

Immunosuppressive components of *Ascaris suum* down-regulate expression of costimulatory molecules and function of antigen-presenting cells via an IL-10-mediated mechanism

Sandriana R. Silva¹, Jacqueline F. Jacysyn¹, Mahasti S. Macedo¹ and Eliana L. Faquim-Mauro²

¹ Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

² Laboratory of Immunopathology, Butantan Institute, São Paulo, Brazil

High-molecular-weight components (PI) of *Ascaris suum* suppress both cell-mediated and humoral responses against ovalbumin (OVA) via an IL-4/IL-10-dependent mechanism. The aim of this work was to investigate the effect of PI on the ability of APC to activate T cells and the role of IL-10 in this process. Flow cytometry analyses of MHC class II, CD80, CD86 and CD40 molecules on LN cells from mice immunized with OVA or OVA+PI showed that PI inhibits expression of these molecules on unfractionated cells and on purified CD11c⁺ cells. A low proliferative response was obtained when OVA-specific TCR-Tg T cells were incubated with CD11c⁺ cells from OVA+PI-immunized mice pulsed with OVA, when compared to those incubated with cells from OVA-immunized mice. Similar results were obtained using as APC CD11c⁺ cells from OVA-immunized mice pulsed with OVA+PI, which also expressed less of the four markers. The inhibitory effect of PI on both the expression of costimulatory molecules and the induction of T cell proliferation was abolished in IL-10-deficient mice. Our data indicate that the potent immunosuppressive effect of *A. suum* extract components on the host immune system is primarily related to their property of down-regulating the Ag-presenting ability of DC via an IL-10-mediated mechanism.

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Introduction

Helminthic infections affect more than two billion people in the world mostly in low-income countries, representing one of the most important health problems. The protective host's immune response is mediated by Th2 cells involving IL-4, IL-5, IL-10 and IL-13 secretion, IgE production and activation of effector

cells such as mast cells, eosinophils and basophils [1–4]. However, some helminths are able to persist in their hosts for many years, creating an anti-inflammatory environment favorable to their survival and maintenance. In this context, it has been demonstrated that helminths down-regulate host's immunity to their own Ag and to a large number of unrelated pathogenic agents [5–7]. More recently, it has been reported that some helminth infections promote the generation of T regulatory cells which are responsible in part for the state of immune hyporesponsiveness [8–10].

We have previously shown that an *Ascaris suum* extract (Asc) from adult worms, as well as its high-molecular-weight components (PI) obtained by gel filtration, inhibit the Th1- and Th2-related immune responses induced by immunization of mice with an

Correspondence: Dra. Eliana Faquim de Lima Mauro, Av. Vital Brasil, 1500, 05503–900 São Paulo, SP, Brasil

Fax: +55-11-3726-1505

e-mail: elianafaquim@butantan.gov.br

Abbreviations: Asc: *Ascaris suum* extract · PI: high-molecular-weight components of Asc

unrelated Ag, OVA, such as delayed-type hypersensitivity reactions, antibody production, *in vitro* lymphoproliferative responses and cytokine secretion induced by *in vitro* stimulation with Ag or mitogen [11, 12]. More recently, it was demonstrated that Asc also suppresses experimental allergic asthma in mice [13]. PAS-1, an affinity-purified protein isolated from Asc, maintains the ability to dampen inflammatory, humoral and pulmonary allergic responses [14–16].

The immunosuppressive effect is accompanied by a Th2-type cytokine profile, represented by IL-4 and IL-10 secretion, in response to Asc, PI or PAS-1 components. Analyzing the role of these Th2 cytokines in this effect, we demonstrated that, in the absence of IL-4 and IL-10, Asc or PI were unable to down-regulate Th1 or Th2 responses against OVA [17, 18]. On the other hand, *in vitro* neutralization of IL-4 and IL-10 did not change the proliferative hyporesponsiveness and the low cytokine secretion by OVA-specific T cells obtained from mice immunized with OVA+Asc [17]. Using a limiting-dilution assay, a low frequency of OVA-specific T cells was demonstrated in mice immunized with OVA+Asc [11]. These results suggest that Asc or PI exert their suppressive effect on the inductive phase of the immune response.

During the development of the adaptive immune response, an efficient T cell activation requires the engagement of a variety of ligand/receptors, in addition to the interactions of TCR with MHC molecule/peptide complexes. The CD80 and CD86 molecules expressed by APC provide important costimulatory signals to augment and sustain a T cell response after their interaction with CD28 expressed by T cells [19, 20]. Likewise, the CD40-CD40L interaction is also essential for the full expression of T cell immunity [21, 22].

The aim of this study was to investigate the effect of PI components on the Ag-presenting process and the role of IL-10 in this mechanism, since this cytokine is described as a modulator of the Ag-presenting capacity of monocytes/macrophages and DC [23, 24]. To this end, we analyzed the expression of MHC class II, CD80, CD86 and CD40 molecules by APC obtained from BALB/c, C57BL/6 WT or IL-10-KO (IL-10^{-/-}) mice previously immunized with OVA or OVA+PI. After this, using an *in vitro* assay of OVA-specific TCR-Tg T cell proliferation, we evaluated the ability of OVA-primed purified CD11c⁺ cells that had been in contact with PI to stimulate T cells.

The results presented here show that PI components inhibit the expression of MHC class II and costimulatory molecules and the function of APC *via* an IL-10-dependent mechanism. The down-regulation of such molecules that play an essential role in Ag presentation impaired the ability of APC to activate OVA-specific T cells, leading to hyporesponsiveness to OVA immunization.

Results

Kinetics of MHC class II, CD80, CD86 and CD40 expression on LN cells after OVA immunization

To determine the number of cells expressing molecules involved in Ag presentation, groups of BALB/c mice were immunized subcutaneously in the base of the tail with 200 µg of OVA in CFA. After 3–7 days of immunization, LN cell suspensions were prepared and the percentage of cells expressing MHC class II, CD80, CD86 and CD40 molecules was evaluated by flow cytometry. The number of labeled cells/animal was then calculated based on the difference in LN cellularity at different time points. Non-immunized mice were used as controls.

