

Schistosoma japonicum infection modulates the development of allergen-induced airway inflammation in mice

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Abstract Asthma, a chronic inflammatory disorder of the airways, is coordinated by Th2 cells in both human asthmatics and animal models of allergic asthma. It has been shown that helminth infections including *Schistosoma mansoni* may modulate atopic diseases including asthma. In the present study, BALB/c mice were infected with bisexual and unisexual (male) *S. japonicum*, respectively, prior to ovalbumin (OVA) sensitization and challenge. Compared to mice with OVA sensitization/challenge alone, *S. japonicum* infection led to a significant decrease of eosinophil accumulation in bronchoalveolar lavage fluid (BALF) collected 48 h postchallenge, as well as to a marked reduction in inflammatory cell infiltration around the airways and pulmonary blood vessels. Compared to OVA-immunized uninfected mice, the level of OVA-specific serum IgE as well as interleukin (IL)-4 and IL-5 in BALF were reduced, but IL-10 was strongly elevated in mice with preexisting *S. japonicum* infection prior to OVA immunization. These results suggest that both bisexual and male *S.*

japonicum infections may modulate the development of allergic asthma.

Introduction

Allergic asthma is Th2 polarization disease. Over the last several decades, the incidence of asthma has substantially increased in industrialized countries, but is lower in developing countries where poverty and lower sanitation are present. A potential explanation for the ongoing epidemic of allergic diseases is that declining family size and improved hygienic conditions have led to reduced exposure to infections including bacteria, viruses, and certain parasites in early childhood and more widespread expression of atopic diseases in industrialized nations, i.e., the hygiene hypothesis (Strachan 1989). An alternative hypothesis holds that certain parasitic helminth infections may protect against allergic disorders because human populations with high rates of parasite infections, which induce an immunological shift toward the “allergic” Th2 response, have a reduced prevalence of allergic disorders (Yazdanbakhsh et al. 2002).

Helminth infections generally induce a strong Th2 immune response (Grzych et al. 1991). In schistosomiasis in South America, asthma was less severe in children infected with *Schistosoma mansoni* (Araújo et al. 2004a). In murine model, *S. mansoni* infection with male worm protected mice from allergen-induced airway hyperresponsiveness (AHR) but not with female and male infection (Mangan et al. 2006). However, the relationship between *S. japonicum* infection and allergic asthma remains unknown. In the present study, the effect of *S. japonicum* infection on the

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development of allergen-induced airway inflammation in mice was observed.

Materials and methods

Animals

Specific pathogen-free 5–7-week-old female BALB/c mice were obtained from the Experimental Animal Center at Tongji Medical College. Food and water were sterilized and ovalbumin (OVA)-free. All animal experiments were performed according to the guidelines of the local authorities.

Infection of mice with *S. japonicum*

BALB/c mice received the *S. japonicum* infection 24 days prior to OVA sensitization. For a conventional bisexual infection where eggs are laid, mice were infected transcutaneously with 25 mixed male and female cercariae. For single-sex infections, mice were exposed to cercariae obtained from snails previously exposed to a single miracidium. The unisexual (male) nature of the infection was confirmed by the absence of eggs in the feces and livers of the animals and the morphology of worms collected from the mesenteric veins of the animals. In order to ensure to obtain enough single-sex mice with male worm infection, the cercariae of six snails were individually used to infect each ten mice. Control groups of mice were without infection and with OVA sensitization and with the bisexual or male worm infection without OVA sensitization.

OVA sensitization and challenge protocol

According to Chung et al. (2003), mice were sensitized and challenged by intraperitoneal (i.p.) injection of 100 µg of OVA (Sigma, USA) in 200 µl of a 10% suspension of Al (OH)₃ adjuvant in phosphate-buffered saline (PBS) on day 0 and boosted i.p. with the same dose on days 14 and 21. On days 28, 29, and 30, mice were anesthetized by an i.p. injection of 0.3% sodium pentobarbital (Sigma, USA) 300 µl and were treated intranasally (i.n.) pipetting with 100 µg OVA in 50 µl sterile PBS. Nonimmunized control mice received PBS only. Each group consisted of five mice, and all experiments were duplicated.

