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Schistosoma mansoni antigens alter activation markers and cytokine profile in lymphocytes of patients with asthma

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ABSTRACT

Asthma is a chronic disease characterized by airway inflammation, obstruction and hyperresponsiveness. Severe asthma affects a small proportion of subjects but results in most of the morbidity, costs and mortality associated with the disease. Studies have suggested that *Schistosoma mansoni* infection reduces the severity of asthma and prevent atopy.

Objective: We evaluated the ability of *S. mansoni* antigens, Sm29 and Sm29TSP-2 to modulate lymphocyte activation status in response to the allergen of the mite *Dermatophagoides pteronyssinus* (*Der p*1) *in* cell cultures of individuals with asthma.

Methods: Thirty four patients were enrolled in this study: seventeen patients with severe asthma (SA group), seventeen patients with mild asthma (MA group) and six controls with no asthma. Peripheral blood mononuclear cells (PBMC) were obtained and stimulated with Sm29 and Sm29TSP-2 in the presence or absence of *Der p1*. The expression of surface markers and cytokines on lymphocytes was evaluated by flow cytometry and the levels of IL-10 in the culture supernatant were determined by ELISA.

Results: The addition of Sm29 and Sm29TSP-2 antigens to PBMC cultures from both groups of subjects with asthma stimulated with *Der p*1 reduced the frequency of $CD4^+CD25^{low}$ cells whereas and increased frequency of $CD4^+CD25^{high}$ population was observed compared to unstimulated cultures. Moreover, cultures stimulated with Sm29TSP-2 showed a reduction in the frequency of T cells expressing CD69, IFN-γ, TNF and TGF-β in the MA group and an increase in the frequency of $CD4^+TSLPR^+$ T cells in the SA group. The addition of Sm29 to the cultures reduced the frequency of $CD4^+CD69^+$ and $CD4^+IL\text{-}5^+$ T cells in all asthmatic groups, and reduced the frequency of $CD4^+$ T cells expressing IL-13 in the MA group. The cultures stimulated with Sm29 and Sm29TSP-2 showed an increase in the level of IL-10 in the supernatants.

Conclusion: These results suggest that the addition of Sm29 and Sm29TSP-2 to the cells cultures from subjects with asthma reduced cell activation markers and altered the cytokine production pattern in a way that can potentialy control the inflammatory response associated with asthma.

1. Introduction

Severe asthma affects approximately 5% to 10% of patients with asthma worldwide and is characterized by the persistence of symptoms, frequent exacerbations, reduced lung function and a need for high doses of inhaled corticosteroids (Antonicelli et al., 2004; Bousquet et al., 2010; Hekking et al., 2014; Moore et al., 2007; Von Bulow et al., 2014). The treatment of severe asthma is difficult, costly and bring the risk of adverse events. Only a small proportion of subjects with severe asthma reaches total control of symptoms and exacerbations, which affect significantly their quality of life and results in higher expenditures with the disease, often related to emergency room visits, hospitalizations and the use of other sort of

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Table 1
Characteristics of the study population.

	Mild Asthma (n = 17)	Severe Asthma (n = 17)	Healthy Controls (n = 6)	P
Age (years)[a] (mean ± DP)	38,8 ± 14,3	46,5 ± 11,4	43,5 ± 13,3	>0,05
Female gender n(%)[b]	13 (76,4)	11 (64,7)	5 (83,3)	>0,05
Positivity to the skin prick test to *Der p*1 n (%)[b]	4 (23,5)	6 (35,2)	0 (0)[c]	<0,0001
SWAP-specific IgE (mean ± SD)	0.12 ± 0.03	0.05 ± 0.04	0.14 ± 0.07	>0.05

Cutoff IgE: 0.36.
[a] ANOVA.
[b] Chi-square.
[c] HC group *vs* MA group and HC group *vs* SA group.

heath resources (Antonicelli et al., 2004; Franco et al., 2009; O'Neill et al., 2015; Santos et al., 2007).

In recent years, many studies have suggested that infection by helminths may modulate the allergic response in asthma, being associated to lower frequency of positive skin prick tests to aeroallergens, and a lower prevalence of atopy in general among individuals living in helminth-endemic areas (Alcantara-Neves et al., 2014; Araujo et al., 2000; Cooper et al., 2003; Hagel et al., 1993; Lynch et al., 1993; Lynch et al., 1987; Medeiros et al., 2004; Van den Biggelaar et al., 2001; Van den Biggelaar et al., 2000). Asthmatics individuals infected with *Schistosoma mansoni* have less severe asthma symptoms as compared to uninfected patients. Moreover, the anthelmintic treatment against *S. mansoni* led to a worsening of asthma symptoms and to an increase in allergen-specific serum IgE (Almeida et al., 2012; Campolina et al., 2013; Medeiros et al., 2003; Van den Biggelaar et al., 2004). Furthermore, infection with *S. mansoni* has been associated with a reduced Th2 response *in vitro* and in murine model with an increase in regulatory mechanisms that may be associated with the control of inflammation and improvement in asthma symptoms (Araujo et al., 2004; Oliveira et al., 2009; Smits et al., 2007).

Regulatory T cell activation have been one of the main hypotheses to explain the inverse relationship between allergy and *S. mansoni* infection (Layland et al., 2013; Van der Vlugt et al., 2012). The literature has described $CD4^+$ T lymphocytes that do not express CD25 ($CD25^{neg}$) as young effector cells that are not yet activated, while those with a low expression of CD25 ($CD25^{low}$) are considered activated responder $CD4^+$T cells (Baecher-Allan et al., 2001). $CD4^+$ T cells with a high expression of CD25 ($CD25^{high}$) are associated with the suppression of the immune response and are, able to control the activation and proliferation of activated cells by cell-cell contact via costimulatory molecules such as CTLA- 4 and PD-1, or by the production of regulatory cytokines, such as IL-10 (Baecher-Allan et al., 2001; Gangi et al., 2005; Okita et al., 2009; Pontoux et al., 2002; Sojka et al., 2009; Uhlig et al., 2006).

The studies mentioned above provided support to further investigating the use of parasite antigens to down-modulate the inflammatory response observed in subjects with asthma. Studies have shown that chronic helminth infections, especially *Schistosoma mansoni*, possesses the ability to modulate the inflammatory response associated to both, Th1 (Bafica et al., 2011; Lima et al., 2013) and Th2 (Cardoso et al., 2012; Cardoso et al., 2010; Cardoso et al., 2006a,b; Pacifico et al., 2009) immune-mediated diseases. These findings have provided the rationale for the use of recombinant *S. mansoni* proteins in *in vitro* studies with cells from patients with asthma in an attempt to modulate the response associated with inflammatory process. Studies have shown that Sm29 and SmTSP-2 antigens are secreted by the membrane and/or tegument of the *S. mansoni* adult worm (Cardoso et al., 2006a,b; Tran et al., 2006). Proteins secreted or localized on the surface of *Schistosoma* spp., which are in intimate contact with host tissues, might be more effective in triggering immunoregulatory processes (Simpson et al., 1990). The Sm29 is a membrane-bound glycoprotein located on the tegument of the adult worm and lung stage schistosomula (Cardoso et al., 2006a,b). SmTSP-2 is a recombinant protein (tetraspanin) from *S. mansoni* tegument (Tran et al., 2006). These antigens have been evaluated by our group regarding their potential to down-modulate inflammatory cytokines and to induces IL-10 production in vitro in PBMC from individuals with cutaneous leishmaniasis, HTLV-1 infection and asthma (Bafica et al., 2011; Cardoso et al., 2010; Lima et al., 2013). Thus, the identification of parasite antigens with the potential to prevent or attenuate the inflammatory response associated with asthma represents a promising strategy for an alternative intervention to control this chronic illness.

2. Materials and methods

2.1. Features of the studied subjects

In this study, we recruited 17 consecutive patients with severe asthma followed up for over one year in the Program for the Control of Asthma of Bahia (ProAR), a reference center for severe asthma in Salvador, Bahia, Brazil, 17 patients with mild asthma and 6 health controls (HC), recruited consecutively from the same communities patients with severe asthma live, invited to volunteer by public advertisement in health facilities and public transportation. Subjects were recruited from January 2013 until July 2015 and the blood samples processed immediately upon collection. Subjects were not included if they had an exacerbation in the last month regardless of using oral corticosteroids or not. At the time of blood samples collection all subjects with severe asthma were treated with a combination of medium to high dose of inhaled corticosteroids (800mcg to 1600 of budesonide or equivalent) and long acting beta 2 agonist. Those with mild asthma were not receiving inhaled corticosteroids. Individuals with severe asthma were identified as having untreated severe asthma at enrollment in ProAR (from 2003), according to the NIH Guidelines for Asthma (NIH-NHBLI. Guidelines for the Diagnosis and Management of Asthma, 1997) and a WHO consultation on severe asthma (Bousquet et al., 2010). In brief, they had any one of the following: (i) symptoms daily, continuous; (ii) activities limited daily (symptoms with minor efforts); (iii) nocturnal symptoms over 2 times a week; (iv) use of bronchodilators ≥2 times a day; (v) Peak Expiratory Flow (PEF) or Forced Expiratory Volume in one second (FEV_1): <60% of predict. Subjects with mild asthma were recruited on the basis of their history of asthma, having their diagnosis validated by a doctor in the research facility. We did not include in this study current smokers individuals and those with a positive serology for Chagas disease, HIV, HTLV-1, or hepatitis virus types B and C, all of which are conditions that could interfere with the immunological response. All participants were submitted to skin prick test to *Dermatophagoides pteronyssinus* antigen 1 (*Der p*1) and a panel of the most common aeroallergens including other house dust mites, coacoroach, molds, cat, dog and grass. To rule out the effect of *S. mansoni* previous exposure in immunological assays, we also excluded individuals who had a positive *S. mansoni* infection or exposure to this parasite any time in his/her life. In the urban area

of Salvador da Bahia there is no report of *Schistosoma* infection in the last decades.

