**Schistosoma mansoni** Egg-induced Early IL-4 Production Is Dependent upon IL-5 and Eosinophils

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Summary

The initial immune response to *Schistosoma mansoni* eggs presumably results in IL-4 production, as schistosome eggs are strong Th2-inducing antigens and the differentiation of antigen-specific Th2 cells is largely dependent on the presence of IL-4 during priming of naive Th cells. Consistent with this concept, intraperitoneal injection of mice with schistosome eggs results in an upregulation of IL-4 production by peritoneal exudate cells (PECs) within 12 h. Egg-induced IL-4 is rapidly bound by its receptor, suggesting that this cytokine is utilized by a cell type present at the site of antigen deposition or is complexed to soluble receptor. The peak of early IL-4 production is accompanied by a local eosinophilia and the apparent disappearance of mast cells. Studies utilizing either IL-4, IL-5, or mast cell-deficient mice indicate that the eosinophilia is dependent on mast cells and IL-5 and independent of IL-4. Strikingly, egg-induced IL-4 production is absent in animals lacking the early peritoneal eosinophilia. Immunocytochemical analysis of PEC following egg injection indicates that the eosinophils themselves make IL-4. These data strongly suggest that egg-induced IL-5 plays an essential role in recruiting eosinophils to the site of antigen deposition and that it is these eosinophils that then directly produce early IL-4.

Blood and tissue eosinophilia are prominent features of immune responses generated during helminth infection, asthma, and allergies. In murine model systems of parasitic infections and airway hyperreactivity (asthma), the in vivo abrogation of IL-5 results in an ablation of the characteristic eosinophil response, demonstrating the central role played by this cytokine in eosinophil mobilization and maturation (1-5). IL-5 is secreted primarily by Th2 cells but other cell types (mast cells, other FceR⁺ cells, and eosinophils themselves) prominent during type 2 immune responses may also contribute to its production (6, 7). Whereas IL-5 is central to eosinophilia, the function of the eosinophils themselves during the course of parasitic infections or as mediators of asthmatic airway hyperreactivity remains controversial (1-5, 8, 9). The majority of studies that have investigated the role of eosinophils have focused on the effluent arm of the immune response; eosinophil mobilization from the bone marrow and migration into tissues has largely been thought of as an antigen-specific Th2 cell-dependent event (10-12). However, recent studies of eosinophils indicate that these cells may play more of an afflent role than previously thought. Eosinophils can express MHC class II and present Ag to Th cells (13, 14); moreover, once activated they also secrete the T cell growth and differentiation factor IL-4 (15, 16).

IL-4, the prototypic type 2 cytokine, plays a crucial role in promoting the differentiation of naive precursor Th (pTh) cells into Th2 cells (17-20). To assert this effect, IL-4 must be available to pTh cells shortly following primary exposure to antigen. Because Th2 cells specific for a given antigen presumably do not exist during priming, other cell types have been proposed to contribute the essential early IL-4 (21-23). Most recently, NK*, CD4+ T cells were shown to play such a role during the induction of a Th2 response by anti-IgD (24). However, in our studies using *Schistosoma mansoni* eggs as a potent Th2 response inducer, early IL-4 production is T cell independent (25). While performing studies designed to identify the cellular source of the egg-induced early IL-4, we have found that eggs profoundly stimulate the IL-4 independent production of early IL-5 with an accompanying IL-5 dependent local eosinophilia. Moreover, the egg-induced IL-5 dependent eosinophilia is essential to allow production of the early IL-4. The IL-4 secreted subsequent to the peritoneal eosinophilia is rapidly bound by IL-4R, indicating that this cytokine is...
being utilized, perhaps by egg-antigen–specific pTh cells. These studies suggest a novel role for eosinophils in the inductive phase of type 2 immune response development and may help to explain previous studies, which have proposed conflicting roles for eosinophils in downstream type 2 responses (4, 5).