Detection of eosinophils in the bronchoalveolar lavage fluid

Mice were deeply anesthetized by i.p. injection of pentobarbital sodium 48 h after the last challenge with OVA or PBS, immediately after which blood samples were collected and the sera were stored for further detection. The lung

was lavaged with one wash of 0.7 ml of sterile PBS and then additional lavage of 0.7 ml three times in and out, together a pooled volume of 1.4 ml. For each mouse, cells were individually pelleted (1,500 ×g, 5 min, 4°C) and the supernatants stored at −70°C until assayed for cytokine levels. Cells were resuspended in PBS 1 ml then were counted, spun onto glass slides, and stained with Giemsa and Wright's staining. A total of 200 cells were counted in each slide to calculate the percentages of eosinophils in bronchoalveolar lavage fluid (BALF; Chung et al. 2003).

Histological analysis

After BALF collection, the left lobes of lungs were removed from *S. japonicum*-infected and/or OVA-immunized mice and fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections (5 µm) were cut and stained using standard histological protocols with hematoxylin/eosin (H&E) stain. The stained sections were visualized by light microscopy. The degree of inflammation was evaluated by assigning a value of 0 for no inflammation, 1 for mild inflammation, 2 for moderate inflammation, and 3 for severe inflammation (Hopfenspirger and Agrawal 2002). A minimum of ten fields was randomly examined by light microscopy by a blinded observer.

Detection of cytokines

Interleukin (IL)-4, IL-5, IL-10, and interferon (IFN)-γ levels in BALF were assayed using enzyme-linked immunosorbent assay (ELISA) Kits (eBioscience, USA) according to the manufacturer's protocol. The optical density (OD) values were read on an ELISA reader (Multiskan Ascent 354 Labsystems, Finland) at λ=450 nm. Cytokine levels were determined by comparison with standards.

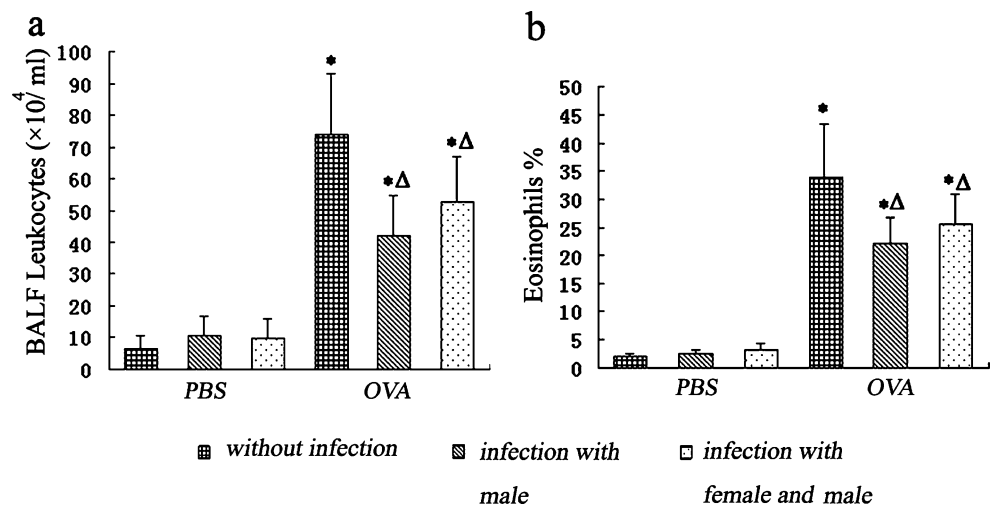
Determination of OVA-specific IgE levels

The levels of OVA-specific IgE in serum were determined by ELISAs as described (Walker et al. 2003). Microtiter plates were coated with 100 µl/well of 100 µg/ml OVA (Sigma, USA). To detect OVA-specific IgE, biotinylated antimouse IgE mAbs (1:500) and horseradish peroxidase conjugated avidin (all from Southernbiotech, USA) were measured.