Furthermore, we measured the levels of serum-specific IgE to *S. mansoni* soluble adult worm antigen (SWAP) and performed parasitological assays by Hoffman et al. technique (Hoffman et al., 1934). There was no significant difference in the mean age, gender and levels of serum-specific IgE to SWAP among groups evaluated. Additionally, there were no significant differences regarding the response to skin prick test (SPT) to *Der p*1 between asthmatics. The HC group was negative to the SPT to *Der p*1 (Table 1). All individuals were negative for *S. mansoni* infection by parasitological exams (not shown).

2.2. Ethical statement

The Ethics Committee of Maternidade Climério de Oliveira, Federal University of Bahia (License Number: 095/2009) approved the present study. Written informed consent was obtained from all patients and controls. All participants who had complaints of asthma and allergies were properly oriented regarding environmental control, prescribed the medication required and referred to the most convenient health facility.

2.3. SWAP-specific IgE measurements in human serum

Levels of SWAP-specific IgE were measured in serum from all studied individuals using an indirect ELISA technique, as previously described (Figueiredo et al., 2012; Souza-Atta et al., 1999).

2.4. Cell culture and flow cytometry assays

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque gradient sedimentation and adjusted to a concentration of 3×10^5/mL in RPMI 1640 medium containing 10% normal human serum (AB positive and heat inactivated), 100 U/mL of penicillin, 100 mg/mL of streptomycin, 2 mmol/L of L-glutamine, and 30 mmol/L of HEPES (all from Life Technologies GIBCO, BRL, Gaithersburg, MS). Cells were cultured *in vitro* either stimulated with 10 μg/mL of the *S. mansoni* antigens Sm29 and Sm29TSP-2 in the presence or absence of *Der p*1 (Cosmo Bio, LTD.; Tokyo, Japan) at the concentration of 5 μg/mL for 48 h at 37 °C in an atmosphere containing 5% CO_2. After incubation, supernatants were harvested for IL-10 measurement by ELISA using commercially available kits (R&D Systems, Inc.), and the cells were stained for flow cytometry as described below.

During the last 4 h of culture, Brefeldin A (10 μg/mL; Sigma, St. Louis, MO), which impairs protein secretion by the Golgi complex, was added to the cultures. Cells were stained with fluorescently conjugated mouse anti-human monoclonal antibodies against human CD3 (clone OKT3, eBioscience), CD4 (clone OKT4, eBioscience), CD25 (clone BC96, eBioscience), CD28 (clone CD28.2, eBioscience), CD69 (clone L78, Becton Dickinson), CTLA-4 (clone 14D3, eBioscience) and TSLPR (clone 1A6, eBioscience), and then analyzed for 100000 events per sample using a flow cytometer (FACSCanto, Becton Dickinson, San Jose, CA). Limits for the quadrant markers were set based on negative populations and controls isotype (data not shown).

Intracellular staining was performed with a PE-labeled monoclonal antibody against human FoxP3 (clone 236A/E7, eBioscience), IL-10 (clone JES3-19F1, eBioscience), TGF-β (clone TW4-2F8, eBioscience), IFN-γ (clone GZ-4, eBioscience), TNF (clone MAb11, eBioscience), IL-17A (clone eBio64DEC17, eBioscience), IL-13 (PVM13-1, eBioscience) and IL-5 (clone JES1-39D10, eBioscience). The Clone TW4-2F8 (eBioscience) evaluated detects LAP/pro-TGF-β1.

The frequency of positive cells was analyzed using the program FlowJo™ (Tree Star, USA). The lymphocyte region was defined by nonspecific fluorescence with forward scatter (FSC) and side scatter (SSC) used to indicate cell size and granularity, respectively. The cells were also gated based on their expression of CD3 and CD4 (Fig. 1A). Fig. 1B and C shows a representative gate strategy to analyse the different $CD4^+CD25^+$ and $CD4^+CD69^+$ T cell populations, respectively.

2.5. Antigen stimulation

The *Schistosoma mansoni* tegument antigens Sm29 and Sm29TSP-2, used in this study were provided by Dr. Sérgio C. Oliveira from the Institute of Biological Science, Department of Biochemistry and Immunology, UFMG, Brazil. The recombinant proteins were cloned in *E. coli* (Cardoso et al., 2006a,b; Pinheiro et al., 2014) and were tested for the presence of lipopolysaccharide (LPS) using a commercially available LAL Chromogenic Kit (CAMBREX).The level of LPS was below the detection limit (data not shown).

2.6. Statistical analysis and sample size

Statistical analysis and graphical representation were performed using Graphpad PRISM 5.0 software (La Jolla, CA, USA). Comparisons among age of groups were performed using ANOVA and for immunological assays we used Kruskal Wallis test with Dunns pos-test. Comparisons among gender and positivity skin prick test and SWAP-specific IgE were performed using chi-square test. All statistical tests were two-tailed and statistical significance was established at the 95 percent confidence interval. *P*-value < 0.05 were considered significant. The sample size calculation was performed based on the frequency of IL-10 expression in $CD4^+CD25^+$ T cells from healthy subjects with mild asthma stimulated with *Der p*1 plus Sm29 (Cardoso et al., 2011). A minimum size of 16 patients per group would be sufficient to detect significant differences.

3. Results

3.1. Effect of S. mansoni antigens on the expression of CD25 molecules on $CD4^+$ T lymphocytes

Different degrees of CD25 expression on $CD4^+$ T lymphocytes have been associated with either activation or regulatory profile in these cells. Therefore, we decided to analyze the expression of this molecule in T cell populations from subjects with mild asthma (MA) and severe asthma (SA) after the addition of *S. mansoni* antigens to the cultures in the presence of *Der p1* antigen.

In the MA group, there was a reduction in the frequency of $CD4^+CD25^{low}$ T cells in cultures stimulated with *Der p1* + Sm29 [median = 2.68% (min–max = 1.16%–10.50%); $p < 0.05$], *Der p1* + Sm29TSP-2 [2.89% (0.76%–9.73%); $p < 0.05$], Sm29 [1.12% (0.49%–3.64%) $p < 0.001$] or Sm29TSP-2 [1.08% (0.53%–2.09%); $p < 0.001$] compared to the frequency in cultures stimulated with *Der p1* alone [3.93% (1.30%–11.60%); Fig. 2A]. Additionally, we observed a lower frequency of $CD4^+CD25^{low}$ T cells in cultures without stimulation (WS cultures) [0.97% (0.34%–3.82%)] than in those stimulated with *Der p1* ($p < 0.001$), *Der p1* + Sm29 ($p < 0.001$), *Der p1* + Sm29TSP-2 ($p < 0.001$), Sm29 ($p < 0.05$) or Sm29TSP-2 ($p < 0.05$; Fig. 2A).

