Role of Eosinophils and Neutrophils in Innate and Adaptive Protective Immunity to Larval *Strongyloides stercoralis* in Mice

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The goal of this study was to determine the roles of eosinophils and neutrophils in innate and adaptive protective immunity to larval *Strongyloides stercoralis* in mice. The experimental approach used was to treat mice with an anti-CCR3 monoclonal antibody to eliminate eosinophils or to use CXCR2^{-/-} mice, which have a severe neutrophil recruitment defect, and then determine the effect of the reduction or elimination of the particular cell type on larval killing. It was determined that eosinophils killed the *S. stercoralis* larvae in naïve mice, whereas these cells were not required for the accelerated killing of larvae in immunized mice. Experiments using CXCR2^{-/-} mice demonstrated that the reduction in recruitment of neutrophils resulted in significantly reduced innate and adaptive protective immunity. Protective antibody developed in the immunized CXCR2^{-/-} mice, thereby demonstrating that neutrophils were not required for the induction of the adaptive protective immune response. Moreover, transfer of neutrophil-enriched cell populations recovered from either wild-type or CXCR2^{-/-} mice into diffusion chambers containing larvae demonstrated that larval killing occurred with both cell populations when the diffusion chambers were implanted in immunized wild-type mice. Thus, the defect in the CXCR2^{-/-} mice was a defect in the recruitment of the neutrophils and not a defect in the ability of these cells to kill larvae. This study therefore demonstrated that both eosinophils and neutrophils are required in the protective innate immune response, whereas only neutrophils are necessary for the protective adaptive immune response to larval *S. stercoralis* in mice.

Roles have been recognized for both eosinophils and neutrophils in immune responses against nematodes in various host-parasite relationships. Eosinophils have been shown to be associated with resistance to helminth parasites infecting humans and animals (6, 37, 45). Evidence that eosinophils can kill nematodes, either alone or in conjunction with other immune components, such as antibody or complement, has been generated in a number of in vitro studies (23, 34, 42, 47, 66). Several approaches have also been used to assess the role of eosinophils in protective immunity in vivo. Eosinophils have been depleted from mice using a monoclonal antibody (MAb) to interleukin-5 (IL-5) (11) which resulted in blocking immunity to some infections (39, 40, 54) yet had no effect on immunity to other infections (11, 30, 56, 64). IL-5^{-/-} mice, which are incapable of augmenting blood and tissue eosinophil levels following exposure to helminths (38), support increased survival of some nematodes (46, 62, 69), while there is no effect on the survival of other nematodes (63). Complementary studies using IL-5 transgenic (TG) mice, which overexpress IL-5 and develop a profound systemic eosinophilia (41), showed that these mice promote decreased survival of several nematodes (12, 16, 27, 57, 62), whereas for other nematodes there is no change in parasite survival (15–17, 31, 61). It is not clear, however, if the effect on parasite survival in all of the experi-

ments described above was mediated by the presence or absence of IL-5 or by the resultant levels of eosinophils.

An alternative approach used to specifically ablate eosinophils in vivo is to block CCR3, the receptor for eotaxin. An anti-CCR3 MAb (6S2-19-4) has been shown to specifically reduce the number of eosinophils in the peripheral blood of mice infected with *Nippostrongylus brasiliensis* to levels below those in naïve mice, without affecting other cell populations (24). Furthermore, treatment of mice with the anti-CCR3 MAb significantly reduced protective immunity to the filarial worms *Onchocerca volvulus* (1) and *Brugia pahangi* (49). A similar observation was made in a study of resistance to *Trichinella spiralis* using CCR3^{-/-} mice, where there was an absence of eosinophil recruitment along with a concomitant increase in larval parasite survival (25).

In vitro studies have shown that neutrophils are effective at killing several nematode parasites in conjunction with antibody and/or complement (10, 14, 32, 55, 67). Neutrophils have also been associated, based on histological analyses, with killing larval *Ancylostoma caninum* in mice (59). Finally, mice deficient in gamma interferon and IL-5 have a defect in neutrophil function which results in increased survival of *Litomosoides sigmodontis* (3, 52, 53). Therefore, the alterations in parasite survival observed in naïve or immunized mice deficient in or depleted of IL-5 may ultimately be caused by a defect in either eosinophils or neutrophils.

