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Role of IL-5 in Innate and Adaptive Immunity to Larval *Strongyloides stercoralis* in Mice¹

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Protective immunity to *Strongyloides stercoralis* infective larvae in mice has been shown to be dependent on IL-5 based on mAb depletion studies. The goal of this study was to determine the functional role of IL-5 during the innate and adaptive immune response to larval *S. stercoralis* in mice. In these studies, three strains of mice were used: wild-type C57BL/6J (WT), IL-5 knockout (KO), and IL-5 transgenic (TG). Innate responses to the larvae indicated that there was enhanced survival in the KO animals and decreased survival in the TG animals compared with WT. Furthermore, killing of larvae in TG mice was associated with eosinophil infiltration and degranulation. In studying the adaptive immune response, it was observed that immunization of KO mice did not lead to the development of protective immunity. Experiments were then performed to determine whether KO mice reconstituted with Abs or cells could then develop protective immunity. KO mice displayed protective immunity via a granulocyte-dependent mechanism following injection of purified IgM from immune wild-type animals. Immunity in KO mice could also be reconstituted by the injection of eosinophils at the time of immunization. These eosinophils did not participate in actively killing the challenge infection, but rather were responsible for the induction of a protective Ab response. We conclude that IL-5 is required in the protective immune response for the production of eosinophils, and that eosinophils were involved in larval killing during innate immunity and in the induction of protective Abs in the adaptive immune response. *The Journal of Immunology*, 2000, 165: 4544–4551.

Interleukin-5, a hematopoietic cytokine, plays a key role in the differentiation, maturation, and survival of eosinophils derived from bone marrow precursors (1, 2). The link between IL-5 and eosinophils is highly conserved in mammals (3, 4), which suggests a selective pressure for maintenance of this function in the immune repertoire. IL-5 has been shown to have an additional role for activation and Ab secretion from mouse, but not human, CD5⁺ B-1 cells, which produce IgM Abs (5, 6). Prominent sources of IL-5 include T cells, mast cells, eosinophils, and NK cells (1, 3), and several immune responses have been associated with an elevation of IL-5, such as allergy and asthma (7), malignant hyperplasia (8), autoimmune disease (4), and parasitic infection (9). The role of IL-5 has been highly debated due to its involvement in various responses considered to be either pathogenic or protective for the host. Parasitic helminth infections have often been correlated with an increase in serum IL-5 levels, peripheral blood eosinophils, and eosinophil infiltration of host tissue where the parasites are located (4, 9), but their role in parasite killing is still unclear.

The importance of IL-5 and eosinophils in protective immunity against parasitic helminths is dependent upon the parasite strain or host species examined. Many studies, in which IL-5 was depleted with the mAb TRFK-5, have shown a decrease in peripheral blood eosinophils, but no significant effect upon the subsequent parasite burden (10–13). Parasites for which there was no correlation between the levels of IL-5 and the survival, growth, or fecundity of the parasite include *Schistosoma mansoni* (12), *Trichinella spiralis* (11), *Toxocara canis* (13), and *Trichuris muris* (10). One report even showed impairment in the successful clearance of *S. mansoni* from IL-5 transgenic mice following immunization (14). These data suggest that, although IL-5 is elevated in response to these infections, the cytokine is not required for immune protection against these species of parasitic helminths.

Conversely, a role for IL-5 has been established in protective immunity against the nematodes *Onchocerca volvulus* (15), *Onchocerca lienalis* (16), *Angiostrongylus cantonensis* (17), *Strongyloides stercoralis* (18), and *Strongyloides ratti* (19). These nematodes showed an increased parasite burden, delayed expulsion, or impaired clearance of infection when the levels of IL-5 were reduced by treatment with the mAb TRFK-5. Differences in dependency on IL-5 have been reported between primary and secondary immune responses. Studies with *S. ratti* showed a dependency upon IL-5 for protection from the primary infection, but not against a challenge infection (19). In contrast, IL-5 was shown to be essential for vaccination-induced protection against the filarial nematode *Litosomoides sigmodontis*, yet had no role during the primary response (20). Although a requirement for IL-5 in immune-mediated resistance has been demonstrated in these models, the specific contribution of IL-5 to the mechanism of parasite control has not been clearly defined.

S. stercoralis is a parasitic nematode of humans causing a spectrum of disease including chronic infection, hyperinfection and

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disseminated disease (21). The third-stage larvae (L3)³ will infect many different strains of inbred mice and can be found migrating through host muscle tissue up to 9 days following primary infection (22). The complete life cycle is not supported in the mouse, and parasite clearance is associated with an eosinophilic infiltrate surrounding dying larvae found within muscle tissue (23). It has been hypothesized that the further development of L3 is prevented by the host immune response, because adults develop in infected SCID mice (24). Adaptive protective immunity against *S. stercoralis* larvae in BALB/cByJ mice was shown to be dependent on a Th-2 response mediated exclusively by CD4⁺ T cells. Larval killing was abrogated by treatment with either anti-IL-5 (TRFK-5) or anti IL-4 (11B11) mAb, or by inoculation of IL-12 during immunization to restrict the response to Th-1 (25). Protective immunity was shown to be dependent on IgM, from serum transfer experiments, and on complement activation, by depletion with cobra venom factor (26). Finally, depletion of granulocytes with the mAb RB6-8C5 eliminated protective immunity in immunized mice, which, combined with the dependency on IL-5, supported the hypothesis that eosinophils were required for larval killing by the adaptive immune response (27).