Statistics

All data were analyzed for statistical significance ($P<0.05$) by Student's two-tailed *t* test for independent means. Statistical analyses were performed using Statistical Package for the Social Sciences 1.2.

Fig. 1 The amount of leukocytes (a) and percentage of eosinophils (b) in bronchoalveolar lavage fluid (BALF) of mice treated with OVA or PBS after bisexual (dotted column) or male worm (hatched column) infections with *S. japonicum*. Control group (squared column) was not infected. * $P < 0.01$ vs. PBS-treated without infection group and $\Delta P < 0.05$ vs. OVA-treated alone ($n = 10$ mice). Data represent mean values with standard deviation from two independent combined experiments



Results

Inflammatory cells recruited into the airways were decreased with *S. japonicum* preexposure

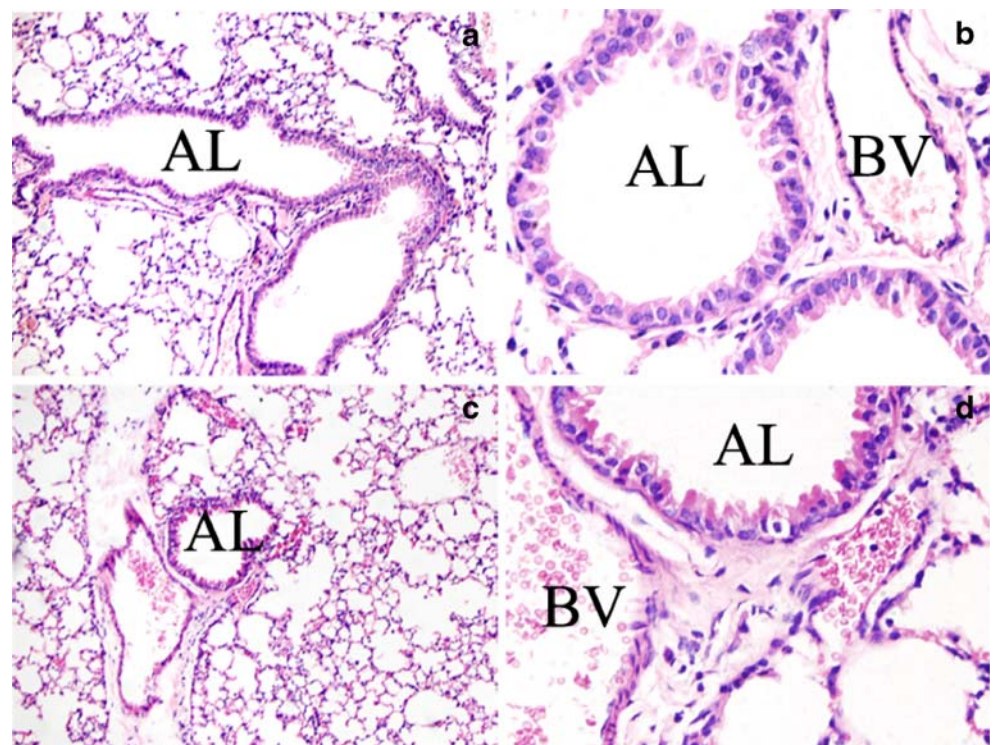
To examine whether *S. japonicum* bisexual infection or male worm infection contribute to the regulation of OVA-induced airway responses, the leukocytes and eosinophils in BALF were counted 48 h after the last i.n. OVA application. As shown in Fig. 1, the leukocytes (Fig. 1a) and eosinophils (Fig. 1b) infiltration were remarkably reduced in both bisexual-infected and male worm-infected mice. Only few leukocytes or eosinophils were observed in the BALF of the

PBS-treated mice and parasite-infected alone mice. Therefore, those mice preexposed to *S. japonicum* bisexual or male worm infections were partially protected from inflammation and asthma development.

