 4) confirm that infected animals are not compromised in their ability to elicit humoral immune responses.

We therefore hypothesize that decreases in allergen-specific Abs are due to increased Ab catabolism. We speculate that the host increases Ab catabolism in an effort to counterbalance the high levels of Abs produced during infection. To this effect, infection may be providing an endogenous source of Abs that produces beneficial effects similar to i.v. Ig administration. To test this, future studies would most likely investigate the role of neonatal FcR for IgG catabolism, or CD23 for IgE catabolism (36), during the course of infection.

In summary, we have shown that chronic infection with the rodent filarial worm *L. sigmodontis* protects the host from type III hypersensitivity. The mechanisms by which this occurs appears to be multifactorial, with protection being associated with fewer neutrophils and macrophages infiltrating the site of allergen challenge, reduced neutrophil-specific chemokine production, and a decrease in eosinophil expression of FcγRI. Because the immunological markers of type I hypersensitivity were decreased in chronically infected animals, *L. sigmodontis* may have the potential to protect against immediate hypersensitivity reactions under some experimental conditions. Future studies would most likely investigate the use of repeated infections to extend the length of infection, as this would allow for mast cell turnover to occur in an immune-regulated setting. Furthermore, future experiments using worm Ag preparations in place of live worm infection would allow for the characterization of the specific Ags responsible for host protection from hypersensitivity diseases. From a translational perspective, the finding that *L. sigmodontis* infection protects against type III hypersensitivity in previously sensitized animals suggests that helminth-derived products may

potentially be developed as therapeutics for immune complex-mediated diseases, such as systemic lupus erythematosus.

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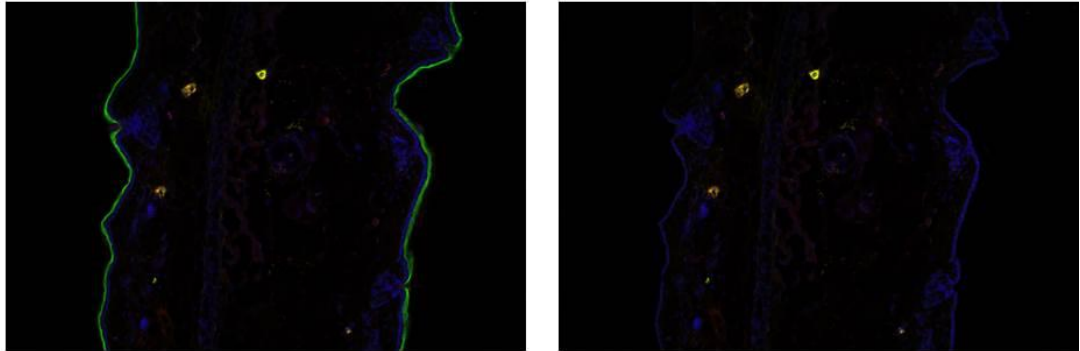
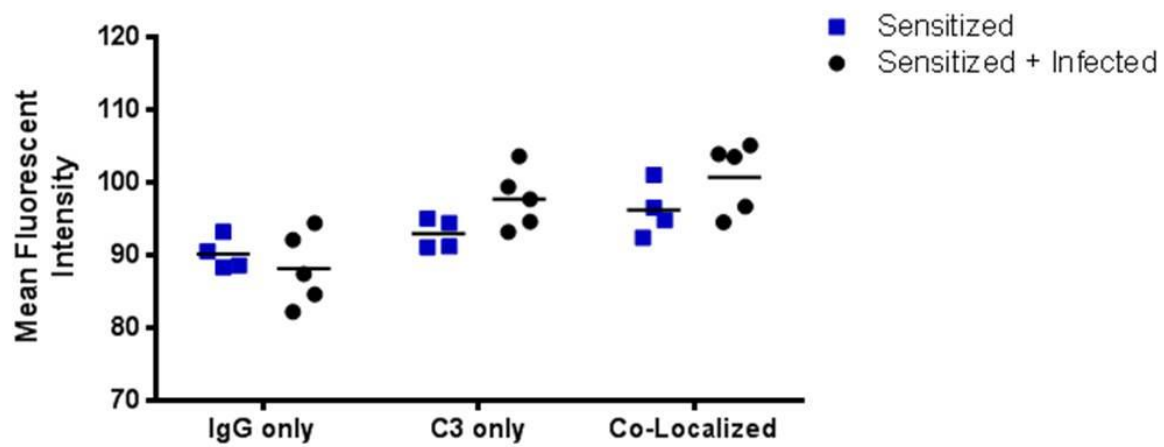
Disclosures