A mouse model has been developed to study innate and adaptive immune responses to the infective third-stage larvae (L3) of *Strongyloides stercoralis*. Naïve IL-5^{-/-} mice, infected with L3 of *S. stercoralis*, have diminished levels of larval killing,

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whereas IL-5 TG mice have an enhanced ability to kill the larvae. Moreover, larval survival was found to be inversely correlated with the number of eosinophils, the level of IL-5, and the level of eosinophil peroxidase found in the larval microenvironment. Therefore, either IL-5 or eosinophils are essential components of the innate protective immune response to larval *S. stercoralis* (27). Eosinophils have also been shown to be crucial as a bridge between the innate and adaptive immune responses. In particular, although immunized IL-5^{-/-} mice did not develop adaptive protective immunity, transfer of eosinophils into immunized IL-5^{-/-} mice restored their ability to produce parasite-specific antibody and thus the adaptive protective response (27).

Adaptive protective immunity to *S. stercoralis* in mice has been shown to be dependent on Th2 cells (50), and roles for complement and immunoglobulin M (IgM) have also been established (8, 28, 44). Depletion of both eosinophils and neutrophils by MAb treatment of immunized animals at the time of the challenge infection resulted in complete ablation of protective immunity (51). However, passive transfer of purified IgM from immunized wild-type mice to naïve IL-5^{-/-} mice at the time of challenge conferred protective immunity. Examination of cells in the larval microenvironment revealed that while eosinophils were absent, the levels of neutrophils in the IL-5^{-/-} mice were comparable to those in wild-type mice, indicating that the transferred IgM killed the larvae through a mechanism independent of an induced eosinophilia (27, 44).

Based on these studies it was hypothesized that eosinophils are required as effector cells in the innate protective immune response and that neutrophils are required for the adaptive protective immune response to *S. stercoralis* L3. The requirement for eosinophils in protective immunity was tested by eliminating these cells from naïve and immunized mice with the anti-CCR3 MAb. Likewise, the necessity for neutrophils was tested in CXCR2 receptor 2 knockout (CXCR2^{-/-}) mice, which are deficient in the IL-8 receptor homologue and have a significant defect in neutrophil recruitment but normal eosinophil responses (9, 18). Using this series of experiments the relative roles of both eosinophils and neutrophils in innate and adaptive protective immunity were determined.

MATERIALS AND METHODS

Experimental animals and parasites. The BALB/cJ and C57BL/6J mice used in experiments were obtained from the Jackson Laboratory (Bar Harbor, ME). CXCR2^{-/-} mice from the Jackson Laboratory and NJ.1638 IL-5 transgenic (41) mice were bred at the Thomas Jefferson University Laboratory Animal Sciences Facility. Male mice that were 7 to 14 weeks old were used in all experiments. All mice were housed at the Thomas Jefferson University Laboratory Animal Sciences Facility in microisolator boxes under temperature- and light-controlled conditions and were provided food and water ad libitum.

S. stercoralis L3 were cultured from the fresh stools of laboratory dogs infected by using previously described methods (2). Larvae were harvested from charcoal cultures and washed via centrifugation and resuspension in a sterile 1:1 mixture of NCTC-135 and IMDM medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml gentamicin (Sigma Chemical Co., St. Louis, MO), and 0.25 mg/ml levaquin (Ortho-McNeil, Raritan, NJ).

Immunization and challenge infection protocol. To investigate the innate immune response, naïve mice received challenge infections of *S. stercoralis* L3 that were in diffusion chambers implanted for 3 or 5 days. For experiments in which adaptive immunity was studied, mice received two injections of 5,000 live L3 given 14 days apart, followed 1 week later by a challenge infection of L3 in an implanted diffusion chamber for 24 h. Diffusion chambers were constructed by using previously described methods (2). Briefly, 14-mm Lucite rings (Millipore,

Bedford, MA) were covered with 2.0- μ m-pore-size membranes (Millipore, Bedford, MA) using cyanoacrylate adhesive (Superglue Corp., Hollis, NY), fused together with an adhesive consisting of a 1:1 mixture of 1,2-dichloroethane (Fisher Scientific, Pittsburgh, PA) and acryloid resin (Rohm and Haas, Philadelphia, PA), and then sterilized using 100% ethylene oxide. Fifty L3 were injected into the diffusion chambers, which were then surgically implanted in subcutaneous pockets created on the flanks of the mice. At the conclusion of the experimental time, larval viability was determined based on motility and morphology, and both sera and diffusion chamber fluids were recovered for further analysis. Cells from diffusion chambers were centrifuged onto slides using a Cytospin 3 (Shandon, Pittsburgh, PA) and were stained for differential analysis using DiffQuik (Baxter Healthcare, Miami, FL).

