

Helminth-Modified Pulmonary Immune Response Protects Mice from Allergen-Induced Airway Hyperresponsiveness¹

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It has been shown that the presence of certain helminth infections in humans, including schistosomes, may reduce the propensity to develop allergies in infected populations. Using a mouse model of schistosome worm vs worm + egg infection, our objective was to dissect the mechanisms underlying the inverse relationship between helminth infections and allergies. We have demonstrated that conventional *Schistosoma mansoni* egg-laying male and female worm infection of mice exacerbates airway hyperresponsiveness. In contrast, mice infected with only schistosome male worms, precluding egg production, were protected from OVA-induced airway hyperresponsiveness. Worm-infected mice developed a novel modified type 2 cytokine response in the lungs, with elevated allergen-specific IL-4 and IL-13 but reduced IL-5, and increased IL-10. Although schistosome worm-only infection is a laboratory model, these data illustrate the complexity of schistosome modulation of host immunity by the worm vs egg stages of this helminth, with the potential of infections to aggravate or suppress allergic pulmonary inflammation. Thus, infection of mice with a human parasitic worm can result in reduced airway inflammation in response to a model allergen. *The Journal of Immunology*, 2006, 176: 138–147.

Asthma is an atopic inflammatory disorder of the airways that is characterized by increased airway hyperresponsiveness (AHR),³ eosinophil infiltration of the airways, and mucus hypersecretion that results in intermittent airway obstruction (1). The immune etiology of asthma is complex, but genetic and immunological analyses of atopic individuals have revealed that Th2-type cytokines are causally associated with allergies (2, 3) with a type 2 cytokine response being characterized by increased (Th2) cell development and production of IL-4, -5, -9, and -13 resulting in IgE production, mucus hyperplasia, and eosinophilia (4).

The prevalence of a range of atopic diseases is rising and with respect to allergic asthma there has been an almost 2-fold increase in incidence in the past two decades (5). The rate at which the incidence of allergic disease is rising implicates a recent change in environmental influences on this process, i.e., the hygiene hypothesis (5). This hypothesis proposes that a shift in the immune system toward type 1 immunity upon early exposure to infections such as bacterial (6, 7) and viral (8) infections protects against allergic diseases by reducing the expression of Th2 cytokines generally evoked by allergens. An alternative explanation holds that certain parasitic helminth infections may protect against allergic disorders because human populations with high rates of parasitic helminth infections, which induce an immunological shift toward

the “allergic” Th2 responses, have a reduced prevalence of allergic disorders (9). *Schistosoma spp.* are tropical helminth parasites, characteristically associated with being potent inducers of Th2 cytokine responses including eosinophilia and IgE responses (10), that have been postulated to ameliorate atopic disorders in humans (9).

Recent experimental studies have shown that mice or rats infected with rodent nematode parasites have reduced allergic responses (11–14). We have previously demonstrated that *Schistosoma mansoni* infection protects mice from anaphylaxis through a regulatory mechanism induced by the worm (15). In this study, we have evaluated whether *S. mansoni* infection of mice, the mouse being the preferred animal model for studies on the immunobiology of schistosomiasis (10), altered susceptibility of the animals to OVA-induced AHR, which is also widely used as a model of human pulmonary inflammation (16). We have identified that the worm stage of *S. mansoni* infection modulates mice so they are refractory to AHR. This is the first formal demonstration of a mechanism that human parasitic worms use to suppress allergen-induced airway inflammation.

Materials and Methods

Mice

Female BALB/c mice were purchased from Harlan at 6–8 wk of age. Outbred male or female Tyler's Original (TO) mice, also from Harlan, were obtained for egg and worm production. IL-13-deficient (IL-13^{-/-}) mice were provided by Dr. A. McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.) and were bred in-house (17). Mice were housed in individually ventilated and filtered cages under positive pressure (Techniplast). Food and water were supplied ad libitum. Sentinel mice were screened to ensure specific pathogen-free status. All animal experiments were performed in compliance with Irish Department of Health and Children regulations.

Parasitology

A Puerto Rican strain of *S. mansoni* was maintained by passage in male or female outbred TO strain mice and albino *Biomphalaria glabrata* snails served as intermediate hosts. Female BALB/c, 6–8 wk of age (Harlan), were infected percutaneously with 30 mixed male and female cercariae for a conventional infection where eggs are laid (worm + egg infections), or mice were infected with 30 male cercariae for a worm infection where no eggs are present. The sex of cercariae shed from individual snails was

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; DPBS, Dulbecco's PBS; Penh, enhanced pause; Mch, methacholine; GL, pulmonary resistance; Cdyn, pulmonary compliance; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; PAS, periodic acid-Schiff; FSC, forward side scatter; SSC, side scatter.

determined by PCR as described (18). To remove worms from worm-infected mice, animals were orally treated with the schistosomicidal drug Praziquantel (Sigma-Aldrich; 100 mg/kg orally for 5 consecutive days).

Antibodies

Cell surface phenotyping was analyzed using Tricolor-conjugated anti-CD19 (6D5; Caltag Laboratories), anti-CD4 (CT-CD4; Caltag Laboratories), anti-CD8 (5H10; Caltag Laboratories), anti-F4/80 (F4/80; Caltag Laboratories), and PE-conjugated anti-CD25 (PC61 5.3; BD Pharmingen), anti-Syndecan-1 (CD138; 281-2 BD Pharmingen), anti-CD5 (Ly-1; BD Pharmingen), anti-IgM (μ -chain specific; The Jackson Laboratory), anti-CD11b (mac-1, M1/70; BD Pharmingen), anti-CCR3 (83101; R&D Systems), and FITC-conjugated anti-GL7 (GL7; BD Pharmingen). Intracellular cytokine staining was with PE-conjugated anti-IL-4 (BVD6-24G2) and FITC-conjugated anti-IL-10 (JES5-2A5) was obtained from Caltag Medisystems.

Hybridoma culturing and Ab production

Anti-IL-10R (1B1.3a), anti-CD25 (PC61 5.3) were purchased from American Type Culture Collection. The anti-CD4 (YTS 191) hybridoma was kindly provided by Prof. A. Cooke (University of Cambridge, Cambridge, U.K.) and Prof. H. Waldman (University of Oxford, Oxford, U.K.). The above hybridoma cell lines were cultured in RPMI 1640 and supernatants were precipitated in 50% ammonium sulfate followed by dialysis against Dulbecco's PBS (DPBS; pH 7.2) (Sigma-Aldrich). Ab was purified on Protein G (Sigma-Aldrich) separation columns and protein was quantified before use. All Abs were tested for endotoxin contamination and were confirmed to have <0.5 endotoxin units/mg (Chromogenic LAL). Macrophages were depleted by the treatment of mice with liposomes containing dichloromethylene bisphosphonate (clodronate liposomes), prepared as described (19). Clodronate was a gift of Roche Diagnostics.

Ag sensitization and challenge

Mice were sensitized with OVA (fraction V; Sigma-Aldrich) and airways challenged with OVA to induce pulmonary allergic inflammation and AHR (see Fig. 1). On days 1 and 14, mice received an i.p. injection of 100 μ l of a sensitization solution containing 20 μ g of OVA and 2 mg of Imject Alum (Pierce). Mice received a 20-min aerosol challenge with 1% OVA in DPBS from days 28–30. Control mice received an aerosol of PBS.

