Leukotrienes are eicosanoid lipid mediators generated from arachidonic acid by the enzyme ALOX5 (5-lipoxygenase; Haeggström and Wetterholm, 2002). Leukotriene B4 (LTB4) contributes to leukocyte accumulation in many inflammatory diseases, and its production by neutrophils is triggered by multiple stimuli (Sadik et al., 2012). Dual expression of ALOX5 and the LTB4 receptor LTB4R1 in neutrophils is required for their recruitment in a model of arthritis, revealing a paracrine amplification loop in this setting (Chen et al., 2006; Sadik et al., 2012). LTB4R1 is also highly expressed on human and mouse eosinophils (Tager et al., 2000), and human eosinophils produce LTB4 (Henderson et al., 1984), raising the possibility of paracrine leukotriene signaling between eosinophils analogous to that established for neutrophils.

Eosinophil accumulation is highly associated with infection by multicellular endoparasites, and this association is conserved from zebrafish to humans (Klion and Nutman, 2004; Balla et al., 2010). Sentinel cells in tissues are thought to instigate eosinophil accumulation by releasing the cytokines TSLP, IL-25, and IL-33 in response to worm-induced injury (Licona-Limón et al., 2013). These cytokines stimulate innate lymphoid cells, which enable eosinophil survival through IL-5 production. However, the signals that attract eosinophils into tissues during infection, and whether any are helminth derived, remain unclear. Mice deficient in CCL11 (eotaxin-1) exhibit reductions in eosinophil accumulation in some models of helminth infection (Klion and Nutman, 2004). However, the eotaxin receptor CCR3 is also important in regulating basal numbers of eosinophils in tissues, confounding these studies (Matthews et al., 1998). Furthermore, eosinophil recruitment is intact in Ccr3−/− mice during Nippostrongylus brasiliensis infection, indicating that other unknown recruitment factors are involved (Knott et al., 2009).

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RESULTS AND DISCUSSION

Eosinophils migrate toward diverse nematode species

To determine whether live nematodes induce eosinophil migration directly, we suspended *Caenorhabditis elegans*, *Heterorhabditis bacteriophora*, or *N. brasiliensis* larvae in a three-dimensional (3D) Matrigel matrix along with mouse eosinophils (cultured from bone marrow progenitors). After 1 h, thousands of eosinophils had accumulated around worms of all three species (Fig. 1 A). Eosinophils did not accumulate around agarose (Fig. 1 A), chitin, or Teflon beads (not depicted), ruling out a general response to foreign surfaces. Accumulation also occurred in collagen gels, with heat- or formaldehyde-killed worms, and with eosinophils isolated from the blood or spleen of IL-5Tg mice (not depicted).

To determine whether directed migration of eosinophils was involved, we performed time-lapse imaging of eosinophils mixed with *C. elegans* dauer (Fig. 1 B and Video 1). Eosinophils migrated en mass over distances of up to 300 µm toward each nematode (Fig. 1 B and Video 1), but these points differed from worm to worm. This indicated that no single anatomical structure was responsible for triggering migration. Eosinophil cell spreading was evident on the worm cuticle and was accompanied by migration along its length (Videos 1 and 2).

Collective migration is dependent on eosinophil–derived LTB₄

Neutrophils attract other neutrophils via LTB₁ in vitro (Afonso et al., 2012), as well as in several models of inflammation (Sadik et al., 2011; Lämmermann et al., 2013). We found that MK886, a leukotriene synthesis inhibitor (Rouzer et al., 1990), prevented the accumulation of eosinophils around *C. elegans* in a dose–dependent manner (IC₅₀ of ~0.5 µM; Fig. 3 A). Furthermore, eosinophils derived from *Alox5*⁻/⁻ bone marrow (Fig. 3 B) were >90% impaired in accumulating around *C. elegans* and *N. brasiliensis* (Fig. 3, C and D). To determine whether paracrine leukotriene signaling accounted for eosinophil migration toward nematodes, we attempted to rescue the migration of *Alox5*⁻/⁻ cells by mixing in wild-type cells. We combined fluorescently labeled *Alox5*⁻/⁻ cells in equal proportion with fluorescently labeled wild-type cells in the presence of abundant (70%) unlabeled wild-type cells. The migration of *Alox5*⁻/⁻ eosinophils was indistinguishable from wild-type cells (Fig. 3 E and Video 5).

