

## *Schistosoma mansoni* antigens modulate the allergic response in a murine model of ovalbumin-induced airway inflammation

L. S. Cardoso,\* S. C. Oliveira,<sup>†</sup>  
A. M. Góes,<sup>†</sup> R. R. Oliveira,\*  
L. G. Pacífico,<sup>†</sup> F. V. Marinho,<sup>†</sup>  
C. T. Fonseca,<sup>†</sup> F. C. Cardoso,<sup>†</sup>  
E. M. Carvalho\*<sup>‡§</sup> and M. I. Araujo\*<sup>‡§</sup>

\*Serviço de Imunologia, Hospital Universitário Prof Edgard Santos, Universidade Federal da Bahia, Salvador, BA, <sup>†</sup>Departamento de Imunologia e Bioquímica, ICB, Universidade Federal University de Minas Gerais, Belo Horizonte, MG, <sup>‡</sup>Escola Bahiana de Medicina e Saúde Pública, Salvador, BA, Brazil, and <sup>§</sup>Instituto Nacional de Ciência e Tecnologia de Doenças Tropicais (INCT-DT), Salvador, BA, Brazil

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Correspondence: M. I. Araujo, Serviço de Imunologia, Hospital Universitário Prof Edgard Santos, Universidade Federal da Bahia, Rua João das Botas, s/n Canela, 40110-160 Salvador – Bahia, Brazil.  
E-mail: mia@ufba.br

### Introduction

Allergic atopic disorders, such as asthma and rhinitis, result from genetic and environmental factors; there is a deregulated immune response, involving the T helper type 2 (Th2) cytokines interleukin (IL)-4, IL-5 and IL-13 and the Th1/proinflammatory cytokines interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$ . Asthma is a chronic inflammatory disease with high morbidity and mortality, characterized by recurrent episodes of airway obstruction and wheezing [1,2]. Currently, more than 300 million people have asthma worldwide, and the numbers are increasing [3].

Human populations with high rates of parasitic helminth infections have a low prevalence of allergic disorders [4–7]. Also, treatment with anti-helminthic drugs leads to an increase in the skin prick test response to aeroallergens [8,9].

### Summary

*Schistosoma mansoni* infection has been associated with protection against allergies. The mechanisms underlying this association may involve regulatory cells and cytokines. We evaluated the immune response induced by the *S. mansoni* antigens Sm22·6, PIII and Sm29 in a murine model of ovalbumin (OVA)-induced airway inflammation. BALB/c mice were sensitized with subcutaneously injected OVA-alum and challenged with aerolized OVA. Mice were given three doses of the different *S. mansoni* antigens. Lung histopathology, cellularity of bronchoalveolar lavage (BAL) and eosinophil peroxidase activity in lung were evaluated. Immunoglobulin (Ig)E levels in serum and cytokines in BAL were also measured. Additionally, we evaluated the frequency of CD4<sup>+</sup>forkhead box P3 (FoxP3)<sup>+</sup> T cells in cultures stimulated with OVA and the expression of interleukin (IL)-10 by these cells. The number of total cells and eosinophils in BAL and the levels of OVA-specific IgE were reduced in the immunized mice. Also, the levels of IL-4 and IL-5 in the BAL of mice immunized with PIII and Sm22·6 were decreased, while the levels of IL-10 were higher in mice immunized with Sm22·6 compared to the non-immunized mice. The frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells was higher in the groups of mice who received Sm22·6, Sm29 and PIII, being the expression of IL-10 by these cells only higher in mice immunized with Sm22·6. We concluded that the *S. mansoni* antigens used in this study are able to down-modulate allergic inflammatory mediators in a murine model of airway inflammation and that the CD4<sup>+</sup>FoxP3<sup>+</sup> T cells, even in the absence of IL-10 expression, might play an important role in this process.

**Keywords:** allergy, asthma, vaccines

Among helminths associated with protection against allergies, *Schistosoma mansoni* appears to induce particularly strong down-modulation of the inflammatory response that mediates atopic disorders [10]. In a 1-year follow-up study, we reported that asthmatics from a rural area endemic for schistosomiasis had fewer asthma symptoms when compared to those from a rural area in which there was no transmission of *S. mansoni* [11]. We also demonstrated that peripheral blood mononuclear cells (PBMC) from asthmatic individuals infected with *S. mansoni* produce higher levels of the anti-inflammatory cytokine IL-10 and lower levels of IL-4 and IL-5 after restimulation *in vitro* with the allergen *Dermatophagoides pteronyssinus* antigen 1 (Der p1), compared to asthmatics without helminthic infections [12].

Although the immune responses in both allergies and *S. mansoni* infection are predominantly of the Th2 type, high

IL-10 production has been found in *S. mansoni* infection [13,14], while there is reduced IL-10 production in asthma patients [15]. A number of anti-inflammatory effects have been reported for IL-10; it appears to protect against allergy [12,16–19]. Support for this idea was provided by the observation that immunotherapy success is associated with increased IL-10 levels [20,21]. The induction of regulatory responses and disease prevention by helminths or their products has been observed not solely for allergic diseases, but also for autoimmune disorders [19,22–24].

Several *S. mansoni* antigens have been tested as vaccines to prevent *S. mansoni* infection and to prevent liver pathology, including Sm22·6, PIII and Sm29 [25,26]. We tested the potential of these three antigens to down-modulate the inflammatory response in an ovalbumin (OVA)-induced model of airway inflammation. The Sm22·6 antigen is a soluble protein associated with the tegument of *S. mansoni*, present throughout the life cycle of this helminth, with the exception of the egg stage [27]. Pacifico *et al.* found that recombinant Sm22·6 induces partial protection (34·5%) against experimental *S. mansoni* infection and also induces high levels of IL-10 production [28]. PIII is a multivalent antigen obtained from the *S. mansoni* adult worm antigen (SWAP); it modulates granuloma size in mice infected with *S. mansoni* [29,30]. The third antigen used in this study, Sm29, is a membrane-bound glycoprotein found on the tegument of the adult worm during the lung stage of *S. mansoni* infection [31]. This protein induces a Th1 cytokine profile in mice and provides 50% protection against infection [32].