OVA has high levels of endotoxin contamination (~ 1.5 – 3 endotoxin units/mg) which previously has been shown to alter the development of AHR (20). It is impossible to completely remove endotoxin from OVA preparations (20), while the addition of polymyxin B, to inactivate LPS, is also inappropriate due to direct effects of the antibiotic on cell activation (21). Therefore, every effort was made to maintain sterile procedures to minimize the endotoxin contamination in all OVA preparations. OVA was prepared using endotoxin-free reagents and all glassware and equipment were endotoxin decontaminated (0.5 mM NaOH). Endotoxin was partially removed (Detoxi-gel endotoxin removing gel columns; Pierce) and OVA preparations were extensively dialyzed against sterile DPBS (Sigma-Aldrich) using dialysis cassettes (Pierce). After these measures, the OVA used in this study had <0.5 (range 0.186–0.447) endotoxin units/mg (Chromogenic LAL).

Pulmonary assessment of enhanced pause (Penh)

Lung reactivity of OVA-sensitized mice was assessed using a protocol by Hamelmann et al. (22). On day 32, airway responsiveness of mice was assessed by barometric whole body plethysmography in response to a methacholine (Mch) challenge. In brief, spontaneously breathing, nonanesthetized mice were placed in the plethysmograph (EMKA Technologies) and PBS aerosol was administered to establish baseline readings over 3 min. Mch (acetyl β -methylcholine chloride; Sigma-Aldrich) was then administered by nebulization in increasing, serial 2-fold concentrations from 3.125–50 mg/ml for 3 min each dose to induce bronchoconstriction. In this model, the extent of airway reactivity of individual mice was quantified as Penh. Airway responsiveness of mice was expressed as the fold increase for each concentration of Mch compared with Penh values after PBS challenge. Twenty-four hours later, mice were sacrificed by terminal anesthesia and samples were taken for further immunological analysis.

Airway conductance and dynamic compliance studies

Pulmonary conductance (GL) and compliance (C_{dyn}) in response to Mch were measured as described previously (23). In brief, mice were assessed on day 31 after OVA sensitization and challenge using the same protocol as outlined above. Mice were anesthetized with 105 mg/kg pentobarbitone, tracheostomized and then mechanically ventilated (EMKA Technologies)

for airway assessment. A two-way connector was attached to the tracheal cannulation tube to allow inspiration and expiration via the ventilator. Breaths were stabilized at a rate of 60 breaths/min and the tidal volume was fixed at 250 μ l. Increasing doses of Mch (3.125–250 μ g/kg) were administered i.p. and readings were taken continuously for 4 min after injection. Maximum pulmonary resistance (GL is the inverse of resistance) and compliance (C_{dyn}) values were taken after each dose for statistical analysis to express changes in these functional parameters as compared against saline challenge.

Bronchoalveolar lavage (BAL)

BAL fluids were collected by cannulating the trachea and lavaging lungs with 1 ml of cold PBS. Supernatant was removed for measurement of cytokines by ELISA. BAL cells were pelleted, washed, and counted for immunofluorescence staining for surface marker expression and cytopins. The numbers of eosinophils, neutrophils, lymphocytes, and macrophages was determined by performing a differential count on at least 400 cells/slide of Giemsa-stained cytopins.

Lung processing and histology

For analysis of cells by FACS, lungs were removed from the mice and digested in 1 mg/ml collagenase D (Roche Diagnostics) in RPMI 1640 with FCS for 30 min at 37°C with shaking. Lung digests were then filtered through 100- μ m Falcon cell strainers (BD Biosciences) followed by three washes and further filtering through 40- μ m Falcon cell strainers. In some experiments, the left lobes of the lungs, stored snap-frozen, were removed for assessment of tissue cytokines and chemokines (IL-4, IL-5, IL-13, IL-10, IFN- γ , and eotaxin). Each lung lobe was homogenized using an Ultra-Turrax homogenizer (IRK-WERKE) in 800 μ l of homogenization buffer (2% FCS, 0.5% cetyltrimethylammonium bromide; Sigma-Aldrich). The homogenate was microcentrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was aliquoted and the tissue extract was stored at -20° C for protein estimation, cytokine analysis, and eosinophil peroxidase (EPO) assay. The protein content of tissue extracts was determined by the BCA protein estimation kit. EPO levels were determined based on a method previously described (24). The remaining lung lobes were fixed in Formalin (10% formaldehyde in 0.9% saline solution) for histological analysis. Lung sections were stained with H&E, Giemsa for eosinophils, periodic acid-Schiff (PAS) for goblet cell counts and Martius Scarlet Blue for collagen. Pulmonary collagen was quantified by differential staining, and is expressed as micrograms of collagen per milligram of lung protein, as described (25). Goblet cells were counted on PAS-stained lung sections using an arbitrary scoring system (26, 27). PAS-stained goblet cells in airway epithelium were measured double-blind using a numerical scoring system (0: $<5\%$ goblet cells; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: $>75\%$). The sum of airway scores from each lung was divided by the number of airways examined, 20–50 airways/mouse, and expressed as mucus cell score in arbitrary units.

Cell preparation

Spleens and the lung draining mediastinal lymph nodes were removed and cells were isolated for cell culture and reactivation for cytokine measurements. Single cell suspensions were prepared from spleens and mediastinal lymph nodes and depleted of erythrocytes by lysis with 0.87% ammonium chloride solution. For in vitro experiments, cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated FCS (Labtech), 100 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen Life Technologies). Mediastinal lymph node or spleen cells were plated at 5×10^6 cells/ml. Cells were unstimulated (media) or stimulated with plate-bound anti-CD3 (clone 145-2C11) plus anti-CD28 (4 μ g/ml), OVA (5–200 μ g/ml), in a 24-well plate (Greiner) at 37°C for 72 h. Plates were precoated with anti-CD3 mAb at 10 μ g/ml for 2 h at 37°C and then washed in sterile DPBS before addition of cells. Supernatants were harvested after 72 h and cytokine levels (IL-4, IL-5, IL-13, IFN- γ , IL-10, and TGF- β) were measured by ELISA. For cell proliferation analysis, cells were exposed to a range of concentrations of OVA, in triplicate wells on 96-well plates. Cultures were pulsed with 1 μ Ci/well [3 H]thymidine (Amersham) for the last 14 h of culture. Cells were harvested with a Tomtec cell harvester and [3 H]thymidine incorporation was measured by a Wallac beta counter.

Flow cytometry

Surface marker expression and intracellular phenotyping of cells was assessed by flow cytometry as described (15, 28). Cells were counted and resuspended in ice-cold FACS buffer (2% FCS, 0.05% sodium azide in PBS) at 2×10^6 cells/ml on a 96-well plate. Cells were stained with surface

Abs for 30 min on ice at the recommended concentration and then washed three times in FACS buffer. For intracellular cytokine staining, unstimulated cells were incubated with Brefeldin A (10 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) for 4 h. Following surface staining, cells were fixed and permeabilized using the Fix and Perm Cell Permeabilization kit (Caltag Laboratories) with the anti-cytokine Ab added upon permeabilization. Data were collected on a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software. In all experiments, appropriate isotype controls were used to set gates and were plotted on logarithmic scales.

Various cell populations in the lung digests were identified by flow cytometry, as described (29). Lung cells were first gated on CD19, CD4, and CD8 vs forward side scatter (FSC). Lymphocytes were identified as FSC^{low} , side scatter (SSC^{low}) CD19^+ , CD4^+ , CD8^+ . Eosinophils distinguished as SSC^{high} , CD19^- , CD4^- , CD8^- nonautofluorescent granulocytes that stained positive for CCR3. Alveolar macrophages were characterized as CD19^- , CD4^- , CD8^- mononuclear cells that were highly autofluorescent and F4/80^+ .