Inflammatory infiltration induced by OVA was diminished in the lungs of *S. japonicum* infected mice following induction of allergic asthma

Histology section of lungs showed PBS and *S. japonicum* infection alone did not induce pulmonary inflammation (Fig. 2), while OVA induced a marked lymphocytic infiltrate (Fig. 3a,b). Mice infected with schistosome prior

Fig. 2 Lung histopathology of mice without OVA immunization (H&E stain). a and b The lung sections of PBS challenged control mice. a The airways lumen (AL) and pulmonary blood vessels (BV) are of normal appearance (100 $\times$). b Inflammatory cells are absent in the bronchiolar and alveolar parenchyma, and mucus release is not present in the airway lumen (400 $\times$). c and d Normal appearance of the airways and pulmonary blood vessels in mice with schistosome infection (100 $\times$, 400 $\times$). This experiment was performed twice with similar results and the histological changes of all mice in the respective experimental groups had closely similar results



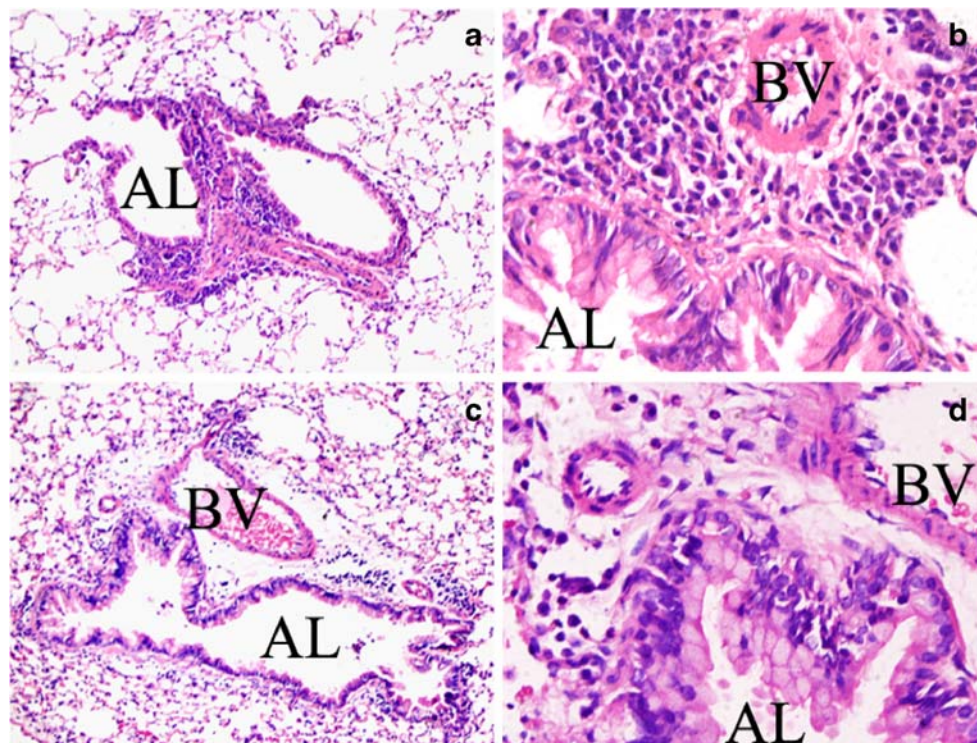


Fig. 3 Histological evidence of airway inflammation in mice with OVA sensitization and challenge (H&E stain). **a** and **b** Lung sections from mice with OVA sensitization and challenge alone. **a** The interstitial space surrounding the airway is characterized by a dense mixed cellular infiltrate. Marked narrowing of the airway lumen (AL) is observed (100 $\times$). **b** Massive infiltration of the interstitial tissue by eosinophils and other inflammatory cells around blood vessels (BV) and airways and goblet cell hyperplasia are noted (400 $\times$). **c** and **d**

Lung section from mice with schistosome infection prior to OVA treatment. **c** Reduction in the cellular infiltration surrounding the airways and blood vessels is observed (100 $\times$). **d** Little cellular infiltration is found surrounding the airways compared with OVA sensitization and challenge alone (400 $\times$). This experiment was performed twice with similar results and the histological changes of all mice in the respective experimental groups had closely similar results

to OVA challenge showed a dramatic reduction of both inflammatory cell infiltration of the airway parenchyma and surrounding blood vessels and airway mucus production compared to murine asthma model without schistosome infection (Fig. 3c,d).

