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Modulation of murine experimental asthma by *Ascaris suum* components

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Summary

Background We have recently isolated two distinct components from *Ascaris suum* adult worms with different effects on the immune system: the allergenic protein of *A. suum* (APAS-3), which induces IgE antibody production, and suppressive protein of *A. suum* (PAS-1), which inhibits humoral and cellular immune responses induced by unrelated antigens. In this study, we investigated the immunomodulatory effect of PAS-1 on a murine model of asthma induced by APAS-3.

Methods BALB/c mice were immunized twice with APAS-3 or APAS-3 plus PAS-1 by the intraperitoneal and subcutaneous route (on days 0 and 7) and challenged twice with the same antigens intranasally (days 14 and 21). Two days after the last challenge, the allergic airway inflammation was evaluated by cellular migration, eosinophil peroxidase (EPO) activity, cytokine and chemokine production and pulmonary mechanical parameters.

Results The allergenic properties of APAS-3 were confirmed by the stimulation of anaphylactic IgE and IgG1 antibody production and eosinophilic airway inflammation and hyper-responsiveness. On the other hand, PAS-1-treated mice showed a marked suppression of cellular migration and EPO activity that correlated well with a significant reduction in the levels of IL-4, IL-5, eotaxin and RANTES in the bronchoalveolar lavage (BAL) fluid. In contrast, considerable amounts of IL-10 were observed in the BAL fluid of PAS-1-treated mice. Airway hyper-responsiveness was obtained in APAS-3-immunized mice, but the conductance of the respiratory system was restored to normal values in the presence of PAS-1.

Conclusion These results indicate that *A. suum* allergenic protein APAS-3 induces a T helper 2-type immune response and, consequently, eosinophilic airway inflammation and hyper-responsiveness. Moreover, the modulatory protein PAS-1 has a marked suppressive effect on this response, and the inhibition of cytokine (IL-4, IL-5) and chemokine (eotaxin and RANTES) release, probably because of the presence of IL-10, may contribute to this effect.

Keywords anaphylactic antibodies, *Ascaris suum*, eosinophilic inflammation, hyper-responsiveness, immunomodulation

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Introduction

Allergic asthma is a disease of the airways characterized by an inappropriate immune response to innocuous proteins or allergens. The immune response to allergen is represented by eosinophilic inflammation of the airways, airway hyper-reactivity (AHR) and antigen-specific IgE production.

The inflammatory reaction in the lung is mediated by T helper type 2 (Th2) cells that produce particularly the cytokines IL-4, IL-5 and IL-13 [1–5]. IL-4 is thought to have an important, but not obligatory, role in the regulation of eosinophilia, AHR and mucus production [6]. IL-5 appears to be involved in the differentiation, recruitment and survival of eosinophils and therefore plays an important role in the development of pulmonary eosinophilia during allergic disorders [7].

AHR is another important feature of asthma and is related to high sensibility to a variety of stimuli, including allergens, pollutants, cold air and non-physiological challenges including bradykinin, histamine and methacholine [8]. Th2 cytokines can induce AHR indirectly by promoting the recruitment of inflammatory cells, but the direct action of these molecules on resident airway cells has also been shown [9].

Helminth infections stimulate a type-2 immune response with the production of both parasite- and non-specific IgE, occurrence of immediate hypersensitivity reactions and recruitment of eosinophils. The ascaroid nematodes are particularly active in inducing atopic reactions [10, 11].

Working with the *Ascaris suum* nematode, we have previously shown that high-molecular-weight components of an extract obtained from adult worms suppress the antibody production to an unrelated antigen in the mouse system [12]. This effect is attributed to a protein component, designated

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suppressive protein of *A. suum* (PAS-1) [13]. Moreover, we have also demonstrated that the suppressive component can be isolated from a low-molecular-weight component that induces high levels of IgE antibody, named allergenic protein of *A. suum* (APAS-3) [14].

In the present study, we investigated the effect of APAS-3 on the induction of allergic airway inflammation and hyperresponsiveness in the mouse system. The suppressive effect of PAS-1 on APAS-3-induced antibody production, cytokine and chemokine release, cellular migration and AHR was also analysed. It was demonstrated that PAS-1 suppressed all these parameters, probably because of the induction of regulatory cytokines (IL-10). These results indicate that *A. suum* has a potent immunomodulatory activity mediated by two distinct protein components.