Figure 1 shows that the kinetics of MHC class II and CD40 expression in OVA-immunized mice is distinct from that of CD80 and CD86 molecules. The number of CD80⁺ and CD86⁺ cells rose until day 5 and declined sharply later on. In contrast, MHC class II⁺ cells reached a plateau around day 6 and 7, whereas CD40⁺ cells increased between day 3 and 4 and remained so. The percentage of labeled cells showed a similar profile (data not shown). In addition, LN obtained from CFA-injected control mice also had increased expression of these same markers but at numbers that were half of those obtained for OVA+CFA-immunized mice (data not shown). This indicates that both adjuvant and Ag enhance the expression of these activation markers on the cell surface.

Inhibition of MHC class II, CD80, CD86 and CD40 expression by PI

The next experiment was to investigate the effect of PI on the expression of these molecules. To this end, BALB/c mice were immunized with OVA or OVA+PI in CFA and after 4–5 days, LN cell suspensions were prepared and stained with anti-MHC class II, anti-CD80, anti-CD86 or anti-CD40 mAb labeled with FITC or PE.

The flow cytometry analyses (Fig. 2A) showed significant reduction in the number of cells/animal expressing MHC class II, CD80, CD86 and CD40 in OVA+PI-immunized mice compared with OVA-immunized mice. Likewise, the percentage of cells bearing these markers was reduced (data not shown). These effects were detected on either day 4 or 5 of immunization. In addition, the fluorescence intensity of staining for the same molecules on the surface of cells from OVA+PI-immunized mice on day 5 was also decreased (Fig. 2B). Together, these results indicate that the helminth-suppressive components diminished both the number of cells and their expression of MHC class II, CD40, CD80 and CD86.

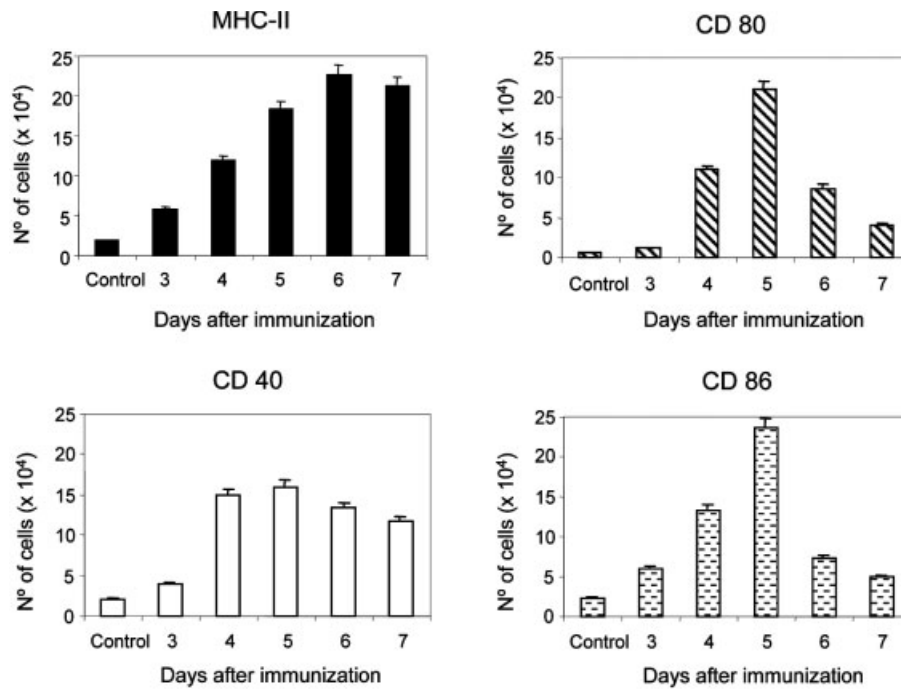


Figure 1. Kinetics of MHC class II, CD40, CD80 and CD86 expression on LN cells after immunization with OVA. Groups of BALB/c mice were immunized with OVA in CFA and LN cells obtained after 3–7 days were labeled with FITC- or PE-conjugated anti-MHC class II, anti-CD40, anti-CD80 or anti-CD86 mAb. Non-immunized mice were used as controls. The results are expressed as the mean $\pm$ SD of the number of labeled cells/animal in duplicate samples ($n=4-5$), and are representative of three experiments.

In comparison, a similar experiment was performed with the low-molecular-weight components from Asc extract (PIII) and no difference was observed between cells from mice immunized with OVA+PIII or OVA alone regarding the expression of such molecules (data not shown).

Inhibitory effect of PI on MHC class II, CD80, CD86 and CD40 expression on purified CD11c⁺ cells

Since DC have a crucial role in the primary induction of the adaptive immune response, and the CD11c integrin is a marker for most DC in the mouse [25], we evaluated the effect of PI on expression of these molecules in purified CD11c⁺ cells obtained from mice immunized with OVA or OVA+PI in CFA. Consistent with the results obtained for whole LN cell suspensions, purified CD11c⁺ cells from OVA+PI-immunized mice showed a reduction of the expression of MHC class II by 16%, CD40 by 50%, CD80 by 55% and CD86 by 46%, when compared to CD11c⁺ cells from OVA-immunized mice (Fig. 3).

Role of IL-10 in the inhibitory effect of PI

Since IL-10 plays a major role in the suppression of immune responses inhibiting the Ag-presenting capacity of APC [23, 24], and we had previously observed that PI induces large amounts of this cytokine [12], we

investigated whether the observed inhibitory effect of PI was IL-10-mediated. To this end, groups of WT and IL-10^{-/-} C57BL/6 mice were immunized as described before with OVA or OVA+PI. After 5 days, LN cell suspensions were prepared and the expression of MHC class II and costimulatory molecules was analyzed.

The geometric MFI data in Figure 4 show that, in the absence of IL-10, there was no reduction in expression of any of the four markers caused by immunization with PI. In clear contrast, the data of WT mice confirmed the inhibitory effect of PI on MHC class II (46%), CD40 (43%), CD80 (59%) and CD86 (55%) expression. The percentage of cells expressing the respective markers is shown in the insert to Figure 4. As can be observed, a marked difference was again obtained between WT mice immunized with OVA or OVA+PI, but not between IL-10-deficient mice immunized in the same ways.