The expression of Th2 cytokines in the BALF decreased while the production of IL-10 increased markedly in schistosome infection plus OVA immunization mice

To evaluate the potential role of the bisexual infection and male worm infection of *S. japonicum* in OVA-induced Th1 or Th2 responses, the protein level of IFN- γ , IL-4, IL-5, and IL-10, a potent regulatory cytokine, in BALF were quantitated. Bisexual-infected mice showed elevated level of IL-4 and IL-5 in BLAF without OVA challenge, while IL-4, not IL-5, increased in male worm infection alone. OVA immunization/challenge induced remarked low production of IL-10 compared to *S. japonicum* infection alone. A significant increase over control values in IL-4 and IL-5 levels in the BALF of OVA-sensitized and challenged mice was observed. While mice infected with schistosome and

immunized with OVA, the levels of IL-4 and IL-5 decreased and IL-10 increased greatly (Fig. 4a–c). IFN- γ was not detectable in BALF (data not shown).

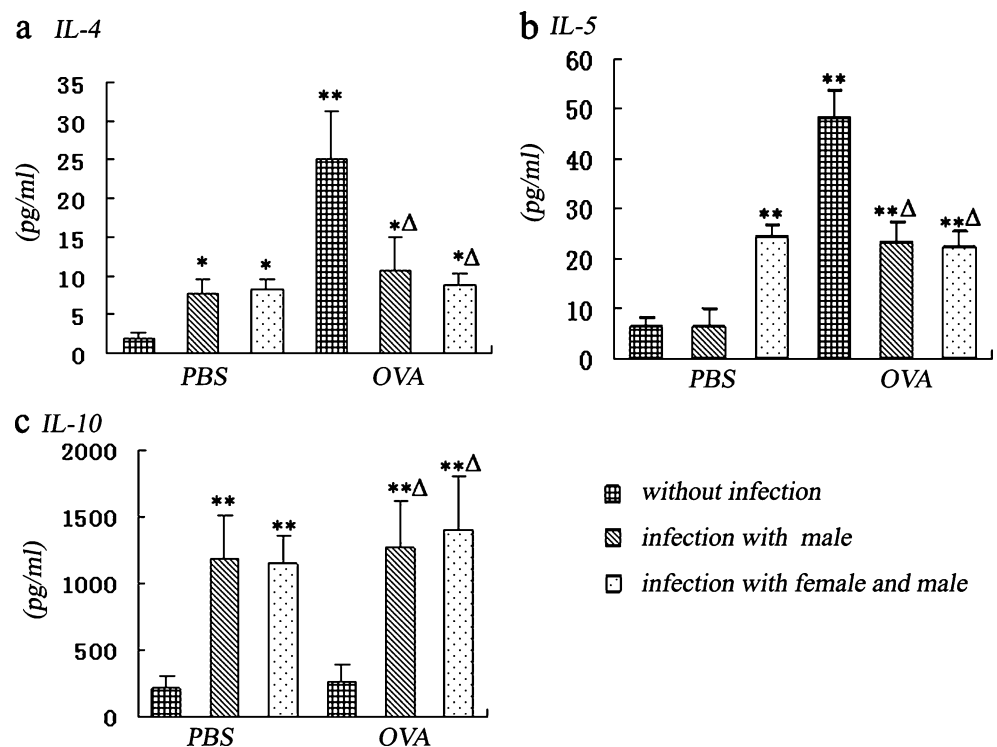
The sera level of OVA-specific IgE decreased in mice infected with *S. japonicum* prior to OVA sensitization

OVA immunization/challenge markedly elevated sera level of allergen-specific IgE, which was significantly reduced by both male worm infection and bisexual infection of *S. japonicum* prior to OVA immunization, although the reduction in bisexual infection and male worm infection were not different (Fig. 5).