In the SA group we found similar results with a reduction in the frequency of $CD4^+CD25^{low}$ T cells in cultures stimulated with *Der p1* + Sm29 [2.77% (0.90%–10.60%); $p < 0.05$], *Der p1* + Sm29TSP-2 [2.63% (1.07%–9.92%); $p < 0.05$], Sm29 [1.78% (0.44%–3.78%) $p < 0.001$] or Sm29TSP-2 [1.36% (0.97%–2.46%); $p < 0.001$] compared

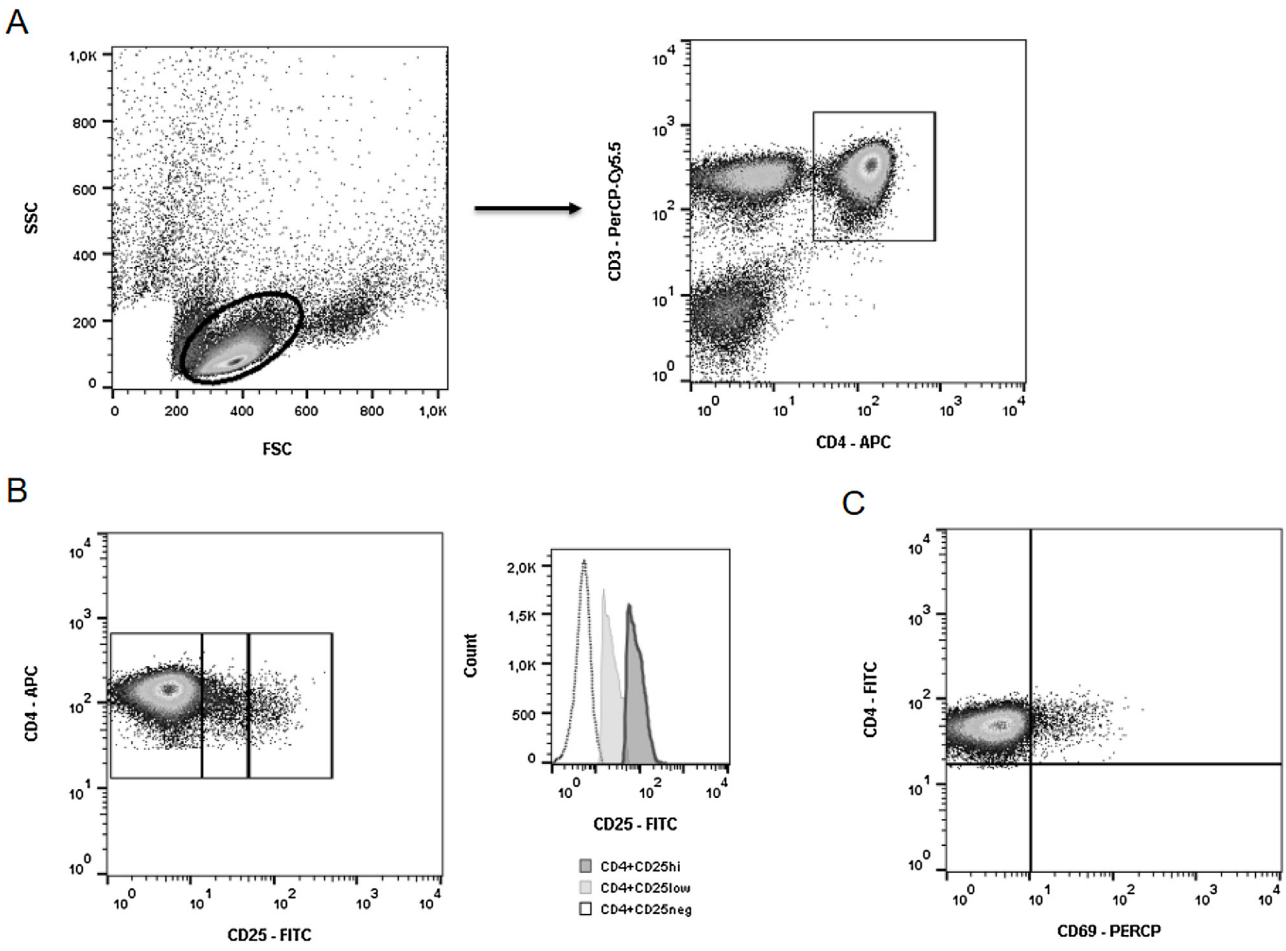

Fig. 1. Gate Strategy for the identification of lymphocytes, defined by nonspecific fluorescence with forward scatter (FSC) and side scatter (SSC). The cells were also gated based on their expression of CD3 and CD4 (A). Representative gate strategy to analyse the different $CD4^+CD25^+$ and $CD4^+CD69^+$ T cell populations (B and C, respectively).

to the frequency in cultures stimulated with *Der p1* alone [3.93% (1.30%–11.60%); Fig. 2B]. We also observed a lower frequency of $CD4^+CD25^{low}$ T cells in cultures without stimulation [0.96% (0.29%–3.04%)] than in those stimulated with *Der p1* ($p < 0.001$), *Der p1* + Sm29 ($p < 0.001$), with *Der p1* + Sm29TSP-2 ($p < 0.001$), Sm29 ($p < 0.05$) or Sm29TSP-2 ($p < 0.05$; Fig. 2B).

In the HC group the addition of Sm29 or Sm29TSP-2 to the cultures stimulated with *Der p*1 did not alter the frequency of $CD4^+CD25^{low}$ T lymphocytes compared to the frequency in cultures stimulated with *Der p*1 alone (Fig. 2C). We observed a lower frequency of $CD4^+CD25^{low}$ T cells in WS cultures [1.47 (0.45–2.68)] compared to the cultures stimulated with *Der p*1 alone [3.30 (1.76–9.16), $p < 0.05$; Fig. 2C].

When we analyzed the $CD4^+CD25^{hi}$ T cells population in the MA group (Fig. 2D), we found a higher frequency of these cells in cultures stimulated with *Der p1* + Sm29 [0.97% (0.18%–1.83%); $p < 0.001$], *Der p1* + Sm29TSP-2 [0.96% (0.39%–1.68%); $p < 0.001$], Sm29 [0.68% (0.29%–1.27%); $p < 0.01$], Sm29TSP-2 [0.73% (0.22%–1.20%); $p < 0.01$] or *Der p1* alone [0.76% (0.28%–1.4%); $p < 0.001$] compared to the frequency in WS cultures [0.28% (0.13%–0.78%)].

Similar to results from the MA group, in the SA group (Fig. 2E) there was an increase in the frequency of $CD4^+CD25^{hi}$ T cells in cultures stimulated with *Der p1* + Sm29 [0.97% (0.44%–2.05%); $p < 0.001$], *Der p1* + Sm29TSP-2 [0.79% (0.44%–1.94%); $p < 0.001$] or *Der p1* alone [0.64% (0.36%–1.45%); $p < 0.001$] compared to the frequency in WS cultures [0.32% (0.07%–0.68%)]. An increase in the population of $CD4^+CD25^{hi}$ T lymphocytes was also observed in cultures stimulated with Sm29 [0.70% (0.26%–1.66%); $p < 0.001$] or Sm29TSP-2 [0.63% (0.26%–1.06%); $p < 0.01$] compared to the frequency in WS cultures.

In the HC group there was a higher frequency of $CD4^+CD25^{hi}$ cells in cultures stimulated with *Der p*1 + Sm29 [0.75% (0.54%–1.12%)] with *Der p*1 + Sm29TSP-2 [0.76% (064%–1.31%)] and *Der p*1 [0.65% (0.46%–0.98%)] compared to the frequency in WS cultures [0.39% (0.24%–0.47%); $p < 0.01$; Fig. 2F].

The MA group showed a higher frequency of $CD4^+CD25^{neg}$ T cells in WS cultures [98.70% (95.70%–99.50%)] compared to the frequency in cultures stimulated with *Der p*1 [95.35% (87.30%–98.40%); $p < 0.001$], *Der p*1 + Sm29 [96.50% (88.40%–98.60%); $p < 0.001$] or *Der p*1 + Sm29TSP-2 [96.30% (89.40%–98.90%); $p < 0.01$; Fig. 2G]. An increase in the frequency of $CD4^+CD25^{neg}$ T cells was also observed in cultures stimulated with Sm29 [98.30% (95.70%–99.20%)] or Sm29TSP-2 [98.30% (97.20%–99.20%)] compared to the frequency in those stimulated with *Der p*1 ($p < 0.001$; Fig. 2G).

In the SA group, the addition of Sm29TSP-2 to the cultures stimulated with *Der p*1 led to an increase in the frequency of $CD4^+CD25^{neg}$ T cells [96.10 (89.30%–98.10%); $p < 0.05$] compared to the frequency in those stimulated with *Der p*1 alone [95.40% (85.40%–96.80%); $p < 0.001$]. This increase was also observed in cultures stimulated with Sm29 [97.40% (95.30%–99.10%)] or with Sm29TSP-2 [97.95% (96.20%–98.60%)] compared those stimulated with *Der p*1 ($p < 0.001$; Fig. 2H).