Proliferative hyporesponsiveness of OVA-specific T cells cultured with DC from OVA+PI-immunized mice

The previous results demonstrated that PI down-regulates the expression of molecules involved in Ag presentation by APC. Thus, we next verified the ability of APC from OVA+PI-immunized mice to *in vitro* activate OVA-specific TCR-Tg T cells. CD11c⁺ cells were purified from LN of BALB/c mice immunized with OVA or

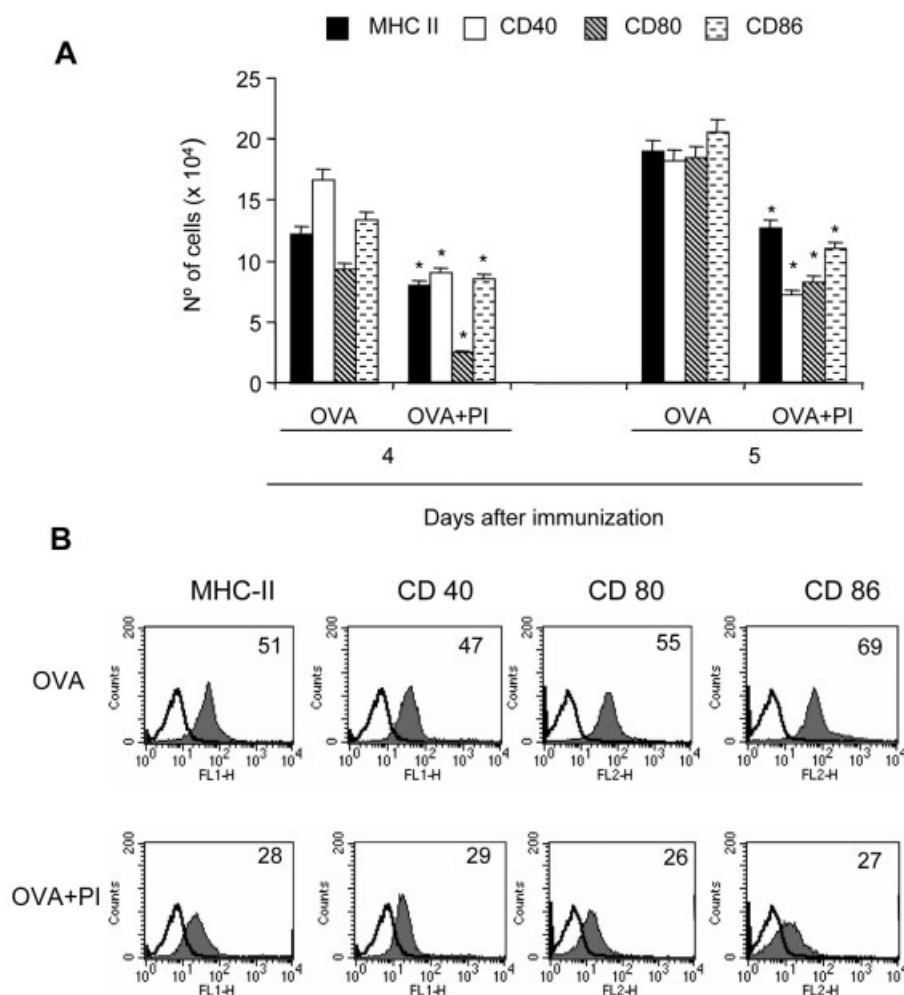


Figure 2. Suppressive effect of PI on MHC class II, CD40, CD80 and CD86 expression. Groups of BALB/c mice were immunized with OVA or OVA+PI in CFA. After 4 or 5 days, LN cells were labeled with FITC- or PE-conjugated anti-MHC class II, anti-CD40, anti-CD80 or anti-CD86 mAb and analyzed by flow cytometry. (A) The results are expressed as the mean $\pm$ SD of the number of labeled cells/animal in duplicate samples ($n=4-5$) from three experiments; * $p<0.05$ compared with OVA-immunized cells. (B) Geometric MFI of staining on cells from OVA- or OVA+PI-immunized mice (day 5) is shown inside each box. The filled histograms represent the cells labeled with the specific mAb; the empty histograms represent the same cell suspension labeled with isotypic control mAb (MFI <6).

OVA+PI in CFA 5 days before. These cells were pulsed with 100 μ g/mL of OVA for 18 h, washed with complete RPMI and X-irradiated (2500 rad). Purified CD3⁺ T cells (3×10^5) from Tg BALB/c mice (DO11.10) were then cultured with these Ag-pulsed CD11c⁺ cells (0.6×10^5) for 48 or 72 h. Non-pulsed and OVA-pulsed CD11c⁺ cells from non-immunized mice were also used as controls. The proliferation was measured by [³H]thymidine incorporation.

OVA-pulsed CD11c⁺ cells from OVA-immunized mice induced a strong proliferative response by T cells at 48 and 72 h when compared with the OVA-pulsed CD11c⁺ cells from non-immunized mice (Fig. 5). However, when APC from OVA+PI-immunized mice were used as a substitute for APC from OVA-immunized

mice, a marked reduction in T cell proliferation was seen at either time point.

The *in vitro* contact with PI reduces the Ag-presenting ability of activated DC

The next question was whether the *in vitro* contact with PI would inhibit the Ag-presenting ability of activated CD11c⁺ cells. To answer this question, CD11c⁺ cells from OVA-primed mice were pulsed with OVA or OVA+PI for 18 h, washed, X-irradiated and 0.6×10^5 cells were added to 3×10^5 T cells for 48 h.