We have shown previously that Sm22·6 and PIII are able to induce IL-10 production in *S. mansoni*-infected individuals [33]; in the current study, we investigated whether these two antigens, as well as Sm29, are able to down-modulate the inflammatory allergic response in an experimental murine model of OVA-induced airway inflammation. We used the antigen IL-4-inducing principle of *S. mansoni* eggs (IPSE), which is a bioactive glycoprotein present in the soluble egg antigen (SEA), as a positive control because it induces activation of basophils and production of IL-4 and IL-13 [34], which are involved in the allergic inflammatory process.

**Materials and methods**

***S. mansoni* antigens**

The *S. mansoni* recombinant proteins, Sm22·6 and Sm29, and an *S. mansoni* soluble adult worm antigen fraction, PIII, were tested. The recombinant protein IPSE was used as control antigen. The recombinant proteins were produced in *Escherichia coli* and were tested for lipopolysaccharide (LPS) using a commercially available chromogenic LAL end-point assay kit (Cambrex, Charles City, IA, USA). The levels of LPS in Sm22·6, Sm29 and IPSE were below 1·2 endotoxin units (EU)/mg of protein. The antigen PIII were also tested for

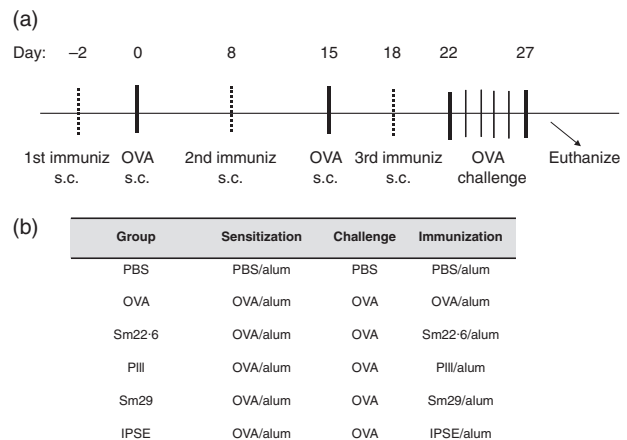
LPS contamination; the levels were under the detection limit of 0·01 EU/ml.

**Animals**

We used 6–8-week-old female BALB/c mice obtained from the Federal University of Minas Gerais (UFMG) animal facility. All protocols were reviewed and approved by the Ethics Committee on Animal Experiments of the Federal University of Minas Gerais.

**Immunization protocol**

To promote allergic airway inflammation, mice (five per group) were sensitized with 10 µg of OVA (Sigma-Aldrich, St Louis, MO, USA) in 1 mg of aluminium hydroxide gel (alum) by subcutaneous injection (days 0 and 15). On days 22–27, they were challenged with aerosolized OVA (1% solution for 30 min). The phosphate-buffered saline (PBS) group received PBS-alum instead of OVA-alum. The mice were immunized with 25 µg of the *S. mansoni* antigens Sm22·6, PIII, Sm29 and IPSE or PBS in 1 mg of alum through subcutaneous injection 2 days before and 8 and 18 days after injecting OVA (Fig. 1a). They were euthanized at day 28 and the immune response evaluated. The different groups of mice were designated according to the immunization protocol, as follows: OVA-sensitized non-immunized mice (OVA), OVA-sensitized Sm22·6-immunized mice (Sm22·6), OVA-sensitized PIII-immunized mice (PIII), OVA-sensitized Sm29-immunized mice (Sm29) and OVA-sensitized IPSE-immunized mice



**Fig. 1.** Induction of airway inflammation in mice and immunization with *Schistosoma mansoni* antigens. BALB/c mice were sensitized with subcutaneous injections of 10 µg of ovalbumin (OVA) in 1 mg of alum on days 0 and 15, and then challenged with OVA aerosol (1% OVA for 30 min) from days 22 to 27. The animals were euthanized on day 28 for the analyses (a). Different groups of mice were immunized with the antigens Sm22·6, PIII, Sm29 and IL-4-inducing principle of *S. mansoni* eggs (IPSE). The negative control group was immunized with phosphate-buffered saline (PBS)/alum (b). Five mice were included in each group and two independent experiments were performed.

(IPSE). Mice that received PBS-alum instead of OVA and *S. mansoni* antigens in the sensitization and immunization protocol were designated the PBS group (Fig. 1b).

### Brochoalveolar lavage

The lungs were washed by cannulating the trachea and gently injecting/recovering (3×) 1.0 ml of PBS. The bronchoalveolar lavage fluid (BAL) was centrifuged at 300 g at 4°C for 5 min and the supernatants were stored at -20°C for cytokine analysis. The cell pellet was resuspended in 0.1 ml of 3% bovine serum albumin (BSA) and cells counted using a haemocytometer. The cells were then cytocentrifuged and stained with haematoxylin and eosin (H&E) for differential counting based on cell morphology and staining patterns. The means of three independent counts of 100 cells in a randomized field were shown.

### Lung histopathology

Following bronchoalveolar lavage, the lungs were fixed with formalin. Serial sagittal sections of whole lung (3–4 µm thick) were cut and stained with Gomori trichrome for light microscopy. At least 10 fields were selected randomly and examined. The severity of the inflammatory process in the lungs was scored by two pathologists who were blinded to group identity. The scale varied from 0 to 5 as follows: 0, no inflammation; 1, minimal; 2, mild; 3, medium; 4, moderate; and 5, marked [35,36].

### Eosinophil peroxidase (EPO) assay

The EPO assay was performed as described previously [37]. Briefly, a 100-mg sample of tissue from each lung was homogenized in 1.9 ml of PBS and centrifuged at 12 000 g for 10 min. The supernatant was discarded and the erythrocytes were lysed. The samples were centrifuged, the supernatant discarded and the pellet resuspended in 1.9 ml of 0.5% hexadecyltrimethyl ammonium bromide in PBS saline. The samples were frozen in liquid nitrogen and centrifuged at 4°C at 12 000 g for 10 min. The supernatant was used for the enzymatic assay. Briefly, *o*-phenylenediamine (OPD) (10 mg) was dissolved in 5.5 ml distilled water, and then 1.5 ml of OPD solution was added to 8.5 ml of Tris buffer (pH 8.0), followed by addition of 7.5 µl H<sub>2</sub>O<sub>2</sub>. In a 96-well plate, 100 µl of substrate solution was added to 50 µl of each sample. After 30 min, the reaction was stopped with 50 µl of 1 M H<sub>2</sub>SO<sub>4</sub> and the absorbance was read at 492 nm.