A discrepancy was previously observed in the ability of TRFK-5 to eliminate protective immunity to *S. stercoralis* in mice as a function of where the challenge infection was inserted. It was shown that TRFK-5 ablated immunity if the challenge larvae were localized within diffusion chambers, but not if the larvae were injected into the tissues of the mouse and allowed to migrate systemically (18). One interpretation of these results is that the anti-IL-5 treatment functioned in elimination of immunity against parasites contained within diffusion chambers because of an artifact caused by the diffusion chamber preventing parasite migration away from effector cells. Alternatively, it may have been that the mAb treatment was unable to completely eliminate IL-5 in the s.c. tissues and the remaining IL-5 with eosinophils participated in killing the systemic challenge larvae.

Mouse strains with genetically altered IL-5 expression have been used to understand what changes occur to the immune system as a result of its dysregulation. Depending on the transgenic construct, IL-5 overexpression has been shown to cause moderate (28) to massive eosinophilia, lower bowel inflammation, and enhanced autoantibody production (29, 30). In the present study, two of these IL-5 transgenic mouse lines were utilized. One was generated (NJ.1638) that produces IL-5 under a CD3 restriction element resulting in a large increase of peripheral blood eosinophils and B220⁺ cells and lower bowel inflammation (30). The second IL-5 transgenic mouse line (1726) produced IL-5 under a lung epithelium promoter and also had a significant increase of peripheral blood eosinophils (31). A single line of IL-5 knockout mice has also been generated, which possess a transient deficiency in the CD5⁺ B-1 cell subset and are severely deficient in generating eosinophilic responses following helminth infection. These IL-5 knockout mice can, however, produce specific IgG responses to foreign Ags and T cell functions are unaffected (32).

In the present study, experiments were designed to determine the role of IL-5 and eosinophils during the innate and adaptive immune response to larval *S. stercoralis* in C57BL/6J mice. Immune responses to the helminth were compared between IL-5 knockout and IL-5 transgenic mice to allow us to understand how IL-5 functions in vivo during innate and adaptive immunity. Re-

sults show that naive IL-5 transgenic mice were naturally resistant to infection characterized by eosinophil infiltration and granule release in the larval microenvironment, whereas naive IL-5 knockout mice were more susceptible than wild-type mice. Adaptive immunity in wild-type mice was characterized by CD4⁺ T cell-restricted IL-5 production and increased titers of parasite-specific IgM. Immunized IL-5 knockout mice were deficient in the development of protective immunity, but could be passively immunized with IgM, which functioned through a granulocyte-dependent mechanism. Finally, reconstitution of IL-5 knockout mice by the adoptive transfer of eosinophils at the time of immunization restored their ability to develop protective immunity. The immune response in the IL-5 knockout mice reconstituted with eosinophils was characterized by an increase in the parasite-specific IgM response and an absence of eosinophils associated with parasite killing. We conclude from these studies that IL-5 is required for eosinophil production to mediate larval killing during the innate immune response and for IgM production during adaptive immunity.

Materials and Methods

Experimental animals and parasites

All mice used in these experiments were on the C57BL/6J background. Wild-type (WT) mice obtained from The Jackson Laboratory (Bar Harbor, ME) were 6–9 wk of age at the beginning of each experiment. NJ.1638 IL-5 transgenic (TG) mouse line (30) and 1726 lung-specific IL-5 transgenic mouse line (31) were obtained from our original animal stocks. IL-5 knockout (KO) mice were a generous gift from Manfred Kopf (Basel Institute for Immunology, Basel, Switzerland) and have been described elsewhere (32). TG and KO mice were bred in the Laboratory of Animal Sciences facility at Thomas Jefferson University (Philadelphia, PA) and housed in filter-top microisolator boxes under light- and temperature-controlled conditions. Male mice were used for all experiments.

S. stercoralis L3 were obtained from the fresh stools of a laboratory dog infected with the parasite according to methods previously described (27). Larvae were collected from charcoal cultures and washed by centrifugation and resuspension in sterile RPMI with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml gentamicin (antibiotics) (Sigma, St. Louis, MO)

Immunization and challenge infections

Mice were immunized with live *S. stercoralis* L3 as described previously (27). The immunization protocol consisted of two s.c. injections with 5,000 live L3 administered 2 wk apart, followed by a challenge infection given 1 wk later consisting of 50 live L3 contained within diffusion chambers or 1000 live L3 injected s.c. Construction of diffusion chambers and challenge infection of mice with L3 followed previously published methods (27). Fourteen-millimeter Lucite (Millipore, Bedford, MA) rings were covered with 2.0- μ m Isopore membranes (Millipore). The membranes were attached to the rings with cyanoacrylate adhesive (Super Glue Corporation, Hollis, NY) and the Lucite rings cemented to each other with a compound consisting of equal parts of 1,2-dichloroethane (Fisher Scientific, Pittsburgh, PA) and acryloid resin (Rohm and Hass, Philadelphia, PA), and the completed diffusion chambers were sterilized in 100% ethylene oxide. Diffusion chambers containing 50 L3 were implanted for 24 or 72 h in a s.c. pocket on the lower flank of the mice during the challenge infection. Recovery of live larvae consisted of removal of the diffusion chambers from the mice and quantitation of live parasites within under a dissecting microscope. Cells found within diffusion chambers were centrifuged onto slides using a Cytospin 3 (Shandon, Pittsburgh, PA) and then stained for differential counts with DiffQuik (Baxter Healthcare, Miami, FL). In some experiments, mice were given a challenge infection consisting of 1000 L3 injected s.c., and, after 24 h, mice were killed, skin and muscle minced, and L3 recovered from tissue after incubation in sterile PBS. In these experiments, the challenge infection with 1000 L3 was given on day 35 to allow for all of the immunization larvae to die before the challenge larvae were injected.