Ab and cytokine ELISA

OVA-specific serum Abs were detected by direct ELISA (25). Total serum IgE was measured using Pharmingen Abs (BD Pharmingen). Sandwich ELISAs were performed to quantify levels of specific cytokines in the supernatants from lung tissue homogenates, BAL fluid, and in vitro cell stimulation cultures. Reagents for quantification of IL-4, IL-5, IL-13, from BD Pharmingen and IL-10 and IFN- γ were purchased as a DuoSet ELISA development system from R&D Systems. Total TGF- β (acidified samples) was measured by ELISA according to the manufacturer's instructions (Promega). Eotaxin detection reagents were also purchased from R&D Systems. Cytokines in lung homogenates are expressed as nanograms of cytokine per milligram of lung protein.

Statistics

GraphPad Prism and GraphPad Instat software was used to analyze the data. Differences were considered significant when $p < 0.05$.

Results

S. mansoni worm + egg infection of mice exacerbates AHR

To experimentally investigate whether schistosome infection modulates immune responses following exposure to an allergen schistosome, infected mice were immunized with alum-adsorbed OVA, a model-type cytokine 2-inducing allergen, and OVA-specific immunity was analyzed. Mice were exposed to a conventional *S. mansoni* male and female worm infection, where eggs are laid, and thus called here worm + egg infections. Worm + egg-infected mice were sensitized with OVA systemically and in the lungs during the acute (between 7 and 11 wk of infection) and chronic (between 12 and 16 wk of infection) stages of infection (Fig. 1). Spleen cells from worm + egg-infected mice immunized with

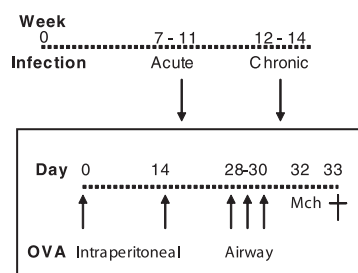


FIGURE 1. Protocol for OVA sensitization and challenge in mice for induction of AHR. Infected mice were sensitized in the acute, starting week 7, or chronic, starting week 12, stages of infection. Mice were systemically sensitized with an i.p. injection of 20 μg of OVA (plus 2 mg of alum) on days 0 and 14. Mice were challenged via the airways from days 28–30 with an aerosol of OVA (1% OVA for 20 min). On day 32, lung function in response to methacholine (Mch) was tested using either restrained or unrestrained plethysmography, as described in *Materials and Methods*. Cell depletion regimens were performed on days 27 and 30. Mice were killed on day 33 (+).

OVA during acute or chronic infection both had elevated in vitro production of allergen-specific type 2 cytokines (IL-4, IL-5, and IL-13) and IL-10 compared with production of these cytokine by cells from uninfected OVA-immunized mice (Fig. 2A). The increased type 2 cytokine response in worm + egg-infected mice was associated with greater levels of OVA-specific IgE (Fig. 2B).

To analyze whether the increased allergen-induced immune response in worm + egg-infected mice altered pulmonary inflammation, we used whole body plethysmography on conscious and unrestrained mice to determine airway function, which was quantified as Penh (22). In this model, uninfected mice sensitized with OVA develop AHR, demonstrated by a dose-dependent elevation in Penh in response to Mch when compared with uninfected PBS-treated mice (Fig. 3A). Unexpectedly, both PBS- and OVA-sensitized worm + egg-infected mice developed severe respiratory distress following exposure to the lowest dose of Mch (3.125 mg/ml) aerosol (Fig. 3A). This distress was manifested by significant elevated Penh values at all doses of Mch when compared with uninfected OVA mice ($p < 0.001$), with all worm + egg-infected mice dying in the plethysmograph chamber at doses of 12.5–25 mg/ml Mch (Fig. 3A). There was no difference in the rate of mortalities between acute vs chronically worm + egg-infected mice, but in both groups, sensitization with OVA accelerated the death of mice, with acute and chronically infected OVA-sensitized animals having elevated AHR and dying after exposure to 12.5 mg/ml Mch (Fig. 3A). Therefore, following allergen challenge schistosome

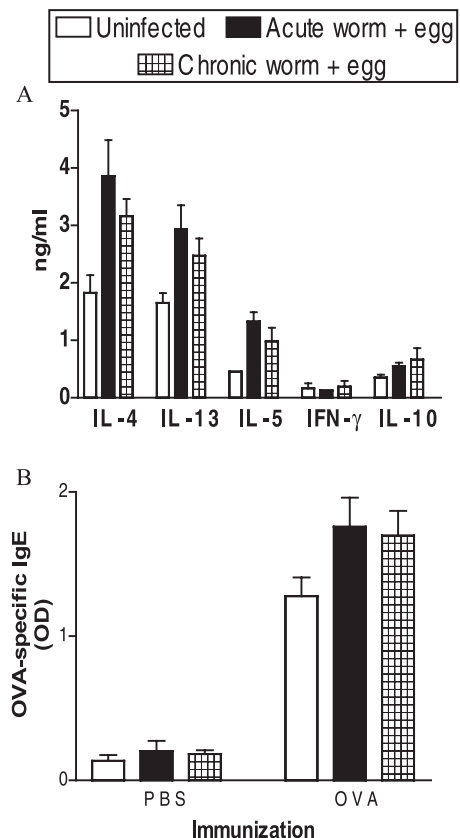


FIGURE 2. *S. mansoni* worm + egg-infected mice produce elevated type 2 responses to OVA sensitization. OVA was administered to uninfected mice and to animals during the acute or chronic stages of a worm + egg-infection, as described in Fig. 1. *A*, Detection of in vitro production of cytokines from spleen cells stimulated with OVA (200 $\mu\text{g}/\text{ml}$). Spleens from three mice were pooled. *B*, Levels of OVA-specific IgE. All data are mean \pm SD. Data are representative of two to three separate experiments.