Material and methods

Animals

Female BALB/c mice weighing 18–22 g and Wistar rats weighing 250–300 g were provided by the animal house of Institute Butantan. The experimental protocols were approved by Institute Butantan Ethical Committee for Animal Research.

Preparation of antigens

The APAS-3 and PAS-1 proteins were isolated from adult worms extract by affinity chromatography, as described earlier [13, 14].

Immunization protocols

Mice were immunized intraperitoneally (day 0) and subcutaneously (day 7) with 20 μg of APAS-3 or 20 μg of APAS-3+300 μg of PAS-1 in 7.5 mg of aluminium hydroxide gel. Fourteen and 21 days later, the mice were intranasally challenged with the same amount of antigens. The control groups were just challenged with APAS-3 or APAS-3+PAS-1.

Bronchoalveolar lavage

Forty-eight hours after the last challenge, mice were sacrificed by intraperitoneal injection of 10% chloral hydrate (1 mL/mouse). The tracheas were cannulated, and the airway lumen was washed with 2×0.5 mL of phosphate-buffered saline (PBS). The recovered fluids were immediately centrifuged at 800 ***g*** for 10 min at 4 °C. The cell pellets were resuspended in 0.5 mL of PBS for cell counts and eosinophil peroxidase (EPO) assay. The total cell counts were performed in a haemocytometer, and differential cell counts in cytocentrifuge preparations stained with a haematoxylin–eosin HEMA-3 system (Biochemical Sciences, Swedesboro, NJ, USA). The lungs were previously washed with 10 mL of saline through the pulmonary artery. After that, they were removed, weighed and homogenized to give a 5% (wt/vol) suspension in Hank's balanced salt solution (HBSS) and centrifuged (1950 ***g***, 10 min). The red blood cells were submitted to lysis by hypotonic shock and the suspension centrifuged again (1950 ***g***, 10 min). The pellet was homogenized in HBSS+0.5% hexadecyltrimetilammonium bromide (Sigma Chemical Co., St Louis, MO, USA) for EPO assay.

Eosinophil peroxidase assay

The EPO activity was measured in bronchoalveolar lavage (BAL) fluid and lung tissue according to the method described by Strath et al. [15]. The suspension of BAL cells and the pulmonary homogenates were frozen/thawed three times using liquid nitrogen and a 37 °C water bath to obtain the EPO. The BAL fluid and lung extracts were centrifuged to 4 °C for 10 min and serially diluted in a 96-well plate (75 μL/well) followed by the addition of 150 μL of substrate (1.5 mM *o*-phenylenediamine and 6.6 mM H_2O_2 in 0.05 M Tris-HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped by the addition of 75 μL of 30% H_2SO_4, and absorbance of the samples was determined at 492 nm on an ELISA reader (Multiskan Labsystems, Helsinki, Finland).

Cytokine and chemokine measurements

Cytokines and chemokines were evaluated in BAL fluid from mice immunized and challenged with APAS-3 or APAS-3+PAS-1. IL-4, IL-5, IFN-γ, IL-10, eotaxin and RANTES were measured by a sandwich ELISA (R&D systems Inc., Minneapolis, MI, USA).

Passive cutaneous anaphylaxis

Serum levels of anaphylactic IgE and IgG1 anti-APAS-3 were measured by passive cutaneous anaphylaxis (PCA) according to Mota and Wong [16]. PCA reactions were performed in mice (for IgG1) or rats (for IgE), using a sensitization period of 2 or 24 h, respectively. The challenge was performed intravenously by the injection of 300 μg of APAS-3 in Evan's blue solution. The PCA titre was expressed as the reciprocal of the highest dilution of pooled sera that gave a lesion of more than 5 mm in diameter in triplicate tests. The detection threshold of the technique was established at 1 : 5 dilutions.

Determination of airway responsiveness

The measurement of conductance (Grs) and dynamic compliance (Crs) of the respiratory system was performed in anaesthetized, tracheostomized and ventilated mice in a Harvard 683 apparatus (Harvard Apparatus, South Natick, MA, USA). Different doses of methacholine (3–10 000 μg/kg) were administered via a sylastic catheter tied into the jugular vein, and data were stored at 30 s, 1, 2 and 3 min after agonist injection. Whole-body plethysmograph pressure (from which changes in lung volume were derived) and tracheal pressure were transduced by the differential pressure transducers. The outputs were amplified and transferred to a computer in which 16 consecutive breaths were stored to average one data point. The Grs and Crs peak response to each agonist dose was expressed as the percentage of baseline value obtained before the next dose of agonist. The dose of methacholine required to reduce Grs to 50% and Crs to 40% of their control values was estimated from the log dose–peak response curve and chosen as our data point.