Enzyme-linked immunospot (ELISPOT) analysis

Spleens were removed from mice at the end of the immunization protocol (day 22), RBC lysis was performed (ammonium chloride-Tris, pH 7.3), and lymphocytes were restimulated with 50 μ g/ml of a sodium deoxycholate-soluble (DOC) L3 extract. The DOC-soluble L3 Ags were obtained following a two-step purification procedure, in which PBS soluble Ags are

³ Abbreviations used in this paper: L3, third-stage larvae; DOC, sodium deoxycholate-soluble L3 extract; EPO, eosinophil peroxidase; KO, IL-5 knockout; TG, NJ.1638 IL-5 transgenic; WT, wild-type C57BL/6J; ELISPOT, enzyme-linked immunospot.

first extracted, followed by extraction of detergent soluble protein in 0.5% DOC in a protease inhibitor cocktail (2 mM leupeptin, 2 μ g/ml pepstatin A, 28 μ g/ml aprotinin, and 5 mM EDTA), and finally dialyzed overnight against PBS. All experiments in which splenocyte cytokine production was determined used individual spleens with three mice per group. Those experiments in which CD4⁺ T cell cytokine secretion was examined, pooled spleen cells from three mice were used for passage through a mouse CD4⁺ T cell affinity column (collect; Cytovax Biotechnologies, Edmonton, Alberta, Canada). Purity of CD4⁺ T cell was >90% as determined by FACS. CD4⁺ T cells were cultured with APC at a ratio of 3:1 (stimulator to responder) for 72 h at 37°C and 5% CO₂. Source of APC were splenocytes from naive C57BL/6J mice pulsed with 50 μ g/ml of DOC-soluble Ag for 3 h and then fixed with 1% paraformaldehyde. Multiscreen plates (Millipore) were coated with capture Ab for IFN- γ (R46A2) (Sigma), IL-4 (11B11) (Sigma), or IL-5 (TRFK-5) (Sigma) at 10 μ g/ml in borate buffer, pH 8.5, overnight at 4°C. Plates were then washed and blocked with 5% BSA for 2 h at room temperature. Cells were plated in triplicate wells in RPMI 1640 supplemented with 10% FCS and 10 U/ml recombinant IL-2 (National Cancer Institute, Biological Resources Branch, Frederick, MD). Following 72 h of culture at 37°C, cells were removed by washing with PBS/Tween 20 and incubated with the appropriately matched biotinylated anti-mouse secondary Abs diluted in PBS 1% BSA (pH 7.3). Plates were washed before incubation with alkaline phosphatase (Sigma) for 2 h at room temperature. Finally, plates were washed with PBS/Tween 20 before developing the colorimetric assay by the addition of Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) tablets for 30 min. The plates were then washed with distilled water and air-dried. Spots were quantitated by examination under a dissecting microscope.

Eosinophil peroxidase (EPO) assay

The method of Strath et al. (33) as modified by White et al. (34) was used for this assay. Briefly, 50 μ l of diffusion chamber fluid was combined with 75 μ l of a 16 mM *o*-phenylenediamine solution in 100 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and 0.01% hydrogen peroxide. Polystyrene 96-well Nunc microtiter plates containing the reactions were placed at 37°C until a color change could be detected, and absorbance at 492 nm was measured using a Dynatech Microtiter Plate Reader (Dynatech Laboratories, Chantilly, VA). HRP (Sigma) was used as a standard with sensitivity ranging from 200 μ l/ml to 1 μ l/ml peroxidase activity.

Serum transfer

Blood was obtained from the exsanguinated mice on the same day as the recovery of the challenge infection. Sera from naive mice challenged for 24 h with 50 L3 in diffusion chambers was defined as control serum. Serum from immunized mice that successfully eliminated greater than 90% of the challenge infection was defined as immune serum. All sera were pooled by group, sterilized through a 0.2- μ m pore size filter, and stored at -80°C until used. Purification of IgM was performed according to previously published methods (26). Briefly, mouse serum was passed through a Gamma-bind Plus protein G Sepharose column (Pharmacia LKB, Uppsala, Sweden) to separate the total IgG (bound) from IgM, IgA, and IgE (flow through). The IgG fraction was eluted using 0.5 M acetic acid, pH 3.0, and the fractions immediately neutralized with concentrated Tris-HCl, pH 9.8. The IgM, IgA, and IgE fraction was further separated by passing the sera sequentially through anti-mouse IgE and anti-mouse IgA affinity columns. IgM was obtained by negative selection and dialyzed with PBS overnight. The Ab concentration before and after fractionation was determined by ELISA.

The IgM fraction was injected into mice at the time of challenge into the s.c. pocket where the diffusion chamber was implanted. The amount of Ab transferred was the same quantity found in 100 μ l of either unfractionated control or immune serum as determined by ELISA and diluted 1:1 in sterile PBS. In those experiments where granulocytes were depleted, mice were given an i.p. injection of 1 mg RB6-8C5 (35) 3 days before the challenge infection and on the day of challenge to deplete granulocytes.

ELISA

Nunc Maxisorp 96-well plates (Nunc, Naperville, IL) were coated with 50 μ l of either 10 μ g/ml of rat anti-mouse IgM or 10 μ g/ml of DOC L3 protein extract overnight at 4°C. Plates were blocked with borate blocking solution (0.17 M boric acid, 0.12 M NaCl, 0.05% Tween 20, 0.25% BSA, 1 mM EDTA, pH 8.5) at 37°C for 1 h. Wells were washed with distilled water and test samples, diluted in PBS 1% BSA (pH 7.3), were placed in wells at serial dilutions, and incubated at 37°C for 2 h. Appropriately matched biotinylated goat anti-mouse isotype detection Abs were added and plates incubated at 37°C for 2 h. Biotinylated goat-anti mouse IgM, IgG1, IgG2a, IgG2b were all obtained from PharMingen (San Diego, CA).