### Cytokine measurements

Levels of IL-4, IL-5, IL-10, TNF-α and IFN-γ were determined by bronchoalveolar lavage (BAL) of the different groups of mice with an enzyme-linked immunosorbent assay (ELISA) sandwich technique using commercially available kits (OptEIA; BD Bioscience, San Jose, CA, USA), according to the manufacturer's protocol. The optical density (OD) values

were read at 450 nm. The results were expressed as picograms per millilitre, compared to a standard curve.

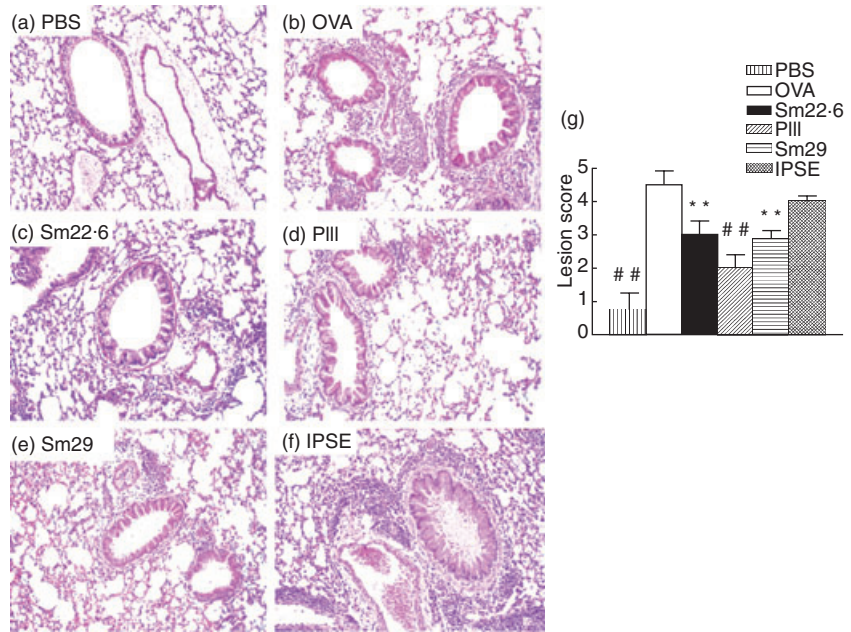
### Determination of OVA-specific immunoglobulin (Ig)E levels

The levels of OVA-specific IgE in serum were determined by ELISA, as described previously [38,39]. Briefly, Maxisorp 96-well microtitre plates (NUNC, Roskilde, Denmark) were coated with rat anti-mouse unlabelled IgE (1 : 250; Southern Biotechnology, AL, USA) in pH 9.6 carbonate-bicarbonate buffer for 12–16 h at 4°C and then blocked for 1 h at room temperature with 200 µl/well of 0.25% PBS-casein. Fifty microlitres of each serum was added per well and incubated for 2 h at room temperature. The samples were then incubated with 50 µl/well of OVA-biotin (1 mg/ml; Sigma, St Louis, MO, USA) at room temperature for 1 h. Plate-bound antibody was detected by treatment with 50 µl/well of streptavidin-horseradish peroxidase (1 : 10 000; Southern Biotechnology) for 1 h at room temperature. The colour reaction was developed by adding 100 µl/well of 200 pmol of OPD (Sigma) in pH 5.0 citrate phosphate buffer plus 0.04% H<sub>2</sub>O<sub>2</sub> for 10 min and stopped with 50 µl of 5% sulphuric acid per well. The plates were read at 492 nm in an ELISA reader (Bio-Rad, Hercules, CA, USA).

### Cell staining and flow cytometry

The lungs of five mice per group were removed and treated with 100 U/ml of collagenase from *Clostridium histolyticum* (Sigma) for 30 min at 37°C. Subsequently, the digested lung tissue was filtered through a 70 micrometre cell strainer and the red blood cells were lysed with ACK buffer (0.15 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA, pH 7.2; Invitrogen, CA, USA). The cell suspension was washed twice in RPMI-1640 and adjusted to 1 × 10<sup>6</sup> cells per well for surface staining and to 2.5 × 10<sup>6</sup> cells for the intracellular cytokine experiment. For CD4 and forkhead box P3 (FoxP3) staining, the cells were generally blocked with anti-mouse CD16/CD32 monoclonal antibodies (mAbs) (Fc-block) and stained for surface marker using fluorescein isothiocyanate (FITC)-labelled anti-mouse CD4 (BD Bioscience) mAb or isotype control, which were incubated for 20 min at 4°C with antibody dilution solution (PBS 0.15 M, 0.5% BSA, 2 mM NaN<sub>3</sub>). The cells were then washed with 0.15 M PBS and incubated with streptavidin-phycoerythrin-cyanine 5 (PE-Cy5) (1 : 200) for an additional 20 min at 4°C. Surface-stained cells were washed twice with 0.15 M PBS and incubated with fixation/permeabilization buffer (eBioscience) for 30 min at 4°C. Anti-FoxP3-PE-labelled antibodies in permeabilization buffer (eBioscience) were added to cells and then incubated for 30 min at 4°C. Cells were washed twice with 150 µl permeabilization buffer (eBioscience) and fixed with 2% paraformaldehyde. For IL-10 and FoxP3 intracellular staining, cells were cultured for 14 h in medium or OVA (25 µg/ml). After this stimulation period, 1 mg/ml of brefeldin A was

**Fig. 2.** Representative lung sections of ovalbumin (OVA)-induced airway inflammation in BALB/c mice at 20× magnification. Lung sections of mice that received only phosphate-buffered saline (PBS)/alum (a). Lung section of non-immunized mice (OVA group) showing marked infiltration of inflammatory cells in peribronchiolar space (b). Lung sections of mice immunized with Sm22-6, PIII, Sm29 and IL-4-inducing principle of *S. mansoni* eggs (IPSE) (c–f, respectively). The inflammatory process was scored from 0 to 5, 5 being the most severe inflammation (g). The OVA group had significantly higher scores compared to the groups immunized with Sm22-6, PIII, Sm29 and to the control group. \*\* $P < 0.01$  and #  $P < 0.001$  versus OVA group.



added to the cell culture, which was incubated for an additional 4 h in a CO<sub>2</sub> incubator at 37°C. Before CD4 staining, the cells were treated with anti-CD16/CD32 (Fc-block). Cell surface and intracellular staining were performed as described above for surface experiments; however, the cells were stained for CD4, IL-10 and FoxP3 using anti-CD4 FITC-labelled, anti-IL-10 PE-labelled, and anti-FoxP3 biotin-labelled plus streptavidin-PE-Cy5 antibodies. Data acquisition was performed using fluorescence activated cell sorter (FACScan) (Becton Dickinson, San Jose, CA, USA). Data analysis was performed using a FlowJO interface (Becton Dickinson).