The authors have no financial conflicts of interest.

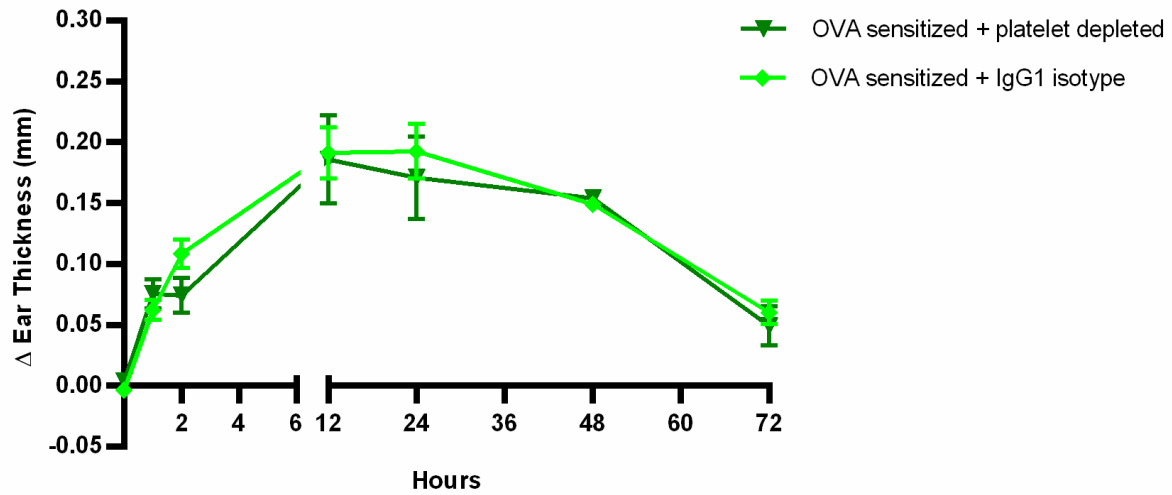
References

- Bager, P., J. Arned, S. Rønberg, J. Wohlfahrt, L. K. Poulsen, T. Westergaard, H. W. Petersen, B. Kristensen, S. Thamsborg, A. Roepstorff, et al. 2010. *Trichuris suis* ova therapy for allergic rhinitis: a randomized, double-blind, placebo-controlled clinical trial. *J. Allergy Clin. Immunol.* 125: 123–130.e1–3.
- Feary, J. R., A. J. Venn, K. Mortimer, A. P. Brown, D. Hooi, F. H. Falcone, D. I. Pritchard, and J. R. Britton. 2010. Experimental hookworm infection: a randomized placebo-controlled trial in asthma. *Clin. Exp. Allergy* 40: 299–306.
- Evans, H., and E. Mitre. 2015. Worms as therapeutic agents for allergy and asthma: understanding why benefits in animal studies have not translated into clinical success. *J. Allergy Clin. Immunol.* 135: 343–353.
- Hoffmann, W., G. Petit, H. Schulz-Key, D. Taylor, O. Bain, and L. Le Goff. 2000. *Litomosoides sigmodontis* in mice: reappraisal of an old model for filarial research. *Parasitol. Today (Regul. Ed.)* 16: 387–389.
- Pfaff, A. W., H. Schulz-Key, P. T. Soboslay, D. W. Taylor, K. MacLennan, and W. H. Hoffmann. 2002. *Litomosoides sigmodontis* cystatin acts as an immunomodulator during experimental filariasis. *Int. J. Parasitol.* 32: 171–178.
- Taylor, M. D., L. LeGoff, A. Harris, E. Malone, J. E. Allen, and R. M. Maizels. 2005. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. *J. Immunol.* 174: 4924–4933.
- Taylor, M. D., N. van der Werf, A. Harris, A. L. Graham, O. Bain, J. E. Allen, and R. M. Maizels. 2009. Early recruitment of natural CD4⁺ Foxp3⁺ Treg cells by infective larvae determines the outcome of filarial infection. *Eur. J. Immunol.* 39: 192–206.
- Dittrich, A. M., A. Erbacher, S. Specht, F. Diesner, M. Krokowski, A. Avagyan, P. Stock, B. Ahrens, W. H. Hoffmann, A. Hoerauf, and E. Hamelmann. 2008. Helminth infection with *Litomosoides sigmodontis* induces regulatory T cells and inhibits allergic sensitization, airway inflammation, and hyperreactivity in a murine asthma model. *J. Immunol.* 180: 1792–1799.