Statistical analysis

The results were expressed as the mean ± SD, and differences between groups were analysed by the Mann–Whitney *U*-test (PRISM 3.0). Differences were considered statistically significant at $P<0.05$.

Results

Cellular influx into airway lumen induced by Ascaris suum *components*

The effect of *A. suum* modulatory factors on the recruitment of inflammatory cells into the airway lumen was evaluated 48 h after challenge comparing mice sensitized with APAS-3 or APAS-3+PAS-1. Figure 1 represents the total number of cells and eosinophils recovered in BAL fluid in the different groups of mice. The data show that the total number of cells in BAL from APAS-3-immunized mice was about 11-fold higher than the amount of cells in BAL from non-immunized mice. These mice developed an important eosinophilia (about 30% of the cells) as shown by the differential cell counts. In the presence of PAS-1, however, the number of total cells and eosinophils observed after challenge was significantly lower in comparison with the APAS-3 group. The cells recovered from mice immunized and challenged with APAS-3+PAS-1 were equivalent in number to control groups (animals that were solely challenged).

Dampening of eosinophil peroxidase activity in the bronchoalveolar lavage fluid and lung tissue

The accumulation of eosinophils was also evaluated by the measurement EPO activity in BAL fluid and homogenates of lung tissue. The data obtained 48 h after the last challenge showed that immunization with APAS-3 induced significantly higher levels of EPO activity than immunization with APAS-3+PAS-1 in lung tissue and in the BAL fluid (Fig. 2). These results indicate that the suppressive protein PAS-1 inhibited the migration of eosinophils to airway lumen, corroborating cellular data, and also to the lung tissue.

Suppression of anti-allergenic protein of Ascaris suum *immunoglobulins E and G1 production*

The production of IgE and IgG1 anaphylactic antibodies was analysed in the serum, 48 h after last intranasal instillation, by the PCA method. Figure 3 shows that immunization with APAS-3 increased the production of IgE and IgG1 anaphylactic antibodies, and the simultaneous administration of PAS-1 inhibited the secretion of these antibodies.

Inhibition of T helper type 2 cytokines by suppressive protein of Ascaris suum*-1*

PAS-1 down-regulated the production of Th2 cytokines (IL-4 and IL-5) in the APAS-3 group compared with control groups, as shown in Fig. 4. In contrast, the production of IL-

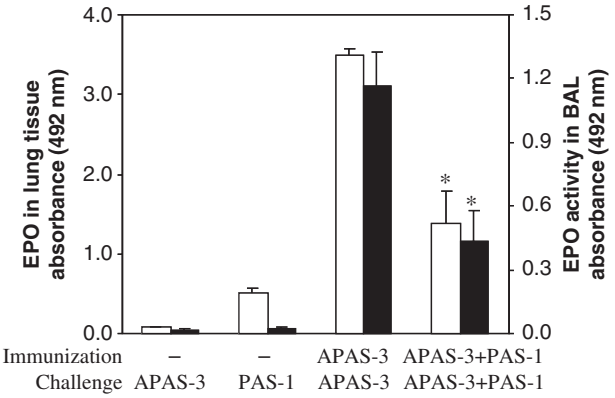

Fig. 2. Effect of suppressive protein of *Ascaris suum* (PAS-1) on eosinophil peroxidase (EPO) activity. Bronchoalveolar lavage (BAL) (□) and lung homogenates (■) from mice described in Fig. 1 were obtained 48 h after challenge and assayed for EPO activity. Data represent mean+SEM for six to sevenanimals/group. *$P<0.05$ compared with allergenic protein of *A. suum*-immunized mice.