### Statistical analysis

Statistical analysis was performed using the software GraphPad Prism (GraphPad Software, San Diego, CA, USA). The mean ± standard deviation (s.d.) of the number of cells and the antibody, EPO and cytokine concentrations were calculated. A one-way analysis of variance (ANOVA) was used to compare the levels of cytokines, IgE and EPO between groups. Fisher's exact test was used to compare proportions. The alpha level for statistical significance was established as 5%.

## Results

### Immunization with *S. mansoni* antigens Sm22-6, PIII and Sm29 results in modulation of OVA-induced airway inflammation in mice

The severity of the inflammatory response to OVA was evaluated in the lungs of mice immunized with *S. mansoni* antigens and in control mice. A dense mixed-cellular infiltrate surrounding the airway was observed in the

sensitized non-immunized mice (Fig. 2b) and in the IPSE-immunized group (Fig. 2f). Comparatively, much less peribronchial airway inflammation was observed in OVA-sensitized mice immunized with Sm22-6, PIII and Sm29, and in non-sensitized mice that were treated with PBS (Fig. 2c,d,e,a, respectively).

### Modulatory effect of *S. mansoni* antigen immunization on cellularity in BAL

Mice immunized with the *S. mansoni* antigens Sm22-6, PIII and Sm29 had significantly fewer total cells and eosinophils in the BAL fluid than did non-immunized mice and mice immunized with IPSE, while there was no significant difference in the number of neutrophils, lymphocytes and macrophages between groups (Table 1).

### Immunization with *S. mansoni* antigens down-regulates OVA-specific IgE production and peroxidase activity in eosinophils

The serum levels of OVA-specific IgE were measured in sensitized non-immunized mice and in those immunized with the different *S. mansoni* antigens. The levels of this isotype were markedly lower in *S. mansoni* antigen-immunized mice than in sensitized non-immunized mice (Fig. 3a).

The levels of eosinophil peroxidase (EPO) were also significantly lower in the lungs of mice immunized with Sm22-6 and PIII than in the non-immunized group (Fig. 3b).

### Cytokine response induced by *S. mansoni* antigens

We measured the cytokines IL-4, IL-5 and IL-10 in BAL fluid. The levels of IL-4 and IL-5 were lower in mice immunized with Sm22-6 and PIII compared to non-immunized mice

**Table 1.** Total cells and differential cell count ( $\times 10^5$ ) in BAL of OVA-sensitized immunized and non-immunized mice.

	Total cells	Eosinophils	Neutrophils	Lymphocytes	Macrophages
PBS	10.2 $\pm$ 6.8	0.1 $\pm$ 0.05 <sup>#</sup>	2.8 $\pm$ 1.3	3.9 $\pm$ 3.2	5.9 $\pm$ 3.6
OVA	69 $\pm$ 18	55 $\pm$ 19	2.2 $\pm$ 1.4	7.0 $\pm$ 2.2	4.0 $\pm$ 1.9
Sm22-6	30 $\pm$ 12*	12 $\pm$ 3*	3.9 $\pm$ 2.2	10.4 $\pm$ 7.4	7.7 $\pm$ 6.6
PIII	27 $\pm$ 8*	6.9 $\pm$ 5*	3.5 $\pm$ 1.0	10.1 $\pm$ 3.4	6.7 $\pm$ 3.2
Sm29	36 $\pm$ 6*	16 $\pm$ 3*	3.3 $\pm$ 1.8	14 $\pm$ 1.8	5.3 $\pm$ 0.8
IPSE	68 $\pm$ 21	52 $\pm$ 29	3.2 $\pm$ 1.9	7.5 $\pm$ 3	5.4 $\pm$ 2.0

Values expressed by mean  $\pm$  standard deviation; # $P < 0.001$  and \* $P < 0.05$  versus OVA group. PBS, phosphate-buffered saline; OVA, ovalbumin; IPSE, IL-4-inducing principle of *S. mansoni* eggs.

(Fig. 4a,b, respectively). The levels of IL-10 were higher in BAL of Sm22-6 immunized mice than in non-immunized mice (Fig. 4c). In order to evaluate the imbalance of the regulatory and the Th2 profile of cytokine, we performed the ratio between the levels of IL-10 and IL-4 in BAL. We observed that in mice immunized with Sm22-6 and with PIII the ratio IL-10/IL-4 was higher than in non-immunized mice (Fig. 4e).

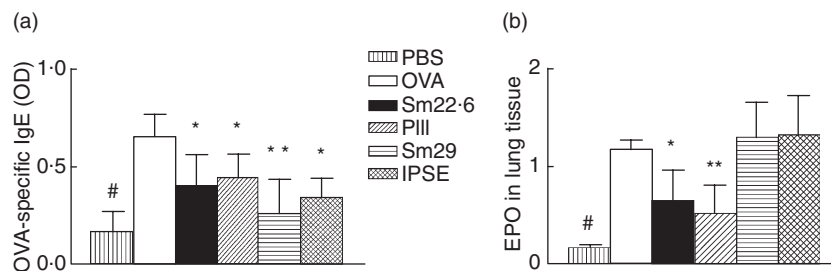
Along with the Th2 and regulatory cytokines, we also measured IFN- $\gamma$  and TNF- $\alpha$  in BAL fluid. The levels of IFN- $\gamma$  were lower in mice immunized with Sm29 (40  $\pm$  10 pg/ml) when compared to the non-immunized mice (120  $\pm$  40 pg/ml), while in the other groups the levels of this cytokine did not differ significantly from what was observed in non-immunized mice (Fig. 4d). The levels of TNF- $\alpha$  were below 50 pg/ml in all groups of mice.

#### Frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells and CD4<sup>+</sup>FoxP3<sup>+</sup>IL-10<sup>+</sup> expressing cells

The frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells and of CD4<sup>+</sup>FoxP3<sup>+</sup>IL-10<sup>+</sup> T cells in cultures stimulated with OVA was evaluated in the different groups of mice. We found that the frequency of the CD4<sup>+</sup>FoxP3<sup>+</sup> T cells was significantly higher in mice immunized with Sm22-6 and PIII. There was a tendency of higher expression of these cells in mice immunized with Sm29 ( $P = 0.06$ ) (Fig. 5a). Conversely, the frequency of these cells expressing IL-10 was higher only in mice immunized with Sm22-6 when compared to the OVA group (Fig. 5b).

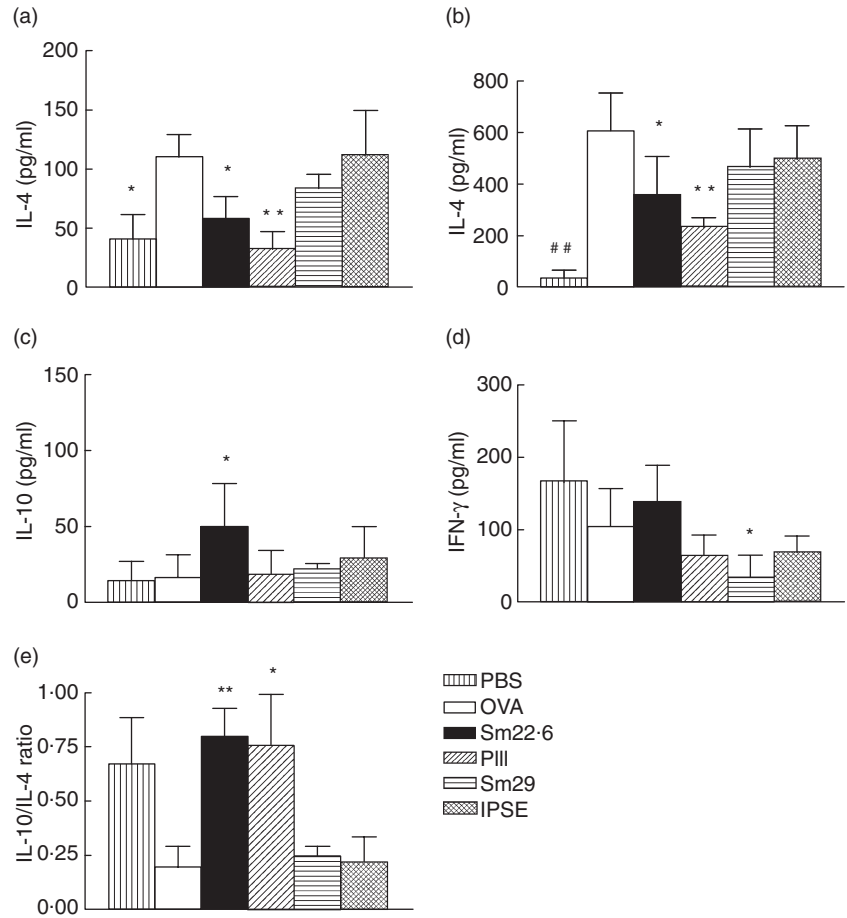
#### Discussion

There is evidence that *Schistosoma* sp. infection protects humans [11] and mice against allergic asthma [36,38,40]. We evaluated whether the *S. mansoni* antigens Sm22-6, PIII and Sm29 could down-modulate the inflammatory allergic response in a murine model of OVA-induced airway inflammation. We found that immunization with these *S. mansoni* antigens protected mice against allergic inflammation; there was a strong reduction in the number of inflammatory cells and eosinophils recruited to the airways. Moreover, the levels of OVA-specific IgE production were also decreased in mice immunized with Sm22-6, PIII and Sm29, and the levels of EPO in the lungs were lower in mice immunized with Sm22-6 and PIII, compared to the non-immunized mice. Additionally, we found that these two antigens have an important immunomodulatory effect on the production of the Th2 cytokines IL-4 and IL-5, evidenced by the lower levels of these cytokines in the BAL of immunized compared to non-immunized mice. IL-5 is a well-known cytokine that induces the production of eosinophils and activates these cells, while IL-4 acts on B cells, inducing IgE synthesis. This antibody participates in the pathogenesis of asthma by binding to mast cells, basophils and eosinophils, which release inflammatory mediators upon contact with the allergen. We observed lower levels of allergen-specific IgE in the groups of *S. mansoni* antigen-immunized mice compared to non-immunized mice. This is additional evidence that these antigens are able to prevent Th2-mediated inflammation.

