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ORIGINAL ARTICLE

Effect of the signaling lymphocytic activation molecule (SLAM) in the modulation of T cells in immune response to *Leishmania* braziliensis *in vitro*

Efeito da molécula de sinalização para ativação linfocítica (SLAM) na modulação de células T na resposta imune à *Leishmania* braziliensis *in vitro*

Zirlane Castelo Branco Coêlho[1], Maria Jania Teixeira[2], Maria do Livramento Leitão Vilar[3], Jesamar Correia Matos[4], Ivo Castelo Branco Coêlho[2], Geanne Matos de Andrade[5], Margarida Maria de Lima Pompeu[2].

1. Docente da Faculdade de Farmácia, Odontologia e Enfermagem, Universidade Federal do Ceará, Ceará, Brasil. 2 Docente da Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, Ceará, Brasil. 3. Docente da Faculdade de Medicina, Faculdade Christus, Fortaleza, Ceará, Brasil. 4. Médico do Hospital Infantil Albert Sabin, Centro de Referência do Diagnóstico do Câncer da Criança e do Adolescente Dr. Murilo Martins, Fortaleza, Ceará, Brasil. 5. Docente da Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, Ceará, Brasil.

Abstract

Introduction: Signaling lymphocyte activation molecule (SLAM) is a self-ligand receptor on the surface of activated T- and B-lymphocytes, macrophages, and DC. Studies have shown PBMC from healthy individuals exposed to *Leishmania* differ in IFN-γ production. **Objective:** We investigated the role of SLAM signaling pathway in PMBC from high (HP) and low (LP) IFN-γ producers exposed to *L. braziliensis in vitro*. **Methods**: PBMC from 43 healthy individuals were cultured with or without antigen, α-SLAM, rIL-12 and rIFN-γ. The cytokines production was evaluated by ELISA, and SLAM expression by flow cytometry. **Results**: *L. braziliensis* associated with rIFN-γ or rIL-12 reduced early SLAM but did not modify this response later in HP. α-SLAM did not alter $CD3^+SLAM^+$ expression, and not affected IFN-γ and IL-13 production, in both groups, but increased significantly IL-10 in HP. *Leishmania* associated with α-SLAM and rIL-12 increased IFN-γ in LP, as well as IL-13 in HP. LP group presented low IFN-γ and IL-13 production, and low SLAM expression. **Conclusion**: Collectively, these findings suggest that when PBMC from healthy individuals are sensitized with L. *braziliensis in vitro*, SLAM acts in modulating Th1 response in HP individuals and induces a condition of immunosuppression in LP individuals.

Key words: Leishmania braziliensis. SLAM. Cytokines. Immunosuppression.

Resumo

Introdução: A molécula de sinalização para ativação linfocítica (SLAM) é um receptor autoligante na superfície de linfócitos T e B ativados, macrófagos e DC. Estudos têm mostrado que PBMC de indivíduos saudáveis expostos à *Leishmania* diferem na produção de IFN-γ. **Objetivo:** Nós investigamos o papel da via de sinalização de SLAM em PMBC de altos produtores de IFN-γ (AP) e baixos (BP) expostos à L. *braziliensis in vitro*. **Métodos**: PBMC de 43 indivíduos saudáveis foram cultivadas com ou sem antígeno, α-SLAM, rIL-12 e rIFN-γ. Foi avaliada a produção de citocinas por ELISA e expressão de SLAM por citometria de fluxo. **Resultados**: *L. braziliensis* associado a rIFN-γ ou rIL-12 reduziu a expressão inicial de SLAM, mas não modificou esta resposta mais tarde em AP. α-SLAM não alterou a expressão de $CD3^+SLAM^+$, e não afetou a produção de IFN-γ e IL-13, em ambos os grupos, mas aumentou significativamente IL-10 em AP. *Leishmania* associada a α-SLAM e rIL-12 aumentou IFN-γ em BP, assim como IL-13 em AP. BP apresentaram baixa produção de IFN-γ e IL-13 e baixa expressão de SLAM. **Conclusão**: Coletivamente, esses achados sugerem que quando PBMC de indivíduos saudáveis são sensibilizados por *L. braziliensis in vitro*, SLAM atua na modulação da resposta Th1 em indivíduos AP e induz uma condição de imunossupressão em indivíduos BP.

Palavras-chave: Leishmania braziliensis. SLAM. Citocinas. Imunossupressão.