- Larson, D., M. P. Hübner, M. N. Torrero, C. P. Morris, A. Brankin, B. E. Swierczewski, S. J. Davies, B. M. Vonakis, and E. Mitre. 2012. Chronic helminth infection reduces basophil responsiveness in an IL-10-dependent manner. *J. Immunol.* 188: 4188–4199.
- Evans, H., K. E. Killoran, and E. Mitre. 2014. Measuring local anaphylaxis in mice. *J. Vis. Exp.* 92: e52005. Available at: <http://www.jove.com/video/52005/measuring-local-anaphylaxis-in-mice>.
- Hübner, M. P., M. N. Torrero, J. W. McCall, and E. Mitre. 2009. *Litomosoides sigmodontis*: a simple method to infect mice with L3 larvae obtained from the pleural space of recently infected jirds (*Meriones unguiculatus*). *Exp. Parasitol.* 123: 95–98.
- Shannon, J. G., A. M. Hasenkrug, D. W. Dorward, V. Nair, A. B. Carmody, and B. J. Hinnebusch. 2013. *Yersinia pestis* subverts the dermal neutrophil response in a mouse model of bubonic plague. *MBio* 4: e00170–e13.
- Mukai, K., K. Matsuoka, C. Taya, H. Suzuki, H. Yokozeki, K. Nishioka, K. Hirokawa, M. Etori, M. Yamashita, T. Kubota, et al. 2005. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 23: 191–202.
- Pandey, M. K. 2013. Molecular basis for downregulation of C5a-mediated inflammation by IgG1 immune complexes in allergy and asthma. *Curr. Allergy Asthma Rep.* 13: 596–606.
- Jönsson, F., D. A. Mancardi, M. Albanesi, and P. Bruhns. 2013. Neutrophils in local and systemic antibody-dependent inflammatory and anaphylactic reactions. *J. Leukoc. Biol.* 94: 643–656.
- Nakajima, H., G. J. Gleich, and H. Kita. 1996. Constitutive production of IL-4 and IL-10 and stimulated production of IL-8 by normal peripheral blood eosinophils. *J. Immunol.* 156: 4859–4866.
- Finkelman, F. D. 2007. Anaphylaxis: lessons from mouse models. *J. Allergy Clin. Immunol.* 120: 506–515, quiz 516–517.
- Negrão-Corrêa, D., M. R. Silveira, C. M. Borges, D. G. Souza, and M. M. Teixeira. 2003. Changes in pulmonary function and parasite burden in rats