Eosinophil depletion. MAb 6S2-19-4 (anti-CCR3) (cell lines were a gift from D. L. Coffman, DNAX Corp.) is specific for the mouse CCR3 receptor and was used to selectively deplete eosinophils from mice, as demonstrated in previous studies (24). Mice received two intraperitoneal injections of 350 μ g of antibody, which were given 1 day before challenge and 2 days after challenge to deplete eosinophils in studies of innate immunity. At the time of challenge, 100 μ g of the anti-CCR3 MAb was also injected subcutaneously in the vicinity of the implanted diffusion chamber. In studies of adaptive protective immune responses immunized mice received an intraperitoneal injection of 150 μ g of the MAb 5 days and 3 days prior to challenge and a subcutaneous injection of 50 μ g in the vicinity of the diffusion chamber at the time of challenge. MAb GL117 (anti- β -galactosidase) was used as an isotype control immunoglobulin in these studies.

Eosinophil and neutrophil purification and implantation. Eosinophils were purified from the spleens of IL-5 transgenic mice using a previously described protocol (7). Briefly, mice were sacrificed via cervical dislocation, and the spleens were collected, homogenized, and then separated on a Percoll density gradient column by centrifugation. The eosinophil-lymphocyte layer was then transferred to a tube containing 2% bovine serum albumin (BSA)-phosphate-buffered saline (PBS). Red blood cells were lysed by mixing the cells in cold sterile water and then adding an equal volume of 2 $\times$ PBS. Cells were pelleted by centrifugation and resuspended in 2% BSA-PBS. Cells were incubated with anti-mouse CD90 microbeads (MACS, Miltenyi Biotec, Auburn, CA) to eliminate T cells and with anti-mouse CD45R microbeads (MACS, Miltenyi Biotec) to eliminate B cells, and eosinophils were collected following passage through a magnetic cell sorter (MACS, Miltenyi Biotec). The purified cell population contained 92 to 95% eosinophils.

A neutrophil-enriched population was prepared by injecting intraperitoneally 1 ml of 10% thioglycolate medium (Becton Dickinson, Sparks, MD) into naïve BALB/cJ, C57BL/6J, and CXCR2^{-/-} mice. Peritoneal exudate cells (PEC) were collected by lavage after 12 h, and differentials were performed. The PEC recovered from BALB/cJ mice were 76% neutrophils, 15% macrophages, and 9% lymphocytes, the PEC recovered from C57BL/6J mice were 84% neutrophils, 12% macrophages, 1% lymphocytes, and 3% eosinophils, and the PEC recovered from CXCR2^{-/-} mice were 74% neutrophils, 11% macrophages, and 15% lymphocytes.

Diffusion chambers were constructed with 0.1- μ m-pore-size membranes that prevented cells inside the diffusion chamber from leaving and prevented any host cells from entering. The diffusion chambers were loaded with 50 L3 and (i) 5×10^4 or 5×10^5 purified eosinophils and implanted subcutaneously in naïve C57BL/6J mice for 3 days, (ii) 5×10^4 , 5×10^5 , or 5×10^6 PEC and implanted subcutaneously in naïve C57BL/6J mice for 5 days, (iii) 5×10^6 PEC and implanted alone or with 5×10^4 or 1×10^5 purified eosinophils subcutaneously in naïve C57BL/6J mice for 5 days, or (iv) 4×10^6 PEC derived from BALB/cJ or CXCR2^{-/-} mice and implanted subcutaneously in naïve and immunized BALB/cJ mice for 24 h. At the conclusion of the experiment cell viability and larval viability were assessed.

ELISA. Deoxycholate (DOC)-soluble L3 proteins used as antigens in enzyme-linked immunosorbent assays (ELISA) were prepared as previously described (29). Briefly, *S. stercoralis* L3 were incubated with a 2% solution of low-melting-point agarose (type I-A; Low EEO; Sigma). After the agar solidified, PBS supplemented with 0.1 mg/ml streptomycin and 0.1 mg/ml gentamicin (Sigma) was added, and larvae that migrated into the PBS were harvested. L3 were homogenized on ice in the presence of a protease inhibitor cocktail, which consisted of 2 mM leupeptin, 2 μ g/ml pepstatin A, 28 μ g/ml aprotinin, and 5 mM EDTA. After homogenization, larvae were sonicated, and the PBS-soluble fraction was obtained following overnight incubation at 4°C. DOC-soluble proteins were extracted from the remaining pellet by incubation with 20 mM Tris-Cl-0.5% deoxycholic acid (Sigma) in the presence of the protease inhibitor cocktail described above. After 24 h, DOC-soluble proteins were dialyzed against PBS overnight and then passed through a 0.2- μ m syringe filter, quantitated by a Micro

BCA protein assay (Pierce, Rockford, IL), and stored at -80°C until they were used.