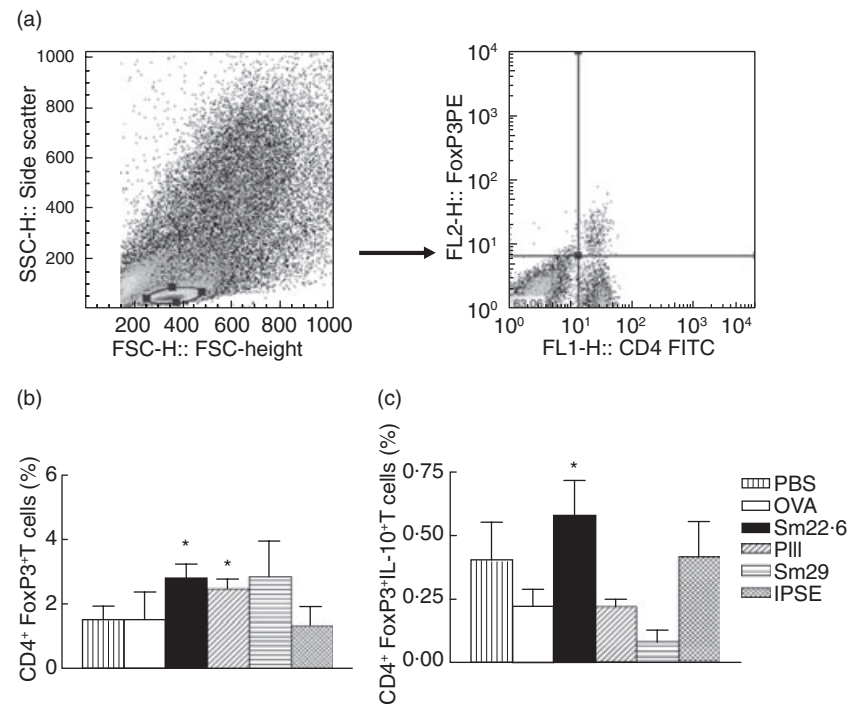


**Fig. 3.** Levels of ovalbumin (OVA)-specific immunoglobulin (IgE) in the serum (a). The data are presented as the mean optical density (OD) at 492 nm  $\pm$  standard deviation (s.d.). Levels of eosinophil peroxidase (EPO) in the lungs of non-immunized mice and mice immunized with the antigens Sm22-6, PIII, Sm29 and phosphate-buffered saline (PBS), respectively (b). The results are expressed as mean  $\pm$  s.d. Five mice per group were evaluated and two separate experiments were performed. \* $P < 0.05$ , \*\* $P < 0.01$  and # $P < 0.005$  versus OVA group.

**Fig. 4.** Effect of *Schistosoma mansoni* antigen immunization on cytokine production in bronchoalveolar lavage (BAL) fluid. Levels of interleukin (IL)-4 (a), IL-5 (b), IL-10 (c) and interferon (IFN)- $\gamma$  (d) in the different groups of mice. Lower levels of IL-4 and IL-5 were observed in the BAL of mice immunized with Sm22-6 and PIII, while higher levels of IL-10 were found in Sm22-6-immunized mice when compared to the non-immunized group. We observed that in mice immunized with Sm22-6 and with PIII the ratio IL-10/IL-4 in BAL was higher than in non-immunized mice (e). The figures represent the mean  $\pm$  standard deviation of five mice per group. Two separate experiments were performed. \* $P < 0.05$ , \*\* $P < 0.01$  and ## $P < 0.005$  versus ovalbumin (OVA) group.



**Fig. 5.** Frequency of CD4<sup>+</sup>forkhead box P3 (Foxp3)<sup>+</sup> T cells in cultures stimulated with ovalbumin (OVA) (a) and of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells expressing interleukin (IL)-10 (b) in mice immunized with the different *Schistosoma mansoni* antigens. Cell suspensions isolated from lungs of mice were stained for surface expression of CD4 and for intracellular expression of FoxP3 and IL-10 and then analysed by flow cytometry in the lymphocyte region of the forward/side-scatter plot. (a) Represents the gating strategy for the analysis of CD4<sup>+</sup>FoxP3<sup>+</sup> frequency. The lymphocyte region was determined in the forward/side-scatter plot and then a dot-plot was accessed to determine CD4<sup>+</sup>FoxP3<sup>+</sup> T cells frequency. The frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells was higher in mice immunized with Sm22-6 and PIII compared to non-immunized mice (b), while expression of IL-10 was seen only in CD4<sup>+</sup>FoxP3<sup>+</sup> T cells of Sm22-6 mice (c). The figures represent the mean and standard deviation from five mice in each group. \* $P < 0.05$  versus OVA group.



In mice immunized with Sm29, despite the fact that there was a reduction in the number of inflammatory cells, eosinophils and OVA-specific IgE compared to non-immunized mice, the decrease in the levels of IL-4 and IL-5 in BAL did not reach statistical significance. The slight decrease in IL-4 and IL-5 production might be effective to reduce IgE production and eosinophils growth or recruitment without, however, being sufficient to alter EPO activity in lung tissue.

We used the antigen IPSE as our positive control as this antigen is an inducer of the Th2 response in schistosomiasis [34]. As expected, in mice immunized with IPSE the Th2 parameters such as number of eosinophils, levels of EPO, IL-4 and IL-5 were comparable to asthmatic non-immunized mice; unpredictably, however, in IPSE-immunized mice there was a reduction in the levels of OVA-specific IgE.

This study examined inflammatory mediators responsible for lung airway inflammation in a murine model of OVA-induced allergic asthma, and found an eosinophil infiltration of the airway. Eosinophils play a pivotal role in the airway inflammation in asthma and they correlate with the extent of inflammatory process in the lung parenchyma [41]. Lung function could not be evaluated in the present study. Lung inflammation and airway hyperreactivity (AHR) are two distinct characteristics of asthma [42–46]. Indeed, it has been demonstrated that methacholine-induced AHR in mouse models correlates with an antigen-specific Th2 immune response [46–49], but not with severity of eosinophilic lung inflammation [47,50].

It has been reported that IL-10 is the main cytokine involved in suppression of Th2 allergic inflammation due to helminth infection [12,40]. We evaluated the levels of this cytokine in BAL of sensitized mice. Although the levels of this cytokine were higher only in mice immunized with Sm22-6, the ratio IL-10/IL-4 was higher in mice immunized with Sm22-6 and also with PIII compared to non-immunized mice. In fact, it is possible that IL-10 may not be the only mechanism involved in down-modulation of the allergic inflammatory response in *S. mansoni* antigen-immunized mice. Indeed, suppression of inflammatory cell migration to the airways and down-modulation of IgE production were seen in mice immunized with Sm29 compared to non-immunized mice, despite the low levels of IL-10 in BAL. The possibility that there are other modulatory mediators that act independently of IL-10 is supported by our previous demonstration that regulatory T cells of *S. mansoni*-infected mice protect against allergen-induced airway inflammation through an IL-10-independent mechanism [38]. While infection with *Nippostrongylus brasiliensis* has been found to suppress airway inflammation in an IL-10-dependent manner [51], other researchers have found that *N. brasiliensis* products inhibit an allergic response in the airways of mice, independently of the levels of IL-10 [52]. Therefore, for the same parasites, different modulatory mechanisms of the allergic response may exist. In this study

the frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells was significantly higher in mice immunized with Sm22-6 and PIII. There was a trend towards increased frequency of these cells in mice immunized with Sm29, compared to non-immunized mice. However, only in mice immunized with Sm22-6 was there a significantly higher frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells expressing IL-10 compared to non-immunized mice. In agreement with these data, higher levels of IL-10 in BAL relative to non-immunized group was also observed only in mice immunized with Sm22-6. It is possible that the CD4<sup>+</sup>FoxP3<sup>+</sup> T cells could be acting through cell–cell contact to inhibit Th2<sup>+</sup> inflammatory mediators in the other groups of mice. Indeed, in the group of mice immunized with Sm29 we did not observe an increase in IL-10 production; nevertheless, there was a reduction in eosinophil infiltration and in the OVA-specific IgE levels.