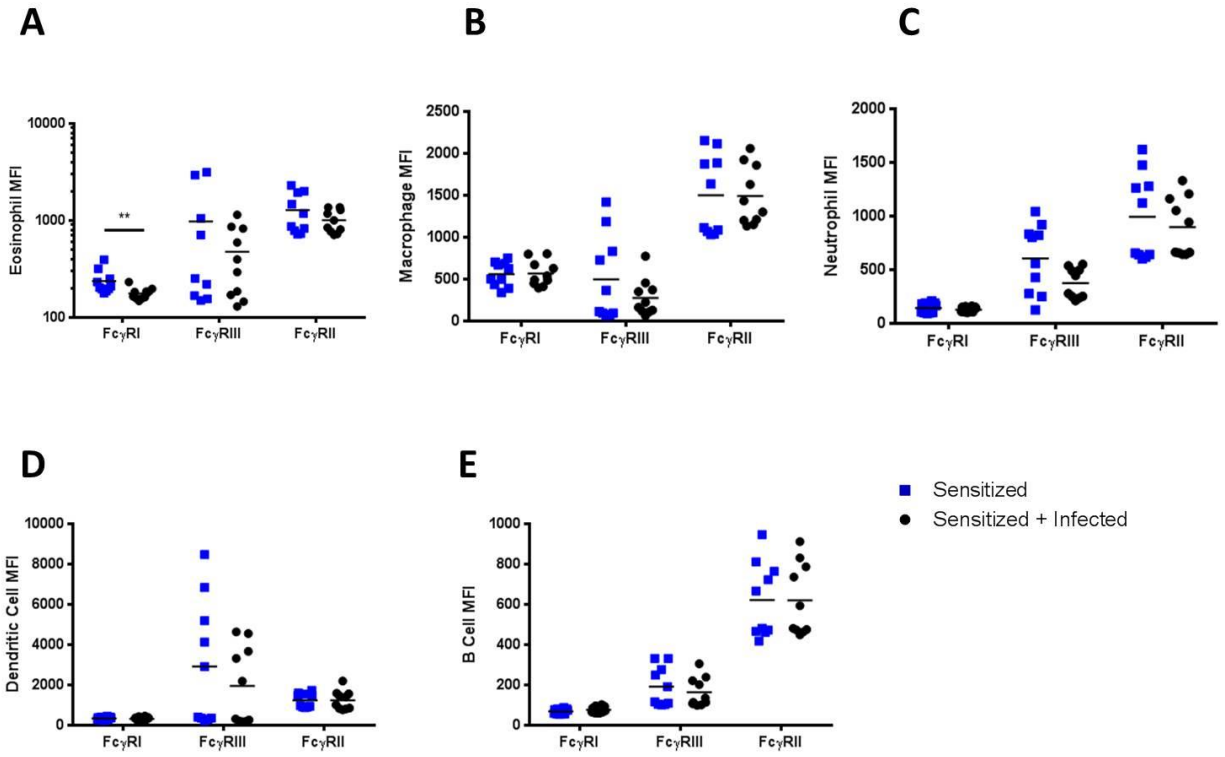
- infected with *Strongyloides venezuelensis* concomitant with induction of allergic airway inflammation. *Infect. Immun.* 71: 2607–2614.
19. Wilson, M. S., M. D. Taylor, A. Balic, C. A. M. Finney, J. R. Lamb, and R. M. Maizels. 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J. Exp. Med.* 202: 1199–1212.
 20. Wohlleben, G., C. Trujillo, J. Müller, Y. Ritze, S. Grunewald, U. Tatsch, and K. J. Erb. 2004. Helminth infection modulates the development of allergen-induced airway inflammation. *Int. Immunol.* 16: 585–596.
 21. Jarrett, E., S. Mackenzie, and H. Bennich. 1980. Parasite-induced 'nonspecific' IgE does not protect against allergic reactions. *Nature* 283: 302–304.
 22. Kiernan, J. A. 1979. Production and life span of cutaneous mast cells in young rats. *J. Anat.* 128: 225–238.
 23. Pieterse, E., and J. van der Vlag. 2014. Breaking immunological tolerance in systemic lupus erythematosus. *Front. Immunol.* 5: 164.
 24. Ko, J. H., and W. H. Chung. 2013. Serum sickness. *Lancet* 381: e1.
 25. Friedman, J., I. van de Rijn, H. Ohkuni, V. A. Fischetti, and J. B. Zabriskie. 1984. Immunological studies of post-streptococcal sequelae: evidence for presence of streptococcal antigens in circulating immune complexes. *J. Clin. Invest.* 74: 1027–1034.
 26. Rose, C. E., Jr., J. A. Lannigan, P. Kim, J. J. Lee, S. M. Fu, and S. S. J. Sung. 2010. Murine lung eosinophil activation and chemokine production in allergic airway inflammation. *Cell. Mol. Immunol.* 7: 361–374.
 27. Alkarmi, T., and K. Behbehani. 1989. *Echinococcus multilocularis*: inhibition of murine neutrophil and macrophage chemotaxis. *Exp. Parasitol.* 69: 16–22.
 28. Leid, R. W., R. F. Grant, and C. M. Suquet. 1987. Inhibition of equine neutrophil chemotaxis and chemokinesis by a *Taenia taeniaeformis* proteinase inhibitor, taeniaestatin. *Parasite Immunol.* 9: 195–204.