Materials and Methods

Parasites, Antigens, and Experimental Inoculations. *S. mansoni* (NMRI strain) eggs were recovered from the livers of infected mice as previously described (26, 27) and resuspended at 10^6/ml in sterile, low endotoxin PBS (Sigma, St Louis, MO). The eggs were determined to be free of endotoxin using a timed-gel endotoxin kit (Sigma) and stored at -70°C until use. Wild type (WT) C57BL/6 mice were from Taconic Farms (Germantown, NY). athymic (nu/nu) C57BL/6 and WBB6F1-W/W^v plus their heterozygote littermates were from the Jackson Laboratory (Bar Harbor, ME). Homozygous C57BL/6 IL-4/-/- mice were derived from 129Sv × C57BL/6 IL-4/-/- mice (28) by eight backcrosses to C57BL/6 WT mice and interbreeding of heterozygote offspring of the final backcross. C57BL/6 IL-5/-/- mice were the offspring of breeding colonies established from previously described animals (29). Female 6–10-week-old mice were injected intraperitoneally with 10,000 *S. mansoni* eggs in 100 μl PBS or with an equivalent volume of PBS alone.

Cell Preparation and Identification. Mice were euthanized with CO2. PECs were collected by lavage with 10 ml of ice-cold HBSS containing 1% FCS and 10 mM Hepes (all from Sigma). Viable PECs were enumerated using Trypan blue exclusion and resuspended at 5 × 10^6/ml in complete tissue culture medium (CTCM) containing DMEM, 10% FCS, 5 × 10^-5 M 2-ME (all from Sigma), plus 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 30 mM Hepes (all from Gibco BRL, Gaithersburg, MD). Cytospin preparations of 10^6 PEC per slide were stained with Wright's stain and cell populations enumerated by light microscopy.

Ex Vivo Cytokine Production. PECs collected from mice injected 12 h previously were aliquoted into 96-well flat-bottomed plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ; 1.25 × 10^6 cells in 250 μl CTCM per well) and cultured at 37°C/5% CO2 in the presence or absence of a blocking mAb directed against the IL-4R (M1 at 2.5 μg/ml; a gift from Immunex, Seattle, WA) and with or without ionomycin (4 μg/ml; Calbiochem Novabiochem, San Diego, CA). Supernatants from 24 h cultures were collected and cytokine levels quantitated using highly specific two-site ELISAs described in detail elsewhere (25, 30). IL-4 levels are expressed in units as defined by Genzyme Diagnostics (Cambridge, MA). Statistical analyses were performed using Student's t test; P values <0.05 are considered significant.

In Vivo Neutralization of IL-5. C57BL/6 mice were intraperitoneally injected 24 h before and again immediately before *S. mansoni* egg injection with 1 mg of neutralizing anti-IL-5 mAb (TRFK5; American Type Culture Collection, Rockville, MD; mAb purified on protein G) or with an equivalent amount of normal rat γ-globulin (Accurate Chemical and Scientific Corp., Westbury, NY). 12 h after intraperitoneal injection, PECs were collected, stimulated in vitro, and cytokine levels in cell culture supernatants analyzed as described above.

Immunocytochemistry. Cytospin preparations of PECs collected from WT or IL-4/-/- mice 12 h following intraperitoneal injection were fixed, permeabilized, and stained with either a biotinylated mAb anti-IL-4 (protein G-purified 11B11 at 4 μg/ml) or a biotinylated isotype control mAb (rat IgG1 at 4 μg/ml; Pharmingen, San Diego, CA), following a previously described protocol (31). Nonspecific Ab binding was blocked by incubation with mAb anti-CD16/CD32 (0.5 μg/ml; Pharmingen) before incubation with biotinylated mAbs. Detection was with alkaline phosphatase-conjugated streptavidin (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) followed by BCIP/NBT (5-bromo-4-chloro-3-indolyl-1-phosphate and nitro blue tetrazolium; Promega, Madison, WI) according to the suggested protocol of the company.

Determination of Cytokine Levels in Serum and Peritoneal Lavage Media. 12 h after intraperitoneal injection, mice were bled from the tail vein and serum stored at -20°C for subsequent quantification of cytokine levels by ELISA. Mice were then euthanized and PECs collected as described above. Cell-free lavage media (9–10 ml/mouse) were concentrated to final volumes of 0.75–1 ml with Centriprep-10 concentrators (Amicon, Beverly, MA) and stored at -20°C for subsequent quantification of cytokine levels.