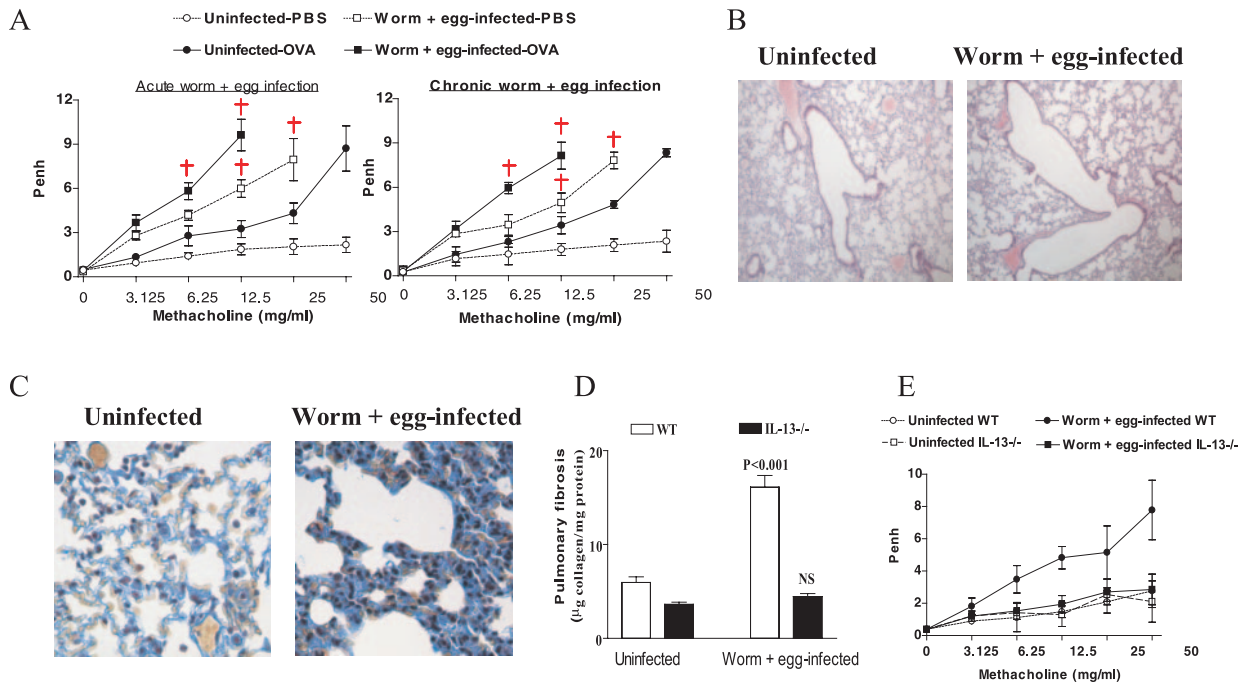


FIGURE 3. *S. mansoni* worm + egg-infected mice have increased susceptibility to AHR via increased IL-13-dependent pulmonary fibrosis. Uninfected and worm + egg-infected (acute and chronic) mice were unsensitized (PBS) or sensitized with OVA, as described in Fig. 1. *A*, Penh responses of acute and chronically worm + egg-infected mice. Data represent the mean change from baseline PBS values for each group. †, Denotes death of infected mice. Penh is presented as mean \pm SEM from three separate experiments ($n = 12\text{--}15$ mice). *B*, Absence of inflammation in lungs of a 10 wk worm + egg-infected mouse. Representative H&E-stained sections of lungs from uninfected and worm + egg-infected mice. *C*, Increased pulmonary fibrosis (blue stain) in worm + egg-infected mice relative to uninfected mice (Martius Scarlet Blue stain). *D*, Quantification of elevated pulmonary collagen levels in worm + egg-infected wild-type (WT) mice relative to uninfected WT mice, and reduced fibrosis in worm + egg-infected IL-13-deficient ($-/-$) mice. Data are mean \pm SEM from 6 to 8 mice per group. Statistical differences between levels of collagen in uninfected vs infected WT or IL-13^{-/-} mice were tested by Student's *t* test. *E*, Worm + egg-infected IL-13^{-/-} mice do not develop spontaneous AHR, whereas worm + egg-infected WT mice have dose-dependent elevations in Penh. Penh values are mean \pm SEM from 7 to 12 mice from two separate experiments. Data in *B*–*D* are from mice infected with a worm + egg infection for 8–10 wk.

worm + egg-infected mice develop elevated type 2 cytokine responses and, independent of allergen sensitization, are also predisposed to fatal AHR.

During a schistosome worm + egg infection of mice, eggs that are laid by the female worms are swept to various organs where they evoke granulomatous inflammation. When schistosome eggs are injected i.v. into naive mice, they are trapped in the lung and stimulate pulmonary inflammation. Therefore, an initial explanation for the increased susceptibility of worm + egg-infected mice to AHR was the presence of eggs in the lungs inducing pathology. However, we have not detected any eggs present in digests of lungs from acute or chronically worm + egg-infected mice (data not shown). The lungs by histology sections from uninfected and worm + egg-infected mice were largely comparable (Fig. 3*B*), with the notable exception of substantial subepithelial and parenchymal collagen deposition in acute and chronic worm + egg-infected mice (Fig. 3*C*; data not shown). Quantification of pulmonary collagen showed that mice with an acute worm + egg infection have a significant, $p < 0.001$ vs uninfected mice, 2- to 3-fold elevation in collagen (Fig. 3*D*). Previously, we have shown schistosome worm + egg-infected IL-13^{-/-} mice do not develop the elevated levels of hepatic collagen seen in comparably infected wild-type mice (30), which is consistent with the role of IL-13 in fibrosis (31). As schistosome worm + egg-infected IL-13^{-/-} mice also do not develop pulmonary fibrosis (Fig. 3*D*), we analyzed AHR in these mice during acute stages of infection. Worm + egg-infected IL-13^{-/-} mice did not develop AHR and had comparable pulmonary function as observed in uninfected mice (Fig.

3*E*). Therefore, during acute worm + egg infection there is elevated IL-13-dependent pulmonary fibrosis that contributes to the exacerbated lung pathology in these mice. In the chronic stages of a worm + egg infection, pulmonary fibrosis is reduced relative to acutely infected mice, but the levels of lung collagen remain significantly elevated above age-matched uninfected mice (data not shown). However, although the increased susceptibility of chronic worm + egg-infected mice to AHR (Fig. 3*A*) is associated with elevated pulmonary collagen, we have not addressed the involvement of IL-13 in AHR and lung fibrosis in these chronically infected mice.

Schistosome worm-infected mice are resistant to OVA-induced AHR

Previously, we have shown that mice infected with schistosome male worms are completely refractory to anaphylaxis, whereas worm + egg-infected mice were only partially resistant (15). We immunized worm-infected mice with OVA, using the protocol described in Fig. 1, to address OVA-induced AHR in these animals. It is important to note that infection of mice with schistosome male worms has been shown to induce a bias toward type 2 cytokine responses (15, 18, 32); thus, worm-infected mice have elevated basal levels of IL-4, IL-5, and IL-13 before OVA challenge. Despite this type 2 cytokine bias in worm-infected mice, spleen cells from these animals, and also from uninfected mice, that were injected with PBS and not OVA, did not produce OVA-specific cytokines in vitro (Fig. 4). In contrast, spleen cells from OVA-immunized worm-infected mice had greater allergen-stimulated type

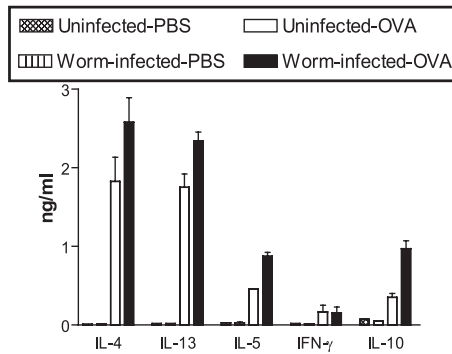


FIGURE 4. Spleen cells from *S. mansoni* worm-infected mice produce elevated allergen-induced IL-10 and type 2 cytokines following OVA sensitization. Uninfected and worm-infected mice were sensitized with PBS or OVA as described in Fig. 1. Spleen cells were stimulated with OVA (200 μ g/ml) in vitro, and cytokines in supernatants were detected by ELISA. Data are mean + SD from pools of spleens from three mice, and are representative of three experiments.

2 cytokine (IL-4, IL-5, and IL-13) production, and also had markedly elevated sera levels of OVA-specific IgE (data not shown), compared with OVA-immunized uninfected mice (Fig. 4). Interestingly, there was greater relative allergen-specific IL-10 release by spleen cells from OVA-sensitized worm-infected mice compared with IL-10 production from spleen cells from comparable treated worm + egg-infected mice ($p < 0.05$; Figs. 2A and 4), which is consistent with previous studies (15, 18).

Mice infected with a worm infection, but not exposed to OVA, had no spontaneous AHR (Fig. 5), which is in marked contrast to what was observed in worm + egg-infected animals (Fig. 2). Strik-

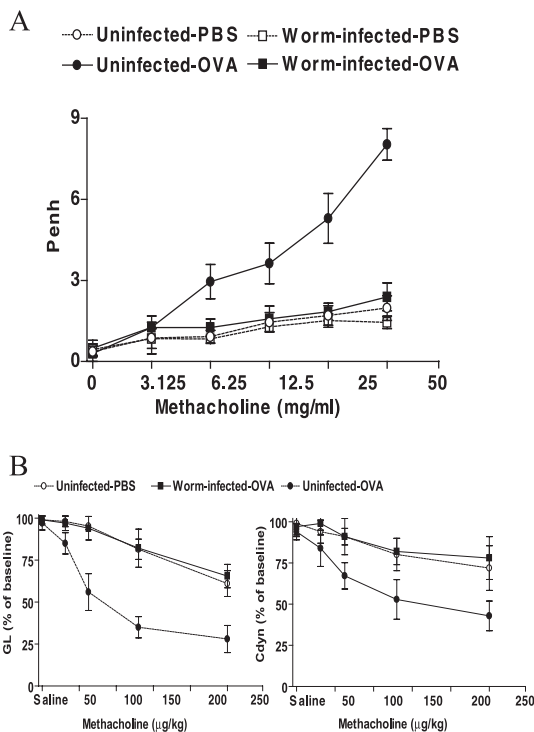


FIGURE 5. *S. mansoni* worm-infected mice are refractory to OVA-induced AHR. Uninfected and worm-infected mice were exposed to OVA or PBS and AHR was determined as (A) Penh, or (B) pulmonary resistance (GL) or compliance (Cdyn). Data are presented as mean \pm SEM. Penh values are from four separate experiments, $n = 16$ –21 mice per group. GL and Cdyn were from two separate experiments, $n = 8$ –10 mice per group.

