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Inflammation Research

Anti-inflammatory activity of PAS-1, a protein component of *Ascaris suum*

T. M. Oshiro[1], M. S. Macedo[2] and M. F. Macedo-Soares[1]

[1] Laboratory of Immunopathology, Butantan Institute, Av. Vital Brasil, 1500, 05503-900, São Paulo, SP, Brasil, Fax: ++ 55 11 3726-1505, e-mail: fmacedo@usp.br

[2] Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, 05508-900, São Paulo, Brazil

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Abstract. *Background*: Recently, we identified a 200 kDa protein (PAS-1) from *Ascaris suum* worms, that suppresses the humoral immune response. Here, the effect of PAS-1 on inflammatory leukocyte migration induced by bacterial lipopolysaccharide (LPS) was investigated.
Methods: Cellular migration and cytokine release, stimulated by LPS or LPS+PAS-1, were analyzed in air pouches induced in the shaved back of BALB/c mice. Cytokines were determined by ELISA and RT-PCR on air pouch exudates and in vitro stimulated peritoneal macrophages.
Results: The significant cellular influx induced by LPS, consisting predominantly of neutrophils, was highly suppressed in the presence of PAS-1, but not a non-related protein. PAS-1 led also to a marked reduction of TNF-α, IL-1β and IL-6 levels in both LPS-stimulated air pouches and peritoneal macrophage cultures. In contrast, PAS-1 induced a significant increase of IL-10 and TGF-β production.
Conclusions: These results demonstrate that PAS-1 has a potent anti-inflammatory activity, probably due to the stimulation of regulatory cytokines in macrophages, thus leading to the inhibition of pro-inflammatory cytokine production.

Key words: *Ascaris suum* – Immunomodulation – Inflammation – Pro-inflammatory cytokine – Regulatory cytokine

Introduction

Helminth infections have been associated with the modulation of the immune responsiveness in man and different animal species. Several studies have demonstrated that these parasites have the ability to interfere in the host's immune system, activating, inhibiting or deviating the immune responses [1–3]. With regard to *Ascaris suum*, it was demonstrated that the crude extract of adult worms strongly suppresses antibody production, induction of delayed type hypersensitivity reactions, lymphoproliferation, and eosinophilic airway inflammation and hyper-reactivity [4–6]. Moreover, we also showed that the immunosuppressive and allergenic effects are due to distinct components of *A. suum* adult worms [7]. While the 29 kDa components induce IgE antibody production (8), the high molecular mass components, like the whole extract, display suppressive effects on the Th1 and Th2 immune responses to unrelated antigens (9). More recently, we isolated from *Ascaris suum* extract a single protein (PAS-1) that is responsible for the suppressive activity of the whole extract on humoral immune response [10].

Among the immunomodulating properties of helminthes, it has been shown that parasite infection or its products are potent modulators of the inflammatory reaction. It was recently demonstrated that a product of filarial nematodes can reduce articular inflammation [11]. Moreover, using a gene delivery strategy, Rao and collaborators [12] showed that the product of an anti-inflammatory cloned gene from *Schistosoma mansoni* suppresses cutaneous inflammation.

Macrophages play key roles in directing the host's immune response to infection. Recruitment and stimulation of macrophages by cytokines and/or microbial products such as LPS result in the release of several immune effector molecules such as IL-1, TNF-α, IL-6 and IL-12, which are required for protective innate and acquired immunity (13). Considering the experimental models of acute inflammation, air pouch is a simple method that allows investigation of cellular and molecular components of the inflammatory response. The cavity produced by injection of air into the subcutaneous tissue of the back in rodents has a lining of organized macrophages and fibroblasts which closely resembles synovial lining tissue, and provides the formation of an effective mechanical barrier that retains the stimulus and the products of the inflammatory response [14]. In the present study, we analyzed the effect of the *Ascaris suum* component PAS-1 on in vivo LPS-induced inflammation in a murine air pouch model.