As can be observed in Figure 6A, CD11c⁺ cells from OVA-immunized mice pulsed with OVA+PI were unable to induce the same amount of T cell proliferation when

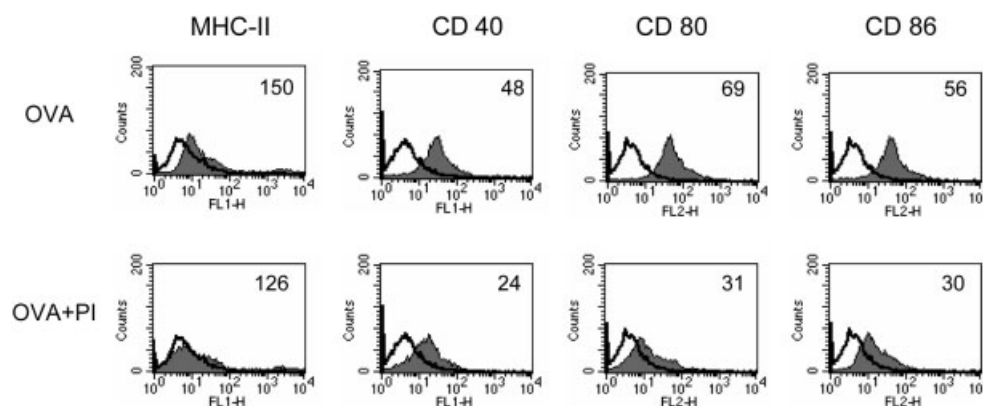


Figure 3. Inhibition of MHC class II, CD80, CD86 and CD40 expression on purified CD11c⁺ cells from mice immunized with OVA+PI. LN cells from BALB/c mice immunized with OVA or OVA+PI in CFA underwent positive selection in Midi-MACS columns to isolate the CD11c⁺ cells. These purified cells were labeled with FITC- or PE-conjugated anti-MHC class II, anti-CD40, anti-CD80 or anti-CD86 mAb and analyzed by flow cytometry. The results are expressed as geometric MFI of MHC class II, CD40, CD80 and CD86 expression on cells from mice immunized with OVA or OVA+PI, and are representative of three experiments. The filled histograms represent the cells labeled with the specific mAb; the empty histograms represent the same cell suspension labeled with isotypic control mAb (MFI < 9).

PERCENTAGE OF POSITIVE CELLS

GROUP	MHC-II	CD 40	CD 80	CD 86
WT/OVA	62	30	56	61
WT/OVA+PI	26	14	28	38
KO/OVA	64	31	60	57
KO/OVA+PI	56	35	58	59

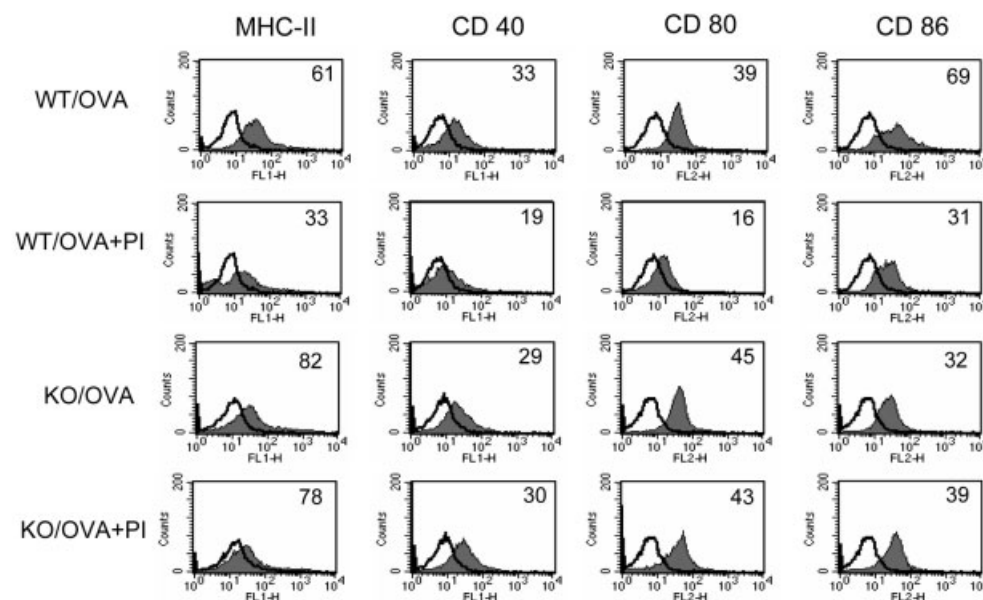


Figure 4. Geometric MFI of MHC class II, CD40, CD80 and CD86 expression on LN cells from WT or IL-10^{-/-} C57BL/6 mice. Insert: Percentage of MHC class II⁺, CD40⁺, CD80⁺ and CD86⁺ cells in LN from WT or IL-10^{-/-} C57BL/6 mice. WT/OVA: WT mice immunized with OVA in CFA; WT/OVA+PI: WT mice immunized with OVA+PI in CFA; KO/OVA: IL-10^{-/-} mice immunized with OVA in CFA; KO/OVA+PI: IL-10^{-/-} mice immunized with OVA+PI in CFA. After 5 days, the LN cells were labeled with FITC- or PE-conjugated anti-MHC class II, anti-CD40, anti-CD80 or anti-CD86 mAb and analyzed by flow cytometry. The results are representative of two experiments. The filled histograms represent the cells labeled with the specific mAb; the empty histograms represent the same cell suspension labeled with isotypic control mAb (MFI < 10).

compared with the same cells pulsed only with OVA. In addition, Figure 6B shows that CD11c⁺ cells pulsed with OVA+PI express less MHC class II and costimulatory molecules than non-pulsed or OVA-pulsed CD11c⁺ cells.

The suppressive effect of PI on the Ag-presenting ability of CD11c⁺ cells is abolished in IL-10-deficient mice

The suppressive effect of PI on the Ag-presenting ability of CD11c⁺ cells was also tested in IL-10^{-/-} C57BL/6 mice. Purified CD3⁺ T cells were obtained from WT mice immunized with OVA in CFA 8 days before. CD11c⁺ cells were purified on day 5 from LN of WT or IL-10^{-/-} mice immunized with OVA or OVA+PI in CFA, pulsed with OVA or OVA+PI for 18 h and X-irradiated. Cultures of 3×10^5 T cells and 0.6×10^5 pulsed or non-pulsed CD11c⁺ cells were set up for 48 h and cell proliferation was measured by [³H]thymidine incorporation.