Because ALOX5 is required for the production of both LTB₁ and cysteinyl leukotrienes (cysLTs), we next asked which leukotrienes were involved in the response to nematodes. Although eosinophils produce cysLTs upon activation (Kanaoka and Boyce, 2004), the CSYLTR₁ antagonist montelukast (10 µM) failed to alter eosinophil migration to nematodes (not
establishing that LTB4 signaling is required for Alox5-dependent migration and accumulation.

Neutrophils are also recruited into tissues during parasite infection, although they are often far outnumbered by eosinophils (Makepeace et al., 2012). The known involvement of LTB4 in neutrophil migration prompted us to test whether neutrophils would respond to nematodes. Neutrophils isolated from mouse bone marrow exhibited >30-fold reduced migration toward C. elegans compared with eosinophils (Fig. 4 E). Sephadex beads, which are recognized by SIGN-R1 (Kang et al., 2003), also induced migration of eosinophils in an Alox5-dependent fashion, whereas neutrophils did not respond at all (not depicted). Neutrophils are known to produce LTB4 in response to Fc receptor engagement (Sadik et al., 2012). We therefore tested whether IgG1-coated beads would induce collective migration of neutrophils. Neutrophils migrated robustly toward beads coated with intact IgG1 (Fig. 4 F) but not to F(ab')2-coated beads, confirming a requirement for Fc receptor engagement. There was no accumulation depicted). Because LTB4 is a chemoattractant for eosinophils in vitro (Spada et al., 1994), we tested whether our eosinophil preparations migrated toward LTB4 or the cysteine leukotrienes in Transwell assays. We confirmed robust migration toward LTB4 as well toward CCL11, but there was no detectable response to LTC4, LTD4, or LTE4 (Fig. 4 A). When we activated eosinophils with ionomycin, there was ~10-fold more LTB4 than LTE4 in the supernatant (Fig. 4 B). Furthermore, mixing of eosinophils with C. elegans stimulated eosinophil LTB4 production, which was completely inhibited by MK886 (Fig. 4 C). In contrast, there was no detectable production of LTE4 by eosinophils in response to worms. Finally, Ltb4r1−/− eosinophils exhibited a >90% reduction in accumulation around worms (Fig. 4 D), establishing that LTB4 signaling is required for Alox5-dependent migration and accumulation.

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Infections and many helminth infections, both eosinophils and neutrophils accumulate (Makepeace et al., 2012). Thus, these granulocytes may have complementary functions during acute inflammation that make common recruitment factors, like LTB₄, advantageous.

Leukotrienes amplify eosinophil accumulation in the lung in response to nematodes

We next investigated the contribution of leukotrienes to eosinophil accumulation in mice in response to nematodes. Within 5 min after i.v. injection, we observed live C. elegans dauers immobilized in the capillaries of the lung (not depicted). After 24 h, abundant eosinophils (identified by eMBP) had accumulated around the worms (Fig. 5A). To quantify eosinophils, we digested lungs with collagenase and analyzed...
Eosinophil accumulation in the absence of bacterial products, TLR4, CCR3, and complement. (A) Flow cytometry analysis of eosinophils in the lung 24 h after injection of C. elegans grown on E. coli or in axenic medium. (B–D) Flow cytometry analysis of eosinophil accumulation in the lungs of Tlr4PT12H/PT12H (B), Ccr3−/− (C), and C3−/− (D) mice after injection of C. elegans dauers or saline. (A–D) Results represent two (A and B) or are pooled from two (C and D) independent experiments. Horizontal bars indicate the mean. **, P < 0.01.