Correspondence to: dra. M. F. Macedo-Soares

Materials and methods

Animals

The experiments were performed in young adult female BALB/c mice weighing 18–22 g, provided by the animal house of Butantan Institute. The animals were kept in a temperature-controlled environment and received food and water *ad libitum*. All experimental protocols were approved by the Butantan Institute Ethical Committee for Animal Research.

Purification of PAS-1

PAS-1 was purified from the whole extract of *Ascaris suum* by affinity chromatography using the monoclonal antibody MAIP-1 coupled to Sepharose 4B, as previously described [10]. Purified PAS-1 was LPS-free, containing less than 1.25 ng of endotoxin per mg of protein according to the Limulus amebocyte lysate test (BioWhittaker Inc., Walkersville, MD, USA)

Injection of air pouches

Air pouches were induced in the shaved back of BALB/c mice according to the method described by Ahluwalia and Perreti [15]. Animals previously anaesthetized were injected into the subcutaneous tissue of the dorsal surface with 2.5 ml of sterile air and, three days after, 2.5 ml of sterile air was re-inflated into the cavity. The 6 days-old pouches were injected with 1 µg of LPS (phenolic extract from *Escherichia coli* 026: B6; Sigma) or 1 µg of LPS mixed with 500 µg of PAS-1 in 0.5 ml of sterile PBS, under aseptic conditions. The control groups received only phosphate buffered saline (PBS), or 1 µg of LPS+500 µg of a non-related protein, ovalbumin (OVA), or 500 µg of PAS-1 alone. Three hours after the stimulus, the inflammatory exudate was collected by washing the air pouches with 1 ml of PBS containing 5% bovine serum albumin (BSA) and 2.5 UI/ml of heparin. The exudate obtained was analyzed regarding number and type of inflammatory cells using Turk's solution and hematoxilin, respectively.

Quantification of cytokines

The levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and regulatory cytokines (IL-10 and TGF-β) were determined by specific two-site sandwich ELISA using specific antibodies (R&D Systems Inc., Minneapolis, USA).

In vitro peritoneal macrophage culture

Thioglycolate-elicited peritoneal macrophages were removed from BALB/c mice by peritoneal washing and enriched by plastic adherence. Adherent peritoneal macrophages were cultured at 37 °C, 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen Corporation, New York, USA), 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. Cells were cultured in 24-well plates (10^6 cells per well) and incubated with 1 µg of LPS or 1 µg of LPS + 50 µg of PAS-1 for 4 h (for RT-PCR analysis) or 24 h (for determination of cytokine levels).

Isolation of mRNA and RT-PCR analysis

The total RNA was extracted from peritoneal macrophages by the single-step isolation method using TRIzol® reagent (Invitrogen Life Technologies, New York, USA), according to the manufacturer's protocol. One µg of mRNA was reverse-transcribed using A3500 reverse transcription system (Promega Corporation, Wisconsin, USA). cDNA was amplified by PCR with DNA-polimerase Ampli Taq Gold® (Applied Biosystems, California, USA) using the following primers: β-actin (3′-CGTGGGCCGCCTAGGCACCAGGG and 5′-CGGAGGAAGAGGATGCGGCAGTGG); TNF-α (3′-AGCTCTGAGACAATGACGC and 5′-GGACATCTCTTCCCCACCC); IL-1β (3′-AAGGAGACC AAGCAACGAC and 5′-GAGATTGAGCTGTCTGCTCA); IL-6 (3′-TGCTGGTGACAACCACGGCC and 5′-GTACTCCAGAGACCAGAGG); IL-10 (3′-TCAAACAAAGGACCAGCTGGACAACATACTG and 5′-CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA) and TGF-β (3′-TGGAGCTGAAGCAATAGTTGGTATCCAGGGCT and 5′-TGGACCGCAACAACGCCATCTATGAGAAAACC). The PCR reaction was carried out in 35 cycles, consisting of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 90 s. The amplified cDNA fragments were resolved electrophoretically on 2% (w/v) agarose gels and visualized by staining with ethidium bromide.