INTRODUCTION

Leishmaniasis presents a wide spectrum of clinical manifestations that depend on parasite species, genetic background and the immune status of the host. Infections range from subclinical cutaneous to more serious disseminating diffuse cutaneous, mucocutaneous and visceral forms[1]. *Leishmania* braziliensis is the major etiologic agent of cutaneous *Leishmania*sis in the New World, a condition that is distinguished from other *Leishmania*sis by its chronicity, latency, and tendency to metastasize in the human host[2].

The early events of *Leishmania*-host interactions are crucial in the outcome of infection, being essential for the infection control or disease progression. Protection and healing in human and experimental cutaneous *Leishmania*sis is mediated by a Th1-type immune response and requires IFN-γ, a macrophage-activating cytokine produced by T cells. In contrast, a Th2 response with IL-4 and IL-10 production often results in disease progression[3,4]. Of note, IFN-γ production following *Leishmania* contact differs among naive individuals, some are high IFN-γ producers whereas others produce low amounts of this cytokine[5]. This observation has been documented with several species of *Leishmania*, as well as in individuals naturally

Correspondence: Zirlane Castelo Branco Coêlho. Faculdade de Medicina, Universidade Federal do Ceará, Rua Alexandre Baraúna, 949, Fortaleza-CE, CEP 60430-160, Brasil. E-mail: zirlanecastelo@gmail.com Phone.: +55-85-33668310/8311
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infected[6,7].

At the initial phase of the immune response cells can communicate with each other or through mediators such as cytokines, chemokines, growth factors or hormones. This communication occurs through the transmission of signals from the extracellular microenvironment by cell-surface receptors. Some of these receptors have intrinsic components with enzyme activity in cytoplasmic domains, however, signal transduction pathway involves most common interactions between membrane receptors and transmembrane proteins and cytoplasmic adapter proteins[8,9].

Immune cells are modulated following the triggering of receptors that bind to their ligands. These receptors include antigen receptors on B (BCR) and T cells (TCR), Fc receptors on mast cells and macrophages, stimulatory natural killer (NK) cell receptors and dendritic cell (DC) receptors[10]. The receptor-ligand interaction results in a phosphorylation signal cascade essential for the activation of immune cells[11].

Although Th cells go through a differentiation process that "programs" their cytokine production upon TCR stimulation, additional factors can influence the level and pattern of cytokines produced by activated T cells. One of these factors is signaling lymphocytic activation molecule (SLAM, CD150), a transmembrane type I glycoprotein of the CD2 subfamily expressed on lymphocytes and immature thymocytes that boosts IFN-γ production and proliferation[12]. SLAM in T cells associates with the small Sh2-containing adaptor protein 1A (SH2D1A), also called Duncan's disease SH2-protein (DSHP) or SLAM-associated protein (SAP). The expression of SLAM is rapidly induced on naive T cells after activation and ligation of SLAM redirects Th2 responses to a Th1 or Th0 phenotype[13].

In order to ascertain whether signaling through SLAM modulates the immune response in individuals low IFN-γ producers after infection by *Leishmania*, we investigated the role of SLAM taking advantage of the *in vitro* priming system using peripheral blood mononuclear cells (PBMC) from healthy individuals stimulated by live promastigotes of L. braziliensis[5]. As previously demonstrated, PBMC from high producers secretes IFN-γ at concentrations ranging from 505.6 to 1,099 pg of IFN-γ/10^6 cells after 96 h of antigen sensitization[5], therefore herein we decided to investigate the production of IFN-γ at both an earlier time, 6 h and at a later time, 120 h. We believe elucidation of the mechanisms for reduced IFN-γ production in individuals that develop the disease will enhance our knowledge of the pathogenesis of *Leishmania*sis and suggest strategies for developing vaccines.

MATERIALS AND METHODS

Aspects Ethical

The study was approved by the Human Research Ethics Committee of the *Universidade Federal do Ceará*, Brazil (protocol nº 310/2004).

Study subjects

Forty three buffy coats were obtained from healthy individuals by Centro de Hemoterapia e Hematologia do Ceará (HEMOCE), Brazil. It was included only subjects who had negative serology for *Leishmania*sis, Chagas disease, hepatitis, syphilis, and human immunodeficiency virus. All these exams are part of routine serological of HEMOCE. All individuals had proliferation index of ≤ 4 when stimulated with *Leishmania* antigens, as previously described by[14].

Parasites

Leishmania braziliensis (MHOM/BR/94/H3227), characterized previously by PCR[15] was used for infection. Parasites were cultivated in Schneider's medium (Sigma, St. Louis, MO, USA) supplemented with 20% inactive fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg gentamicin (all from Gibco, Grand Island, NY, USA), and 2% sterile human urine. *In vitro* stimulation of PBMC was performed with stationary-phase promastigotes, which were washed three times in sterile saline (1,800 g, 15 min, 4°C) and concentration adjusted in RPMI 1640 medium (Sigma).