Plates were then washed and avidin peroxidase (Sigma) added for 30 min followed by the peroxidase substrate ABTS (2,2'-azinodi(3 ethylbenzthiazoline-6-sulfonate)) (Kirkgaard & Perry Laboratories, Gaithersburg, MD). ABTS color reaction was measured at 410 nm on a Dynatech MR5000 microplate reader.

Adoptive transfer

Peripheral blood eosinophils were purified from NJ.1638 IL-5 TG mice 6–8 wk of age that have 50–70% blood eosinophilia (30). Mice were anesthetized and exsanguinated, and blood was collected in a 50-ml conical tube with 10 U/ml heparin. Blood was separated on a Percoll column density gradient by centrifugation for 1 h at 4°C. The eosinophil/lymphocyte layer was transferred to a fresh tube with 2% FCS in PBS, and RBC lysis was performed with ammonium chloride-Tris, pH 7.3. Cells were pelleted by centrifugation and resuspended in 2% FCS in PBS and then incubated with 10 μ g/ml mouse IgG (Sigma) to block nonspecific binding of Fc receptors, anti-mouse CD45R to eliminate B220⁺ cells (PharMingen), and anti-mouse CD3 to eliminate T cells (PharMingen). The last step included incubation for 45 min and addition of streptavidin microbeads before passage through a magnetic cell sorting column (MACS; Miltenyi Biotec, Auburn, CA). Eosinophils were collected by negative selection, and purity was >97% as determined by differential cell staining. In addition, eosinophils were collected from 1726 IL-5 transgenic mice as described above, to control for the effect of the cytokine promoter on the ability of the eosinophil to adoptively transfer immunity.

Statistical analysis

Experiments consisted of five mice per group unless otherwise noted. All experiments described, with one exception, were performed at least twice, and data shown are from a representative experiment. The experiment in which there was the adoptive transfer of eosinophils from 1726 IL-5 transgenic mice was performed a single time. Statistical analysis of the data was performed using MGLH multifactorial ANOVA with Systat version 5.2 software (Systat, Evanston, IL). Probability values of less than 0.05 were considered significant.

Results

IL-5 is involved in the innate immune response against *S. stercoralis* L3

Experiments were performed to determine the impact of endogenous IL-5 levels upon parasite survival in naive C57BL/6J mice. Primary infections, consisting of 50 L3 contained within diffusion chambers, were implanted s.c. in WT, TG, and KO mice. Diffusion chambers removed 24 h after infection revealed no significant change in parasite survival between any groups. However, at 72 h postinfection, there was a negative correlation between parasite survival and endogenous levels of IL-5. KO mice had high parasite survival, WT a moderate level, and TG mice had the lowest number of live parasites recovered (Table I). The magnitude of eosinophil infiltration into the diffusion chamber also negatively correlated with parasite survival (Table I). Assays for EPO performed on the diffusion chamber fluid revealed that the levels of EPO detected correlated with the number of eosinophils present (Table I). Finally, ELISAs were performed on the fluid recovered from the diffusion chambers. It was determined that there was no parasite Ag-specific IgM present in the fluid recovered from any of the mice. Thus, endogenous levels of IL-5 correlated with the number of infiltrating eosinophils and EPO levels, and high levels of these three factors were associated with diminished parasite survival.

IL-5 plays an essential role in the adaptive immune response against *S. stercoralis*

ELISPOT analysis was performed to determine the cytokines secreted by splenic T cells in response to immunization with *S. stercoralis* larvae. WT mice were immunized with 5000 L3 on day 0 and day 14 followed by a challenge infection on day 21 consisting of 50 L3 contained within a diffusion chamber. The percentage of live larvae recovered 24 h later was 55 \pm 14 from the control

Table I. Comparison between WT, KO, and TG mice in levels of IL-5 in serum, their ability to support survival of larval *S. stercoralis*, the percentage of cells that migrate into the diffusion chambers that are eosinophils, and the quantity of EPO measured in the fluid recovered from the diffusion chambers^a

	IL-5 Levels in Serum	% Viable Larvae	% Eosinophils	EPO (mU/ml)
WT	Detectable	22 ± 11	6 ± 8	1 ± 3
KO	Absent	46 ± 13*	0.3 ± 1*	0.4 ± 0.08
TG	400 pg/ml	1 ± 2*	64 ± 28*	60 ± 94*

^a Results were obtained 72 h after implantation of diffusion chambers into naive animals. *, Statistical difference as compared to WT value. Values are means ± SD.

animals as compared with 5 ± 4 from the immunized animals. Splens were removed on day 22 for analysis of cytokine production. There was an elevation in the frequency of IL-5-secreting cells from the splens of immunized mice compared with nontreated controls. IL-4-secreting cells showed a slight but nonsignificant increase following immunization; however, IFN- γ -secreting cells did not increase following immunization (Fig. 1A). ELISPOT analysis was also performed using purified CD4⁺ T cells from a pool of spleen cells from immunized or nontreated control animals. Primed CD4⁺ T cells produced IL-5 at a frequency 10-fold higher than control values, whereas IFN- γ secretion was completely absent from this T cell population (Fig. 1B).