Statistical analysis

Values were expressed as means ± standard deviation, using groups of 6 animals and at least three independent experiments. Differences between groups were analyzed by the Mann-Whitney U-test (PRISM 3.0). The differences were accepted as significant for $p<0.05$.

Results

1. Effect of PAS-1 on leukocyte migration into air pouches stimulated by LPS

Initially, the kinetics of LPS-induced cell migration into 6 days-old air pouches was performed in mice at different time-points after stimulation. The highest level of leukocyte accumulation, including neutrophils, was observed at 3 h, declining slowly later on (data not shown). To investigate the effect of PAS-1, air pouches were stimulated with LPS, with or without PAS-1. The exudates were collected 3 h after the stimuli and analyzed regarding total and differential cell counts. The results, shown in Table 1, demonstrate that LPS induced a marked leukocyte migration, which consisted predominantly of neutrophils. Few neutrophils were found in the lavage fluid of air pouches that were infused with PBS

Table 1. Cellular composition of the air pouch exudate.

Air pouch stimulus	Cell number ($\times 10^6$/mL)			
	total	mononuclear	neutrophils	eosinophils
PBS	1.21±0.40	0.92±0.41	0.11±0.06	0.11±0.07
LPS	5.21±1.30*	0.77±0.38	4.08±1.12*	0.62±0.59*
LPS+OVA	4.02±1.34*	0.86±0.29	2.66±1.39*	0.58±0.17*
LPS+PAS-1	1.58±0.87#	0.37±0.14	0.57±0.32*#	0.25±0.21
PAS-1	1.47±0.3#	0.57±0.20	0.65±0.3*#	0.05±0.00

Total and differential cell number in the air pouch exudate of BALB/c mice stimulated with LPS, LPS+OVA, LPS+PAS-1 or PAS-1. The negative control group received only PBS. The exudate was obtained 3 h after the stimulus. Data represent mean ± SD of 5–6 animals/group.
* $p<0.01$ compared with control group.
# $p<0.03$ compared with LPS-stimulated group.

only. The presence of PAS-1, but not OVA, resulted in a great suppression of the LPS-induced inflammatory cell influx, mainly due to inhibition of neutrophil migration. The exudates of air pouches infused with PAS-1 alone did also contain the same number of total cells and neutrophils as those from the LPS+PAS-1-infused group, indicating that PAS-1 is hardly inflammatory by itself.

2. Effect of PAS-1 on cytokine production

Because the inflammatory leukocyte migration is closely related to cytokine release, the role of PAS-1 in the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and regulatory cytokines (IL-10 and TGF-β) was also evaluated in the exudates of air pouches stimulated with LPS or LPS+PAS-1. As shown in Figure 1, PAS-1 strongly suppressed the production of TNF-α, IL-1β and IL-6, induced by the stimulus with LPS (approximately 81%, 43% and 45%, respectively). Stimulation with PAS-1 alone induced very low amounts of these cytokines. In contrast, the synthesis of IL-10 and TGF-β increased two-fold or more upon LPS+PAS-1 stimulation and almost the same was obtained only with PAS-1.