Cell preparation and culture condition

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat of 43 healthy donors of blood by density gradient centrifugation on Ficoll-Hypaque (Sigma), 800 g, 30 min, 21°C. Cells were submitted to three cycles of washes with sterile saline, 450 g, 15 min, 5°C, and resuspended (5 x 10^6 or 10^6 cells/mL) in RPMI 1640 medium (Sigma) supplemented with 10 mM HEPES (Sigma), 50 mg/mL gentamicin (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (all from Sigma), and 10% human heat-inactivated AB serum. Cells were cultured in 48-well plates (Nunc, Roskilde, Denamark) at 37°C in a humidified 5% CO_2 atmosphere and stimulated in the presence or absence of different stimuli.

SLAM expression

PBMC (5 x 10^6 cell/mL) were cultured with or without L. braziliensis promastigotes (2 x 10^6 promastigotes/well) in the presence or absence of: anti-SLAM mAb (A12; 10 µg/mL: eBioscience, San Diego, CA, USA), rIL-12 (500 pg/mL, Peprotech Mexico, D.F., Mexico), and rIFN-γ (7.5 ng/mL, Chemicon, San Diego, CA, USA). Also PBMC (5 x 10^6 cell/mL) were cultured without L. braziliensis promastigotes in the presence of 5 µg/mL of phytohemagglutinin (PHA, Sigma). Cells were incubated in a humidified 5% CO_2 atmosphere for 6 to 120 h, and after that, were harvested and staining with specific antibodies: CD3+ (PE-Phycoerythrin, BD Biosciences, Rockville, MD, USA) and anti-SLAM (A12, FITC-Fluorescein isothiocyanate, eBiosciences). After staining, cells were fixed in 1% paraformaldehyde and, for each sample, 10^4 cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) as previously described by[16], and

data were analyzed using WinMDI 2.9 software (Joseph Trotter, La Jolla, CA, USA).

Blockade of SLAM signaling pathway

The blockade of SLAM signaling pathway was performed using anti-SLAM mAb (A12, eBioscience) and evaluated at different concentrations (5, 10 e 15 µg/mL), and at different times (6 and 120 h) in PBMC stimulated or not with antigen. After 5 days of culture, cells were washed and examined for SLAM expression on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using WinMDI 2.9 software (Joseph Trotter). From these tests we selected the concentration of 10 µg/mL of anti-SLAM mAb for use in all of the following experiments.

Cytokine assays: PBMC (5 x 10^6 células/mL) were dispensed into 48-well plates in a 300-µL volume, and cultured at 37ºC, 5% CO_2, in the presence or absence of L. braziliensis (2 x 10^6 promastigotes/well); anti-SLAM mAb (10 µg/mL, eBioscience) rIL-12 (500 pg/mL, Peprotech); rIFN-γ (7.5 ng/mL, Chemicon). PHA (5 µg/mL; Sigma) was used as control in the absence of L. braziliensis. After 5 days, IFN-γ, IL-10, and IL-13 productions were determinate in cell-free supernatants by enzyme-linked immunoabsorbent assay (ELISA), following the manufacture's instructions (BD Biosciences).

Statistical analysis

Analysis between high and low producers was performed by using the nonparametric Wilcoxon signed rank test for paired samples and the Mann-Whitney rank sum test for independent samples. For all statistical analysis we used GraphPad Prism, version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Characterization of immunological status of the groups

To define individuals high and low IFN-γ producers (HP and LP, respectively) we used as criterion the same adopted by[6], defining as HP individuals whose PBMC produced concentrations equal or greater than 160 pg/mL, and those with concentrations below this value as LP.

Expression of SLAM induced by L. braziliensis in the initial immune response:

To evaluate the expression of SLAM induced by L. braziliensis, we analyzed $CD3^+$ $SLAM^+$ expression in PBMC from 43 individuals, without prior exposure to *Leishmania*, after 6 h (earlier) and 120 h (later) of stimulation with live promastigotes of L. braziliensis. In the first 6 h, no change was observed in $SLAM^+$ expression in L. braziliensis-stimulated cells as compared to non-stimulated cells. However, when we compared the frequency of these cells at 6 h and 120 h of culture with L. braziliensis, there was a significant reduction in this frequency (from 8.7% to 2.6%; p=0.0114) (Fig. 1A). SLAM expression behaved differently in PMBC of individuals HP and LP stimulated by L. braziliensis (Fig. 1B and 1C). At 6 h under stimulation with L. braziliensis, LP presented a lower frequency than HP, both constitutively (p=0.0434) (Fig. 1B). After 120 h, there was a reduction in the frequency of $SLAM^+$ T cells expression in HP group when compared to control (p=0.0085) (Fig. 1C), and when compared to the period of 6 h (p=0.0031) (Fig. 1B and 1C). It is noteworthy that the response of LP group remained unchanged in both periods evaluated (Fig. 1B and 1C).