Results and Discussion

IL-4 Secreted by Peritoneal Exudate Cells Is Rapidly Bound by Its Receptor. Previously, we described the induction of thymic-independent IL-4 gene transcription in PECs within 2–12 h of intraperitoneal injection of *S. mansoni* eggs (25).

![Figure 1](https://example.com/image1.png)

Figure 1. IL-4 accumulates in PEC culture supernatants only when the IL-4R is blocked. 12 h after intraperitoneal injection of PBS or egg, PECs from three mice per group were combined and cultured in single wells or in duplicate, if cell numbers allowed, for an additional 24 h. IL-4 levels in supernatants from cells cultured with or without anti-IL-4R alone (A) and in the presence of ionomycin (B) were measured twice by ELISA. Results shown are the mean ± SE of data obtained from five separate experiments.
Because cytokine production is primarily regulated at the mRNA level (32, 33), we were surprised when we were unable to demonstrate IL-4 protein accumulation in PEC culture supernatants using an ELISA. It occurred to us that IL-4 secreted into culture supernatants may be rapidly utilized, thus precluding its measurement by conventional ELISA. To address this issue, PECs collected 12 h after intraperitoneal injection were cultured in the presence or absence of a mAb directed against the IL-4R (cxlL-4R; 34). Only when cxIL-4R was included in the culture medium were we able to measure constitutive IL-4 secretion by PECs collected from egg-injected mice (2.29 ± 0.62 U/ml; Fig. 1 A). PECs from PBS-injected mice failed to yield measurable levels of IL-4 spontaneously, regardless of whether cxL-4R was present (Fig. 1 A).

Although we have consistently observed constitutive production of IL-4 by PECs 12 h after intraperitoneal egg injection, the absolute quantitites of cytokine secreted into culture supernatants are always low. To increase our ability to measure IL-4, we stimulated PECs in vitro with the calcium ionophore, ionomycin (since IL-4 production by T cells [35] and FcεR+ cells [36, 37] can be promoted by increased cytoplasmic Ca2+). The amount of IL-4 secreted by PECs from egg-injected animals following in vitro ionomycin stimulation (in the presence of cxIL-4R) increased 150 to 250-fold over that produced spontaneously (434.13 ± 101.12 versus 2.29 ± 0.62 U/ml; compare Fig. 1, B with A). Under these same conditions, IL-4 can be detected in the culture supernatants of PECs from PBS-injected mice, albeit at significantly lower levels than from egg-injected mice (55.09 ± 7.22 versus 434.13 ± 101.12, P <0.005; Fig. 1 B). Moreover, ionomycin stimulation promotes sufficient IL-4 secretion to allow IL-4 to accumulate to detectable levels even in the absence of cxIL-4R (Fig. 1 B).

The increase in measurable levels of egg-stimulated IL-4 production by PECs in the presence of cxIL-4R strongly suggests that secreted IL-4 is rapidly bound by its receptor. Receptors specific for IL-4 can be found either in the extracellular milieu in a soluble form or on the surface of such IL-4 responsive cells as T cells, B cells and mast cells (38). If the IL-4 produced early following intraperitoneal injection of S. mansoni eggs is important for the induction of Th2 cell differentiation, then it might be expected that cell surface IL-4R on pTh cells would rapidly bind the IL-4 secreted into the peritoneal fluid during the initial exposure to egg antigens. In addition, soluble IL-4R may serve as an IL-4 carrier protein (39, 40), extending the half-life of this cytokine and allowing IL-4 produced by PECs at the site of antigen deposition to circulate easily to the draining thoracic lymph node, where both antigen presentation and Th2 cell differentiation may occur. We are currently investigating whether soluble IL-4R concentrations increase in the peritoneal fluid at times coincident with peak egg-induced IL-4 production.