There was no significant difference in the frequency of $CD4^+CD25^{neg}$ T cells in cultures stimulated with *Der p* 1 in the

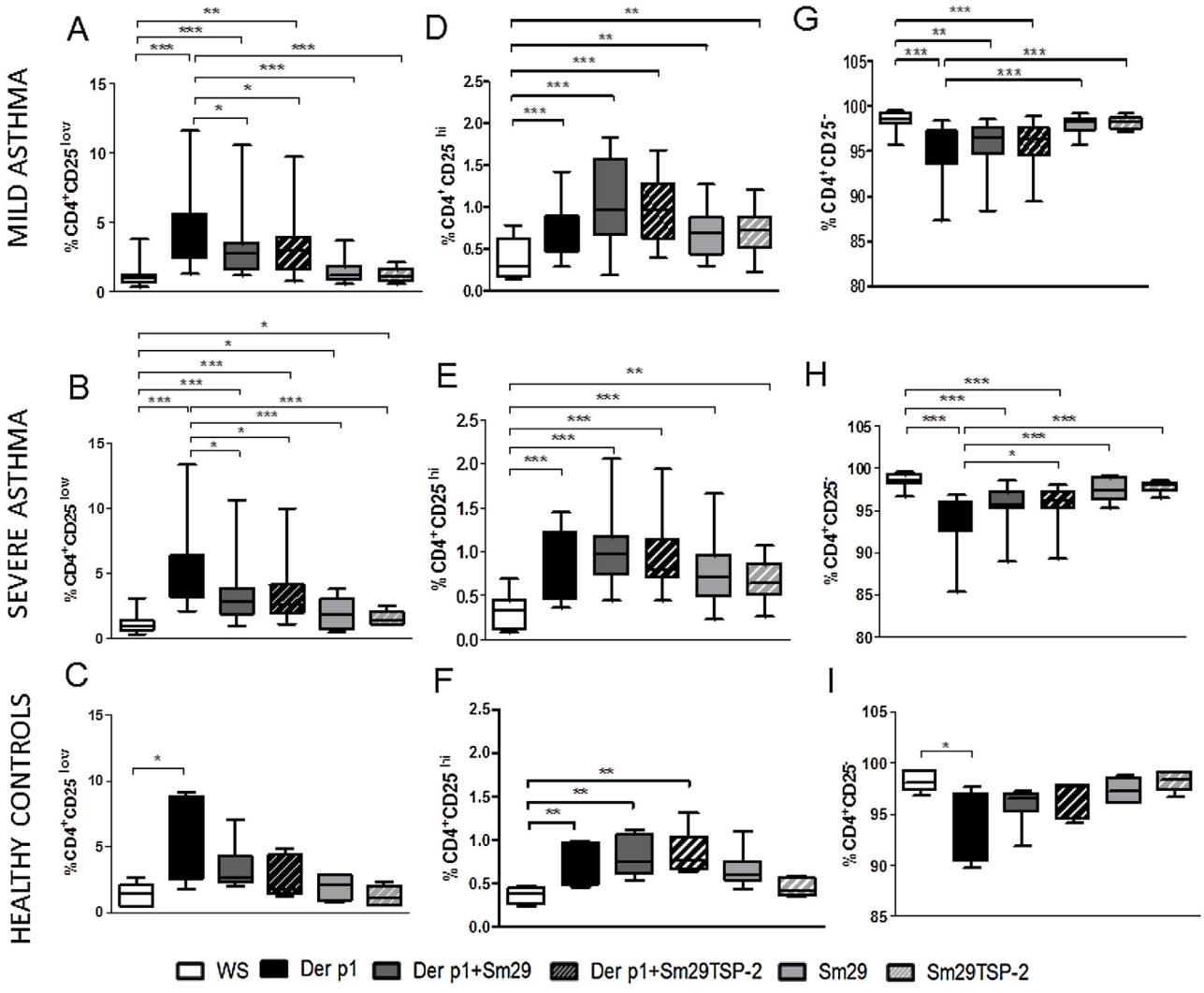

Fig. 2. Frequency of subpopulations of CD4⁺ T lymphocytes expressing CD25^{low} (A–C), CD25^{hi} (D–F) and CD25- (G–I) in cell cultures from patients with asthma and healthy controls. WS = without stimulation. *Der p1* = *Dermatophagoides pteronyssinus* antigen 1. Sm29 = *S. mansoni* antigen Sm29. Sm29TSP-2 = *S. mansoni* antigen Sm29TSP-2. The results are expressed as median, min-max values. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, Kruskal-Wallis test.

presence or absence of *S. mansoni* antigens in cultures from HC group (Fig. 2I). In this group we observed a higher frequency of CD4⁺CD25^{neg} T cells in WS cultures [98.00 (96.80–99.20)] compared to the frequency in cultures stimulated with *Der p*1 [95.80 (89.70–97.70), p < 0.05; Fig. 2I]

Since the expression of transcription factor Foxp3 is essential to the regulatory function of T CD4⁺CD25^{hi} cell population, we decided to evaluate the expression of the molecule in this group of cells (Fig. 3). We did not observe significant difference in the frequency of T CD4⁺CD25⁺ cell expressing Foxp3 in the cultures stimulated with *Der p*1 in the presence or absence of *S. mansoni* antigens in all evaluated groups (Fig. 3A–C).

3.2. Activation CD28 and CD69 molecules expressed by CD4⁺T lymphocytes in response to the S. mansoni antigens

The frequency of activation molecules CD28 and CD69 in CD4⁺ T lymphocytes in PBMC cultures from asthmatic subjects in response to the *S. mansoni* antigens was evaluated. There were no significant difference in the frequency of CD4⁺ CD28⁺cells in the MA group in the presence of Sm29 and Sm29TSP-2 antigens (p > 0.05; Fig. 4A). On the other hand, in the SA group we observed a lower frequency of CD4⁺CD28⁺ T cells in cultures stimulated with Sm29TSP-2 [93.60% (74.60%–98.50%)], compared to the frequency in WS cultures [96.25% (82.60%–98.90%); p < 0.05; Fig. 4B].

We observed a decrease in the frequency of cells expressing CD69 in PBMC cultures from the MA group that were stimulated with *Der p*1 + Sm29 [0.46% (0.09%–1.7%); p < 0.05], with *Der P*1 + Sm29TSP-2 [0.46% (0.13%–1.56%); p < 0.01], compared to the frequency in cultures stimulated with *Der p*1 [1.48% (0.04%–4.91%); Fig. 4D]. In this same group, cultures stimulated with Sm29 [0.37% (0.12%–2.32%); p < 0.01], or with Sm29TSP-2 [0.35% (0.12%–1.84%); p < 0.01] showed a decrease in the frequency of CD4⁺CD69⁺ T cells, compared to the frequency in cultures stimulated with *Der p*1 alone [0.41% (0.04%–1.29%); Fig. 4D].

In the SA group there was also a reduction in the frequency of CD4⁺CD69⁺ T cells in cultures stimulated with *Der p*1 + Sm29 [0.72% (0.38%–1.6%); p < 0.05], compared to the frequency in cultures stimulated with *Der p*1 alone [1.27% (0.38%–3.41%); Fig. 4E]. In cultures stimulated with Sm29 [0.52% (0.17%–2.15%); p <0.01] or Sm29TSP-2 [0.36% (0.15%–1.69%); p < 0.001] and in WS cultures [0.29% (0.07%–2.24%); p < 0.001], there was also a reduction in the frequency of CD4⁺CD69⁺ T cells, compared to the frequency in cultures stimulated with *Der p*1. A lower frequency of CD4⁺CD69⁺ T cells was also observed in the cultures stimulated with *Der p*1 + Sm29 (p < 0.01) and *Der p*1 + Sm29TSP-2 [0.81% (0.17%–3.16%)

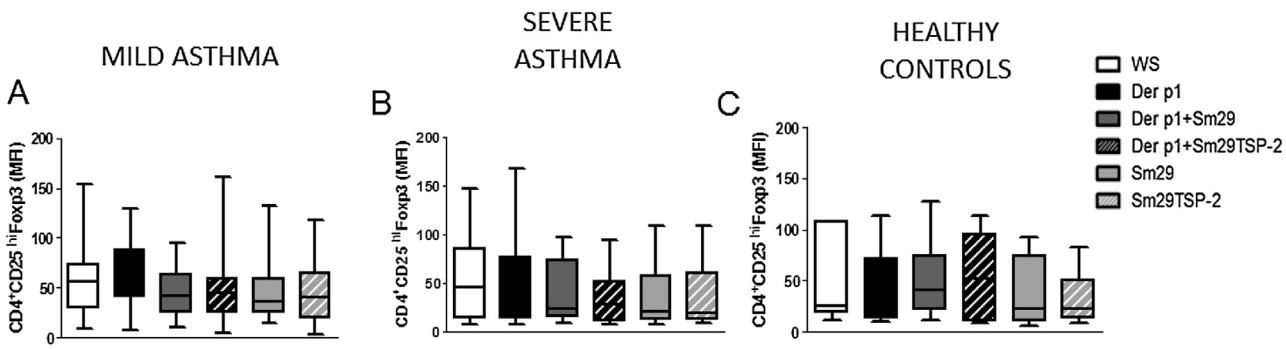

Fig. 3. Mean fluorescence intensity (MFI) of Foxp3 in the $CD4^+CD25^{hi}$ T lymphocytes from asthmatic patients and healthy controls. WS = without stimulation. *Der p*1 = *Dermatophagoides pteronyssinus* antigen 1. Sm29 = *S. mansoni* antigen Sm29. Sm29TSP–2 = *S. mansoni* antigen Sm29TSP-2. The results are expressed as median, min-max values.