Experiments were performed to determine whether IL-5 was essential for adaptive immunity against *S. stercoralis* L3. WT and KO mice were immunized with 5000 L3 on day 0 and day 14 followed by a challenge with 1000 L3 injected s.c. on day 35. Twenty-four hours after challenge infection, the live parasites were recovered from the mouse tissues. Immunized KO mice failed to eliminate a significant number of the systemically migrating larvae, whereas WT immunized mice eliminated the challenge infection (Fig. 2). ELISAs were performed to measure the parasite-specific Ab responses in the serum of immunized mice. The quantity of parasite-specific IgM was significantly lower in KO mice compared with the WT following immunization (Fig. 3). In contrast, there was an increase in the parasite-specific IgG2a and IgG2b in KO mice following immunization, with no alterations in

IgG1, as compared with the WT mice (Fig. 4). These data indicate that IL-5 is crucial for elimination of challenge parasites in an immunized animal and suggests that in its absence, alteration in the type of Ab response generated occurs.

Passive immunity in IL-5 KO mice is granulocyte dependent

Serum transfer experiments were performed to determine whether the Ab responses generated following immunization of WT and KO mice could protect naive mice against initial infection. Naive WT mice were implanted with 50 L3 contained within diffusion chambers and simultaneously injected s.c. with purified IgM from immunized or nontreated mice. Twenty-four hours after infection, diffusion chambers were removed and live parasites quantitated. IgM from immunized WT mice was able to passively transfer immunity to naive WT animals, whereas IgM from immunized KO mice could not (Table II, experiment A). Furthermore, naive KO mice that received IgM from immunized WT mice were protected from infection (Table II, experiment B).

To determine the mechanism of passive immunity in WT and KO mice, depletion of granulocytes was performed. WT and KO mice were treated with mAb RB6-8C5 to eliminate granulocytes before the passive transfer of IgM from immunized WT mice. These experiments revealed that passive protection could be ablated following treatment with an anti-granulocyte mAb in both WT and KO recipients (Table II, experiment B).

Experiments were performed to determine whether the lower titer of parasite-specific IgM in the immunized KO mice contributed to the deficiency in immune protection observed in these mice. Furthermore, this would explain why the IgM fraction from immunized KO mice could not transfer immunity. To test this hypothesis, IgM from immunized KO mice was transferred at two different concentrations into WT mice: 1) the amount found in 100 μ l of serum from immunized KO mice, and 2) the amount of Ag-specific IgM from immunized KO mice that was equivalent to that measured in 100 μ l of serum from immunized WT mice. The concentrated IgM was prepared by purifying IgM from immunized

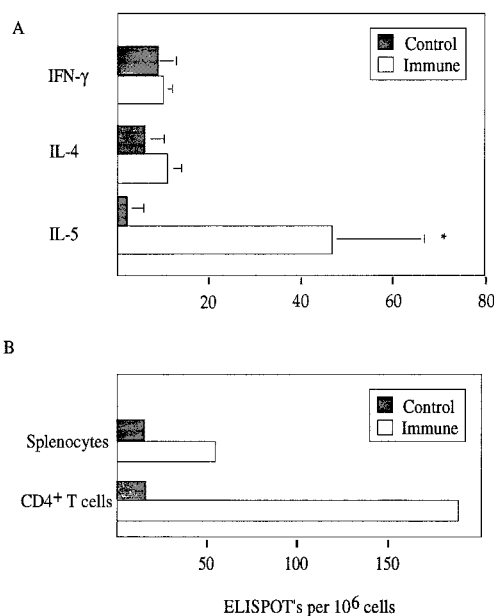


FIGURE 1. Cytokine ELISPOT assays. A, Number of splenocytes secreting IFN- γ , IL-4, and IL-5 from control and immunized C57BL/6J WT mice. B, Comparison of the number of IL-5-secreting splenocytes and CD4⁺ T cells from control and immunized C57BL/6J WT mice.

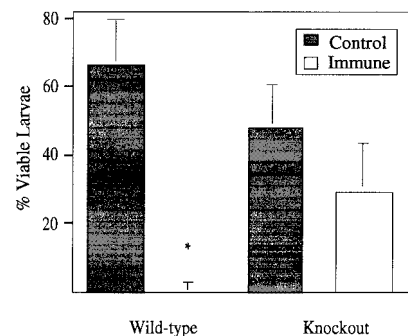


FIGURE 2. Effect of immunization of C57BL/6J WT mice and IL-5 KO mice on the survival of challenge larvae injected s.c. into the mice and recovered from the skin and muscles of the mice.

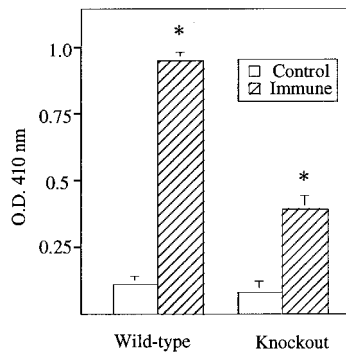


FIGURE 3. Parasite-specific IgM Ab responses of control and immunized WT C57BL/6J and IL-5 KO mice. Values listed are means \pm SDs of OD measured at a serum dilution of the 1:100. *, Statistical difference between control and immune values.

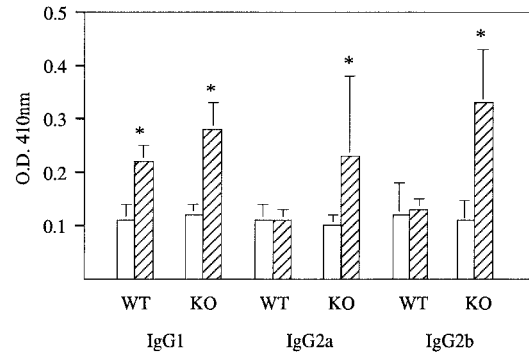


FIGURE 4. Parasite-specific IgG1, IgG2a, and IgG2b Ab responses of control and immunized WT C57BL/6J and IL-5 KO mice. Values listed are means \pm SDs of OD measured at a serum dilution of the 1:100. *, Statistical difference between control and immune values.