Egg-Induced Early IL-4 Is Absent in Mast Cell-Deficient Mice. Intraperitoneal injection of C57BL/6 mice with S. mansoni eggs not only results in early IL-4 gene tran-

scription and protein production but also, over a similar time frame, in a decrease in the number of recoverable peritoneal mast cells and an increase in eosinophil numbers (25). Similar alterations in peritoneal eosinophil and mast cell numbers occur in egg-injected nude and CD4+ cell-depleted animals (25); PECs from T cell-depleted mice also make early IL-4 (for nude versus WT mice; 12 h PEC from egg-injected animals constitutively made 0.89 ± 0.13 versus 0.89 ± 0.11 U/ml IL-4). Together, these data strongly suggest that the egg-induced early changes in peritoneal cell composition and IL-4 production are T cell independent. Having excluded thymus-dependent T cells as the source of early IL-4, we asked which other cell types in the PEC population might be responsible for the production of this cytokine. Because murine mast cell lines can secrete IL-4 upon activation with calcium ionophores or other appropriate stimuli (36), and because the apparent decrease in recoverable peritoneal mast cells after egg injec-

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Figure 2. Egg-induced early IL-4 production and eosinophil infiltrate a blocked in mast cell-deficient mice. WBB6F1-W/Wv (W/Wv) or heterozygote intermutes (W/+), W/Wv or heterozygote intermutes (W/+), were injected intraperitoneally with S. mansoni eggs. 12 h later, PECs from a total of six mice per group were cultured and cultured in duplicate in the presence of nn-IL-4R alone (BKG) or plus ionomycin (IONO) for an additional 24 h. IL-4 levels in culture supernatants from PEC were measured twice by ELISA (A, note different scales on x axes). Differential counts were performed on Wright's stained cytoplasmic preparations of PECs before culturing and percentage of eosinophils were shown in (B). IL-4 levels are the mean ± SE from a total of six distinct fields of 100 cells from the same experiment described in (A).
Mice carrying a mutation in the gene encoding c-kit and thus congenitally deficient of mast cells (W/Wv) or their heterozygote mast cell intact littermates (W/+ ) were injected with eggs, and 12 h later the ability of their PECs to secrete IL-4 when recovered at 12 h after intraperitoneal egg injection and stimulated in vitro with ionomycin (74.97 ± 102.26 U/ml, P < 0.05; Fig. 2 A). IL-4 production by PECs from egg-injected W/Wv mice was also significantly less than that of W/+ mice following stimulation in vitro with ionomycin (74.97 ± 27.66 versus 346.98 ± 102.26 U/ml, P < 0.001; Fig. 2 B).

The most straightforward explanation for the defect in early IL-4 production observed in the W/Wv mice is that mast cells play a critical role in this process. However, several lines of evidence suggest that this may not be the case. First, PECs (which are 4%-5% mast cells) transferred from naive WT mice to the peritoneal cavities of IL-4-/- mice are unable to make detectable levels of IL-4 when recovered at 12 h after intraperitoneal egg injection and stimulated in vitro with ionomycin (data not shown).

Secondly, mast cell deficiency alters the egg-stimulated changes in PEC composition and it may be that another PEC lacking from the egg-injected W/Wv mice is responsible for early IL-4 production. In this context, the most striking difference between PEC composition in egg-injected W/Wv mice and W/+ mice is the relative lack of eosinophils in the former (6% eosinophils versus 34% eosinophils; P < 0.001; Fig. 2 B).

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nophils. The experiments described above reveal that in mast cell–deficient mice there is a significant decrease in both egg-induced IL-4 production and peritoneal eosinophilia compared with that seen in mast cell–replete animals. Several recent studies have demonstrated the ability of human eosinophils to make, store, and secrete IL-4 (15, 16). It may be that mast cells, through the release of eosinophil chemoattractants including IL-5 (36, 37), are necessary for eosinophil recruitment and that the eosinophils themselves that directly make early IL-4. To address this issue experimentally, two types of eosinophil–deficient mice were utilized: IL-5-/- mice and C57BL/6 mice injected with a neutralizing anti-IL-5 mAb (TRFK5).