Discussion

The development of allergies and asthma is influenced by both genetic and environmental factors. There is already evidence suggesting the helminth infection may be one such environment modifier in that it plays a role in preventing the onset of allergic or autoimmune diseases in rodent models and

Fig. 4 Level of cytokines in BALF. **a** IL-4, **b** IL-5, and **c** IL-10. * $P<0.05$ and ** $P<0.01$ vs. PBS-treated without infection group and $\Delta P<0.01$ vs. OVA-treated alone ($n=10$ mice). Data represent mean values with standard deviation from two independent combined experiments



possibly also in humans (Baxter et al. 1994; Gale 2002). Asthma is an allergic disorder that is characterized by increased AHR, elevated IgE, and eosinophil infiltration of the airways. Previous studies have shown elevated serum IgE level, inflammatory reactions in the airways associated with the presence of eosinophils (Corrigan and Kay 1996), and high level of Th2 cytokines such as IL-4 and IL-5 in allergic asthma (Kay et al. 1995; Humbert et al. 1997). Pulmonary eosinophilia is associated with increased IL-5 level (Tavernier et al. 1996) and elevated IgE is related to

high IL-4 level in asthma (de Vries et al. 1999). During human and murine schistosome infection, IL-4 and IL-5 are also elevated (Grzych et al. 1991; Roberts et al. 1993). OVA-induced mouse is considered as a good model of allergic asthma. Although the suitability of the OVA sensitization mice as a model for asthma was recently questioned because of the limitation of this mouse model as a completely representation of the lung dysfunction associated with asthma (Zosky et al. 2007), this OVA-induced murine model, which exhibits AHR perfectly, is still increasingly used in many experimental studies on immunoregulation (Yang et al. 2006; Dittrich et al. 2008) and therapeutic potential (Naura et al. 2008) of allergic asthma.

In this study, we have experimentally investigated the role of a *S. japonicum* bisexual vs. male worm infection in modulating airway inflammation. We have demonstrated that the preexisting *S. japonicum* infection either bisexual or male worm infection suppressed the development of airway inflammation in a murine model. Tissue-infiltrating inflammatory cells recruitment of both eosinophils and leukocytes into airway was significantly reduced by preinfection with the parasite. The development of OVA-specific IgE response was also impaired in schistosome-infected mice. Other studies evaluating the interrelationship among helminth infection and allergic diseases have provided similar results. Preexisting *Strongyloides stercoralis* suppressed pulmonary allergic responses to ovalbumin (Wang et al. 2001) and absence of inflammation in lungs was reported in OVA-induced mice with *S. mansoni* worm and egg infection (Mangan et al. 2006). In *S. venezuelensis* infection rat

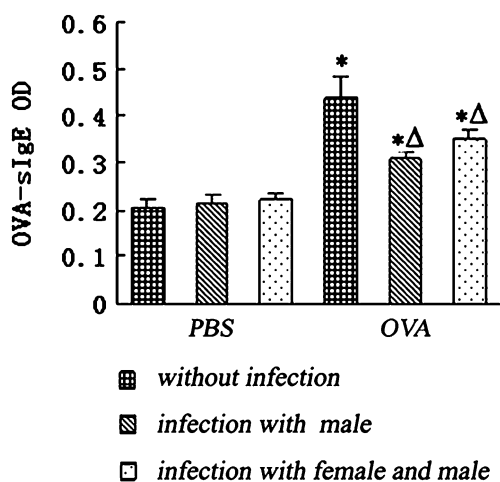


Fig. 5 Levels of OVA-specific IgE in serum. The data are presented as the mean OD at 491 nm $\pm$ standard deviation at a 1:10 dilution of serum. * $P<0.01$ vs. PBS-treated without infection group and $\Delta P<0.05$ vs. OVA-treated alone ($n=10$ mice). Data are from two independent combined experiments

concomitant with induction of allergic inflammation, airway inflammation was unchanged, but the AHR in the experimental asthma model was significantly suppressed (Deborah et al. 2003). These discrepancies can be explained by the fact that the development of pulmonary inflammation and airway hyperresponsiveness are not totally parallel-related processing. Furthermore, various helminthes infection and their various stages may cause effects on the development of allergic response in different degree (Eduardo et al. 2007).