Involvement of leukotrienes in eosinophil accumulation in the lung. (A and B) Flow cytometry analysis of eosinophil accumulation in the lungs (left; three experiments pooled) and blood (right) of Alox5−/− (A) and Ltb4r1−/− (B) mice 24 h after injection of C. elegans dauers or saline. (C) Flow cytometry analysis of neutrophil accumulation in the lungs of Alox5−/−, Ltb4r1−/−, and wild-type mice 24 h after injection of C. elegans dauers or saline. (D) Flow cytometry analysis of eosinophil accumulation in the lungs (left) and blood (right) of Alox5−/− and wild-type mice 9 d after infection with N. brasiliensis larvae. Numbers of eosinophils in uninfected mouse lung (from A) are shown for comparison. (A–D) Results are pooled from three (A–C) or represent two (D) independent experiments. Horizontal bars indicate the mean. **, P < 0.005; ***, P < 0.0005.

found that eosinophil accumulation in Car3−/− and C3−/− mice did not differ from that in wild-type mice (Fig. 6, C and D).

In light of our 3D culture findings, we next investigated the contribution of leukotriene signaling. The number of eosinophils that accumulated in the lung 24 h after C. elegans injection was decreased by 70% in Alox5−/− mice (Fig. 7 A). This decrease was not likely caused by systemic defects in eosinophil production or survival because Alox5−/− mice had normal numbers of eosinophils in peripheral blood. In Ltb4r1−/− mice, eosinophils were 55% reduced in the lung (Fig. 7 B), whereas there was no reduction in the blood. Neutrophil accumulation did not exhibit this dependency on leukotriene signaling (Fig. 7 C). To determine whether leukotrienes were also important for the response to a bonafide rodent parasite,
we infected Alox5−/− mice with *N. brasiliensis*. Again, there was no difference in the number of peripheral blood eosinophils between wild-type and Alox5−/− mice during infection (Fig. 7 D). However, leukotriene deficiency resulted in a >80% reduction in eosinophils in the lung 9 d after infection.

It is possible that the residual eosinophil accumulation in *Lbt*Δr1+/− mice is caused by cysLTs because human eosinophils can migrate toward cysLTs in Transwell assays (Spada et al., 1994). However, we do not favor an involvement of cysLTs in collective migration because mouse eosinophils were not attracted to LTC4, LTD4, or LTE4 in Transwells and exhibited unperturbed migration to *C. elegans* in the presence of a CYSLTR1 antagonist. Moreover, mice deficient in cysLTs do not have defects in eosinophil recruitment to the skin in a model of atopic dermatitis (Oyoshi et al., 2012). It is conceivable that cysLTs exert effects on eosinophils by promoting their survival in the lung (e.g., by modulating IL-5).

This study establishes that eosinophils exhibit collective migration toward both parasitic and nonparasitic nematode species in the absence of adaptive immunity and stromal cell injury. This strongly points to recognition of conserved features of nematodes during eosinophil accumulation and raises intriguing questions regarding the nature of these features. The in vitro assay that we describe is amenable to genetic screens in *C. elegans*. Used in conjunction with the ability of *C. elegans* to eliciting eosinophil accumulation in mouse lung, this model provides a methodology for the discovery of nematode factors that initiate immune responses during helmhnt infection.

**MATERIALS AND METHODS**

**Mice.** YARKC(Arg1)c(1) (Reese et al., 2007), Thb4−/−(Dumont and Barrois, 1996), Cx3−/−(Humbles et al., 2002), C.F.+/−(Klion and Nutman, 2004), Alox5−/− (Chen et al., 1994), Ltb4r1−/−(Tager et al., 2000), and B55−(Lee et al., 1997) mice have been described previously. All strains were maintained on the C57BL/6J background except Cx3−/−, which was maintained on the BALB/c background. Wild-type C57BL/6J and BALB/cj mice were obtained from the Jackson Laboratory. All animal procedures were approved by the University of California, San Francisco (UCSF), Institutional Animal Care and Use Committee and performed in accordance with the guidelines established by the National Institutes of Health.