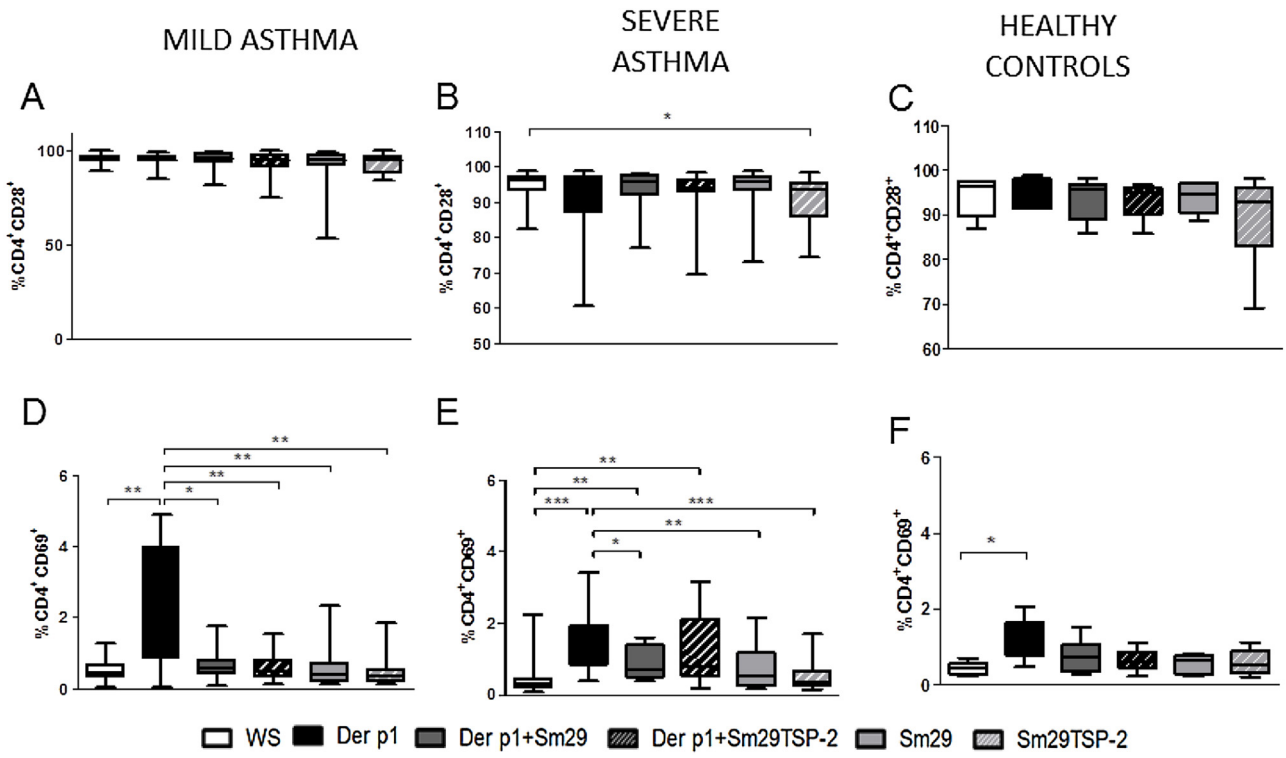

Fig. 4. Frequency of $CD4^+$ T lymphocytes expressing CD28 (A, B and C) and CD69 (D, E and F) in cell cultures from asthmatic patients and healthy controls. WS = without stimulation. *Der p*1 = *Dermatophagoides pteronyssinus* antigen 1. Sm29 = *S. mansoni* antigen Sm29. Sm29TSP–2 = *S. mansoni* antigen Sm29TSP-2. The results are expressed as median, min-max values. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, Kruskal-Wallis test.

$p < 0.01$], compared to the frequency in those stimulated with *Der p*1 (Fig. 4E).

In the MA group the frequency of $CD4^+$ T cells expressing CD69 in the cultures stimulated with *Der p*1 + Sm29TSP-2 was higher [0.46 (0.13–1.56)] than in those observed in SA group [0.72 (0.38–1.60), $p < 0.05$; Table 3], while there was no significant difference in the frequency of $CD4^+$ T cells expressing CD28 (Fig. 4C) or CD69 (Fig. 4F) in cultures stimulated with *Der p* 1 in the presence or absence of *S. mansoni* antigens in cultures from HC group.

3.3. Reduced expression of IFN-γ or TNF by $CD4^+$T lymphocytes after the addition of Sm29 and Sm29TSP-2 to cultures from asthmatic patients

The expression of intracellular cytokines TNF, IFN-γ and IL-17A by $CD4^+$ T lymphocytes was also evaluated in this study, because these cytokines have been associated with a worsening of the inflammatory process in asthma. A lower frequency of $CD4^+TNF^+$ T cells was observed in the cultures stimulated with *Der p*1 + Sm29TSP-2 [0.09% (0.02%–0.87%); $p < 0.05$] or Sm29TSP-2 [0.09% (0.04%–0.73%); $p < 0.01$] and in WS cultures [0.13% (0.01%–0.73%); $p < 0.05$], compared to the frequency in cultures stimulated with *Der p*1 alone [0.20% (0.05%–0.93%); Table 2].

There was a reduction in the frequency of $CD4^+IFN\text{-}\gamma^+$ T cells in the MA group in cultures stimulated with *Der p*1 + Sm29TSP-2 [0.21% (0.03%–1.38%); $p < 0.05$], compared to the frequency in cultures stimulated with *Der p*1 alone [0.32% (0.10%–1.38%); Table 2]. In cultures stimulated with Sm29 [0.21% (0.06%–0.70%); $p < 0.05$] or Sm29TSP-2 [0.18% (0.08%–1.51%); $p < 0.05$], there was also a reduction in the expression of this cytokine, compared to the expression in cultures stimulated with *Der p*1 (Table 2).

There was no significant difference in the frequency of $CD4^+$ T cells expressing IL-17A in cultures stimulated with *Der p*1 in the presence or absence of *S. mansoni* antigens in cultures from either

Table 2
Frequency of $CD4^+$ T lymphocytes expressing TNF, IFN-γ, IL-17A, IL-5, IL-13, TSLPR, CTLA-4, IL-10 and TGF-β in cell cultures from asthmatics patients.

	MILD Asthma						SEVERE Asthma					
	WS	*Der p*1	*Der p*1 + Sm29	*Der p*1 + Sm29TSP-2	Sm29	Sm29TSP-2	WS	*Der p*1	*Der p*1 + Sm29	*Der p*1 + Sm29TSP-2	Sm29	Sm29TSP-2
$CD4^+TNF^+$ (%)	0.13 (0.02–0.73)	0.20 (0.06–0.93) †	0.15 (0.04–0.89)	0.10 (0.02–0.87) *	0.12 (0.05–0.48)	0.09 (0.04–0.73) **	0.14 (0.06–1.97)	0.16 (0.05–2.96)	0.19 (0.04–0.36)	0.16 (0.07–0.28)	0.12 (0.02–0.85)	0.16 (0.07–0.74)
$CD4^+IFN\text{-}\gamma^+$ (%)	0.37 (0.03–1.02)	0.32 (0.10–1.38)	0.30 (0.05–1.77)	0.21 (0.03–1.38) *	0.21 (0.07–0.70) *	0.18 (0.08–1.51) *	0.12 (0.05–2.06)	0.23 (0.03–1.24)	0.21 (0.07–1.03)	0.20 (0.06–0.99)	0.12 (0.04–1.00)	0.18 (0.08–0.74)
$CD4^+IL\text{-}17A^+$ (%)	0.16 (0.01–1.37)	0.29 (0.02–1.49)	0.14 (0.01–2.44)	0.17 (0.02–2.03)	0.16 (0.01–1.61)	0.11 (0.05–1.52)	0.31 (0.02–1.28)	0.23 (0.02–2.28)	0.21 (0.02–1.69)	0.31 (0.02–4.23)	0.19 (0.01–1.46)	0.20 (0.01–1.65)
$CD4^+IL\text{-}5^+$ (%)	0.26 (0.02–1.79)	0.42 (0.07–2.35)	0.55 (0.14–1.36)	0.14 (0.02–1.64)	0.19 (0.02–1.21) *	0.22 (0.03–0.84)	0.28 (0.07–1.47)	0.25 (0.12–1.7)	0.40 (0.05–1.52)	0.32 (0.05–1.73)	0.11 (0.04–0.53)*	0.16 (0.01–0.35)
$CD4^+IL\text{-}13^+$ (%)	0.93 (0.07–2,65)	0.78 (0.06–2.82)	0.78 (0.13–1.86)	1.07 (0.02–2.07)	0.69 (0.01–1.72)	0.42 (0.01–0.90)	0.31 (0.03–2.77)	0.45 (0.05–2.19)	0.43 (0.08–2.86)	0.66 (0.09–3.00)	0.19 (0.05–2.11)*	0.37 (0.05–2.46)
$CD4^+TSLPR^+$ (%)	0.49 (0.09–0.78)	0.36 (0.04–4.31)	0.51 (0.15–2.40)	0.46 (0.14–1.23)	0.29 (0.08–1.02)	0.25 (0.11–0.45)	0.29 (0.08–0.95)	0.25 (0.10–0.91)	0.43 (0.12–1.21)	0.62 (0.12–1.52)*†	0.23 (0.13–1.84)	0.18 (0.07–0.92)
$CD4^+CTLA\text{-}4^+$ (%)	1.35 (0.07–4.85)	1.73 (0.19–6.13)	2.03 (0.37–7.75)	2.49 (0.03–7.83)	1.6 (0.16–4.40)	1.5 (0.18–6.20)	0.72 (0.18–7.71)	0.50 (0.11–2.83)	1.45 (0.15–9.09)	1.30 (0.10–6.37)	0.97 (0.14–5.16)	1.36 (0.20–4.62)
$CD4^+IL\text{-}10^+$ (%)	0.43 (0.04–1.78)	0.47 (0.04–2.22)	0.58 (0.04–3.09)	0.54 (0.05–2.07)	0.41 (0.05–2.03)	0.28 (0.03–1.89)	0.30 (0.07–2.60)	0.59 (0.07–1.93)	0.56 (0.05–2,27)	0.56 (0.04–2.44)	0.39 (0.07–2.26)	0.38 (0.05–1.49)
$CD4^+TGF\text{-}\beta^+$ (%)	0.32 (0.09–1.79)	0.71[a] (0.07–3.70)	0.41 (0.07–1.46)	0.27 (0.07–1.36) *	0.36 (0.01–1.42) *	0.38 (0.09–1.68) *	0.27 (0.04–1.48)	0.24 (0.05–1.78)	0.37 (0.03–0.77)	0.28 (0.02–0.93)	0.24 (0.02–1.29)	0.26 (0.04–1.08)