It was also investigated in our study that those parasite infections additionally stimulated a modified pulmonary type 2 responses that prevents OVA-induced hyperreaction, with protection involving reduced production of IL-4, IL-5 in BALF, and allergen-specific IgE in serum. Moreover, elevated IL-10 in BALF was also observed, which might contribute to the helminth-specific suppression of inflammatory type 2 responses in airway. Our findings from the present study are consistent with *S. japonicum* egg antigens treatment leading to the modified airway inflammation in a murine model of asthma by inducing a population of CD4⁺CD25⁺ T regulatory cells (Treg) which elicited immunosuppressive activity characterized by increased production of IL-10, which may play an important role on modulating excessive activation of Th2 response (Yang et al. 2006). We have previously demonstrated that the percentage of CD4⁺CD25⁺ Treg was increased in the spleens of *S. japonicum* egg-exposed mice with Trinitrobenzene sulfonic acid (TNBS)-induced colitis compared to non-egg-exposed animals. Our data suggest that Tregs may be activated by *S. japonicum* eggs and play a role in restoring immune disorders in TNBS-induced colitis of mice (Mo et al. 2007). In contrast, Mangan's data showed *S. mansoni* worm infection elevated, not reduced IL-4 and IL-13 although IL-10 were significantly increased (Mangan et al. 2006). Similarly, our previous studies on TNBS-induced colitis have demonstrated elevated IL-4 and IL-5 after egg administration. It may be concluded as bias of modification of IL-10 on Th2 cytokines in various models with different parasitic infections.

IL-10, a potent regulatory/suppressive cytokine, probably helps suppress the immunopathologic complications of such infection (Yazdanbakhsh and Matricardi 2004; Moore et al. 2001) and slows the progression of autoimmune and allergic diseases in experimental models (Wynn et al. 1997; Grunig et al. 1997). Helminth-infected mild asthmatics produce higher IL-10 in response to *Dermatophagoides pteronyssinus* antigen alongside lower Th2 responses than severe uninfected asthmatics (Araújo et al. 2004b). *Nippostrongylus brasiliensis* infection is able to inhibit lung inflammation and airway hyperresponsiveness (Wohleben et al. 2004), while IL-10-deficient mice remarkably aggravated allergen-induced airway eosinophilia and

other inflammatory parameters compared with control mice. The established schistosome infections induce an elevated IL-10 level which has been observed both in our study and other reports (Grzych et al. 1991). It has been argued that Th2 cells, monocytes, macrophages, and CD4⁺/CD25⁺ Treg are the potential source of IL-10 production following schistosome infection (Wohleben et al. 2004; Yang et al. 2006). These antigen-specific Tregs can regulate immune responses and inflammatory pathologies and it has been shown that CD4⁺/CD25⁺ Tregs protect mice against airway hyperreactivity in an IL-10-dependent manner (McKee and Pearce 2004). In our research, *S. japonicum* infection either with male or with female and male suppressed the development of allergen-induced airway inflammation. This effect may be mediated by IL-10 and the elevated IL-10 may be the result of modulation of Tregs, which is as part of an anti-inflammatory network.

Our data confirm and extend previous reports about the modulation of helminthes on the development of Th2 response. Furthermore, our results reveal both bisexual and male worm alone infection of *S. japonicum* can protect the mice from airway disorders induced by allergen. The suppression of parasitic infections should be potently induced by increasing production of regulatory cytokine IL-10. Previous studies have shown that lacto-*N*-fucopentase III and lacto-*N*-neotetraose, the schistosome oligosaccharide, function as inducing factors of both Th2-type response and production of IL-10 (Okano et al. 2001; Taylor et al. 2005), thereby suggest that oligosaccharide-associated mechanism may involved in the generation of anti-inflammatory mediators that suppress inflammatory response (Terrazas et al. 2001). The interaction between helminth infections and allergic disorders is complex and the regulatory mechanisms by which helminths suppress allergic disorders vary from parasite to parasite. Further studies are required to address between IL-10, Tregs, and the helminth-modulated type 2 response, and the identification of certain parasitic molecule(s) that may arise the protection against allergic and autoimmune diseases will lead to the development of new perspectives for the prevention of diseases characterized by an excessive immune response.

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