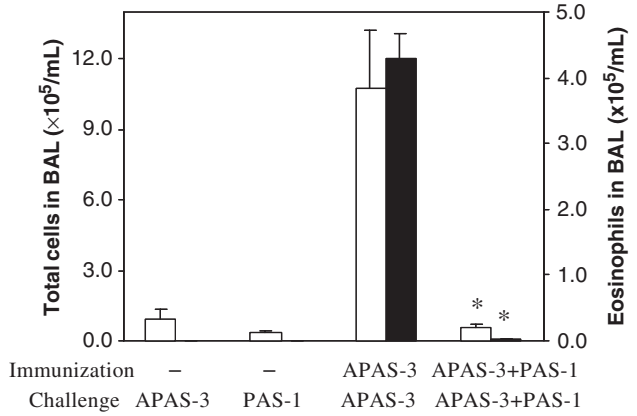

Fig. 1. Effect of suppressive protein of *Ascaris suum* (PAS-1) on airway eosinophilic inflammation induced by allergenic protein of *A. suum* (APAS-3). Mice were immunized as depicted in the figure. Two days after challenge, bronchoalveolar lavage (BAL) fluid was collected, and total cell (□) and eosinophil (■) numbers were estimated. Data represent mean+SEM for six to seven animals/group. *$P<0.05$ compared with the APAS-3 group.

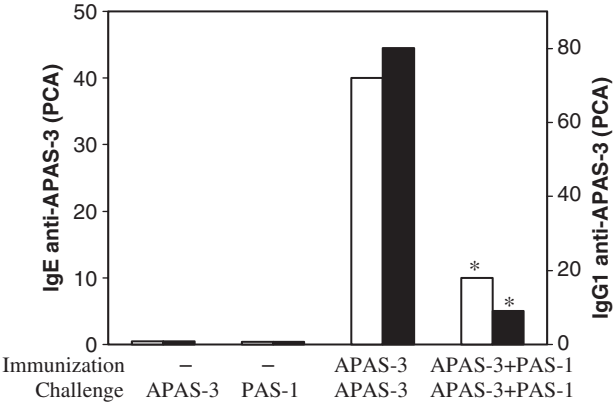

Fig. 3. Suppressive effect of suppressive protein of *Ascaris suum* (PAS-1) on anaphylactic antibody production. Plasma from mice immunized and challenged with allergenic protein of *A. suum* (APAS-3) or APAS-3+PAS-1 ($n=7$) were used to measure the levels of IgE (□) and IgG1 (■) anaphylactic antibodies. The passive cutaneous anaphylaxis (PCA) titre represents the highest dilution of pooled plasma that gave a positive reaction (diameter ⩾5 mm). *$P<0.05$ compared with the APAS-3 group.