KO mice and then concentrating it so that it contained the same amount of Ag-specific IgM in 100 μ l as the WT immunized mice, as determined by ELISA. The quantity of total IgM transferred for WT control was 12 μ g, for WT immune 15 μ g, for KO control 6 μ g, for KO immune 7 μ g, and for the concentrated IgM from KO mice 120 μ g. These passive transfer experiments demonstrated that injection of Ag-specific IgM from KO mice in quantities equal to that found in immunized WT mice resulted in the passive transfer of immunity (Fig. 5). Furthermore, this was not due to non-specific killing due to the increased amount of total IgM, because concentrated IgM from naive WT mice had no effect upon parasite survival (data not shown). This suggested that the quantity of parasite-specific IgM is a major factor in active and passive immunity and that the deficiency in the KO mice could be corrected with the appropriate concentration of parasite-specific Ab in cooperation with granulocytes for larval killing.

Eosinophils restore protective immunity in IL-5 KO mice

Adoptive transfer experiments were performed to determine whether eosinophils could restore the development of immune protection to KO mice. WT and KO mice were immunized with 5000 L3 on day 0 and day 14 and given a challenge infection of 50 L3 contained within diffusion chambers on day 21. At the same time and location of the larval immunization, 1×10^7 purified peripheral blood eosinophils from TG mice were injected. Results showed that the immunized KO mice, reconstituted with eosinophils, eliminated the challenge larvae, whereas immunization of

KO mice with L3 alone led to an insignificant decrease in parasite survival. Furthermore, WT mice were able to eliminate L3 at the same rate regardless of the eosinophil transfer (Table III). This experiment was repeated using 1726 IL-5 transgenic mice as an alternative eosinophil donor. The results were identical with those obtained with the transfer of cells from NJ.1638 IL-5 transgenic mice; KO mice receiving the eosinophils at the time of immunization developed protective immune responses. The microenvironment of the challenge larvae was examined to determine the number of eosinophils that were present in immune protected animals. Differential staining of the cellular infiltrate following challenge infection revealed that the percentage of eosinophils did not increase in KO mice immunized with L3 or in immunized KO mice that were reconstituted with eosinophils. This was in contrast to the WT, which had a significant increase of infiltrating eosinophils in response to the challenge infection that was enhanced by eosinophil reconstitution (Table III). There was no detectable EPO in the diffusion chamber fluid of eosinophil reconstituted KO mice, which further confirms the total absence of eosinophils at the site of the challenge infection. In contrast, there was EPO in the diffusion chambers of immunized WT mice, which doubled in mice that received the eosinophil transfer (Table III). Neutrophils were seen in diffusion chambers recovered from immunized WT and KO mice and in immunized mice reconstituted with eosinophils. There were no statistically significant differences in the total number of neutrophils entering the diffusion chambers of these mice. Therefore, these data indicate that eosinophils are essential during

Table II. Survival of larval *S. stercoralis* in WT and KO mice after transfer of IgM from naive or immunized WT or KO mice (experiment A) and effect of elimination of granulocytes by mAb RB6-8C5 on passive transfer of immunity (experiment B)^a

Expt.	Donor	Recipient	Source of IgM	Treatment	% Viable Larvae
A	WT	WT	Naive	—	51 \pm 21
	WT	WT	Immune	—	16 \pm 13*
	KO	WT	Naive	—	52 \pm 2
	KO	WT	Immune	—	51 \pm 18
B	WT	WT	Naive	—	30 \pm 7
	WT	WT	Immune	—	3 \pm 2*
	WT	WT	Naive	RB6-8C5	41 \pm 10
	WT	WT	Immune	RB6-8C5	45 \pm 36
	WT	KO	Naive	—	45 \pm 15
	WT	KO	Immune	—	10 \pm 7*
	WT	KO	Naive	RB6-8C5	37 \pm 15
	WT	KO	Immune	RB6-8C5	23 \pm 13

^a Values are means \pm SD of the percentage of larvae recovered alive. *, Statistical difference between control and immune values.

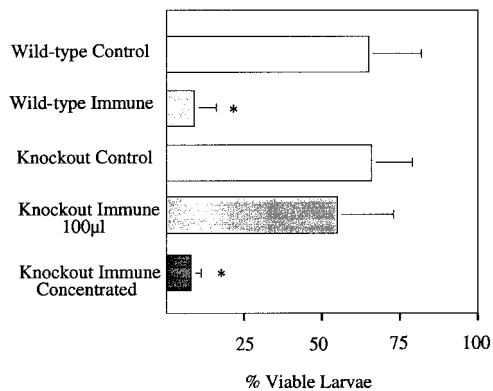


FIGURE 5. Effect of transfer of IgM from immunized IL-5 KO mice to naive WT C57BL/6J mice on challenge parasite survival. Two different concentrations of IgM was transferred: (100 μ l), the amount found in 100 μ l of serum from immunized IL-5 KO mice. (concentrated), the amount of total IgM from immunized IL-5 KO mice that had an OD for parasite-specific IgM comparable to that measured in 100 μ l of immunized WT C57BL/6J mice. Values listed are means \pm SDs of the percentage of larvae recovered alive. *, Statistical difference between control and immune values.

the induction of adaptive immunity and that they are nonessential as end stage effector cells for larval killing.