WS = without stimulation. Der p1 = *Dermatophagoides pteronyssinus* antigen 1. Sm29 = *S. mansoni* antigen Sm29. Sm29TSP–2 = *S. mansoni* antigen Sm29TSP-2. The results are expressed as median, min-max values. $^*P < 0.05$,$^{**}P < 0.01$ compared to *Der p*1 stimulated cultures. $^\dagger P < 0{,}05$ compared to WS cultures, Kruskal-Wallis test.
[a] $p < 0.05$ *Der p*1 (MA group) *vs Der p*1 (SA group)

Table 3
Expression of CD25, Foxp3, CD69 and CD28 by $CD4^+$ T lymphocytes stimulated with *S. mansoni* antigens and the levels of IL-10 in supernatants of cell cultures from asthmatic patients.

	MILD Asthma						SEVERE Asthma					
	WS	*Der p*1	*Der p*1 + Sm29	*Der p*1 + Sm29TSP-2	Sm29	Sm29TSP-2	WS	*Der p*1	*Der p*1 + Sm29	*Der p*1 + Sm29TSP-2	Sm29	Sm29TSP-2
$CD4^+CD25^{low}$ (%)	0.97 (0.34–3.8)	3.93 (1.30–11.6)	2.68 (1.16–10.5)	2.89 (0.76–9.73)	1.12 (0.49–3.64)	1.08 (0.53–2.09)	0.96 (0.29–3.04)	3.85 (2.06–13.30)	2.77 (0.90–10.60)	2.63 (1.07–9.92)	1.78 (0.44–3.78)	1.36 (0.97–2.46)
$CD4^+CD25^{hi}$ (%)	0.28 (0.13–0.8)	0.76 (0.28–1.4)	0.97 (0.18–1.8)	0.96 (0.39–1.68)	0.68 (0.29–1.27)	0.73 (0.22–1.2)	0.32 (0.07–0.68)	0.64 (0.36–1.45)	0.97 (0.44–2.05)	0.79 (0.44–1.94)	0.70 (0.22–1.66)	0.63 (0.26–1.06)
$CD4^+CD25^{hi}$ Foxp3(MIF)	56.30 (9.65–154.0)	51.25 (7.67–130.0)	41.81 (10.90–94.90	45.20 (5.00–161.0)	36.80 (14.5–132.0)	40.70 (3.49–118.0)	45.10 (6.98–147.0)	25.20 (6.99–168.0)	22.60 (7.74–97.30)	27.60 (7.19–93.60)	19.90 (6.68–109.0)	18.80 (8.24–109.0)
$CD4^+CD25^-$ (%)	98.70 (95.70–99.5)	95.35 (87.30–98.7)	96.50 (88.4–98.6)	96.30 (89.40–98.90)	98.30 (95.7–99.2)	98.30 (97.2–99.2)	98.60 (96.7–99.6)	95.40 (85.40–96.8)	95.70 (89.0–98.5)	96.10 (89,30–98.1)	97.40 (95.3–99.1)	97.95 (96.40–98.60)
$CD4^+CD69^+$ (%)	0.41 (0.04–1.2)	1.48 (0.04–4.9)	0.56 (0.09–1.76)	0.46 (0.13–1.56)*	0.37 (0.12–2.3)	0.35 (0.12–1.84)	0.29 (0.07–2.24)	1.27 (0.38–3.4)	0.72 (0.38–1.60)	0.81 (0.17–3.16)	0.52 (0.17–2.15)	0.36 (0.15–1.69)
$CD4^+CD28^+$ (%)	95.10 (88.60–99.4)	95.15 (84.8–98.8)	95.85 (81.50–98.8)	94.65 (74.50–99.50)	94.95 (53.5–99.2)	94.65 (83.90–99.60)	96.25 (82.60–98.90)	94.35 (60.80–98.90)	95.50 (76.90–8.20)	94.10 (69.60–98.30)	95.50 (73.10–8.70)	93.60 (74.60–98.50)
IL-10 (pg/ml)	15.62 (15.6–15.6)	15.62 (15.6–291.9)	780.30 (15.62–1000)	392.10 (15.62–1000)	704.40 (15.6–1000)	356.00 (15.6–1000) †	15.62 (15.6–17.10)	15.62 (15.6–39.40)	431.60 (15.6–1000)	233.30 (15.6–81.90)	407.90 (15.6–1000)	35.72 (15.6–793.20)

WS = without stimulation. *Der p*1 = *Dermatophagoides pteronyssinus* antigen 1. Sm29 = *S. mansoni* antigen Sm29. Sm29TSP–2 = *S. mansoni* antigen Sm29TSP-2. The results are expressed as median, min-max values. *p < 0.05 *Der p*1 + Sm29TSP-2 (MA group) *vs Der p*1 + Sm29TSP-2 (SA group). †p < 0.05 Sm29TSP-2 (MA group) *vs* Sm29TSP-2 (SA group).

group of asthmatic subjects (Table 2). In the SA group there was no significant difference in the frequency of cells expressing the different cytokines in cultures stimulated with *Der p*1 in the presence or absence of *S. mansoni* antigens (Table 2).

We did not observe significant difference in the frequency of $CD4^+$ T cells expressing the cytokines TNF, IFN-γ and IL-17A in cultures stimulated with *Der p*1 in the presence or absence of *S. mansoni* antigens in the HC group (data not shown).

3.4. Expression of molecules associated with the Th2-profile after the addition of S. mansoni antigens to the cultures

The frequency of cells expressing the Th2-cytokines IL-5 and IL-13 and the surface receptor TSLPR, was evaluated in PBMC cultures from asthmatic patients. In the MA group there was a lower frequency of T $CD4^+$ cells expressing IL-5 in cultures stimulated with Sm29 [0.19% (0.01%–1.2%)], compared to the frequency in those stimulated with *Der p*1 [0.42% (0.06%–3.35%); $p < 0.05$, Table 2]. Similarly, in the SA group, a lower frequency of $CD4^+IL\text{-}5^+$ T cells was observed in cultures stimulated with Sm29 [0.11% (0.04%–0.53%)], compared to the frequency in cultures stimulated with *Der p*1 [0.25% (0.12%–1.70%); $p < 0.05$; Table 2]

There was no difference in the frequency of $CD4^+IL\text{-}13^+$ T cells after the addition of *S. mansoni* antigens to the cultures from the MA group (Table 2). In the SA group, however, there was a lower frequency of these cells in cultures stimulated with Sm29 [0.19% (0.05%–2.11)], compared to the frequency in those stimulated with *Der p*1 [0.45% (0.05%–2.29%); $p < 0.05$; Table 2].

The addition of Sm29 or Sm29TSP-2 antigens did not change the expression of TSLPR in cultures stimulated with *Der p*1 in the MA group (Table 2). However in the SA group there was an increased frequency of TSLPR expression by $CD4^+$ T cells in cultures stimulated with *Der p*1 + Sm29-TSP2 [0.62% (0.12%–1.5%)], compared with the frequency in cultures stimulated with *Der p*1 [0.25% (0.09%–0.91%); $p < 0.05$]; $p < 0.05$; Table 2].

We did not observe significant difference in the frequency of $CD4^+$ T cells expressing the cytokines IL-5, IL-13 and TSLPR in cultures stimulated with *Der p*1 in the presence or absence of *S. mansoni* antigens in the HC group (data not shown).

3.5. Regulatory molecules expressed by $CD4^+$ T lymphocytes in PBMC cultures of asthmatic patients

In this study, we assessed the frequency of $CD4^+$T cells expressing the regulatory markers CTLA-4, IL-10 and TGF-β in PBMC cultures from the patients in response to *S. mansoni* antigens.