Blockade of SLAM expression with anti-SLAM mAb in response to L. braziliensis

To further understand the activation of SLAM and its role in the presence of L. braziliensis, we evaluated by flow cytometry the frequency of T lymphocytes expressing SLAM in the cytoplasmic membrane, after PBMC have been cultivated with anti-SLAM mAb and L. braziliensis, for 6 h and 120 h. In Fig. 1D, after 6 h, L. braziliensis associated with anti-SLAM reduced by 30% SLAM expression in T lymphocytes as compared to L. braziliensis alone (p=0.0039), while no change was observed in SLAM expression after 120 h. Interestingly, we observed a significant reduction in SLAM expression after 120 h, when cells were stimulated with L. braziliensis alone (p=0.0114) (Fig. 1D).

Regulation of SLAM expression by proinflammatory cytokines after L. braziliensis and anti-SLAM stimulation in vitro

To evaluate if the expression of SLAM could be modulated by proinflammatory cytokines during L. braziliensis stimulation, PBMCs were cultured with rIFN-γ or rIL-12. After 6 h, in the presence of rIFN-γ associated to L. braziliensis, a significant (p=0.0166) inhibition of the parasite-induced SLAM expression was observed in the cultures (Fig. 2A). Under the same conditions, LP group showed no significant change in SLAM expression. In the first 6 h, treatment of HP group with rIL-12 showed that this cytokine was unable to block SLAM expression as observed with rIFN-γ. LP group showed no significant change under the same conditions (Fig. 2A). After 120 h, we observed a reduction in the frequency of T lymphocytes expressing SLAM. In addition, the effect of anti-SLAM did not alter the expression of $CD3^+$ $SLAM^+$, in both groups (Fig. 2B).

Effect of anti-SLAM on cytokines production induced by L. braziliensis in individuals high or low IFN-γ producers

To evaluate the effect of anti-SLAM on cytokines productions, PBMC from individuals HP or LP were cultured with anti-SLAM associated with L. braziliensis and after five days, the supernatants were collected and evaluated for IFN-γ, IL-13, and IL-10. L. braziliensis associated with anti-SLAM showed no additional effect on IFN-γ production (Fig. 3A and 3B) as compared to antigen alone, which in turn was sufficient to cause a significant increase in IFN-γ in both groups (HP, p<0.0001; LP, p<0.0033) (Fig. 3A and 3B). This production was more significant in HP (mean=3,750.0) than in LP individuals (mean=40.51) (Fig. 3A and 3B). Unlike expected, in the presence of L. braziliensis, the levels IL-13 were approximately 29-fold

lower in LP group than the levels in HP group (HP mean = 524.3; LP mean = 17.99; p = 0.0046) (Fig. 3C and 3D). L. braziliensis associated with anti-SLAM induced a tendency to reduce IL-13 production in HP group as compared to antigen alone (Fig. 3C). Under these same conditions, we observed a slight tendency to increase IL-13 production in LP group (Fig. 3D). IL-10 production was lower in LP group (HP mean = 95.17, LP mean = 49.3; p=0.0023) (Fig. 3E and 3F). However, we have observed that L. braziliensis associated with anti-SLAM promoted the increase of IL-10 production in HP group (mean = 142.1, p = 0.0056) when compared to stimulation by L. braziliensis alone (mean = 32.6) (Fig. 3E). On the contrary, L. braziliensis associated with anti-SLAM was unable to determine an increase in IL-10 production in LP group (Fig. 3F).

Figure 1. SLAM expression in the initial and later immune response (A), and in individuals high (HP) and low (LP) producers of IFN-γ (B and C), and after blockade of SLAM expression (D) induced by L. braziliensis. For SLAM expression, PBMC from 43 healthy individuals were cultured with or without promastigotes of L. braziliensis. For the blockade of SLAM expression, PBMC of 43 healthy individuals were stimulated with L. braziliensis and anti-SLAM mAb (10 µg/mL). Mean values ± SEM are shown. Wilcoxon test was used for comparison within groups and Mann-Whitney test for comparison between groups. P values < 0.05 were considered significant. HP = High producer of IFN-γ; LP = Low producer of IFN-γ.