IL-5-/- mice maintain a near normal basal number of blood and bone marrow eosinophils while being unable to mount a blood or tissue eosinophilia (29). 12 h after intraperitoneal egg injection in these mice, IL-4 production by PECs is significantly less than that seen in egg-injected WT animals, regardless of the in vitro stimulus (P < 0.005; Fig. 3 A). As with the W/Wv mice, we were unable to detect spontaneous secretion of IL-4 by PECs from IL-5-/- mice, while PECs from WT animals did secrete this cytokine without further in vitro stimulation (1.44 ± 0.32 U/ml; Fig. 3 A). As expected, IL-5-/- mice fail to mount a peritoneal eosinophilia in response to egg injection (Fig. 3 C).
Because a lack of IL-5 during development may have as yet undetermined effects on the IL-5-/- animals which might then influence their ability to respond to S. mansoni egg antigens, we also neutralized in vivo IL-5 by intraperitoneally injecting WT C57BL/6 mice with the mAb TRFK5 immediately prior to schistosome egg injection. This resulted in a significant decrease in the peritoneal eosinophil infiltrate compared with that seen in controls injected with normal rat γ-globulin and eggs (P < 0.001; Fig. 3 C). Concomitantly, PECs collected from egg-injected anti-IL-5-treated animals also exhibited a decreased ability to secrete IL-4 when compared with control PECs, again regardless as to the in vitro stimulus (Fig. 3 B). Moreover, we were unable to measure spontaneous egg-induced IL-4 production by PECs from TRFK5-treated animals, while constitutive IL-4 produced by control PECs was easily measurable (2.23 ± 0.99 U/ml; Fig. 3 B).

Taken together, the above data suggest that the egg-induced early IL-4 production is dependent on IL-5. A plausible explanation for this is that IL-5 made at the site of egg deposition is acting on bloodstream eosinophils to promote their ability to migrate into the peritoneal cavity (41, 42), and that it is these eosinophils which make the early IL-4. IL-5 could additionally be acting as an eosinophil chemoattractant, but data supporting such a role for this cytokine are conflicting (41, 43, 44), raising the possibility that another molecule, for example eotaxin (45, 46), C5a (47), or IL-4 (48, 49), is fulfilling this function.

Support for the hypothesis that egg injection stimulates IL-4 production specifically by eosinophils is provided by immunocytochemical analysis of permeabilized PECs, since mAb anti-IL-4 bound only to a subpopulation (12%) of cells with the nuclear morphology of eosinophils (Fig. 3 D, center and right). An isotype control Ab failed to bind to cells with this nuclear morphology (Fig. 3 D, left). Furthermore, mAb anti-IL-4 did not bind to PEC from egg-injected IL-4-/- mice (data not shown). That only a small percentage of PEC was positive for IL-4 (<0.05%) is consistent with previous ELISPOT results (25).

Egg-induced Peritoneal Eosinophilia Is Dependent on IL-5 and Independent of IL-4. If IL-5 is acting directly as an eosinophil chemoattractant to promote peritoneal eosinophilia, then the data presented above would predict that PECs from egg-injected mice should secrete more IL-5 than those from control PBS-injected animals. Surprisingly, we were unable to measure any differences in the ability of PEC from egg- versus PBS-injected C57BL/6 animals to make IL-5; both secreted IL-5 in response to ionomycin (data not shown). However, we were able to measure significantly elevated levels of IL-5 in both serum and lavage fluid from egg-injected compared with PBS-injected mice (lavage fluid values, 24.56 ± 11.11 versus 0 ± 0 pg/ml; P < 0.05; serum values, 297.23 ± 55.68 versus 492.14 ± 22.51 pg/ml; P < 0.001; lavage fluid values, 32.99 ± 20.91 versus 93.15 ± 9.69 pg/ml, P < 0.05).