The frequency of $CD4^+$ cells expressing CTLA-4 and IL-10 did not differ in cultures stimulated with *Der p1* in the presence or absence of the *S. mansoni* antigens in either group of asthmatic patients ($p > 0.05$; Table 2). On the other hand, the addition of Sm29TSP-2 to the cultures stimulated with *Der p*1 [0.27% (0.07%–1.36%); $p < 0.05$] led to a reduction in the frequency of $TCD4^+$ cells expressing TGF-β in the MA group, compared with the frequency in those stimulated with *Der p*1 alone [0.71% (0.06%–3.7%); Table 2]. Cultures stimulated with Sm29 [0.36% (0.01%–1.42%); $p < 0.05$] or Sm29TSP-2 [0.38% (0.08%–1.68%); $p < 0.05$] showed a reduction in the frequency of cells expressing TGF-β when compared to the frequency in cultures stimulated with *Der p*1 alone (Table 2). In the SA group there was no significant difference in the frequency of these cells in cultures stimulated with *Der p*1 in the presence or absence of the *S. mansoni* antigens (Table 2).

Regarding the frequency of $CD4^+$ T cells expressing TGF-β, it was higher in the group of individuals with mild asthma [0.71 (0.07–3.70)] than in to the group with severe asthma [0.24 (0.05–1.78), $p < 0.05$; Table 2].

In the HC group the mean frequency of $CD4^+$ T cells expressing CTLA-4, IL-10 and TGF-β did not differs in cultures stimulated with *Der p*1 in the presence or absence of *S. mansoni* antigens (data not shown).

3.6. IL-10 levels in supernatant of PBMC cultures after the addition of S. mansoni antigens

Since there was no difference in the IL-10 expression by $CD4^+$ T lymphocytes, we evaluated the levels of this cytokine in PBMC supernatants from the two different groups of asthmatic patients.

In the MA group, the addition of Sm29 [780.3 pg/mL (15.6–1000)] or Sm29TSP-2 antigens [392 pg/mL (15.6–1000)] to the cultures stimulated with *Der p*1 resulted in increased levels of IL-10, compared to the levels in cultures stimulated with *Der p1* alone [15.6 pg/mL (15.6–291); $p < 0.001$; Fig. 5A]. The levels of IL-10 were also greater in cultures stimulated with Sm29 [704 pg/mL (15.6–1000)] or Sm29TSP-2 [356 pg/mL (15.6–1000)], when compared to the levels of those stimulated with *Der p*1 ($p < 0.001$). Additionally, all cultures stimulated with *S. mansoni* antigens, independent of the presence of *Der p*1, showed an increase in IL-10 levels, compared to the levels in WS cultures [15.6 pg/mL (15.6–15.6) $p < 0.01$, Fig. 5A].

In SA group, it was observed that in the cultures stimulated with *Der p*1 + Sm29 [432 pg/mL (15.6–1000)] or *Der p*1 + Sm29TSP-2 [233 pg/mL (15.6–982)] showed higher levels of IL-10 compared to the levels in cultures stimulated with *Der p*1 [15.6 pg/mL (15.6–139); $p < 0.001$; Fig. 4B]. There was also a higher production of IL-10 in cultures stimulated with Sm29 alone [408 pg/mL (15.6–1000); $p < 0.001$] or Sm29TSP-2 alone [36 pg/mL (15.6–793.2); $p < 0.01$], compared to the levels in those stimulated with *Der p*1. Additionally, in cultures stimulated with *S. mansoni* antigens independently of the presence of *Der p*1, there was a higher levels of IL-10 when compared to the levels in WS cultures [15.6 pg/mL (15.6–317); $p < 0.001$; Fig. 5B].

In the HC group there was an increase in IL-10 levels in supernatants from cultures stimulated *Der p*1 + Sm29 [703 pg/mL (15.6–1000)], and Sm29 [657 pg/mL (15.6–1000)] compared to the levels in cultures stimulated with *Der p*1 alone [15.6 pg/mL (15.6–55); $p < 0.05$; Fig. 5C].

The levels of IL-10 in supernatants of PBMC cultures stimulated with Sm29TSP-2 was higher in individuals with mild asthma [356.00 (15.6–1000.00) than in those with severe asthma [35.72 (15.6–793.20), $p < 0.05$; Table 3].

4. Discussion

This study aimed to evaluate the *in vitro* potential of *S. mansoni* antigens to modulate the inflammatory response of blood mononuclear cells from subjects with asthma. Several studies have associated the expression of the CD25 molecule, which is the α chain of the IL-2 receptor, with the phenotype of natural regulatory T cells derived from the thymus. These cells are responsible for important mechanisms of immune self-tolerance and control of the immune response. Therefore, a deficiency in these cells is associated with the development of autoimmune disorders (Baecher-Allan et al., 2001; Crispin et al., 2003; Ehrenstein et al., 2004; Kim et al., 2007; Sakaguchi et al., 1995)

The present study found that the addition of Sm29TSP-2 and Sm29 antigens to PBMC cultures from patients with mild or severe asthma increased the frequency of $CD4^+CD25^{hi}$ T lymphocytes, reduced the frequency of $CD4^+CD25^{low}$ T cells and increased the frequency of $CD4^+CD25^{neg}$ T cells compared to unstimulated cultures. The cultures stimulated with *Der p*1 alone also increased frequency of $CD4^+CD25^{hi}$ T lymphocytes, however this increase

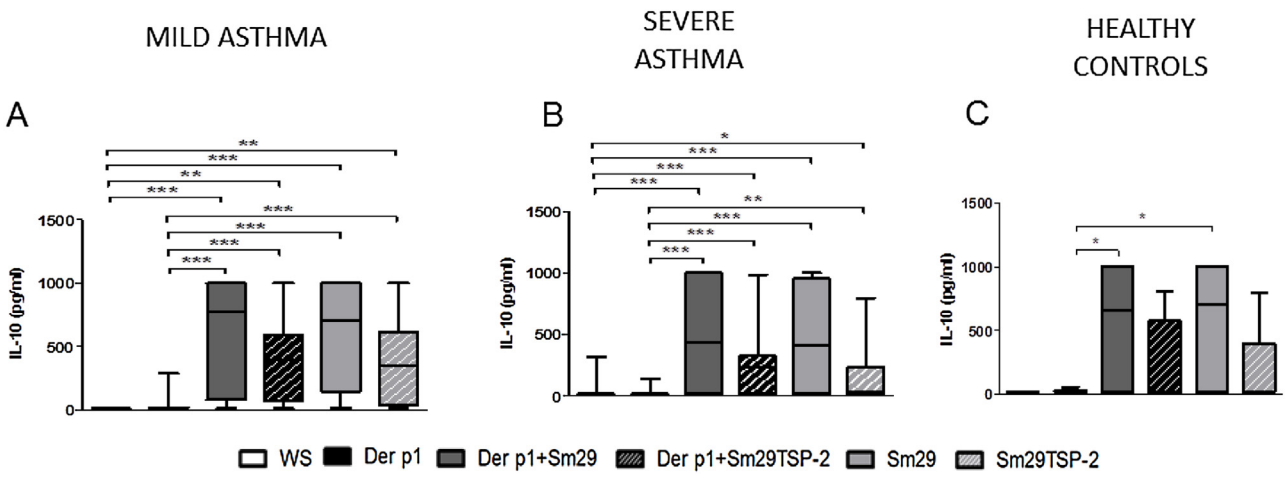

Fig. 5. Levels of IL-10 in supernatant of cell cultures from patients with asthma (A and B) and healthy controls (C). WS = without stimulation. *Der p*1 = *Dermatophagoides pteronyssinus* antigen 1. Sm29 = *S. mansoni* antigen Sm29. Sm29TSP–2 = *S. mansoni* antigen Sm29TSP-2. The results are expressed as median, min-max values. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, Kruskal-Wallis test.

was also accompanied by an increase in $CD4^{+}CD25^{low}$. Important, therefore, was the fact that the addition of Sm29 or Sm29TSP-2 to the cultures increased the frequency of $CD25^{high}$ cells, a marker of T regulatory cell, and different to the observed when *Der p*1 alone was added, reduced the frequency of $CD25^{low}$ cells. It suggests that the increase of $CD25^{high}$ cells in response to *Der p*1 does not result in down modulation of activated T cells. Indeed, one of the hypothesis to explain the existing inflammatory response in asthma and other immune-based diseases is the absence or dysfunction of regulatory T cells.

Studies have shown that individuals with asthma and allergic rhinitis have a deficiency in regulatory $CD4^{+}CD25^{hi}$ T cells in their peripheral blood compared to healthy subjects (Pietruczuk et al., 2012; Rojas-Ramos et al., 2015; Stelmaszczyk-Emmel et al., 2013). Further, the bronchoalveolar lavage fluid from asthmatic children has been shown to have a reduced frequency of $CD4^{+}CD25^{hi}$ T cells compared to the frequency in children without asthma, and this frequency is even lower in untreated children (Hartl et al., 2007). Moreover, $CD4^{+}CD25^{hi}$ T cells are able to suppress the allergic response of airway inflammation in an ovalbumin-induced mouse model of asthma (Xia et al., 2006).