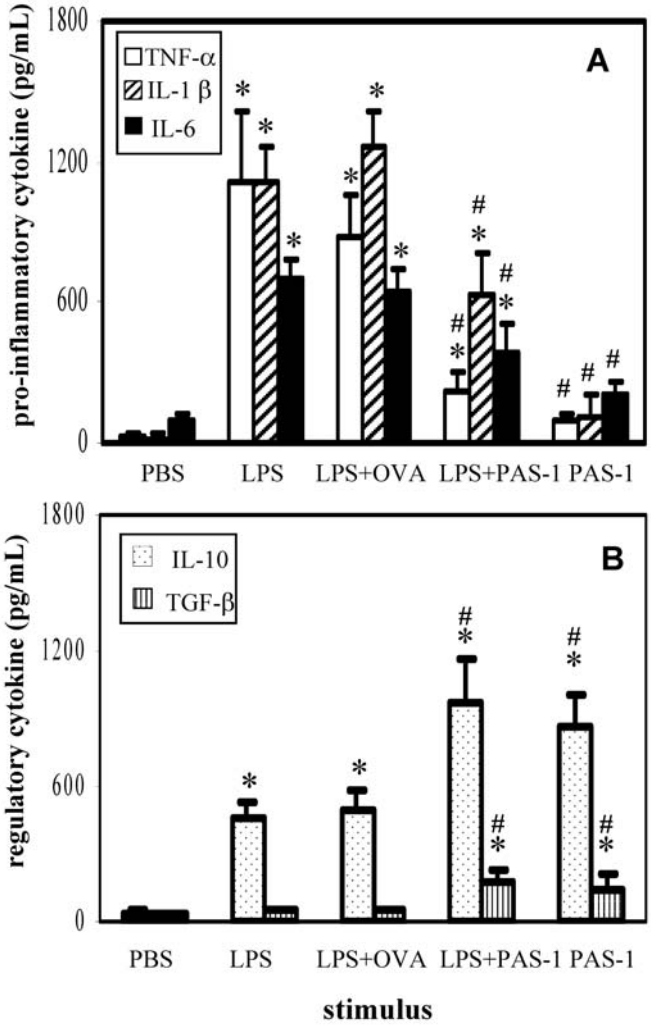

Fig. 1. Effect of PAS-1 on cytokine release. Levels of pro-inflammatory (A) and regulatory (B) cytokines were determined by ELISA in the exudates of air pouches. The results represent the mean value and standard deviation for each cytokine (n=6).* $p<0.03$ compared with control group (PBS); # $p<0.05$ compared with LPS-stimulated group.

3. Cytokine production by macrophages stimulated with PAS-1 in vitro

The effect of PAS-1 on pro-inflammatory cytokine synthesis was also analyzed at the mRNA level by RT-PCR in in vitro LPS-stimulated peritoneal macrophages. Compared with control cells (PBS-stimulated), LPS-stimulated mRNA expression for TNF-α, IL-1 β and IL-6 was markedly inhibited by the presence of PAS-1 (Fig. 2 A). In contrast, the expression of mRNA for IL-10 and TGF-β was increased in LPS+PAS-1-stimulated cells (Fig. 2C). Secretion of pro-inflammatory and regulatory cytokines was also evaluated in supernatants of the macrophage cultures. Consistent with previous results, LPS-stimulated peritoneal macrophages secreted significant amounts of TNF-α, IL-1β and IL-6 (Fig. 2B). Co-stimulation of macrophages with LPS+PAS-1 resulted in about 56%, 67% and 65% inhibition of each cytokine, respectively. On the other hand, secretion of IL-10 and TGF-β by these cells was enhanced in 60% and 125%, respectively (Fig. 2D).

Discussion

Helminth infections are characterized by the initiation of both allergic and immunomodulatory responses [16]. Protective responses to these parasites are associated with the production of the Th2 cytokines IL-4, IL-5 and IL-13, and the resulting mastocytosis, IgE response, and eosinophilia [17]. In addition to promoting Th2-type responses, helminth infections have been shown to modulate immune responses to heterologous antigens. This ability is not dependent on the presence of a live infection, as antigens derived from the parasites display a similar property [4–7].

In this study, we investigated the effect of PAS-1, an *Ascaris suum* protein component, on macrophage function in a murine model of inflammation. In LPS-stimulated air pouches, we could demonstrate that PAS-1 strongly suppresses the leukocyte migration, mainly of neutrophils that were most abundant in the cell infiltrate. Moreover, the pattern of cytokines produced by LPS-stimulated macrophages was altered in PAS-1-treated mice. The synthesis of IL-1β and IL-6 was significantly diminished by PAS-1, but more drastic was the suppression of TNF-α production in this model.

The 6 days-old pouch consists of a cavity covered by lining tissue, new formed vessels, collagen fibers, fibroblasts and macrophages (14). In this model, the leukocyte influx is dependent on the presence of macrophages and TNF-α, because pouches devoided of macrophages (freshly-induced pouches) or infused with anti-TNF antibody have no LPS-induced leukocyte accumulation [18].