We found no increase in the levels of the Th1 cytokines IFN- $\gamma$  and TNF in the BAL of immunized mice compared to non-immunized ones. These data argue in favour that down-modulation of the Th2 response by the parasite antigens was not due to an increase in Th1 response. This is an important finding, because Th1 cytokines could contribute to the inflammatory process in severe asthma [53]. Other studies have demonstrated that helminth infections or antigens down-regulate the allergic response through the action of regulatory T cells, rather than by altering the Th1/Th2 balance [23,38].

In conclusion, we showed that *S. mansoni* antigens Sm22-6, PIII and Sm29 are able to down-modulate the inflammatory response in a model of allergic airway inflammation and we suggest that the CD4<sup>+</sup>FoxP3<sup>+</sup> T cells might be involved in this modulation. Studies evaluating other mechanisms underlying the modulatory effect of *S. mansoni* antigens on the allergic inflammation are in progress; they may contribute to the development of new strategies to prevent allergic diseases.

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## Disclosure

The authors have no financial conflict of interest.

## References

- 1 Pearce N, Douwes J, Beasley R. The rise and rise of asthma: a new paradigm for the new millennium? *J Epidemiol Biostat* 2000; 5:5–16.

- 2 Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000; **161**:1720–45.
- 3 Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA dissemination committee report. *Allergy* 2004; **59**:469–78.
- 4 Lynch NR, Hagel IA, Palenque ME *et al.* Relationship between helminthic infection and IgE response in atopic and nonatopic children in a tropical environment. *J Allergy Clin Immunol* 1998; **101**:217–21.
- 5 Hagel I, Lynch NR, Perez M, Di Prisco MC, Lopez R, Rojas E. Modulation of the allergic reactivity of slum children by helminthic infection. *Parasite Immunol* 1993; **15**:311–15.
- 6 Araujo MI, Lopes AA, Medeiros M *et al.* Inverse association between skin response to aeroallergens and *Schistosoma mansoni* infection. *Int Arch Allergy Immunol* 2000; **123**:145–8.
- 7 Cooper PJ, Chico ME, Rodrigues LC *et al.* Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. *J Allergy Clin Immunol* 2003; **111**:995–1000.
- 8 Lynch NR, Hagel I, Perez M, Di Prisco MC, Lopez R, Alvarez N. Effect of anthelmintic treatment on the allergic reactivity of children in a tropical slum. *J Allergy Clin Immunol* 1993; **92**:404–11.
- 9 Cooper PJ, Chico ME, Bland M, Griffin GE, Nutman TB. Allergic symptoms, atopy, and geohelminth infections in a rural area of Ecuador. *Am J Respir Crit Care Med* 2003; **168**:313–17.
- 10 Araujo MI, de Carvalho EM. Human schistosomiasis decreases immune responses to allergens and clinical manifestations of asthma. *Chem Immunol Allergy* 2006; **90**:29–44.
- 11 Medeiros M Jr, Figueiredo JP, Almeida MC *et al.* *Schistosoma mansoni* infection is associated with a reduced course of asthma. *J Allergy Clin Immunol* 2003; **111**:947–51.
- 12 Araujo MI, Hoppe B, Medeiros M *et al.* Impaired T helper 2 response to aeroallergen in helminth-infected patients with asthma. *J Infect Dis* 2004; **190**:1797–803.
- 13 Araujo MI, de Jesus AR, Bacellar O, Sabin E, Pearce E, Carvalho EM. Evidence of a T helper type 2 activation in human schistosomiasis. *Eur J Immunol* 1996; **26**:1399–403.
- 14 Correa-Oliveira R, Malaquias LC, Falcao PL *et al.* Cytokines as determinants of resistance and pathology in human *Schistosoma mansoni* infection. *Braz J Med Biol Res* 1998; **31**:171–7.
- 15 Borish L, Aarons A, Rumbly J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol* 1996; **97**:1288–96.
- 16 Adachi M, Oda N, Kokubu F, Minoguchi K. IL-10 induces a Th2 cell tolerance in allergic asthma. *Int Arch Allergy Immunol* 1999; **118**:391–4.
- 17 Akdis CA, Joss A, Akdis M, Blaser K. Mechanism of IL-10-induced T cell inactivation in allergic inflammation and normal response to allergens. *Int Arch Allergy Immunol* 2001; **124**:180–2.
- 18 van den Biggelaar AH, van Ree R, Rodrigues LC *et al.* Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. *Lancet* 2000; **356**:1723–7.
- 19 Kitagaki K, Businga TR, Racila D, Elliott DE, Weinstock JV, Kline JN. Intestinal helminths protect in a murine model of asthma. *J Immunol* 2006; **177**:1628–35.
- 20 Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; **102**:98–106.
- 21 Akdis CA, Blaser K. [Principles of specific immunotherapy of IgE-induced allergic reactions]. *Ther Umsch* 2001; **58**:274–7.
- 22 Elliott DE, Li J, Blum A *et al.* Exposure to schistosome eggs protects mice from TNBS-induced colitis. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**:G385–91.
- 23 Itami DM, Oshiro TM, Araujo CA *et al.* Modulation of murine experimental asthma by *Ascaris suum* components. *Clin Exp Allergy* 2005; **35**:873–9.
- 24 Vissers JL, van Esch BC, Hofman GA, Kapsenberg ML, Weller FR, van Oosterhout AJ. Allergen immunotherapy induces a suppressive memory response mediated by IL-10 in a mouse asthma model. *J Allergy Clin Immunol* 2004; **113**:1204–10.
- 25 Oliveira DM, Gustavson S, Silva-Teixeira DN, Goes AM. Nitric oxide and IL-10 production induced by PIII – a fraction of *Schistosoma mansoni* adult worm antigenic preparation – associated with downregulation of *in vitro* granuloma formation. *Hum Immunol* 1999; **60**:305–11.
- 26 Oliveira DM, Silva-Teixeira DN, Gustavson S, Oliveira SM, Goes AM. Nitric oxide interaction with IL-10, MIP-1alpha, MCP-1 and RANTES over the *in vitro* granuloma formation against different *Schistosoma mansoni* antigenic preparations on human schistosomiasis. *Parasitology* 2000; **120**:391–8.
- 27 Jeffs SA, Hagan P, Allen R, Correa-Oliveira R, Smithers SR, Simpson AJ. Molecular cloning and characterisation of the 22-kilodalton adult *Schistosoma mansoni* antigen recognised by antibodies from mice protectively vaccinated with isolated tegumental surface membranes. *Mol Biochem Parasitol* 1991; **46**:159–67.
- 28 Pacifico LG, Fonseca CT, Chiari L, Oliveira SC. Immunization with *Schistosoma mansoni* 22-6 kDa antigen induces partial protection against experimental infection in a recombinant protein form but not as DNA vaccine. *Immunobiology* 2006; **211**:97–104.
- 29 Hirsch C, Goes AM. Characterization of fractionated *Schistosoma mansoni* soluble adult worm antigens that elicit human cell proliferation and granuloma formation in vitro. *Parasitology* 1996; **112**:529–35.
- 30 Hirsch C, Zouain CS, Alves JB, Goes AM. Induction of protective immunity and modulation of granulomatous hypersensitivity in mice using PIII, an anionic fraction of *Schistosoma mansoni* adult worm. *Parasitology* 1997; **115**:21–8.
- 31 Cardoso FC, Pacifico RN, Mortara RA, Oliveira SC. Human antibody responses of patients living in endemic areas for schistosomiasis to the tegumental protein Sm29 identified through genomic studies. *Clin Exp Immunol* 2006; **144**:382–91.
- 32 Cardoso FC, Macedo GC, Gava E *et al.* *Schistosoma mansoni* tegument protein Sm29 is able to induce a Th1-type of immune response and protection against parasite infection. *PLoS Negl Trop Dis* 2008; **2**:e308.
- 33 Cardoso LS, Oliveira SC, Pacifico LG *et al.* *Schistosoma mansoni* antigen-driven interleukin-10 production in infected asthmatic individuals. *Mem Inst Oswaldo Cruz* 2006; **101** (Suppl. 1):339–43.
- 34 Schramm G, Falcone FH, Gronow A *et al.* Molecular characterization of an interleukin-4-inducing factor from *Schistosoma mansoni* eggs. *J Biol Chem* 2003; **278**:18384–92.
- 35 Siddiqui S, Morris J, Avery N, Wyand S, Rood D, Silbart LK. Pulmonary eosinophilia correlates with allergen deposition to the lower respiratory tract in a mouse model of asthma. *Clin Exp Allergy* 2008; **38**:1381–90.
- 36 Mo HM, Lei JH, Jiang ZW *et al.* *Schistosoma japonicum* infection modulates the development of allergen-induced airway inflammation in mice. *Parasitol Res* 2008; **103**:1183–9.