Nunc Maxisorb 96-well plates (Nunc, Naperville, IL) were coated with 10 $\mu\text{g}/\text{ml}$ of DOC-soluble antigen. Plates were blocked using borate blocking buffer consisting of 0.17 M boric acid, 0.12 M NaCl, 0.05% Tween 20, 0.25% BSA, and 1 mM EDTA (pH 8.5) at room temperature for 30 min. Test samples consisted of diffusion chamber fluids serially diluted in blocking buffer placed in wells and then incubated either at room temperature for 2 h or overnight at 4°C . The dilutions for chamber fluids started at 1:200. Samples were then incubated at room temperature for 1 h with biotinylated goat anti-mouse IgM (Vector Laboratories, Burlingame, CA). IgM was assayed because it is an essential component of adaptive protective immunity to *S. stercoralis* in mice (8, 44). ExtrAvidin peroxidase (Sigma) was then added, and samples were incubated for 30 min at room temperature. The final step consisted of addition of the peroxidase substrate 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Kirkgaard & Perry Laboratories, Gaithersburg, MD). The ABTS color reaction was measured at 410 nm with a Dynatech MR5000 microplate reader.

Serum transfer. Pooled control and immune sera from either CXCR2 $^{-/-}$ or BALB/cJ mice were used for serum transfer experiments. The control sera were sera from naïve mice that received a diffusion chamber challenge for 24 h; immune sera were defined as sera from mice that were immunized and challenged using the protocol described above. Sera were pooled and sterilized by passage through a 0.2- μm -pore-size filter prior to use. Serum from each treatment group was diluted 1:1 with sterile PBS, and 200 μl was injected at the time of challenge into the subcutaneous pockets surrounding the diffusion chambers implanted in naïve mice.

Statistical analysis. Statistical analysis was performed using multivariate general linear hypothesis multifactorial analysis of variance with the Systat version 11 software (Systat, Inc., Evanston, IL). *P* values less than 0.05 were considered significant. Fisher's least-significant-difference test was performed for post hoc analyses. All results reported below are the combined results of at least two independent experiments, and in each individual experiment there were at least five animals per group.

RESULTS

Role of eosinophils and neutrophils in the innate protective immune response. Eosinophils were depleted from naïve C57BL/6J and BALB/cJ mice using the anti-CCR3 MAb, and the mice were subsequently infected with L3 of *S. stercoralis*. For both strains of mice there was significantly higher recovery of live larvae following anti-CCR3 treatment than there was for the control animals at 5 days postinfection (Table 1). Analysis of cell populations recovered from diffusion chambers showed that there was no significant difference in the number of neutrophils between treated and untreated mice. A statistically significant decrease in eosinophil levels was seen in MAb-treated C57BL/6J mice. A similar decrease was seen in treated BALB/cJ mice, although the difference was not statistically significant (Table 1). Naïve CXCR2 $^{-/-}$ mice, which have a defect in neutrophil recruitment, were infected with *S. stercoralis* L3. The levels of recovery of live larvae were elevated in the CXCR2 $^{-/-}$ mice and were equal to those seen in mice depleted of eosinophils (Table 1). Differential counting of the cell populations recovered from diffusion chambers revealed a significant decrease in neutrophil recruitment in CXCR2 $^{-/-}$ mice, while the eosinophil levels were not statistically different from the wild-type level (Table 1).

Eosinophils and neutrophils were transferred along with larvae into diffusion chambers that were covered with 0.1- μm -pore-size membranes and then implanted in naïve C57BL/6J mice for 3 to 5 days to confirm that the cells could kill larvae in vivo with the innate immune response. The pore size of the membranes covering the diffusion chambers prevented cellular ingress and egress. Statistically significant reductions in larval survival were seen in diffusion chambers containing eosino-

TABLE 1. Effect of reduced levels of eosinophils (treatment with anti-CCR3 MAb) or neutrophils (CXCR2 $^{-/-}$ mice) on the survival of *S. stercoralis* L3 implanted in diffusion chambers in naïve mice for 5 days