In our experiments, to prompt an acute inflammatory response we utilized LPS, the major virulence factor of Gram-negative bacteria. The recognition of LPS is initialized

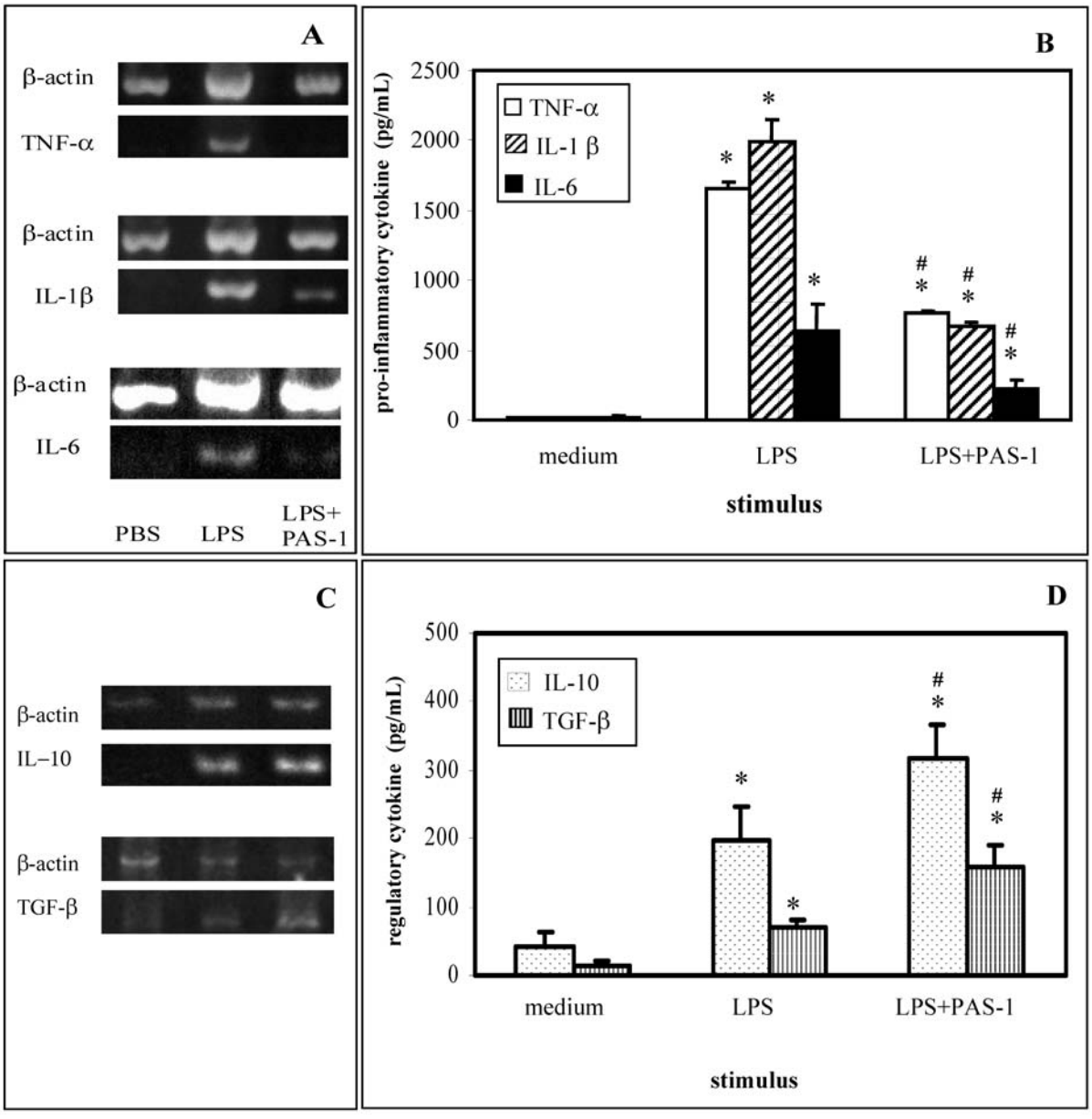

Fig. 2. Effect of PAS-1 on cytokine production by LPS-stimulated peritoneal macrophages. (A) RT-PCR for pro-inflammatory and (C) regulatory cytokines in murine peritoneal macrophages stimulated with LPS or LPS+PAS-1 for 4h. (B) Levels of pro-inflammatory and (D) regulatory cytokines determined by ELISA in the supernatant of peritoneal macrophages, non-stimulated (medium) or stimulated with LPS or LPS+PAS-1 for 24h. The results represent the mean value and standard deviation for each cytokine (n=6). * $p<0.03$ compared with non-stimulated cells (medium) # $p<0.03$ compared with LPS-stimulated cells.

by a complex interaction between LPS-binding protein (LBP), CD14 and TLR4. The recognition of LPS promotes an intracellular signalling pathway, leading to the release of pro-inflammatory mediators by phagocytes [19]. In our model, we observed that the presence of PAS-1 diminished drastically the leukocyte migration accompanied by a reduction of the high levels of pro-inflammatory cytokines induced by LPS. LPS is known to stimulate the production of tumor necrosis factor by macrophages, and this cytokine plays an important role in neutrophil accumulation [20]. Among its effects, TNF increases the adherence of neutrophils to endothelial cells [21], being directly chemotactic for neutrophils [22]. Moreover, TNF has been shown to enhance the production by macrophages of a number of inflammatory cytokines, such as IL-1, macrophages inflammatory protein, monocytederived neutrophil chemotactic factor, platelet-activating factor, and TNF itself [23, 24]. Thus, TNF produced by macrophages in response to LPS could function in an autocrine manner, leading to the production of additional cytokines with inflammatory activities.

We now report that murine macrophages treated with PAS-1, in vivo (cutaneous air pouches) and in vitro, show a substantially reduced ability to produce TNF-α, IL-1β and IL-6 when exposed to their classic inducer, LPS. The pattern