There was no significant difference in the frequency of $CD4^{+}CD25^{hi}$ T cells expressing Foxp3 after addition of the *S. mansoni* antigens. In an experimental model of ovalbumin-induced asthma, the regulation of the exacerbated inflammatory immune response observed by immunization with the Sm29 antigen was not dependent on Foxp3 (Cardoso et al., 2010). It suggests that there are other mechanisms associated to the regulatory property of Sm29antigen.

In the present study the effect of Sm29 and Sm29TSP-2 antigens on lymphocyte activation status was also evaluated. The addition of Sm29 was shown to reduce the frequency of $CD4^{+}CD69^{+}$ T cells in cultures stimulated with *Der p*1 in subjects with severe asthma. This reduction was also observed upon the addition of Sm29 or Sm29TSP-2 to cultures from the group of individuals with mild asthma, suggesting that these antigens have the ability to reduce the activation of $CD4^{+}$ T cells in response to an allergen. In the cultures stimulated with *Der p*1 alone, there was an increase in the frequency of $CD4^{+}$ T lymphocytes expressing CD69 in cultures from patients with severe or mild asthma.

Studies have shown that the development of airway hyperresponsiveness induced by ovalbumin in mice is associated with an increase in the number of $CD4^{+}CD69^{+}$ T lymphocytes in the airways (Zosky et al., 2009). CD69-deficient mice have been shown to have a significant reduction in their Th2 response and in the migration of lymphocytes into the lung (Miki-Hosokawa et al., 2009). Furthermore, both the peripheral blood and sputum of patients with asthma have a high number of $CD69^{+}$ lymphocytes and this number is increased after stimulation by an allergen (Lourenco et al., 2009; Pelikan 2014).

In the group of individuals with mild asthma, we observed that the addition of Sm29TSP-2 to cultures stimulated with *Der p*1 led to a reduction in the proportion of $CD4^{+}$ T cells expressing inflammatory cytokines IFN-γ and TNF.

Studies on the role of IFN-γ in asthma have shown an increase in this cytokine in the sputum of patients with asthma compared to that in non-asthmatics. The association of IFN-γ with disease severity has also been shown (Cho et al., 2005). The role of this cytokine in the immunopathogenesis of asthma in the murine model is highly controversial. Mice deficient in the IFN-γ receptor show a perpetuation of the inflammatory Th2 response along with persistent eosinophilic inflammation (Coyle et al., 1996).

TNF is another cytokine widely involved in the inflammatory process observed in asthma patients, and studies have associated this cytokine with severe and refractory asthma (Berry et al., 2006; Thomas and Heywood, 2002; Thomas et al., 1995). Treatment of asthmatic individuals with a TNF antagonist prevents exacerbations of the disease (Berry et al., 2006; Erin et al., 2006; Morjaria et al., 2008).

Previous studies have demonstrated the ability of *S. mansoni* infection or its antigens (including Sm29), to reduce the production of Th2 cytokines in a murine model of airway inflammation and in PBMC cultures from asthmatic patients (Araujo et al., 2004; Cardoso et al., 2010; Cardoso et al., 2011). In the present study, we observed a lower frequency of $CD4^{+}$ T cells expressing IL-5 and IL-13 in cultures from subjects with severe asthma stimulated with Sm29, compared to the frequency in cultures stimulated with only *Der p*1. This reduction was not observed after the addition of Sm29 to cultures stimulated with *Der p*1. In the mild asthma group, there was also a reduction in the frequency of $CD4^{+}IL\text{-}5^{+}$ T cells in cultures stimulated with Sm29, compared to cultures stimulated with *Der p*1 alone.

These findings agree with previous studies, which have demonstrated the ability of Sm29 and other antigens of *S. mansoni* to reduce the production of IL-5 in PBMC cultures from asthmatics individuals (Cardoso et al., 2011). Additionally, this antigen has been shown to down-modulate the allergic immune response in an experimental model of asthma, decreasing the production of the Th2 cytokines IL-4 and IL-5, the levels of specific serum IgE and

of eosinophil peroxidase in bronchoalveolar lavage fluid (Cardoso et al., 2010).

The literature has described cytokines, such as IL-33, TSLP and IL-25, that act to maintain the Th2 response (Ballantyne et al., 2007; Kondo et al., 2008; Ying et al., 2008; Ying et al., 2005). In the present study, the addition of Sm29TSP-2 to the cultures stimulated with *Der p*1 from the group of patients with severe asthma led to an increase in the frequency of $CD4^+$ T lymphocytes expressing TSLP receptors. TSLP has been associated with the initiation and progression of the allergic inflammation observed in asthma, acting in synergy with IL-33 and IL-25 to support the Th2 response (Saenz et al., 2008; Ying et al., 2008; Ying et al., 2005).

The effect of *S. mansoni* antigens on the expression of molecules associated with the regulation of immune response, was also assessed in this study. In the group of patients with mild asthma, the addition of Sm29TSP-2 to cultures stimulated with *Der p*1 led to a reduction in the frequency of $CD4^+TGF\text{-}\beta^+$, which was also observed in cultures stimulated with Sm29 alone or Sm29TSP-2 alone. Although considered to be a regulatory cytokine, several studies have consistently associated TGF-β with the pathogenesis of asthma, because it is associated with the fibrotic remodeling process of airway epithelium that results in loss of lung function (Al-Alawi et al., 2014; Fichtner-Feigl et al., 2006; Itoigawa et al., 2015; Lee et al., 2001; Xu et al., 2003). Furthermore, there are data showing high levels of TGF-β in the bronchoalveolar lavage fluid and bronchial biopsies from individuals with asthma (Kokturk et al., 2003; Redington et al., 1997).

IL-10 has been described as a key cytokine in the inhibition of inflammatory response in asthma. It suppresses airway hyperresponsiveness, epithelial hyperplasia, eosinophilia and airway neutrophilia in an experimental model of asthma (Akbari et al., 2001; Nabe et al., 2012; Oh et al., 2002; Tournoy et al., 2000). In humans it has been reported that subjects with asthma have low levels of IL-10 in both serum and bronchoalveolar lavage fluid. They have also been show to have a decreased production of this cytokine in PBMC cultures when compared to production in healthy individuals (Borish et al., 1996; Gupta et al., 2014; Raeiszadeh Jahromi et al., 2014).

In the present study, the addition of Sm29 or Sm29TSP-2 led to an increase in the levels of IL-10 in PBMC cultures stimulated with *Der p*1 in the group from individuals with severe asthma. The same effect was observed in cultures from the group with mild asthma. These findings agree with our previous studies demonstrating that patients with asthma infected with *S. mansoni* and other helminths have an increased *in vitro* production of IL-10 and a decreased production of Th2 cytokines, in response to *Der p*1, when compared to the production in uninfected asthmatic patients (Araujo et al., 2004). Moreover, in our previous studies, *S. mansoni* antigens, including Sm29, they were able to induce IL-10 production by PBMCs from uninfected asthmatic patients (Cardoso et al., 2006a,b; Cardoso et al., 2011). These antigens were also able to induce IL-10 production *in vitro* in other disease models, such leishmaniasis and HTLV-1 infection (Lima et al., 2013; Lopes et al., 2014).

When we compared the two groups of asthmatic individuals we did not observe significant differences in the levels of IL-10 in response to *S. mansoni* antigens in cultures stimulated with the allergen *Der p*1. The find of higher levels of IL-10 in cultures stimulated with Sm29TSP-2 alone in the MA group compared to SA group was an isolated finding and does not appear to be sufficient to state that this antigen benefits one or other group. Indeed, the most cell markers evaluated in this study showed a similar pattern between groups.

The increase in the levels of IL-10 in the supernatant of cultures stimulated with *Der p*1 + Sm29 and *Der p*1 + Sm29TSP-2 was not accompanied by the increased expression of this cytokine in lymphocytes, suggesting the existence of other cell sources of IL-10. Previously, Cardoso et al. (2011) showed that monocytes and B lymphocytes of patients with mild asthma produced high levels of IL-10 after PBMC stimulation with Sm29.

Subjects with severe asthma were receiving inhaled corticosteroids at medium to high doses. Although such medications are directly active in the airway mucosa, there may be some small systemic bioavailability and effects. Therefore we cannot rule out the possibility some observations on the *ex-vivo* behavior of their PBMC might bear some influence of these medications. However, it would be unethical to withdraw treatment of patients with severe asthma for the purpose of the study.

Our results demonstrate that *S. mansoni* antigens, Sm29 and Sm29TSP-2, were able to down-modulate the *in vitro* inflammatory asthma response by reducing the levels of activation markers in T lymphocytes, reducing the expression of Th1 and Th2-type cytokines and inducing IL-10 production. Thus, these results suggest that the use of parasite antigens is promising in the development of strategies for controlling the dysregulated immune response that occurs with asthma.

Conflict of interests

There is no conflict of interests.

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