Eosinophil adoptive transfer restores protective IgM in IL-5 KO mice

KO mice reconstituted with eosinophils showed an increase in the titer of parasite-specific IgM following the adoptive transfer of eosinophils, however not to the levels of wild-type immunized mice (Table III). Experiments were designed to determine whether this increase in titer was sufficient for immune protection in these mice. The IgM fraction was pooled from the animals of each group, the concentration of IgM found in 100 μ l of sera determined by ELISA and that equivalent amount passively transferred to naive WT animals. The results from this experiment clearly showed that immunized KO mice that were reconstituted with eosinophils at the time of immunization, produced sufficient quantities of parasite specific IgM to passively transfer immunity (Fig. 6). Therefore, these data suggest that eosinophils reconstitute the protective immune response in KO mice by supporting the induction of a functional Ab response.

Discussion

The objective of these studies was to determine the role of IL-5 in the innate and adaptive immune response to larval *S. stercoralis*. Experiments studying the innate response were performed using

naive mice with different endogenous IL-5 levels. A clear distinction was seen in the survival of the larvae based on the level of IL-5 found in the host mouse. As compared with survival in WT mice, larvae in KO mice had significantly increased survival, and larvae in TG mice had significantly decreased survival. The differences in parasite survival between the strains was absent at 24 h but was seen at 72 h postinfection. The difference in parasite survival among the strains was also reflected in the magnitude of eosinophil infiltration and degranulation, as measured by EPO levels. As compared with levels in WT mice, the number of eosinophils and the quantity of EPO in KO mice was significantly decreased, and in TG mice they were significantly increased. A similar result was obtained using a different IL-5 transgenic mouse strain where it was reported that naive mice infected with infective stage larvae of *Nippostrongylus brasiliensis* showed eosinophil recruitment within 6 h postinfection and a significant decrease in worm burden within 72 h postinfection (36). A direct role for eosinophil killing of larval *S. stercoralis* has been demonstrated in vitro, where it was shown that the human eosinophil granule proteins major basic protein and eosinophil cationic protein were toxic to *S. stercoralis* larvae, whereas EPO and eosinophil-derived neurotoxin were not (18). Killing with major basic protein and eosinophil cationic protein only occurred after the larvae had undergone development under mammalian ambient conditions. In the present study, the time required for killing larvae in TG mice may be a reflection of the time required for sufficient numbers of eosinophils to migrate into the diffusion chamber or it may reflect the time required for the parasite to develop into a stage that is susceptible to the eosinophil granule proteins. It is not known whether EPO from mouse cells is toxic to the larvae; the presence of EPO in the diffusion chamber, is however, evidence that the eosinophils were recruited to the diffusion chamber and that granule products were released. Finally, the correlation between the presence of eosinophils and their degranulation products and larval killing suggests that the eosinophil plays a significant role in controlling the parasite during the innate immune response.

Adaptive protective immunity of mice to larval *S. stercoralis* was previously shown to depend upon the presence of CD4⁺ T cells (25). In this report, it was shown that there was a significant increase in the frequency of CD4⁺ T cells from mice with protective immunity secreting IL-5 following restimulation with *S. stercoralis* larval Ags. It is possible that the role for CD4⁺ T cells in adaptive immunity is to serve as a source of IL-5, because T cells are the major producers of this cytokine (2, 8). The finding that the frequency of IL-4 secreting cells did not significantly increase was surprising due to our previous observation that protective immunity was abolished by anti-IL-4 mAb treatment (25). It is possible that only small amounts of IL-4 are required or,

Table III. Effect of eosinophil reconstitution on the development of protective immunity in WT and KO mice^a

	% Viable Larvae	% Eosinophils	EPO (mU/ml)	IgM
WT control	23 \pm 12	0	0	0.160
WT immune	3 \pm 3*	7 \pm 7	20 \pm 10	0.867
WT immune + eosinophils	1 \pm 1*	13 \pm 7	42 \pm 40	0.763
KO control	30 \pm 11	0.4 \pm 1	0	0.150
KO immune	13 \pm 15	0.2 \pm 4	7 \pm 9	0.280
KO immune + eosinophils	3 \pm 3*	0	6 \pm 8	0.420

^a Results listed are percentage of viable larvae recovered, percentage of cells that migrated into the diffusion chambers that were eosinophils, quantity of EPO measured in the fluid recovered from the diffusion chambers, and optical density measurements of parasite-specific IgM measured in the pooled serum for each group. Values are mean \pm SD. *, Statistically significant difference between control and immune results.

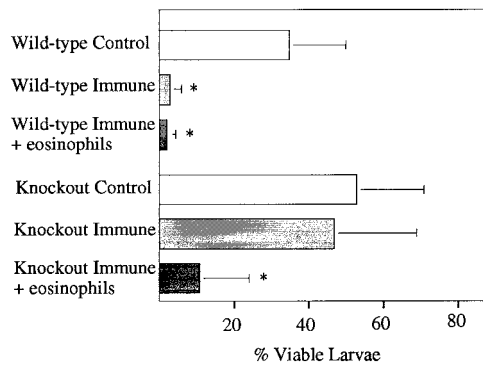


FIGURE 6. Effect of transfer of IgM purified from WT C57BL/6J and IL-5 KO mice, which received eosinophils at the time of immunization, on the survival of challenge larvae in recipient naive WT mice. Values listed are means \pm SDs of the percentage of larvae recovered alive. *, Statistical difference between control and immune values.

alternatively, that the IL-4 is required to initiate the response, but is not required after the response has been effectuated.