 29. Rzepecka, J., K. Donskow-Schmelter, and M. Doligalska. 2007. *Heligmosomoides polygyrus* infection down-regulates eotaxin concentration and CCR3 expression on lung eosinophils in murine allergic pulmonary inflammation. *Parasite Immunol.* 29: 405–413.
 30. Shin, M. H., H. Kita, H. Y. Park, and J. Y. Seoh. 2001. Cysteine protease secreted by *Paragonimus westermani* attenuates effector functions of human eosinophils stimulated with immunoglobulin G. *Infect. Immun.* 69: 1599–1604.
 31. Park, S. K., M. K. Cho, H. K. Park, K. H. Lee, S. J. Lee, S. H. Choi, M. S. Ock, H. J. Jeong, M. H. Lee, and H. S. Yu. 2009. Macrophage migration inhibitory factor homologs of anisakis simplex suppress Th2 response in allergic airway inflammation model via CD4+CD25+Foxp3+ T cell recruitment. *J. Immunol.* 182: 6907–6914.
 32. Makepeace, B. L., C. Martin, J. D. Turner, and S. Specht. 2012. Granulocytes in helminth infection— who is calling the shots? *Curr. Med. Chem.* 19: 1567–1586.
 33. Bashir, M. E. H., P. Andersen, I. J. Fuss, H. N. Shi, and C. Nagler-Anderson. 2002. An enteric helminth infection protects against an allergic response to dietary antigen. *J. Immunol.* 169: 3284–3292.
 34. Price, P., and K. J. Turner. 1984. Immunological consequences of intestinal helminth infections: humoral responses to ovalbumin. *Parasite Immunol.* 6: 499–508.
 35. Torrero, M. N., M. P. Hübner, D. Larson, H. Karasuyama, and E. Mitre. 2010. Basophils amplify type 2 immune responses, but do not serve a protective role, during chronic infection of mice with the filarial nematode *Litomosoides sigmodontis*. *J. Immunol.* 185: 7426–7434.
 36. Cheng, L. E., Z. E. Wang, and R. M. Locksley. 2010. Murine B cells regulate serum IgE levels in a CD23-dependent manner. *J. Immunol.* 185: 5040–5047.