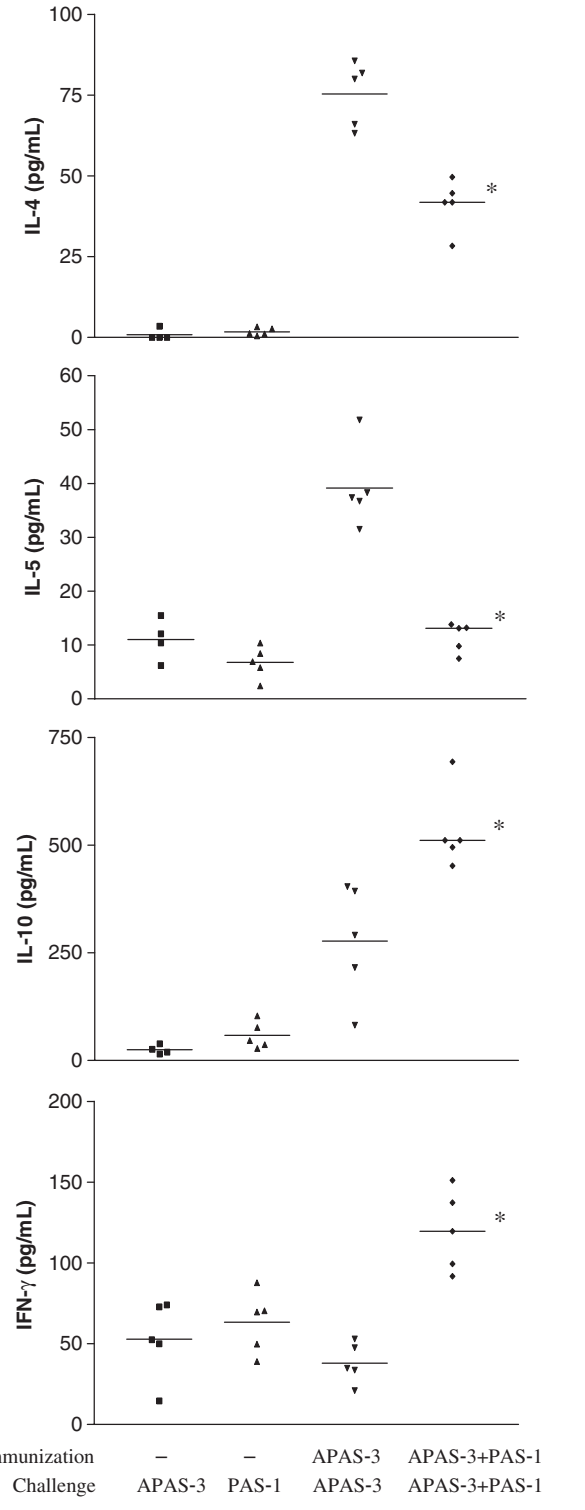

Fig. 4. Effect of suppressive protein of *Ascaris suum* (PAS-1) on cytokine production by bronchoalveolar lavage (BAL) cells. Two days after challenge, BAL fluid from all groups of mice were obtained for individual measurement of IL-4, IL-5, IL-10 and IFN-γ by ELISA. *$P<0.05$ compared with the allergenic protein of *A. suum* group.

10, an anti-inflammatory cytokine, was increased in mice immunized and challenged to APAS-3+PAS-1 in relation to control groups and APAS-3 group. A small amount of IFN-γ was detected in the APAS-3+PAS-1 group.

The production of eotaxin and RANTES was also influenced by PAS-1. The production of both chemokines was diminished in BAL fluid from mice immunized and challenged to APAS-3+PAS-1 compared with the APAS-3 group (Fig. 5).

Inhibition of hyper-reactivity by suppressive protein of Ascaris suum*-1*

The effect of PAS-1 on the development of respiratory hyper-reactivity was studied 48 h after the last intranasal instillation by evaluation of the parameters of pulmonary function. Values of Crs, which represent distal bronchioli and lung tissue reactivity, and Grs, which represent airway mechanical data, were obtained by volume, airflow and pressure data using a dose–response curve to methacholine. The results were expressed as log of the dose of methacholine that reduced Grs to 50% and Crs to 40% of their baseline values.

According to the results showed in Fig. 6, the allergen challenge on the APAS-3 group induced a significant reduction of Grs and Crs compared with control groups (challenged only), indicating a high level of airways and lung reactivity to the administered agonist. On the other hand, Grs but not Crs returned to normal values in mice immunized and challenged with PAS-1.

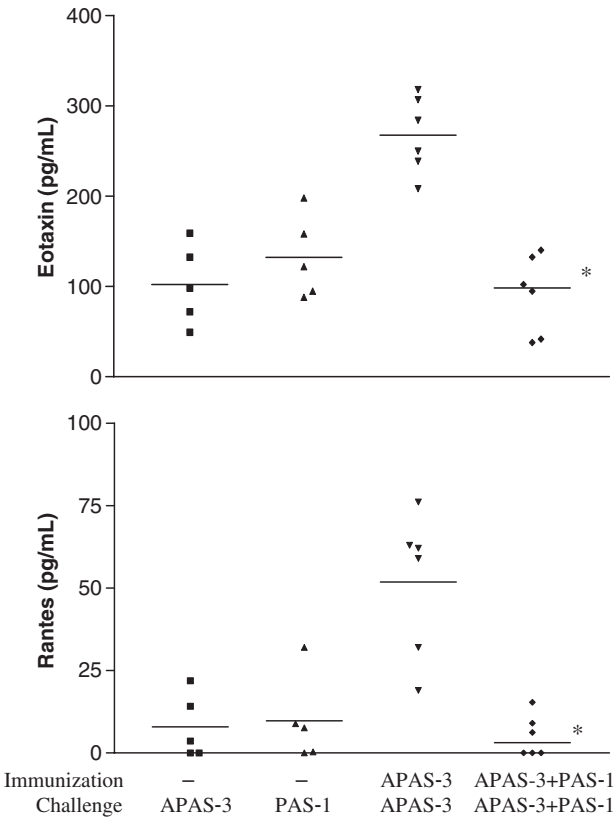

Fig. 5. Effect of suppressive protein of *Ascaris suum* (PAS-1) on chemokine production by bronchoalveolar lavage (BAL) cells. Mice were immunized and treated as depicted in the figure, and 2 days after challenge, BAL fluid was collected for measurement of eotaxin and RANTES. Data represent mean+SEM for six to seven animals/group.*$P<0.05$ compared with the allergenic protein of *A. suum* group.