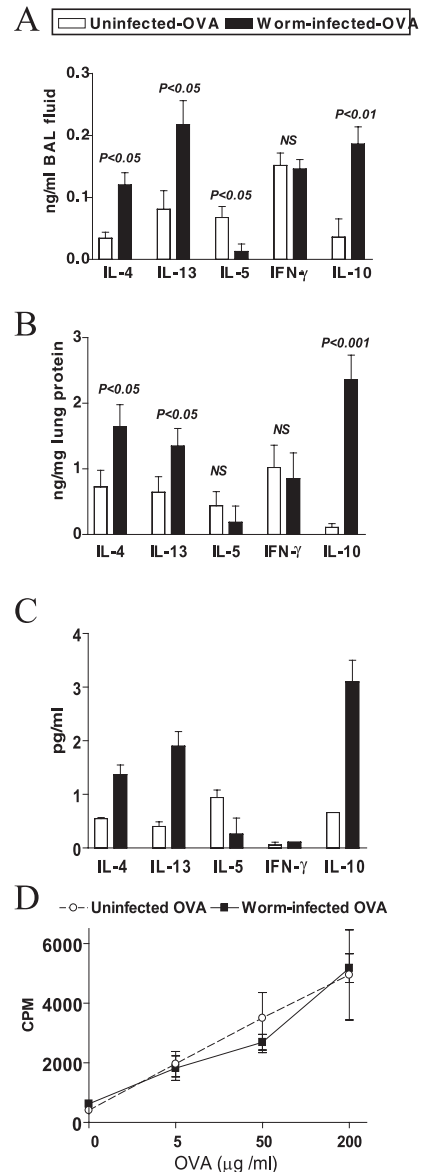


FIGURE 6. Modified type 2 response and elevated pulmonary IL-10 in OVA-sensitized worm-infected mice. A, Levels of cytokines in BAL from OVA-sensitized uninfected and worm-infected mice. Data are mean + SEM from five to eight individual mice, and are representative from two separate experiments. The Student t test was used to determine the difference between uninfected and infected mice. B, Levels of cytokines in lung homogenates from OVA-sensitized uninfected and worm-infected mice. Cytokine values were adjusted to nanograms per milligram of lung protein. Data are mean + SEM from six to nine individual mice, and are representative from three separate experiments. The Student t test was used to determine difference between uninfected and infected mice. C, OVA-specific cytokine responses from mediastinal lymph node cells from OVA-sensitized uninfected and worm-infected mice. Lymph nodes from four to eight mice were pooled and cells were cultured in duplicate or triplicate with OVA (200 μ g/ml). D, Cell proliferation of mediastinal lymph node cells to different concentrations of OVA was measured by [3 H] incorporation and are expressed as cpm. Data are representative of two separate experiments.

ingly, OVA-sensitized worm-infected mice did not develop AHR, with no alterations in Penh following Mch challenge, whereas OVA-sensitized uninfected mice had dose-dependent increases in Penh (Fig. 5A). As the accuracy of Penh as a measure of murine lung function has been questioned (33), we also tested pulmonary

function by analysis of pulmonary resistance (GL) and compliance (Cdyn) in unconscious and restrained animals (23). Similar to the Penh data, mice with worm infections had reduced GL and Cdyn responses to Mch challenge compared with resistance/compliance responses of sensitized uninfected mice (Fig. 5B).

During schistosome infection there is a larval migration phase that passes through the lung (34) that may alter pulmonary function. To address this point, worms were removed from infected mice by drug (praziquantel) treatment, after larval migration, and the mice then became fully susceptible to OVA-induced AHR (data not shown). These data demonstrate that mice infected with *S. mansoni* worm infections do not develop OVA-induced AHR.

Modified pulmonary type 2 response in OVA-sensitized worm-infected mice

We have addressed lung-specific responses in worm-infected mice, as in the OVA pulmonary challenge model used, the elevated AHR in sensitized mice is associated with elevations in pulmonary type 2 cytokines, eosinophil infiltration, and goblet cell hyperplasia (35). OVA-sensitized worm-infected mice had significantly elevated levels of both IL-4 and IL-13 in BAL fluid and lung homogenates ($p < 0.05$) compared with OVA-sensitized uninfected mice, with no differences in IFN- γ levels (Fig. 6, A and B). In contrast, IL-5 levels in BAL and lung homogenates from worm-infected mice were lower than uninfected mice, with BAL IL-5 significantly reduced ($p < 0.05$; Fig. 6, A and B). Thus, worm-infected mice stimulated a modified type 2 cytokine response in

the lungs, with IL-4 and IL-13 levels being significantly elevated whereas IL-5 was reduced. Sensitized worm-infected mice had a striking >3 -fold increase in total IL-10 in both homogenates of lungs and BAL fluid, which was significantly elevated above IL-10 levels detected in lungs of sensitized but uninfected mice ($p < 0.01$ – 0.001 ; Fig. 6, A and B). OVA-specific cytokine production by the lung mediastinal lymph node cells also demonstrated the modified Th2 response, elevated IL-4 and IL-13 but reduced IL-5, in sensitized worm-infected mice compared with sensitized uninfected mice, with an associated pronounced increase in OVA-induced IL-10 (Fig. 6C). There were no differences in OVA-specific proliferation of mediastinal lymph node cells from OVA-sensitized uninfected and worm-infected mice, indicating normal Ag-induced T cell responsiveness (Fig. 6D). Similarly, lung TGF- β levels in worm-infected and uninfected OVA-sensitized mice were not different (data not shown).

Histology sections of lungs of OVA-sensitized uninfected mice showed normal airway inflammation and peribronchial infiltrating eosinophils, and increased eosinophils in BAL and lung digests and goblet cell hyperplasia (Fig. 7, A–D). In sensitized worm-infected mice, there was no lung inflammation or peribronchial eosinophilia (Fig. 7A). Enumeration of cells in the BAL showed that worm-infected mice did not have the normal increase in eosinophils that occurred in OVA-sensitized uninfected mice, with significantly ($p < 0.005$) fewer eosinophils recovered in BAL from infected vs uninfected mice (Fig. 7B) and also reduced lung eosinophils (FSC/SSC^{high}, CD19[−], CD4[−], CD8[−], CCR3⁺ cells)