**Nematodes.** *C. elegans* strains carrying the *daf-2(e1370), mrl126[nec-5::Q0::YFP::Q0::YFP]* alleles were obtained from the Caenorhabditis Genetics Center and crossed with the aid of him-5 RNAi provided by C. Kenyon (UCSF, San Francisco, CA) to generate a *daf-2(e1370); mrl126[nec-5::Q0::YFP]* strain. Worms carrying a *myo3::CFP* extra-chromosomal array on the *daf-2(31368)* background were obtained from A. Bethke (UCSF). *C. elegans* dauers were generated by culturing synchronized L1 *daf-2* mutants at 27°C for 4 d in sealed Petri dishes and then collecting larvae from the sides and lids of the dishes. Dauers were >99% pure based on body morphology and an absence of pharyngeal pumping. For axenic culture, mCelHR was prepared as described previously (Rao et al., 2005). Axenic cultures were routinely tested for sterility by culture on brain heart infusion agar plates (BD) at 30°C and 37°C. Additionally, LPS was not detectable by Limulus amebocyte lysate testing (Pyrotell) in axenic cultures or in supernatants of *E. coli* fed *C. elegans* dauers after washing (as described below). *H. bacteriophora*-infected juveniles were provided by T. Ciche (Michigan State University, East Lansing, MI) and maintained using RET16 *Photorhabdus luminescens* cultures, as described previously (Helfen et al., 2007). *N. brasiliensis* infection was performed as described previously (Patnode et al., 2013). In brief, mice were anesthetized using isoflurane and injected subcutaneously at the base of the tail with 500 *N. brasiliensis* L3 larvae. Mice were maintained on water containing 2 g/liter neomycin sulfate and 100 mg/liter polymyxin B for 5 d and sacrificed after 9 d. For administration of *C. elegans*, 500 live dauers were injected into the caudal tail vein in 150 µl of 0.01% BSA in saline.

**3D and Transwell migration assays.** Nematodes were washed by six rounds of suspension in 1.5 ml of wash buffer (0.01% BSA in 0.9% NaCl), centrifugation for 5 s at ~1,000 g, and aspiration of supernatant. Nematodes were suspended at a concentration of 6,000 larvae/ml in Matrigel with phenol red (BD) or 1 mg/ml collagen (Invitrogen) and kept on ice. For bead-based assays, streptavidin-agarose beads (Sigma-Aldrich) were incubated with 10 µg/ml mouse IgG1-biotin or mouse F(ab′)2-biotin for 1 h. Eosinophils were obtained from bone marrow cultures, as described previously (Patnode et al., 2013). In brief, bone marrow from femurs and tibiae from two mice per group were flushed with RPMI-1640 (RPMI) and treated with water to lyse erythrocytes. Cells were then cultured in the presence of recombinant SCF and Flk3L (R&D Systems) for 4 d and subsequently cultured in the presence of recombinant IL-5 and collected after 10 additional days. Neutrophils were isolated from mouse bone marrow as described previously (Bonzo et al., 2004). Human eosinophils were obtained from peripheral blood using a Human Eosinophil Isolation kit (Miltenyi Biotech) according to the manufacturer’s instructions. Purified human eosinophils were >98% Siglec-8− and <2% CD16+CD66b−, whereas human neutrophils were <1% Siglec-8− and >99% CD16+CD66b−. Eosinophils from two separate donors exhibited similar cell surface staining and migration responses. Mouse eosinophils or neutrophils were incubated in 1 µM chloromethyl tetranethylrhodamine or CFSE in RPMI containing 2% FBS for 25 min at 37°C. Cells were washed, and some samples were treated with 250 µg/ml Bovine leukotriene B4 (Cayman Chemical) or CCL11 (R&D Systems).

**Microscopy.** Images were acquired using a microscope setup that has been described previously (Gilden et al., 2012). In brief, three lasers (argon 488 nm, krypton 568 nm, and indium gallium nitride 406 nm) were connected to a spinning disk confocal scan-head (CSU-10b; Yokogawa Corporation of America; modified by Solamere Technology Group), which was connected to a motorized, inverted microscope (Axiovert 200M inverted fluorescence microscope; Carl Zeiss). Emission light was passed through an automated filter wheel (FW-1000; Applied Scientific Instrumentation) and detected by an electron-multiplying charge-coupled device (EMCCD) camera (iXon3; Andor Technology). Images were collected through 10× fluor 0.5 NA and 20× fluor air objectives. For time-lapse imaging, cultures were maintained at 37°C on a heated stage and imaged starting roughly 15 mm after the start of the culture. For endpoint leukocyte accumulation assays, image volumes were acquired for all nematodes within 100 µm from the bottom of the imaging slice (three to seven larvae per well) from at least two wells per condition. Images were analyzed using ImaJis (BitPlane) software. Volumes representing the accumulated leukocytes were generated in parallel by applying single, manually set fluorescence intensity and volume thresholds to all larvae from all wells. For bead-induced accumulation, volumes were calculated for beads in several random fields. Accumulations of cells that were not in contact with the surface of larvae or beads were rare and omitted from the volume measurement. Immunofluorescence staining of lung tissue sections was performed as described previously (Patnode et al., 2013).