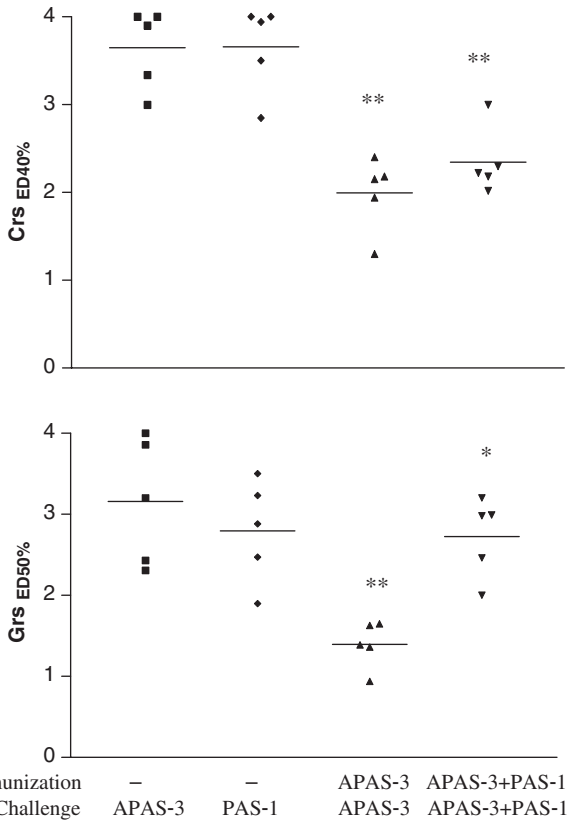

Fig. 6. Effect of suppressive protein of *Ascaris suum* (PAS-1) on lung hyper-responsiveness. Two days after challenge, mice were intravenously injected with different doses of methacoline and results expressed as the dose (log) required to reduce conductance (Grs) to 50% and dynamic compliance (Crs) to 40% of their control values. $^{**}P<0.05$ compared with control groups. $^{*}P<0.05$ compared with the allergenic protein of *A. suum* group.

Discussion

Allergic asthma is a chronic disease characterized by airway eosinophilic inflammation and AHR. There are several animal models of asthma, and in most of them ovalbumin (OVA) or dust mite allergens are used as antigen. Allergens have been isolated from ascaroid nematode extracts, body fluids or culture supernatants, such as a 14 kD protein identified in the body fluid of adult *A. suum*, which induces an IgE antibody response in a rat model [17] or the allergen ABA-1 [18]. However, few studies have been developed with respect to the capacity of these allergens to induce allergic asthma.

In this study, we demonstrated that different protein components from *A. suum* have opposite effects on a murine model of experimental asthma. The APAS-3 protein is able to induce allergic asthma as demonstrated by the eosinophilic inflammatory infiltrate in airways and lung, the production of anaphylactic antibodies, the induction of IL-4, IL-5, eotaxin and RANTES and of pulmonary hyper-responsiveness. The immunomodulatory protein PAS-1 suppresses this response elicited by APAS-3. In the protocol used in this study, we injected PAS-1 in both immunization and challenge steps in order to obtain the greatest suppressive effect. The presence of PAS-1 only at immunization leads to a significant but not complete suppression of cell migration into BAL. In addition, PAS-1 given only at challenge has no suppressive effect (data not shown). These findings indicate that PAS-1 acts mainly in the inductive phase of the immune response, but it suppresses more when also present in the effector phase.

The differential recruitment of eosinophils is a hallmark feature of both asthmatic patients and mouse models and have been associated with preferential induction of Th2 cells that, through the release of IL-5, will induce the recruitment of this cellular type [19]. Eosinophils have a potential destructive and pathogenic role in asthma, releasing toxic granule proteins (EPO, EDN, ECP, etc) that may be responsible for the effector functions of these cells [20].

In addition, EPO activity was detected in BAL fluid from mice immunized and challenged with allergen APAS-3, confirming the results obtained in differential cell counts. Moreover, the high EPO activity measured in homogenates of lung tissue from the APAS-3 group indicates the recruitment of eosinophils into the lung. The suppressive effect of PAS-1 on eosinophil influx into airway and lung tissue was also confirmed by the inhibition of EPO activity detected in these sites.

The suppression of eosinophilic inflammation by PAS-1 seems to be because of reduction of IL-5 and eotaxin production, which are potent chemoattractants for eosinophils [21–23]. Furthermore, RANTES, another eosinophil chemoattractant [24], was also decreased by PAS-1 in our asthma model, indicating that the eosinophil migration might have been diminished because of this suppressive effect too.