IL-4 is also ascribed eosinophil attractant properties due to its ability to upregulate endothelial cell expression of VCAM-1, which in turn increases eosinophil binding to endothelium through cell surface expression of VLA-4 (48, 49). Thus, we were interested in the interplay of IL-5, IL-4, and eosinophils at early time points following intraperitoneal schistosome egg injection, as it was possible that...
Egg

Mast cell

IL-5

OTHER EOSINOPHILS

Figure 5. Model of S. mansoni egg-stimulated early IL-4 production. Within 12 h of intraperitoneal egg injection, mast cell activation and IL-5 production results in a peritoneal eosinophilia. A subset of eosinophils (Eo), which migrate to the site of antigen deposition, are activated to produce IL-4. The IL-5 and eosinophil dependent production of IL-4 is hypothesized to be important for the subsequent development of egg Ag-specific Th2 cells. Also depicted in the model is an IL-5- and eosinophil-independent mechanism of egg-induced IL-4 production.

the peritoneal eosinophilia induced by egg injection was affected by egg-induced early IL-4 production and not vice versa. Consequently, WT and IL-4-/-/- mice were intraperitoneally injected with S. mansoni eggs or PBS, and 12 h later the extent of peritoneal eosinophilia was determined from Wright's stained cytospin preparations of PECs. Compared with PBS-injected mice, egg-injected WT and IL-4-/-/- mice exhibited significantly increased numbers of eosinophils in their PEC populations (P <0.05 for both types of mice), although the absolute magnitude of the eosinophilia was significantly lower in the IL-4-/-/- mice than in the WT animals (P <0.01; Fig. 4A). Consistent with a role for IL-5 in eosinophil recruitment, levels of this cytokine not only increased in the lavage media and sera of WT animals following egg injection, but were also significantly elevated in both the peritoneal lavage media and sera of egg-injected versus PBS-injected IL-4-/-/- animals (lavage media, 11.14 ± 5.17 versus 0 ± 0 pg/ml, P <0.05; serum, 150.13 ± 53.56 versus 2.81 ± 1.64 pg/ml; P <0.05; Fig. 4, B and C). The apparent differences in IL-5 levels between WT and IL-4 mice were not significant (lavage media, P = 0.31; serum, P = 0.22). These results demonstrate that egg-induced IL-5 production and eosinophilia can occur in the absence of IL-4, but suggest that IL-4 may promote eosinophil infiltration through a mechanism unrelated to the upregulation of IL-5 per se. The data do not rule out the possibility that once made, IL-4 plays a role in a positive feedback loop to promote further early production of IL-5.

We describe here studies that indicate that S. mansoni eggs, potent Th2-inducing antigens, promote not only IL-4 secretion but also IL-5 production and tissue eosinophilia within 12 h of intraperitoneal injection into naive mice; previous studies from our laboratory indicate that this eosinophilia is T cell independent. The production of IL-4 by egg-stimulated PECs does not occur if IL-5 is unavailable or if peritoneal eosinophilia does not develop. Moreover, egg-induced IL-4 secreted by PECs is rapidly bound by its receptor, indicating that, at the time of antigen priming, a cell type within the PECs is utilizing IL-4 or is secreting soluble IL-4R. Work is currently underway to investigate the relative importance of the IL-5/eosinophil-dependent production of IL-4 to the developing egg antigen-specific Th2 response. Our hypothesis to account for the data presented here is that exposure to eggs leads to the FceR-independent activation of peritoneal mast cells, which subsequently secrete IL-5 (and possibly other eosinophil chemotactants) and thereby recruit eosinophils to the site of antigen deposition. By immunocytochemical methods, we have shown that a subpopulation of these eosinophils is capable of producing IL-4 in response to egg injection. This model proposes a novel role for eosinophils and/or IL-5 in the inductive phase of an immune response; previous attempts to show such a role for IL-5 have failed to implicate this cytokine in Th response development (2, 4, 50). Since, unlike the situation in mice immunized with anti-IgD (24), S. mansoni egg-specific Th2 cells can develop independently of NK+, CD4+ T cells (unpublished data), there is presumably more than one pathway through which Th2 responses can develop. The model depicted in Fig. 5 allows for the possibility of IL-5/eosinophil-dependent and -independent early IL-4 production. Both pathways may contribute equally to Th2 response development. Alternatively, the nature of the immunogen, the route of exposure, or the mouse strain utilized may allow for the predominance of one pathway over the other.

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References