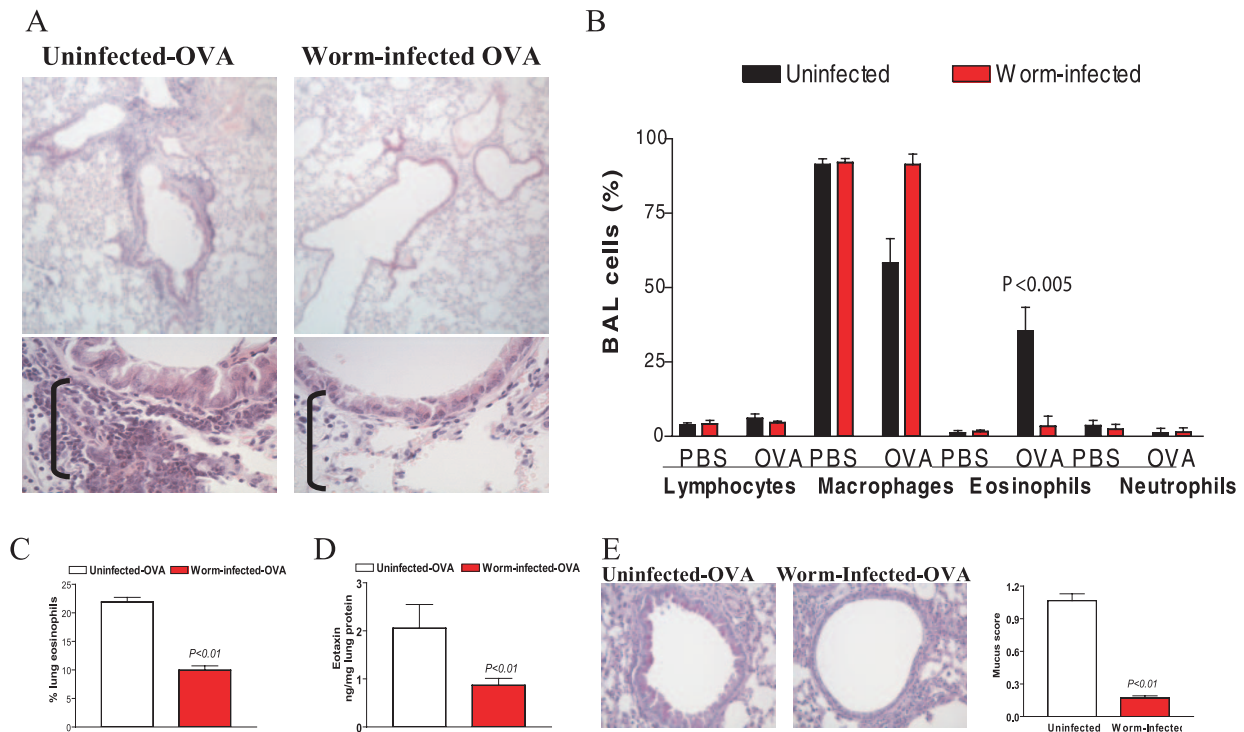


FIGURE 7. Absence of pulmonary inflammation in OVA-sensitized worm-infected mice. *A*, Airway inflammation in OVA-sensitized uninfected mice but not in sensitized worm-infected mice (*top panel*). *Lower panels* are higher magnifications of sections of lungs from OVA-sensitized uninfected and worm-infected mice showing peribronchial eosinophilia in uninfected mice (bracket) but not infected mice (H&E-stained). *B*, Differential cell counts of cytopins of BAL cells from uninfected and worm-infected mice exposed to OVA or PBS. The Student test was used to test for statistical differences in the percentage of BAL eosinophils. *C*, Percentage of granulocytes that are eosinophils in the lungs of OVA-sensitized uninfected and worm-infected mice. Eosinophils were phenotyped as SSC^{high}, CD19[−], CD4[−], CD8[−], CCR3⁺ by flow cytometry. *D*, Detection of eotaxin in lung homogenates from OVA-sensitized uninfected and worm-infected mice. *E*, OVA-induced goblet cell hyperplasia in uninfected mice but not in worm-infected mice (PAS-stained). Graph shows quantification of goblet cell hyperplasia, expressed as mucus score. All data are mean + SEM from four to nine individual mice and are representative of two to three separate experiments. Statistical differences between infected mice and uninfected OVA-sensitized mice was tested by Student's *t* test.

detected by flow cytometry of lung digests (Fig. 7C). In view of the deficit in eosinophils in the lungs of worm-infected mice, we measured eotaxin. The levels of eotaxin were significantly lower in lungs recovered from sensitized worm-infected mice relative to uninfected mice ($p < 0.01$; Fig. 7D). Worm-infected mice also had limited goblet cell hyperplasia, with significantly reduced mucus scores compared with uninfected mice (Fig. 7E).

IL-10 mediates resistance of worm-infected mice to AHR

We have previously demonstrated worm-infected mice are resistant to anaphylaxis through an IL-10-dependent mechanism (15). As there was elevated pulmonary IL-10 in OVA-sensitized worm-infected mice, we used an anti-IL-10R mAb to block IL-10 activity and analyzed lung function. OVA-sensitized worm-infected mice were fully susceptible to AHR when IL-10 was blocked, with significantly greater Penh values in these animals than OVA-sensitized uninfected mice at doses >12.5 mg/ml Mch ($p < 0.05-0.001$; Fig. 8A). The increase in AHR in infected mice with IL-10 blocked was associated with a restoration of OVA-induced eosinophilia in the BAL (Fig. 8B). This increase in susceptibility to AHR and development of pulmonary eosinophilia in worm-infected mice treated with anti-IL-10R mAb was associated with significant elevation ($p < 0.01$) in lung IL-5 levels in the mice; IL-10R mAb-treated mice had 0.46 ± 0.19 ng of IL-5/mg lung protein, vs 0.14 ± 0.08 ng of IL-5/mg lung protein in control Ig-treated mice (data are mean \pm SD, $n = 5$; Student's t test). These data show that schistosome worm infection prevents AHR in a mouse model of allergen-induced pulmonary inflammation via IL-10-dependent suppression of pulmonary eosinophilia.

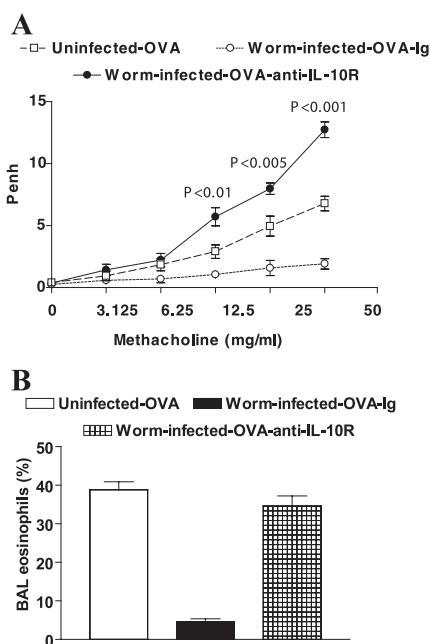


FIGURE 8. Resistance of worm-infected mice from AHR is dependent on IL-10. *A*, Penh responses of OVA-sensitized uninfected mice and sensitized-infected mice treated with anti-IL-10R or control IgG. The Student t test was used to determine the differences between uninfected mice and anti-IL-10R-treated infected mice. Data represent the mean change from baseline Penh values for each group. *B*, Percentage of eosinophils in the BAL of OVA-sensitized mice. All data are presented as group mean \pm SEM ($n = 8-10$) and are representative of at least two separate experiments.

Resistance of worm-infected mice to AHR is independent of CD4⁺, CD25⁺ cells and macrophages

In mice with *S. mansoni* worm + egg or worm-only infections, a number of IL-10-producing cells have been identified, including CD4⁺ cells, CD4⁺CD25⁺ regulatory cells, macrophages, and B cells (15, 36). As we were addressing a pulmonary allergic phenotype, we characterized by flow cytometry which of these different potential cellular sources of IL-10 preferentially infiltrated the lungs of worm-infected mice. We found no major difference in the percentages of CD4⁺ cells, CD25⁺ cells, or macrophages infiltrating the lungs of sensitized worm-infected vs sensitized uninfected mice (Fig. 9A). The absence of a role for CD4⁺ and CD25⁺ cells in the resistance of worm-infected mice to AHR was further corroborated by in vivo depletion studies whereby depletion of either CD4⁺ or CD25⁺ cells in worm-infected mice demonstrated that each cell population has no role in affording protection from OVA-induced AHR (Fig. 9B). Furthermore, depletion of pulmonary macrophages had no effect on the worm infection-mediated airway protection, with OVA-sensitized worm-infected mice having lower Penh values compared with uninfected mice that developed AHR (Fig. 9B).