Strain	Cell deficiency	% Survival	Total cells in diffusion chamber (mean $\pm$ SD)		
			Neutrophils (10^4)	Macrophages (10^4)	Eosinophils (10^2)
C57BL/6		25 $\pm$ 18	31 $\pm$ 23	32 $\pm$ 25	62 $\pm$ 90
C57BL/6	Eosinophils	44 $\pm$ 18 ^a	24 $\pm$ 24	19 $\pm$ 18	0 $\pm$ 0 ^a
BALB/c		18 $\pm$ 7	73 $\pm$ 21	38 $\pm$ 9	47 $\pm$ 66
BALB/c	Eosinophils	55 $\pm$ 9 ^a	78 $\pm$ 84	35 $\pm$ 18	0 $\pm$ 0
CXCR2 $^{-/-}$	Neutrophils	60 $\pm$ 20 ^a	4 $\pm$ 4 ^a	97 $\pm$ 39	59 $\pm$ 82

^a The value is statistically significantly different from the value for control and cell-deficient mice.

phils, and the maximum larval killing was observed in diffusion chambers containing 5×10^5 eosinophils (Fig. 1A). Larval survival was also reduced in diffusion chambers containing neutrophils, and significant larval killing was observed in diffusion chambers containing 5×10^6 neutrophils (Fig. 1B). Furthermore, combining 5×10^6 neutrophils with 1×10^5 eosinophils resulted in maximum killing of the larvae (Fig. 1C). More than 75% of the eosinophils and 90% of the neutrophils implanted in the diffusion chambers were recovered live at the conclusion of the experiments, and the differential distribution within the cell populations was unchanged. These observations confirm that both eosinophils and neutrophils have the capacity to kill larval *S. stercoralis* in vivo during the innate immune response.

Role of eosinophils and neutrophils in the adaptive protective immune response. C57BL/6J mice were immunized with live L3 and treated with the anti-CCR3 MAb at the time of the challenge infection. Differential cell counting performed at the time of larval recovery showed that the antibody effectively depleted eosinophils from the diffusion chambers without affecting neutrophils or macrophages (Fig. 2A). Mice depleted of eosinophils eliminated challenge larvae as effectively as control mice eliminated challenge larvae (Fig. 2B).

CXCR2 $^{-/-}$ mice were immunized to determine the role of neutrophils in the killing of challenge of parasites by the adaptive immune response. Analysis of cell populations recovered from the diffusion chamber fluids revealed a significant decrease in the number of infiltrating neutrophils in both naïve and immunized CXCR2 $^{-/-}$ mice compared to the number in wild-type animals, while there was no difference in the levels of eosinophils and macrophages between knockout and wild-type animals (Fig. 3A). Protective immune responses developed in the immunized CXCR2 $^{-/-}$ mice; however the level of larval killing was significantly decreased compared to the level in immunized wild-type mice (Fig. 3B).

The levels of parasite-specific IgM in the diffusion chamber fluids of immunized CXCR2 $^{-/-}$ animals were comparable to the IgM levels in the diffusion chamber fluids recovered from immunized wild-type mice (Fig. 4). To determine if the quantity and specificity of IgM produced in CXCR2 $^{-/-}$ mice were sufficient to confer immune protection, passive transfer experiments using serum from immunized CXCR2 $^{-/-}$ mice were performed. Naïve BALB/cJ mice were inoculated subcutane-

FIG. 1. Effect of eosinophils and neutrophils on the survival of L3. (A) Eosinophils recovered from naïve IL-5 TG mice were transferred into diffusion chambers covered with 0.1- μ m-pore-size membranes along with *S. stercoralis* L3, and the diffusion chambers were implanted in naïve C57BL/6J mice for 72 h. One asterisk indicates that there was a statistically significant difference between larval recovery from diffusion chambers in which there were no cells and larval recovery from diffusion chambers in which there were 5×10^4 or 5×10^5 eosinophils. Two asterisks indicate that there was a statistically significant difference between larval recovery from diffusion chambers in which there were 5×10^4 eosinophils and larval recovery from diffusion chambers in which there were 5×10^5 eosinophils. (B) Neutrophils recovered from the peritoneal cavity of C57BL/6J mice after injection of thioglycolate medium were transferred into diffusion chambers covered with 0.1- μ m-pore-size membranes along with *S. stercoralis* L3, and the diffusion chambers were implanted in naïve C57BL/6J mice for 5 days. The asterisk indicates that there was a statistically significant difference between larval recovery from diffusion chambers in which there were no cells and larval recovery from diffusion chambers in which there were 5×10^6 neutrophils. (C) Eosinophils and neutrophils, prepared as described above, were transferred into diffusion chambers covered with 0.1- μ m-pore-size membranes along with *S. stercoralis* L3, and the diffusion chambers were implanted in naïve C57BL/6J mice for