- 37 Strath M, Warren DJ, Sanderson CJ. Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. *J Immunol Meth* 1985; **83**:209–15.
- 38 Pacifico LG, Marinho FA, Fonseca CT *et al.* *Schistosoma mansoni* antigens modulate experimental allergic asthma in a murine model: a major role for CD4+CD25+Foxp3+ T cells independent of IL-10. *Infect Immun* 2009; **77**:98–107.
- 39 Fallon PG, Dunne DW. Tolerization of mice to *Schistosoma mansoni* egg antigens causes elevated type 1 and diminished type 2 cytokine responses and increased mortality in acute infection. *J Immunol* 1999; **162**:4122–32.
- 40 Mangan NE, van Rooijen N, McKenzie AN, Fallon PG. Helminth-modified pulmonary immune response protects mice from allergen-induced airway hyperresponsiveness. *J Immunol* 2006; **176**:138–47.
- 41 Gavett SH, Chen X, Finkelman F, Wills-Karp M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994; **10**:587–93.
- 42 Gleich GJ. The eosinophil and bronchial asthma: current understanding. *J Allergy Clin Immunol* 1990; **85**:422–36.
- 43 Gleich GJ, Flavahan NA, Fujisawa T, Vanhoutte PM. The eosinophil as a mediator of damage to respiratory epithelium: a model for bronchial hyperreactivity. *J Allergy Clin Immunol* 1988; **81**:776–81.
- 44 Gonzalo JA, Lloyd CM, Wen D *et al.* The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* 1998; **188**:157–67.
- 45 Negrao-Correa D, Silveira MR, Borges CM, Souza DG, Teixeira MM. Changes in pulmonary function and parasite burden in rats infected with *Strongyloides venezuelensis* concomitant with induction of allergic airway inflammation. *Infect Immun* 2003; **71**:2607–14.
- 46 Epstein MM. Do mouse models of allergic asthma mimic clinical disease? *Int Arch Allergy Immunol* 2004; **133**:84–100.
- 47 Whitehead GS, Walker JK, Berman KG, Foster WM, Schwartz DA. Allergen-induced airway disease is mouse strain dependent. *Am J Physiol Lung Cell Mol Physiol* 2003; **285**:L32–42.
- 48 Takeda K, Haczku A, Lee JJ, Irvin CG, Gelfand EW. Strain dependence of airway hyperresponsiveness reflects differences in eosinophil localization in the lung. *Am J Physiol Lung Cell Mol Physiol* 2001; **281**:L394–402.
- 49 Brewer JP, Kisselgof AB, Martin TR. Genetic variability in pulmonary physiological, cellular, and antibody responses to antigen in mice. *Am J Respir Crit Care Med* 1999; **160**:1150–6.
- 50 Renz H, Smith HR, Henson JE, Ray BS, Irvin CG, Gelfand EW. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J Allergy Clin Immunol* 1992; **89**:1127–38.
- 51 Wohlleben G, Trujillo C, Muller J *et al.* Helminth infection modulates the development of allergen-induced airway inflammation. *Int Immunol* 2004; **16**:585–96.
- 52 Trujillo-Vargas CM, Werner-Klein M, Wohlleben G *et al.* Helminth-derived products inhibit the development of allergic responses in mice. *Am J Respir Crit Care Med* 2007; **175**:336–44.
- 53 Stephens R, Randolph DA, Huang G, Holtzman MJ, Chaplin DD. Antigen-nonspecific recruitment of Th2 cells to the lung as a mechanism for viral infection-induced allergic asthma. *J Immunol* 2002; **169**:5458–67.