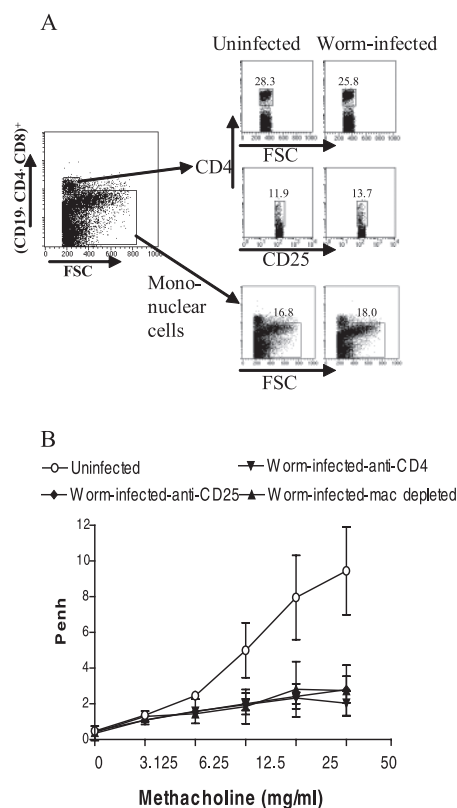


FIGURE 9. Schistosome worm infection-mediated protection from AHR is independent of CD4⁺ and CD25⁺ cells and macrophages. *A*, Flow cytometry of different potential cellular sources of IL-10 preferentially infiltrated the lungs of uninfected and worm-infected mice. Cells were characterized as described in *Materials and Methods*. *B*, No alteration in the Penh responses of worm-infected mice with CD4⁺, CD25⁺ cells or macrophages depleted. OVA-sensitized mice were administered depletion treatments on days 27 and 30 of OVA sensitization and challenge protocol: anti-CD4 (0.5 mg/mouse), anti-CD25 (0.25 mg/mouse), or clodronate-liposomes (50 μ l of liposomes in on day 27 and 0.1 ml i.p. on day 30). All depletions were confirmed to be effective by flow cytometry. Data represent the mean change from baseline Penh values for each group. Data are from two separate experiments; $n = 8-10$ mice per group.

Resistance of worm-infected mice to AHR is dependent on B cells

Previously, we have shown that worm-infected mice are resistant to anaphylaxis via a schistosome-induced splenic IL-10-producing B cell subpopulation (15). Significantly, there was a ~30% increase in the number of infiltrating B cells in the lungs of sensitized worm-infected mice when compared with sensitized uninfected mice (Fig. 10A). Following partial depletion of B cells, via anti-IgM treatment, OVA-sensitized worm-infected mice were rendered fully susceptible to AHR (Fig. 10B), suggesting that a B cell population is intrinsic in the mechanism of resistance of worm-infected mice to AHR. In fact, the airway reactivity of these mice was even more enhanced than in uninfected mice indicating the significance of B cells in the protection from pulmonary in-

flammation. The susceptibility of worm-infected mice to AHR after B cell depletion was associated with a restoration of eosinophil infiltration of the lungs (Fig. 10C).

Discussion

In this study, we have experimentally investigated the role of a helminth worm vs worm and egg infection in modulating allergen-induced pulmonary airway hyperreactivity. We have demonstrated both the protective and the exacerbating roles of schistosome infections in a murine model of allergen-induced pulmonary inflammation. Schistosome worms alone stimulated a modified pulmonary type 2 response that prevents OVA-induced AHR, with protection involving B cell and IL-10-dependent suppression of pulmonary eosinophil infiltration. In contrast, infection of mice with *S. mansoni* egg-laying male and female worms exacerbated AHR.

The failure of schistosome worm-infected mice to develop OVA-induced AHR was not associated with an inability of infected mice to respond to the allergen. On the contrary, cells from infected mice had normal *in vitro* cell proliferation to OVA and their spleen and mediastinal lymph nodes cells produced greater levels of both spleen and lung IL-4 and IL-13 than uninfected mice. Strikingly, worm-infected mice had reduced levels of total and OVA-specific IL-5 in the lungs compared with uninfected animals, suggesting a unique selective Th2 defect in the lungs. Recently, Platts-Mills et al. (37) have suggested that a "modified Th2 response" to cat allergens may explain the reduced levels of asthma in children exposed to cats. We now describe for the first time that schistosome worms induce what we have termed "a helminth-modified pulmonary type 2 response" to OVA that renders mice refractory to allergen-induced AHR. The helminth-modified pulmonary type 2 response is characterized by elevated pulmonary allergen-specific IL-4, IL-13, but reduced IL-5 and elevated IL-10. An important question is why would a worm selectively suppress pulmonary immunity? The answer may relate to the biology of the parasite infection. Humans are repeatedly reinfected with schistosomes, with new infections requiring migration of larvae through the lungs. However, established schistosome infections evoke concomitant immunity, whereby immune responses against the adult worms and the eggs cross-react with larval Ags and thereby invading new larvae killed are killed in the lungs. Interestingly, this schistosome antilarval immunity is thought to involve production of blocking IgG Ab (38), which is similar to what is proposed in the modified Th2 response to cat allergens (37). Therefore, the schistosome parasite may induce a modified pulmonary type 2 response to suppress inflammation in the lung induced by new invading larvae.

There is already evidence from field studies in Africa that schistosome infection of humans can reduce allergic responses. *Schistosoma hematobium*-infected school children in The Gabon have lower prevalence of skin reactivity to house dust mites than those free of this infection (39). Strikingly, when worms are removed from patients by chemotherapy there is an increase in atopy, directly establishing a link between the presence of worms and suppression of allergic responses (40). In the experimental study described here, when adult schistosome worms were killed by drug treatment the previously resistant mice became susceptible to OVA-induced AHR. Therefore, there is a requirement for the continual presence of the worm during infection to sustain a helminth-modified type 2 pulmonary response to suppress allergic inflammation. Therefore, our experimental data support the argument that the chronic down-regulation of the immune system during helminth infections evokes a regulatory environment (41), called here a helminth-modified pulmonary type 2 response, that may impart protection from allergies. However, it is important to stress that schistosome worm-only infection is a laboratory model that