FIG. 2. Role of eosinophils in adaptive immunity: cell recruitment (A) and larval recovery (B) in diffusion chambers implanted for 24 h in immunized C57BL/6J mice treated with anti-CCR3 MAb to eliminate eosinophils. The asterisk indicates that there was a statistically significant difference between larval recovery in naïve mice and larval recovery in immune mice. The data are means and standard deviations. PMN, neutrophils; Mf, macrophages; Eos, eosinophils; α CCR3, anti-CCR3 MAb.

ously with serum from naïve or immunized wild-type and CXCR2^{-/-} animals. Mice that received serum from immunized CXCR2^{-/-} animals exhibited levels of immune protection that were equivalent to the levels for mice that received serum from immunized wild-type mice (Fig. 5A). Cells recovered from diffusion chamber fluids were predominantly neutrophils in all groups, while the eosinophil levels were consistently low in all groups (Fig. 5B).

Neutrophil-enriched cell populations recovered from naïve BALB/cJ and CXCR2^{-/-} mice were transferred (4×10^6 cells) with larvae into diffusion chambers covered with 0.1- μ m-pore-size membranes and implanted for 24 h in naïve and immunized BALB/cJ mice. Larvae survived in naïve wild-type mice when they were implanted with the cells derived from either wild-type or CXCR2^{-/-} mice, but larvae were killed when they

5 days. One asterisk indicates that there was a statistically significant difference between larval recovery from diffusion chambers in which there were no cells and larval recovery from diffusion chambers in which there were eosinophils and/or neutrophils. Two asterisks indicate that the data were statistically significantly different from the data for all other groups. The data are means and standard deviations. PMN, neutrophils; Eos, eosinophils.

FIG. 3. Role of neutrophils in adaptive immunity: cell recruitment (A) and larval recovery (B) in diffusion chambers implanted in immunized BALB/cJ and CXCR2^{-/-} mice for 24 h. One asterisk indicates that there was a statistically significant difference between larval recovery from naïve mice and larval recovery from immune mice. Two asterisks indicate that there was a statistically significant difference between larval recovery from immunized BALB/cJ mice and larval recovery from immunized CXCR2^{-/-} mice. The data are means and standard deviations. PMN, neutrophils; Mf, macrophages; Eos, eosinophils.

were implanted with the cells in immunized mice (Fig. 6). Approximately 90% of the transferred cells, regardless of the source, were observed to be alive at the time of the analysis of the diffusion chamber contents.

FIG. 4. Role of neutrophils in the development of protective IgM: parasite-specific IgM levels measured in diffusion chamber fluids recovered from immunized BALB/cJ and CXCR2^{-/-} mice. An asterisk indicates that there was a statistically significant difference between IgM levels in naïve mice and IgM levels in immune BALB/cJ or CXCR2^{-/-} mice. The data are means and standard deviations. O.D. (410 nm), optical density at 410 nm.

FIG. 5. Passive transfer of immunity from immunized CXCR2^{-/-} mice to naïve BALB/cJ mice: larval recovery (A) and cell recruitment (B) in diffusion chambers implanted in mice for 24 h after transfer of serum from donor naïve and immunized BALB/cJ and CXCR2^{-/-} mice into naïve recipient BALB/cJ mice. An asterisk indicates that there was a statistically significant difference between larval recovery from naïve mice and larval recovery from immune mice. The data are means and standard deviations. PMN, neutrophils; Mf, macrophages; Eos, eosinophils.

DISCUSSION

The present study demonstrated that eosinophils and neutrophils function in protective innate immunity to larval *S. stercoralis*. Eosinophils were shown to participate in larval killing based on increased larval survival in mice treated with the anti-CCR3 MAb to eliminate eosinophils. Treatment with the MAb resulted in a statistically significant reduction in the number of eosinophils in C57BL/6 mice. In both C57BL/6 and BALB/c strains of mice comparatively few eosinophils remained in the diffusion chambers after treatment with the anti-CCR3 MAb, and the reduction in the number of eosinophils correlated with an increase in larval survival.