of cytokine mRNA induction was similar to the pattern of released cytokines, indicating that PAS-1 acts at the transcriptional level of cytokine production. These results are similar to those reported by McInnes et al. [11] who showed that a phosphorylcoline-containing glycoprotein (ES-62) from filarial nematodes suppressed LPS-induced TNF-α and IL-6 release from rheumatoid arthritis synovium-derived tissues.

The reduced ability of macrophages to synthesize pro-inflammatory cytokines upon stimulation with LPS+PAS-1 was accompanied by enhanced production of regulatory cytokines, such as IL-10 and TGF-β. However, no additive effect was observed when the results were compared with the amount of these cytokines synthesized by cells stimulated solely with LPS or PAS-1, probably due to the limited number of cytokine-producing cells within the air pouch and/or their maximal threshold of cytokine production. Among other functions, IL-10 is capable of inhibiting TNF-α, IL-1β, IL-6, IL-8, IL-12, GM-CSF and chemokine production by macrophages [25]. Pro-inflammatory responses mediated by LPS are driven largely by the transcription factor NF-κB, which coordinates the activation of numerous genes. IL-10 receptor signaling has been shown to decrease the binding of NF-κB to DNA, either directly or by decreasing the degradation of the inhibitor of kB (IkB) [26, 27].

The immunomodulatory effects of IL-10 and TGF-β overlap to a great extent, since both suppress T-cell functions and deactivate macrophages. However, TGF-β is a less potent inhibitor than IL-10 and has little or no effect on IL-1 production [28].

Our results suggest that the anti-inflammatory activity of PAS-1 is due to the induction of the regulatory cytokines IL-10 and TGF-β, and consequently suppression of pro-inflammatory cytokine production by macrophages. They also indicate that as we better understand the parasite-mediated anti-inflammatory mechanisms we improve the opportunity to find new therapeutic strategies for allergic and autoimmune diseases.

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