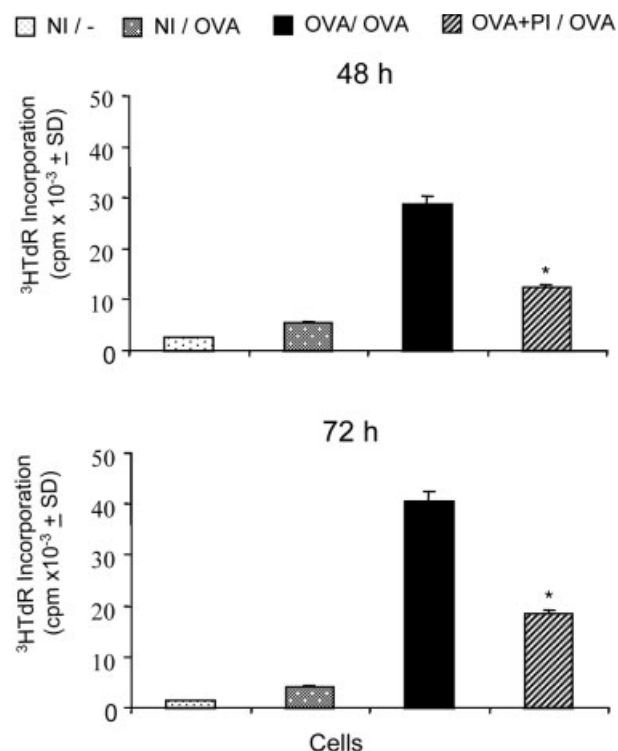


Figure 5. Proliferative response of OVA-specific TCR-Tg T cells incubated with CD11c⁺ cells from mice immunized OVA or OVA+PI. Purified CD3⁺ T cells from DO11.10 Tg mice (3×10^5 cells) were incubated with purified CD11c⁺ cells (0.6×10^5 cells) from BALB/c mice immunized 5 days before with OVA or OVA+PI in CFA. The CD11c⁺ cells were pulsed with OVA (100 µg/mL) for 18 h (OVA/OVA; OVA+PI/OVA), washed, irradiated and then incubated with the CD3⁺ T cells for 48 or 72 h. CD11c⁺ cells from non-immunized mice were non-pulsed (NI/-) or pulsed with OVA (NI/OVA) and used as control APC. The results are expressed as the mean $\pm$ SD of [³H]thymidine incorporation in triplicate cultures from three experiments; * $p < 0.05$ for OVA+PI- compared with OVA-primed cells.

As we observed in the previous two sets of experiments using BALB/c mice, OVA-primed C57BL/6 T cells proliferated much less in the presence of CD11c⁺ cells obtained from C57BL/6 WT mice immunized with OVA+PI and pulsed with OVA. Even without any pulse, T cell proliferation was lower with DC from the OVA+PI group. When PI was added *in vitro* to OVA and the mixture was used to pulse activated CD11c⁺ cells from OVA-primed WT mice, the DC also lost their ability to induce T cell proliferation (Fig. 7A).

In contrast, the suppressive effect of PI on the Ag-presenting ability of DC was not observed in IL-10-deficient mice and T cells proliferated equally well with DC from OVA- or OVA+PI-immunized mice, either pulsed or not with OVA (Fig. 7B). The same results were obtained when PI was added *in vitro* to OVA in order to pulse OVA-activated CD11c⁺ cells. Moreover, even DC from OVA+PI-immunized IL-10^{-/-} mice when pulsed with OVA+PI could stimulate OVA-specific T cell proliferation, demonstrating that the PI suppressive activity was abolished in the absence of IL-10 (Fig. 7B).

Discussion

There is irrefutable evidence that helminth infections can down-regulate immune responsiveness to other types of infection or to unrelated Ag [5–7, 26–29]. We have previously shown that Asc or PI have the same ability as a live infection to strongly suppress the immune response and to induce a typical Th2 response (with IL-4 and IL-10 secretion) which is responsible for the suppressive effect [10–14]. Concerning the mechanisms involved in this effect, we investigated in this work the hypothesis that PI components could act in the inductive phase of the immune response, modulating the Ag presentation process by APC, and consequently T cell activation and proliferation.

As postulated, the T cell response to an Ag is a complex process in which cytokines and costimulatory molecules provide signals that direct the development of adaptive immunity during the contact between lymphocytes and APC [30, 31]. Among APC, DC are highly motile cells that operate at the interface of innate and acquired immunity, possessing the ability to recognize and internalize a wide range of pathogens and Ag through membrane receptors. Subsequently, DC process Ag and present peptides associated to MHC class I or II molecules, as well as provide the costimulatory molecules that are essential for the stimulation of naive T cells [32, 33].

As we demonstrated here, the percentage and the total number of cells/animal that express MHC class II and costimulatory (CD40, CD80 and CD86) molecules increased in LN from mice immunized with OVA+CFA.

In addition, the expression of these activation markers on the cell surface was also up-regulated, the same being observed on purified CD11c⁺ cells, as demonstrated by the fluorescence intensity of labeled cells. CD40 is up-regulated in mature DC and functions in the adaptive immunity as a trigger for the expression of MHC and of several costimulatory and adhesion molecules required for efficient T cell activation [22]. As shown in our results, the number of cells expressing CD40 was the first to increase more than threefold on day 4 of immunization. CD80 and CD86 expression also have a

different timing, with CD86 being expressed earlier [34]. In our results, the number of CD86⁺ cells was fivefold higher than that of CD80⁺ cells on day 3 after immunization.