Larval killing occurred in diffusion chambers covered with membranes allowing cell entry after implantation in naïve mice for 5 days. Approximately 1×10^4 eosinophils were seen in the diffusion chambers, but it is not known how many eosinophils actually entered the diffusion chambers, killed the larvae, and then died or exited. In cell transfer experiments we used numbers of eosinophils that approximated the number of cells seen infiltrating into the parasite microenvironment. Larvae were killed in diffusion chambers covered with membranes which prevented cell trafficking, if as few as 5×10^4 eosinophils were inserted with the larvae. Therefore, eosinophils in the absence

FIG. 6. Neutrophil-mediated killing in adaptive immunity: live larval recovery after neutrophil-enriched cell populations from BALB/cJ and CXCR2^{-/-} mice were transferred (4×10^6 cells) into diffusion chambers (containing larvae) covered with 0.1- μ m-pore-size membranes and implanted in naïve or immunized wild-type mice. An asterisk indicates that there was a statistically significant difference between larval survival levels in diffusion chambers containing cells in naïve recipients and larval survival levels in diffusion chambers containing cells in immune recipients. The data are means and standard deviations.

of significant numbers of other cell types can kill L3 in vivo in the innate immune response. Based on experiments using MAb treatments to deplete eosinophils and the cell transfer experiments, it is clear that eosinophils play an important role in larval killing during the innate immune response.

Previous studies demonstrated a role for IL-5 in innate immunity to larval *S. stercoralis* based on increased larval survival in naïve IL-5^{-/-} mice and decreased survival in naïve IL-5 TG mice. Larval survival was also correlated with eosinophil numbers and the level of the granule protein eosinophil peroxidase in the microenvironment of the larvae, with high levels of eosinophils and eosinophil peroxidase correlated with low levels of parasite recovery (27). In the present study it was observed that larval killing was decreased if eosinophils were eliminated while IL-5 levels were left unchanged. Therefore, we concluded that IL-5 was required, at least in part, in larval killing for its contribution to eosinophil production, activation, and recruitment.

There have been previous reports demonstrating that IL-5 or eosinophils participated in primary protective immune responses to the nematodes *Strongyloides ratti* (68), *Strongyloides venezuelensis* (19), *B. pahangi* (48), *Brugia malayi* (60), and *N. brasiliensis* (12). In vitro studies have demonstrated that the serum components complement and fibronectin are required for eosinophil binding and degranulation resulting in the killing of *N. brasiliensis* larvae (58). Complement component C3 has been shown to be required for innate immunity to *S. stercoralis* (35). It has also been reported that the purified human eosinophil granule products major basic protein and eosinophil cationic protein are directly toxic to larval *S. stercoralis* in the absence of any other cells or factors (51). Therefore, it is still not clear what specific sequence of events is required for eosinophils to kill larval *S. stercoralis* in mice in the innate immune response.

Neutrophils were also required for larval killing in the innate immune response. This conclusion was based on the observation that larval survival increased in naïve CXCR2^{-/-} mice, where there was a significant deficit in neutrophils in the diffusion chambers. Cell transfer experiments were performed using numbers that approximated the number of neutrophils seen infiltrating into the parasite microenvironment. Larvae were killed in diffusion chambers covered with membranes

which prevented cell trafficking when 5×10^6 neutrophils were inserted with the larvae. Therefore, neutrophils in the absence of significant numbers of other cell types can kill L3 in vivo in the innate immune response.

Results of the cell transfer experiments suggest that either eosinophils or neutrophils alone can kill the larvae. However, optimal killing occurred when both cell types were present. Furthermore, the cell depletion experiments suggested that both cell types are required for larval killing, based on the observation that elimination of either eosinophil function or neutrophil function resulted in an equivalent increase in larval survival. These results suggest that either cell type alone is not sufficient to kill the larvae, and therefore innate protective immunity requires both eosinophils and neutrophils. Killing of the microfilariae of *O. volvulus* (23, 33) and *Dirofilaria immitis* (20) has been shown to be mediated by either eosinophils or neutrophils. Similarly, larval *S. stercoralis* is killed by both cell types, and maximal killing occurs when both eosinophils and neutrophils are present.