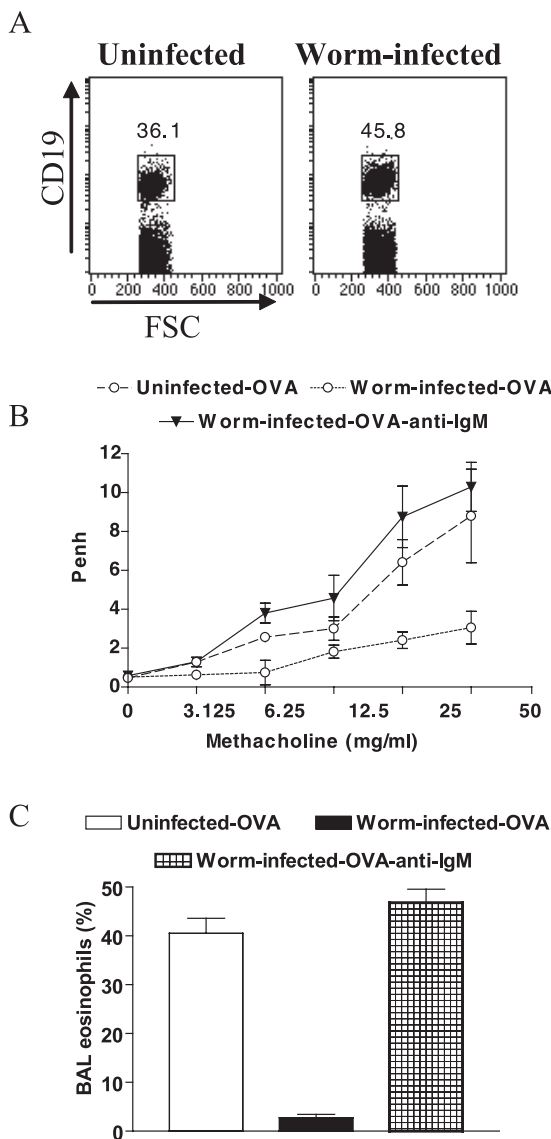


FIGURE 10. B cells have an integral role in schistosome worm-mediated protection of mice from AHR. *A*, Flow cytometry of increased infiltration of B cells (CD19⁺) into lungs of worm-infected mice relative to uninfected mice. *B*, Increased Penh responses worm-infected mice with B cells depleted. Mice were sensitized against OVA and treated with anti-IgM (μ -chain specific; 0.5 mg/mouse) before OVA aerosol challenge. Data represent the mean change from baseline Penh values for each group. *C*, BAL eosinophil counts in OVA-sensitized mice and sensitized-infected mice treated with anti-IgM. Data are presented as group mean \pm SEM ($n = 8-10$) and are representative of two separate experiments.

facilitates intimate functional analysis of modulation of immunity by the worm, in the absence of eggs. Such worm-only infections may not occur in infected humans.

In *S. hematobium*-infected school children, the reduced mite-specific allergic response was associated with production of parasite-specific IL-10 (42). We have shown that worm-infected mice are resistant to allergen-induced AHR via suppression of pulmonary eosinophilia via IL-10. IL-10 is a potent regulatory cytokine suppressing a range of immune-mediated responses (43). In mouse AHR models, there are various data showing a role for IL-10 in suppressing airway inflammation and AHR (44–47). One of these recent studies showed that when IL-10 was administered in vivo by gene delivery it suppresses OVA-induced AHR and airway eosinophilia (47). Although our data showing worm infection-induced IL-10 also blocks AHR and airway eosinophilia unlike the gene delivery of IL-10 in worm-infected OVA-sensitized mice, there is elevated, not reduced, allergen-specific IL-4 and IL-13 cytokines and IgE, and also no alteration in cellular response to OVA. IL-10 may be a regulatory component of the helminth modified pulmonary type 2 response we describe, as pulmonary IL-5 levels are restored in worm-infected mice with IL-10 blocked in vivo.

The production of IL-10 in conventional *S. mansoni* male and female egg-laying infections of mice has been shown to have a central role in preventing infection-induced pathology (36, 48, 49). Indeed, IL-10 also mediates resistance of tapeworm-infected mice to experimental colitis (50). Despite worm + egg-infected mice having elevated IL-10, these animals were highly susceptible to AHR, with worm + egg-infected mice dying, even without allergen sensitization, when exposed to Mch-induced bronchoconstriction (Fig. 2). The predisposition of worm + egg-infected mice to AHR was evident in both the acute stages of infection, which is the peak of Th2 cytokine induction, and also during the chronic stages, when the parasite has down-modulated host immunity. Although worm-infected mice produce relatively more IL-10 than comparably infected worm + egg-infected mice (15, 18), the discrepancy between infection with male worm alone causing mice to be resistant to allergen-induced inflammation whereas a male and female worm and egg-laying infection exacerbated AHR is unlikely to be solely due to IL-10 levels. Worm + egg-infected mice also have the modified type 2 response in the lungs that is observed in worm-infected mice (Fig. 6; data not shown). However, the presence of marked IL-13-dependent fibrosis in the lungs (Fig. 3) of worm + egg-infected mice, and not in mice infected with male worms (data not shown), is relevant due to the effects of IL-13-induced fibrosis on lung inflammation (31). Indeed, the contradictory exacerbating or suppressive influences of schistosomes on AHR, described here, is comparable to the potential negative or positive outcomes from disease following infections with a range of other pathogens (51).

Previous experimental studies have shown that infection with various parasitic worms causes reduced allergic responses (11–14). Using the rodent gastro-intestinal nematode *Nippostrongylus brasiliensis*, it was shown that infection suppressed allergen-induced airway eosinophilia via IL-10 from an unidentified cell source (14). It has been argued that CD4⁺ cells, either Th2 or regulatory, are the potential source of the worm-induced IL-10 (14). As CD4⁺ IL-10-producing cells generated by pathogens or genetically prepared suppress airway inflammation (52, 53), they are an attractive possible source for helminth-induced IL-10. Indeed, we have previously shown that schistosome worm infections of mice induce elevated frequencies of natural CD4⁺CD25⁺ regulatory cell and IL-10-producing CD4⁺ cells (15). However, depletion of CD4⁺ or CD25⁺ cells did not alter the resistance of worm-infected mice to OVA-induced AHR. Similarly, depletion of alveolar macrophages,

which also produce IL-10, did not alter the worm-induced protection against AHR. Previously, we have shown B cell-IL-10 levels were significantly enhanced in worm-infected mice, with B cells having a crucial role in schistosome worm infection-mediated resistance to anaphylaxis (15) and AHR (this study). Earlier studies have already proposed that B regulatory cells or IL-10-producing B cells may function in immune-mediated inflammatory reactions (54–56). For example, in murine experimental autoimmune encephalomyelitis and collagen-induced arthritis IL-10-producing B cells have been shown to have protective function in ameliorating disease (54, 55), and such cells may also suppress intestinal inflammation (56).

Our initial studies have found no major alteration between worm + egg vs worm-only infected mice in different B cell subpopulations we have examined (B1: CD19⁺CD5⁺; B2: CD19⁺CD5⁻; germinal center B cells: CD19⁺GL7⁺; Ab-containing B cells (plasma cells and plasmablasts): syndecan-1⁺CD19^{int} (57)), although both groups had elevated numbers of B cells in the lungs in comparison to uninfected mice. Worm-infected mice have increased frequencies of IL-10-producing B-1 cells in the peritoneum, an observation originally reported in worm + egg-infected mice (58). However, using worm-infected *xid* mice that have defective B-1 cells, we have shown that these B cells have no role in affording protection from anaphylaxis (15) or from OVA-induced AHR (data not shown). We are currently addressing the specific B cell subpopulation that is evoking this IL-10-mediated protection from pulmonary insult in worm-infected mice. Nonetheless, it is of significance that depletion of B cells disrupts the fine signaling balance in immune regulation and thus exacerbates OVA-induced pulmonary inflammation by removal of a potentially critical regulatory cell. Further studies are required to address the interplay between B cells, IL-10, and the helminth-modified pulmonary type 2 response.

In this study, we demonstrate both protective and antagonistic roles for schistosome infections of mice in an experimental test of the hygiene hypothesis (51). In marked contrast, *S. mansoni* worm-only infection of mice diminishes the disease effects in a model of OVA-induced AHR via induction of what we have termed a helminth-modified pulmonary type 2 response. These data highlight the important influence of the helminth parasite *S. mansoni* and its significance in allergic disorders. Importantly, this is the first formal demonstration of protection by a pathogen of humans in a mouse model of allergic pulmonary disease.

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Disclosures

The authors have no financial conflict of interest.

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