PAS-1 also induced the production of high levels of IL-10, an anti-inflammatory cytokine. Inhibition of allergic responses can be mediated by T $CD4^{+}CD25^{+}$ regulatory cells, which produce IL-10 [25], down-regulating the immune response in asthma. So, it is possible that IL-10 detected in our model could be secreted by T regulatory cells. Other regulatory T cells called Tr1 produce IL-10 and transforming growth factor-β (TGF-β) [26]. Although this latter cytokine was not measured in the experiments reported here, a similar protocol performed more recently showed that IL-10, TGF-β and IFN-γ are present in the BAL fluid from mice immunized with APAS-3+PAS-1 or just with PAS-1. These data show that PAS-1 can induce these cytokines by itself and do not need APAS-3 as a co-factor. In addition, we have recently reported that PAS-1 has a potent anti-inflammatory activity because of the stimulation of IL-10 and TGF-β in macrophages, thus leading to the inhibition of pro-inflammatory cytokine production [27]. Therefore, the source of IL-10 in our asthma model might also be alveolar macrophages.

In addition, PAS-1 suppressed IgE and anaphylactic IgG1 antibody production induced by APAS-3. IL-4 and IL-13 are involved in the induction of the allergen-specific IgE and IgG1 synthesis [28, 29], and the reduction of IL-4 in APAS-3+PAS-1-injected mice may well be correlated with the suppression of these antibodies. In addition, the presence of IFN-γ in this group could down-regulate the production of anaphylactic IgG1, as previously reported [30]. We have demonstrated in another asthma model that anaphylactic antibodies enhance eosinophilic inflammation and AHR [31, 32], and, therefore, their reduced levels in APAS-3+PAS-1-immunized mice may also contribute to the inhibition of the inflammatory parameters in the lung. The role of IL-10 induced by PAS-1 in reducing the allergic response is further

supported by two sets of experiments performed in IL-10-deficient mice. In the first [33], PI had less or no suppressive effect on the OVA-specific IgG1 and IgE antibody response, respectively, in OVA+PI-immunized IL-10$^{-/-}$ mice. In the second [unpublished results], the IgE and anaphylactic IgG1 responses against PI were markedly enhanced in IL-10$^{-/-}$ treated with anti-IFN-γ antibodies, indicating that IL-10 and IFN-γ also down-regulate the PI-specific response.

The pulmonary inflammation in asthma is frequently associated with the development of AHR. However, the participation of the IgE-mast cell pathway or of the IL-5-eosinophil pathway in the pathophysiology of altered airway responsiveness produces intense debate. Kraneveld et al. [34] verified that the induction of AHR in guinea-pigs is dependent on IL-5 and that the infiltration and activation of eosinophils in the bronchial tissue depend on the adhesion of very late antigen-4 molecules expressed on the eosinophil surface. The same degree of dependence between eosinophilia and bronchial hyper-responsiveness was demonstrated in studies developed by Hogan et al. [35]. On the other hand, mice immunized with Der p 1 and exposed to a low concentration of this antigen aerosol developed AHR but not airway eosinophilia [36].

In our model, the sensitization and challenge with APAS-3 led to both eosinophilic inflammation and reduction of airway Grs and Crs, meaning high levels of airway and lung reactivity. The simultaneous administration of PAS-1 resulted in enhancement of Grs but not of Crs values, indicating that it abolished airway reactivity, but did not affect the elastic properties of the lung tissue significantly.

Altogether, our results demonstrate that *A. suum* has allergenic components, and also suppressive components that have the ability to down-regulate allergic responses, mostly because of the stimulation of IL-10. All the data obtained so far in this and previous papers [12, 37] support the idea that the action of suppressive components is prevalent in the whole extract. PAS-1 can be detected by immunofluorescent monoclonal antibodies in all stages of the worm's life cycle obtained from infected mice. Its functional activity can also be visualized in infected mice that exhibit dampened immune response against heterologous antigens and reduced inflammation induced by lipopolysaccharide. In addition, IgE and IgG1 antibodies against APAS-3 are detected in *A. suum*-infected mice and pulmonary inflammation is observed in these mice when they are challenged intranasally with APAS-3 (unpublished results). These data reinforce the biological importance of these parasite components in the expression of the host response.

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