Previous studies of the adaptive protective immune response to *S. stercoralis* in mice have shown that granulocytes are required for larval killing. This conclusion was based on the observation that larval killing did not occur in immunized mice treated with anti-granulocyte MAb (51). Furthermore, passive transfer experiments with IL-5^{-/-} mice demonstrated that antibody-dependent killing of larvae occurred in these eosinophil-deficient animals (27, 44). Collectively, these results suggest that neutrophils are the effector cells required for killing larvae in adaptive immunity. Results of the present study confirm that neutrophils are essential in the adaptive immune response to larval *S. stercoralis*. This conclusion is based on the observation that immunized CXCR2^{-/-} mice kill statistically fewer parasites than wild-type mice. The reduction in protective immunity correlates with a concomitant reduction in the number of neutrophils migrating into the diffusion chambers in the immunized CXCR2^{-/-} mice. Immunized CXCR2^{-/-} mice develop protective antibody responses that can passively transfer immunity to naïve wild-type mice. This observation demonstrates that neutrophils are not required to induce B-cell production of protective antibody. This is different from the IL-5^{-/-} mice immunized against *S. stercoralis* that also failed to develop adaptive protective immunity. The deficit in IL-

5^{-/-} mice was associated with diminished production of parasite-specific IgM (27). Therefore, it can be concluded that IL-5 is required at the induction phase of the adaptive immune response for the production of protective antibody and that CXCR2 is required at the effector end of the adaptive response for neutrophil function.

Further confirmation that neutrophils were competent effector cells in the adaptive immune response was obtained from neutrophil transfer experiments, in which neutrophils were placed with L3 in diffusion chambers that blocked cell ingress and egress. These diffusion chambers were implanted in immunized wild-type mice, and it was determined that larval killing occurred in immunized mice only if neutrophils were implanted with the L3. In subsequent experiments the question was asked whether the absence of immunity in CXCR2^{-/-} mice was caused by a defect in neutrophil recruitment or there was a defect in the ability of the neutrophils to kill L3. Neutrophils recovered from naïve CXCR2^{-/-} mice were injected along with L3 into diffusion chambers covered with membranes that blocked cell migration. These diffusion chambers were implanted in immunized wild-type mice, and it was determined that larval killing occurred in both groups of immunized mice, demonstrating that the defect in the CXCR2^{-/-} mice was in neutrophil recruitment and not in activity. CXCR2^{-/-} mice have been used in other studies, where they demonstrated that there were increases in susceptibility to the protozoan *Toxoplasma gondii* (13), various bacteria (22, 36), viruses (5), and fungi (4). The present study adds nematodes to the list of pathogens in which immunity is dependent on CXCR2 and therefore presumably neutrophils. With regard to neutrophil recruitment to the site of infection, it has been reported that *Ascaris suum*-derived products attract and activate human neutrophils through an IL-8 receptor (CXCR2) pathway (21). Furthermore, CXCR2^{-/-} mice did not recruit neutrophils to the corneas of mice after injection of proteins from *O. volvulus* (26). Therefore, it appears that CXCR2 functions in nematode infections, as it does in other infections (9, 18), as a crucial factor for neutrophil recruitment.

Treatment of immunized mice with the anti-CCR3 MAb at the time of the challenge infection did not adversely affect protective immunity. Thus, even though eosinophil levels were significantly elevated in immunized mice, these cells were apparently not required for killing of the larvae in the adaptive immune response. This observation confirms similar observations with the nematode *B. malayi*, where eosinophils were required only in the primary response and not in the secondary response (60), and the nematode *B. pahangi*, where it was observed that IL-5 and presumably eosinophils were required for clearance of primary infections but not secondary infections (48). In contrast to these two studies, eosinophils have been shown to function in adaptive immunity to *O. volvulus* (1), *T. spiralis* (65), and *L. sigmodontis* (43).

IL-5 has been associated with both eosinophil-mediated (27) and neutrophil-mediated (3, 52, 53) killing of nematode parasites. Therefore, it is possible that in the *S. stercoralis* mouse model IL-5 is required for eosinophils and neutrophils in the innate response and for neutrophils in the adaptive response. Correlations have been found between IL-5 levels, eosinophil levels, and larval killing in innate immunity (27). With regard to adaptive immunity, it has been reported that treatment of

mice immunized against *S. stercoralis* with an MAb to block IL-5 at the time of the challenge infection blocked protective immunity (51). Based on the current study, which demonstrated that eosinophils were not effector cells in the adaptive immune response, it is possible to explain the requirement for IL-5 at the time of the challenge infection as a factor that is essential for neutrophil function.

In conclusion, the present study demonstrated that the protective immune response utilizes different granulocytes in innate protective immunity and adaptive protective immunity. Eosinophils and neutrophils are required in the innate response, and neutrophils are required in the adaptive response.

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