The concomitant presence of PI in the immunization of mice with OVA+CFA, however, strongly inhibited the expression of the four types of molecules. This result was not due to a delay in the expression of MHC class II and costimulatory molecules, because on day 6 of immunization with OVA+PI the expression of MHC class II and CD40 on LN cells was lower than on day 5, and CD80

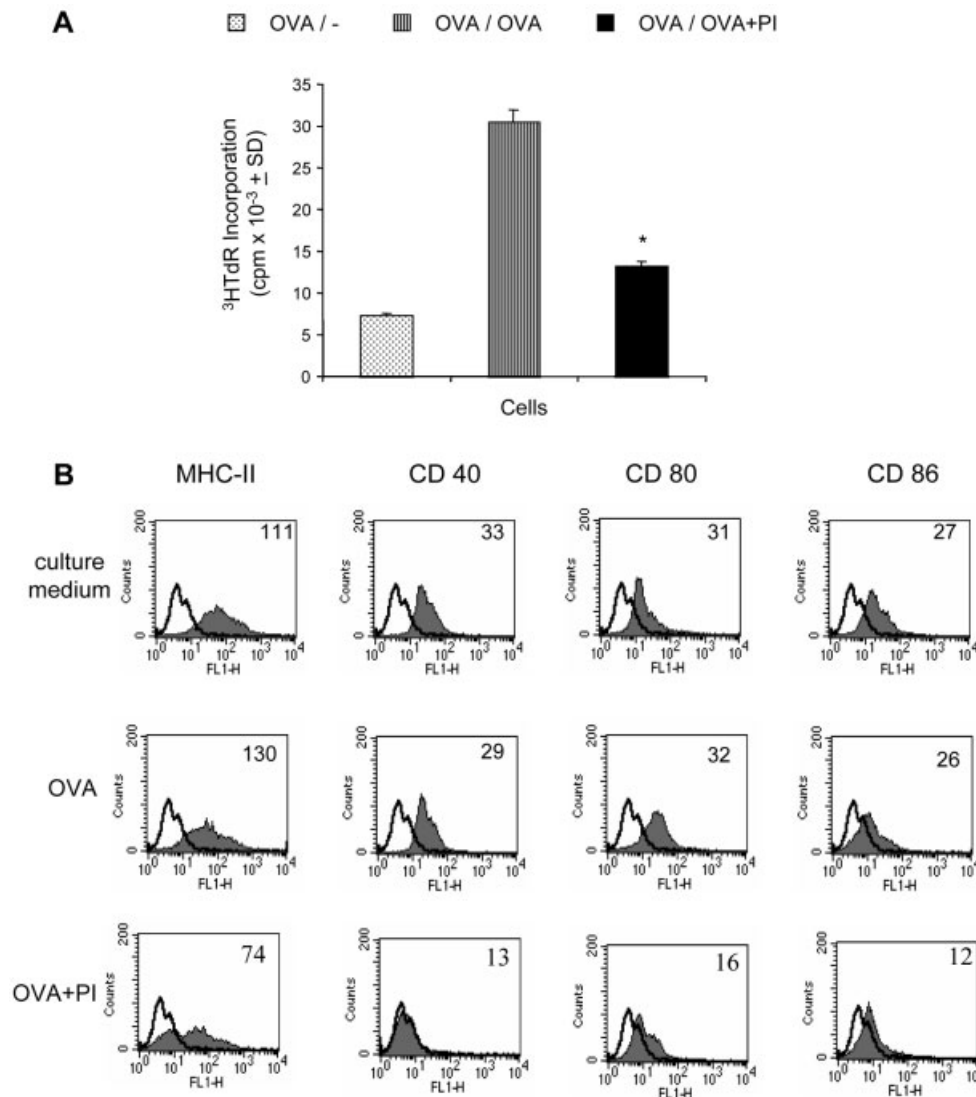


Figure 6. Effect of *in vitro* contact with PI on the expression of activation markers and function of purified CD11c⁺ cells. Purified CD3⁺ T cells from DO11.10 Tg mice (3×10^5 cells) were incubated with purified CD11c⁺ cells (0.6×10^5 cells) from OVA-immunized BALB/c mice. The CD11c⁺ cells were pulsed only with culture medium, or with OVA (100 μ g/mL) or OVA+PI (100 μ g/mL of each) for 18 h, washed, X-irradiated and incubated with the CD3⁺ T cells for 48 h. (A) The results are expressed as the mean $\pm$ SD of [³H]thymidine incorporation in triplicate cultures from three experiments; * $p < 0.05$ for OVA+PI- compared with OVA-pulsed cells. (B) Geometric MFI of MHC class II, CD40, CD80 and CD86 molecules on purified CD11c⁺ cells after 18 h of *in vitro* incubation with culture medium, OVA or OVA+PI. The results are representative of two experiments. The filled histograms represent the cells labeled with the specific mAb; the empty histograms represent the same cell suspension labeled with isotypic control mAb (MFI < 6).

and CD86 expression had fallen to baseline values of control group cells (data not shown). Since the ability of APC to induce T cell activation in lymphoid organs is directly related to the high expression of peptide-MHC complexes and costimulatory molecules [35], the high frequency of APC lacking costimulatory molecules in OVA+PI-immunized mice may prevent adequate Ag presentation to T cells. Indeed, CD11c⁺ DC purified from LN of these mice had their Ag-presenting ability compromised as shown by the significantly reduced proliferation of OVA-specific TCR-Tg T cells. Furthermore, these results may explain the immunosuppressive effect of Asc on both Th1- and Th2-mediated responses, because its action is primarily on the APC function.

The activation state of the APC, and in particular of DC, can be influenced by a range of stimuli including

pathogen-derived products and inflammatory mediators like cytokines, that modulate Ag presentation, costimulation and cytokine production [36, 37]. IL-10 is an important immunoregulatory cytokine produced by numerous cell types that has a crucial role in the differentiation of DC subsets and their function in the immune response [38].

As shown here, this cytokine, which is induced in large amounts by PI components [12], is a major effector molecule in the down-regulation of MHC and costimulatory molecules in our model, because in the complete absence of IL-10 (IL-10-KO mice) the down-regulation simply did not occur. Furthermore, the Ag-presenting ability of DC was not impaired when these cells were obtained from IL-10-deficient mice immunized with OVA+PI, indicating that, in the absence of this cytokine, DC were functionally preserved from the suppressive effects of PI. As to the molecular mechanisms that underlie IL-10 effects on Ag presentation, it has been reported that IL-10 delays the traffic of endosomes containing peptide-MHC class II complexes to the cell surface and reduces the expression of CD80 and CD86 in IL-10-treated myeloid cells [39].