Immunization of KO mice did not lead to the development of protective immunity regardless of whether the challenge infection was injected into the tissues or was implanted within a diffusion chamber. These findings confirm previous reports in which elimination of IL-5 by mAb treatment (TRFK-5) was shown to abrogate *S. stercoralis* larval killing in immunized BALB/cByJ mice (18). In contrast, many other groups have found no role for IL-5 in protection from other parasites, however measurable levels of eosinophils could still be found in the peripheral blood, cutaneous tissues, cerebrospinal fluid, liver, and lungs of TRFK-5-treated mice (12, 35, 37–43). Thus, the eosinophils still present in mouse tissues after mAb treatments might still have been able to mediate systemic L3 destruction. It was only in the KO mouse, where tissue eosinophil levels are further reduced or are absent, that a dependency on IL-5 for the immune elimination of systemic infections could be demonstrated. These data show that deletion of the IL-5 gene is linked with the inability of mice to clear *S. stercoralis* L3 infections, regardless of the challenge site.

The next objective of this study was to determine what the specific function of IL-5 was in the adaptive immune response. IL-5 has been shown to be required for Ab secretion by B-1 cells (5, 6), so, therefore, the effect of IL-5 depletion on Ab responses was assessed. Immunization of KO mice led to the appearance of parasite specific IgG2a and IgG2b which was not observed in immunized WT mice and decreased levels of IgM as compared with the levels measured in immunized WT mice. The observation that IgG2a and IgG2b responses become elevated suggested that, in the absence of the Th2 cytokine IL-5, the Ab response shifted to a Th1 type of response characterized by IgG2a and IgG2b. A similar observation was noted where immunization of IL-5 α knockout mice with the nematode *Angiostrongylus cantonensis* also led to an increase in the total levels of serum IgG2a following infection as compared with control animals (17).

It was hypothesized that the decrease in parasite-specific IgM in immunized KO mice was one of the reasons why protective immunity was not evident. This hypothesis was based on the report that immunity to larval *S. stercoralis* is dependent on parasite-specific IgM (26). This hypothesis was confirmed by the experiment which showed that IgM from immunized KO mice could not transfer immunity. The deficiency in the IgM was quantitative and not qualitative; increasing the amount of IgM from immunized KO mice to levels seen in immunized WT mice allowed passive transfer of immunity.

The next question addressed was whether IgM was the only deficit in the immunized KO mice. IgM from immunized WT mice was found to passively transfer immunity to naive KO mice. In previous studies, it was shown that IgM required granulocytes to kill *S. stercoralis* larvae in vivo (26). This finding was confirmed in KO mice by treating the mice with RB6–8C5, a mAb that recognizes the common granulocyte marker (Gr-1) (44). Elimination of granulocytes from KO mice prevented the passive transfer of immunity with WT IgM. It can be concluded from these studies that the defect in immunized KO mice is in their ability to produce a sufficient quantity of parasite-specific IgM. Furthermore, the results suggest that the effector cells collaborating with IgM in KO mice are granulocytes. These effector cells may be neutrophils, residual eosinophils found in KO mice, or both cell types may be functional.

IL-5 has been shown to be essential for eosinophil maturation and survival (1, 2). It was therefore hypothesized that the defect in IL-5 KO mice might also include a role for eosinophils. Experiments in this study suggested that eosinophils were not likely to be required as effector cells for the adaptive immune response. The next series of experiments sought to determine whether they were required as inducing cells for the beginning of the immunization process. It was clearly demonstrated that adding eosinophils to KO mice at the time of immunization supported the development of a protective immune response. This protective immune response was characterized by an elevated parasite-specific IgM response that functioned in passive transfer experiments. Finally, experiments were performed to confirm that the transferred eosinophils were responsible for the development of the protective immune response and not possible IL-5 transgenic CD3⁺ cells contaminating the transfer preparation. Eosinophils were purified from a different line of IL-5 transgenic mice in which IL-5 overexpression was restricted by a lung-specific promoter (31). Transfer of these eosinophils at the time of immunization into KO mice led to the identical finding, that recipient KO mice developed protective immunity.

Eosinophils were completely absent from the microenvironment of the challenge larvae in immunized reconstituted KO mice. This conclusion was based on analysis of the contents of the diffusion chambers where neither eosinophils nor EPO was found. The absence of eosinophils from the challenge site may be explained by the lack of IL-5 in these animals, which is an essential factor for eosinophil survival (5, 45). The other closely related hemopoietins IL-3 and GM-CSF can promote eosinophil function and survival (8, 46); however, eosinophils undergo apoptosis within 72 h in the absence of IL-5 (45). Therefore, the eosinophils transferred at the time of immunization only survived and functioned for a limited period of time. This period of time coincided with the induction phase of the immune response and not with the effector phase.

There are several hypotheses that could explain the role of eosinophils during the initiation of the immune response against larval *S. stercoralis*. One possibility is that the transferred eosinophils serve as a time limited source of IL-5. Eosinophils can produce and store IL-5 in their electron dense granules (47). After transfer to KO mice, they may release enough IL-5 to promote B cell activation for IgM production. Alternatively, the transferred eosinophils may have been required to initiate the immune response. They may function as cells responsible for the disruption or the solubilization of the larval parasites into a form that can be processed by the APCs. Evidence from the studies looking at the role of the eosinophil in the innate response suggests that the eosinophil could serve this function. This is based on the observation that, after 3 days in vivo in the presence of degranulating eosinophils, the larvae were killed and appeared to be solubilized (D. Abraham,

unpublished observations). In addition, the eosinophil could function as an APC. Eosinophils have been shown to process inhaled Ags and function in vivo as APCs to stimulate CD4⁺ T cell responses (48). Finally, it is possible that the eosinophil might be required for all three functions, a source of cytokines, a source of molecules that will disrupt the larvae, and as the predominant APC.

The experiments described in this study show that IL-5 is required during the innate and adaptive immune response against *S. stercoralis* L3. IL-5 is required in the protective immune response for the production of eosinophils, and that eosinophils were involved in larval killing during innate immunity and in the induction of protective Abs in the adaptive immune response.

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