A**B**

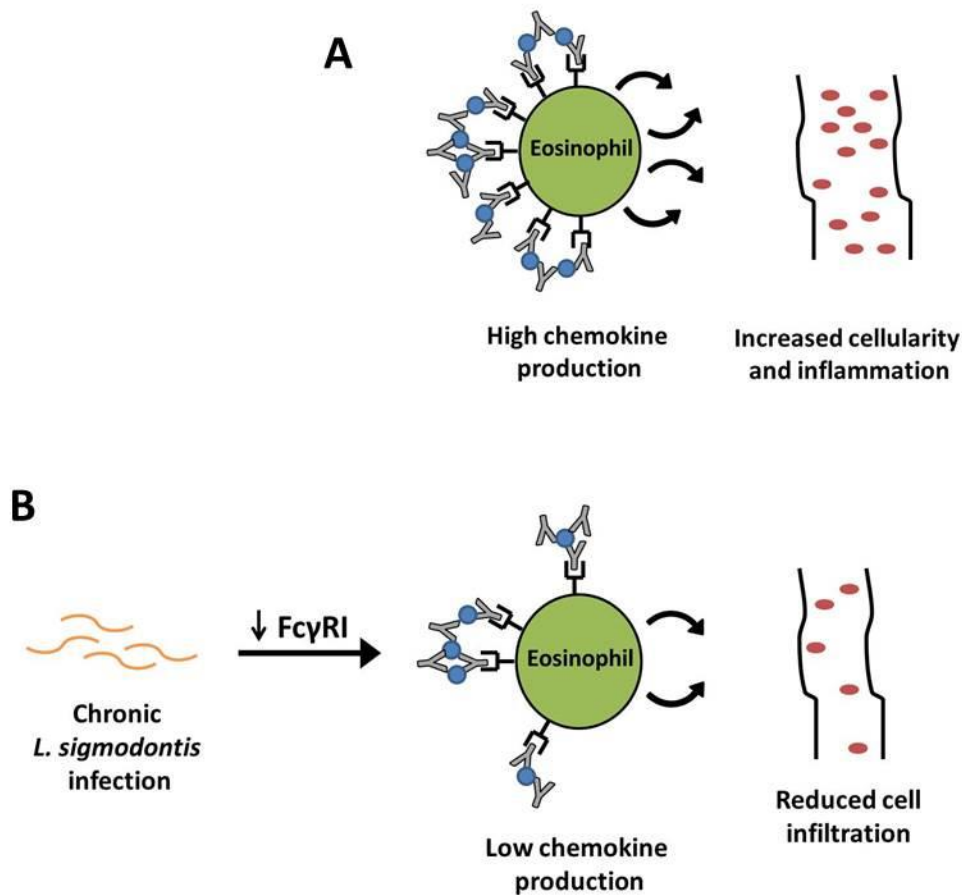
Supplement 1. Masking of confocal images and mean fluorescent intensity. (A) Masking of non-specific C3 staining. Left panel represents image before masking, and right panel represents image after masking. (B) Mean fluorescent intensity of puncta was calculated by the Puncta Analyzer plugin on ImageJ.



Supplement 2. Ear thickness assay following platelet depletion. Ear thickness assay was performed on OVA sensitized BALB/c mice following platelet depletion. Platelets were depleted by i.p. injection of anti-CD41 24 hours prior to ear challenge. Depletion was confirmed by CBC analysis. Data are representative of two independent experiments with 3 mice per group.



Supplement 3. Mean fluorescent intensity of FcγRI, FcγRII, and FcγRIII. MFI for FcγRI, FcγRII, and FcγRIII on eosinophils (A), macrophages (B), neutrophils (C), dendritic cells (D), and B cells (E). Data are representative of two independent experiments with 4-5 BALB/c mice per group.



Supplement 4. Working model for helminth protection from immune complex-mediated inflammation. (A) In sensitized animals, antibodies bind to OVA to form immune complexes. These complexes then ligate FcγRs on the surface of eosinophils to induce chemokine production. Neutrophils migrate to the tissue to induce inflammation and swelling. (B) In Sensitized + Infected animals, there is less FcγRI expression on eosinophils, therefore fewer receptors are ligated by immune complexes. Due to weaker stimulation, chemokine production is reduced, resulting in fewer neutrophils infiltrating to the site of immune complex deposition.