IL-10 has previously been shown by us [17, 18] to be crucial in the suppression of Th2 and, together with IL-4, also suppresses Th1 responses in mice injected with Asc or PI. In murine *Schistosoma mansoni* infections [40], IL-10 also seems to be an endogenous down-regulator of type 1 and type 2 cytokine synthesis. Recently, IL-10 has been associated with the induction of tolerance. In this context, it was demonstrated that DC cultured in the presence of IL-10 suppress the response of Ag-specific naive or activated CD4⁺ T cells and promote the generation of T regulatory cells [41, 42]. CD4⁺CD25⁺ regulatory T cells down-regulate costimulatory molecules on APC even in the presence of stimuli that would normally increase their expression [43]. Helminth-induced T regulatory cells play also an important role in the general state of immunosuppression observed in some infections [8–10]. These cells are also induced by the Asc component PAS-1 (C. A. Araújo and M. F. Macedo-Soares, unpublished observations).

As we demonstrated for the first time here, PI can also down-regulate the expression of MHC class II and costimulatory molecules on already activated DC. Thus, CD11c⁺ cells from OVA-immunized mice incubated with OVA+PI for a short period of time (18 h) exhibited less cell surface activation markers. Consequently, they had reduced Ag-presenting ability and did not stimulate T cell proliferation as did similar CD11c⁺ cells pulsed only with OVA. Again, IL-10 proved to be an important mediator of this effect of PI, because OVA-activated DC from IL-10-deficient mice incubated with OVA or OVA+PI were not suppressed and both were equally effective in Ag presentation.

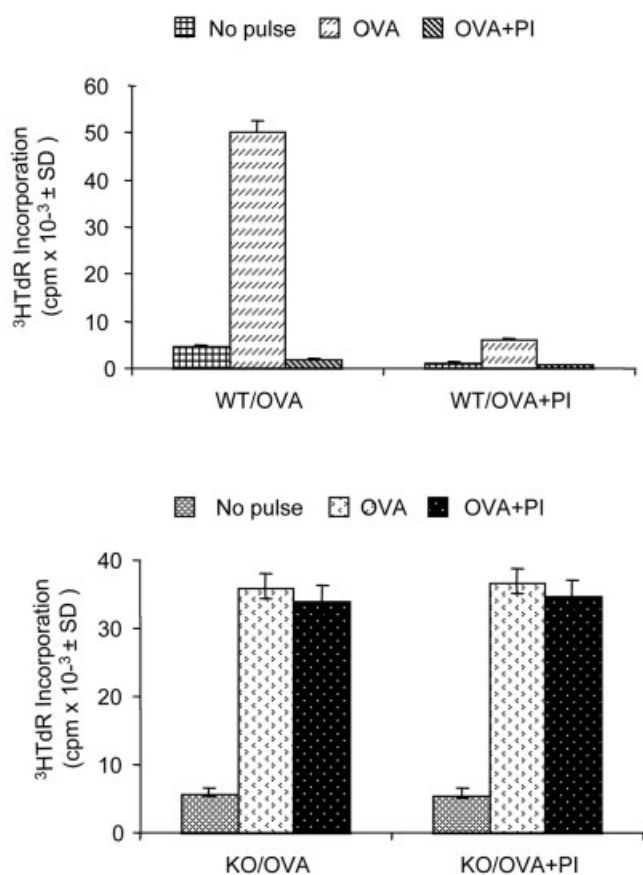


Figure 7. Role of IL-10 in the Ag-presenting ability of CD11c⁺ cells. These cells were purified on day 5 from LN of WT or IL-10^{-/-} C57BL/6 mice immunized with OVA (WT/OVA; KO/OVA) or OVA+PI (WT/OVA+PI; KO/OVA+PI) in CFA, pulsed with OVA or OVA+PI for 18 h, washed and X-irradiated. Purified CD3⁺ T cells were obtained from WT C57BL/6 mice immunized with OVA in CFA 8 days before. Cultures of 3 × 10⁵ T cells and 0.6 × 10⁵ pulsed or non-pulsed CD11c⁺ cells were set up and cell proliferation was measured after 48 h. The results are expressed as the mean ± SD of [³H]thymidine incorporation in triplicate cultures and are representative of two experiments.

The stability of DC maturation/activation depends on the initial signaling. Nakamura *et al.* [44] have shown transient maturation and subsequent deactivation of DC by two different TLR stimuli, in contrast to the stable maturation after CD40 stimulation by anti-CD40 mAb. Since we used CFA as adjuvant in the OVA immunization protocol, it is conceivable that PI might also act to deactivate DC by signaling through TLR. Recently, diminished expression and function of TLR in APC and T cells have been reported in human filarial infection [45, 46]. Experiments to check these aspects of TLR on *Ascaris*-mediated suppression are already under way. Altogether, our results indicate that the immunosuppressive effect of Asc components might be due to an interference with the ability of APC to activate and stimulate Ag-specific T cell proliferation, *via* an IL-10-dependent mechanism.

Materials and methods

Animals

C57BL/6 mice that had targeted disruption of the IL-10 gene (IL-10^{-/-}), WT C57BL/6 mice, BALB/c mice and OVA-specific TCR-Tg BALB/c mice (DO11.10) were bred in the animal house facilities of the Department of Immunology, ICB/University of São Paulo (São Paulo, Brazil). Eight-week-old male mice of each type were used for immunization and proliferation assays. All the animals were maintained under SPF conditions. The experimental protocols were approved by the Biomedical Sciences Institute/USP Ethical Committee for Animal Research.