**Leukotriene ELISAs.** Detection of leukotrienes was performed using LTB4 and LTE4 Enzyme Immunoassay kits (Cayman Chemical) according to the manufacturer’s instructions. Purified human eosinophils were >98% Siglec-8− and <2% CD16+CD66b−, whereas human neutrophils were <1% Siglec-8− and >99% CD16+CD66b−. Eosinophils from two separate donors exhibited similar cell surface staining and migration responses. Mouse eosinophils or neutrophils were incubated in 1 µM chloromethyl tetranethylrhodamine or CFSE in RPMI containing 2% FBS for 25 min at 37°C. Cells were washed, and some samples were treated with 250 µg/ml Bovine leukotriene B4 (Cayman Chemical) or CCL11 (R&D Systems).

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to the manufacturer’s instructions. Bone marrow–derived eosinophils (10⁶/well) were incubated with 1 µM ionomycin or DMSO in 0.1% BSA in RPMI for 2 h at 37°C. Incubation with C. elegans dauers (1,000/well) was performed in parallel on wells coated with Matrigel. For all experiments, separate groups of cells were treated with 10 µM MK886 or DMSO before the start of the culture.

Flow cytometry. Mice were sacrificed and all lobes of lungs, except the left, were collected. Lungs were injected with digestion buffer consisting of 0.2 U/ml Liberase DL (Roche) and 10 µg/ml DNase (Sigma–Aldrich) in HBSS (without Ca²⁺/Mg²⁺). Lungs were minced using razor blades and incubated with digestion buffer for 25 min at 37°C. Digestion was stopped by adding EDTA to 10 mM and 2% FBS in RPMI, and tissue was crushed onto 70-µm cell strainers (BD). Cells were treated with ammonium chloride buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 100 mM EDTA) to lyse erythrocytes. Cells were incubated with 10 µg/ml anti–mouse CD16/32 (clone 93; eBiosciences) to block Fc receptors before staining with PE anti–Siglec–F (E56; BD) and APC anti–Ly-6G (1A8; BioLegend). Viability was determined by adding 50 µl/ml 7-aminoactinomycin D solution (BD) or 0.5 µg/ml DAPI. Cells were analyzed using a FACSort cytometer (BD) equipped with CellQuest software (BD). Further analysis was performed using FlowJo software (Tree Star).

Statistical analysis. Bar graphs are plotted as means plus the standard error of the mean using Prism 5 software (GraphPad Software). Where individual mice are plotted, horizontal bars represent the mean. Student’s t test was used to evaluate the statistical significance of differences between groups.


We thank Jakob von Molkte and Sebastian Peck for technical assistance, Creg Darby for helpful discussions, James Lee, Cynthia Kenyon, Todd Ciche, Axel Bethke, and Iqbal Hamza for advice and reagents. Some strains were provided by the C. elegans Genetic Center, which is funded by the National Institutes of Health (NIH) Office of Research Infrastructure Programs (P40 OD010440). Access to imaging equipment and analysis software was provided by the Biological Imaging Development Center at the University of California, San Francisco, which is funded in part by the Sandler Asthma Basic Research Center. This work was supported by NIH grants GM-23547 and GM-57411 (to S.D. Rosen), P01 HL024136 (to M.F. Krummel), and AI-26918 and AI-30663 (to R.M. Locksley). The authors declare no competing financial interests.

Submitted: 8 November 2013
Accepted: 25 April 2014

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