Antigens and antibodies

OVA (grade V) was obtained from Sigma Chemical Co. (St. Louis, MO). Asc was prepared from adult *A. suum* bodies and fractionated by gel filtration under conditions previously described [11]. Fractions eluted in the first peak (PI) corresponding to high-molecular-weight components were concentrated with Centriprep 100 (Amicon) concentrator (100 cut-off point). Protein content was measured by the Lowry's method [47]. FITC-labeled or biotinylated anti-CD11c (integrin α_x chain, clone HL3), anti-CD11b and anti-CD3 mAb were obtained from BD Pharmingen (San Diego, CA). FITC- or PE-labeled anti-CD80 (clone 1G10), anti-CD86 (clone GL1), anti-CD40 (clone 1C10) and isotype control mAb were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). Anti-MHC class II (I-A^b and I-A^d) mAb obtained from hybridomas HB3 and HB163 were kindly provided by Dr. R. L. Coffman (Dynavax Technologies, Berkeley, CA) and labeled with FITC (Sigma Chemical Co).

Immunization

Groups of four to five BALB/c mice, or WT or IL-10^{-/-} C57BL/6 mice were injected subcutaneously with OVA (200 μ g of

protein/animal) or an equal amount of OVA+PI (200 μ g of protein/animal) emulsified in CFA (Sigma Chemical Co.), in the base of the tail (0.2 mL/animal).

Preparation of cell suspensions

For the flow cytometry analyses, inguinal and periaortic LN were collected from mice immunized 3, 4, 5 or 6 days before and used to prepare cells suspensions in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10 mM HEPES, 0.05 mM 2-ME, 216 mg of L-glutamine and 5% FCS (complete RPMI). The cell suspensions were centrifuged and adjusted at 1×10^6 cells/30 μ L in PBS plus 1% BSA and after this stained with different mAb. For the proliferative assays, cell suspensions were prepared from inguinal and periaortic LN from non-immunized or previously immunized mice and maintained in complete RPMI. The total cell number in LN from OVA- or OVA+PI-immunized mice was similar at any post-immunization time point, as well as the percentage of CD4⁺, CD8⁺, B220⁺, CD11b⁺ and CD11c⁺ cells.

CD3⁺ T cell and CD11c⁺ cell purification

OVA-specific TCR-Tg CD3⁺ T cells from DO11.10 BALB/c mice or CD3⁺ T cells from WT C57BL/6 mice immunized with OVA in CFA (day 8) were purified with magnetic microbeads. For this, LN from groups of six to eight mice were collected and the cell suspension prepared in complete RPMI. Briefly, total cells were incubated with biotinylated anti-CD3 mAb (1 μ g/10⁶ cells) for 30 min at 4°C. After washing, the cells were resuspended in 800 μ L of PBS containing 0.5% BSA/2 mM EDTA, pH 7.2, with streptavidin microbeads at 20 μ L/10⁶ cells (Milenyi Biotech, Bergisch, Gladbach, Germany) and incubated for 20 min at 4°C. The cells were washed and diluted in 1 mL of PBS containing 0.5% BSA/2 mM EDTA, pH 7.2, and separated in Midi-MACS columns according to manufacturer's instructions by positive selection. The purified CD3⁺ T cells were washed and resuspended in complete RPMI.

CD11c⁺ cells were purified in the same way by positive selection using biotinylated anti-CD11c mAb and streptavidin-conjugated magnetic microbeads. For this, LN from BALB/c, WT C57BL/6 or IL-10^{-/-} C57BL/6 mice immunized 5 days before with OVA or OVA+PI, or from non-immunized mice, were removed and digested in 5.0 mL of collagenase type IV (Sigma Chemical Co.) diluted in RPMI 1640 medium. After this, the suspensions obtained were submitted to the same procedure described above. The fraction of CD11c⁺ cells was washed and resuspended in complete RPMI. The purified CD11c⁺ cells were used in proliferative assays and to analyze the MHC class II, CD80, CD86 and CD40 molecules by flow cytometry. The purity of cell preparations was determined by FACScalibur using Cell Quest software (BD Biosciences, Franklin Lakes, NJ) analysis and was routinely 85–90%. The purity of OVA and OVA+PI samples within an experiment was similar.

Analysis of cell surface molecules by flow cytometry

Total cell suspensions or purified CD11c⁺ cells (10^6) were stained with anti-mouse CD80, CD86, CD40 or MHC class II

mAb conjugated to FITC or PE in PBS with 1% BSA and incubated for 30 min at 4°C. After the incubation, the cells were centrifuged and resuspended in 0.2 mL of PBS with 1% paraformaldehyde. All the cell suspensions were also incubated with FITC- or PE-labeled isotype control mAb. Flow cytometric analyses of duplicate samples (10^4 events per data acquisition file) were performed with FACScalibur using Cell Quest software (BD Biosciences). The percentage of positive cells for each marker was multiplied by the total cell number obtained per animal and the result divided by 100. The results were expressed either as the mean $\pm$ SD of the total number of labeled cells/animal ($n=4-5$) or as the geometric MFI of labeled cells.

T cell proliferation assay

For the antigenic pulse, purified CD11c⁺ cells were incubated with OVA (200 μ g/mL) or OVA (200 μ g/mL) plus PI (200 μ g/mL) diluted in complete RPMI (Sigma Chemical Co.) at 37°C in an humidified 5% CO₂ incubator for 18 h. After this time, the cells were centrifuged, resuspended in complete RPMI and X-irradiated (2500 rad). Some cells were only incubated with culture medium.

For the proliferation assays, purified CD3⁺ T cells (3×10^5 /well) were cultured in 96-well flat-bottomed microplates (Falcon BD Biosciences) with CD11c⁺ cells (0.6×10^5 cells/well) in complete RPMI at 37°C in 5% CO₂ for 48 or 72 h. Cell proliferation was measured by [³H]thymidine incorporation (0.5 μ Ci/well; DuPont, São Paulo, Brazil) added 18 h before cell harvesting. The results were obtained by liquid scintillation spectrometry and reported as the mean cpm $\pm$ SD of triplicate cultures from three experiments.

Statistical analysis

Results were expressed as mean $\pm$ SD. Statistical differences (significance levels of $p < 0.05$) between groups were assessed using the Mann-Whitney U-test (GraphPad Instat software).

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