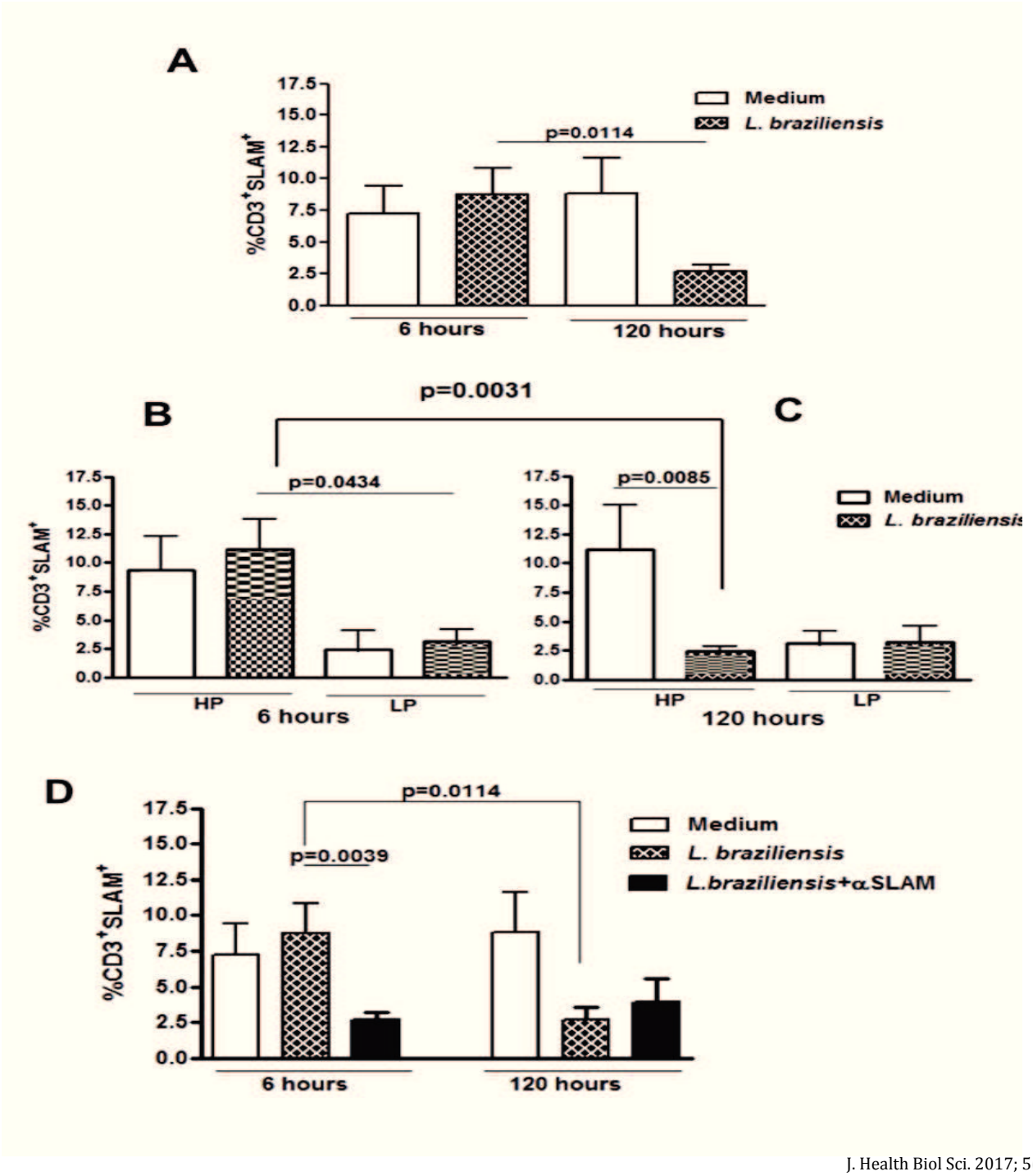


Figure 2. Regulation of SLAM expression by proinflammatory cytokines *in vitro* after L. braziliensis stimulation (A), and effect of anti-SLAM on SLAM expression in PBMC (B) from individuals high (HP) and low producers (LP) of IFN-γ. PBMC were stimulated with live promastigotes of L. braziliensis, rIFN-γ (7.5 ng/mL), rIL-12 (500 pg/mL) or anti-SLAM (10 µg/mL). Mean values ± SEM are shown. Wilcoxon test was used for comparison within groups. P values < 0.05 were considered significant.

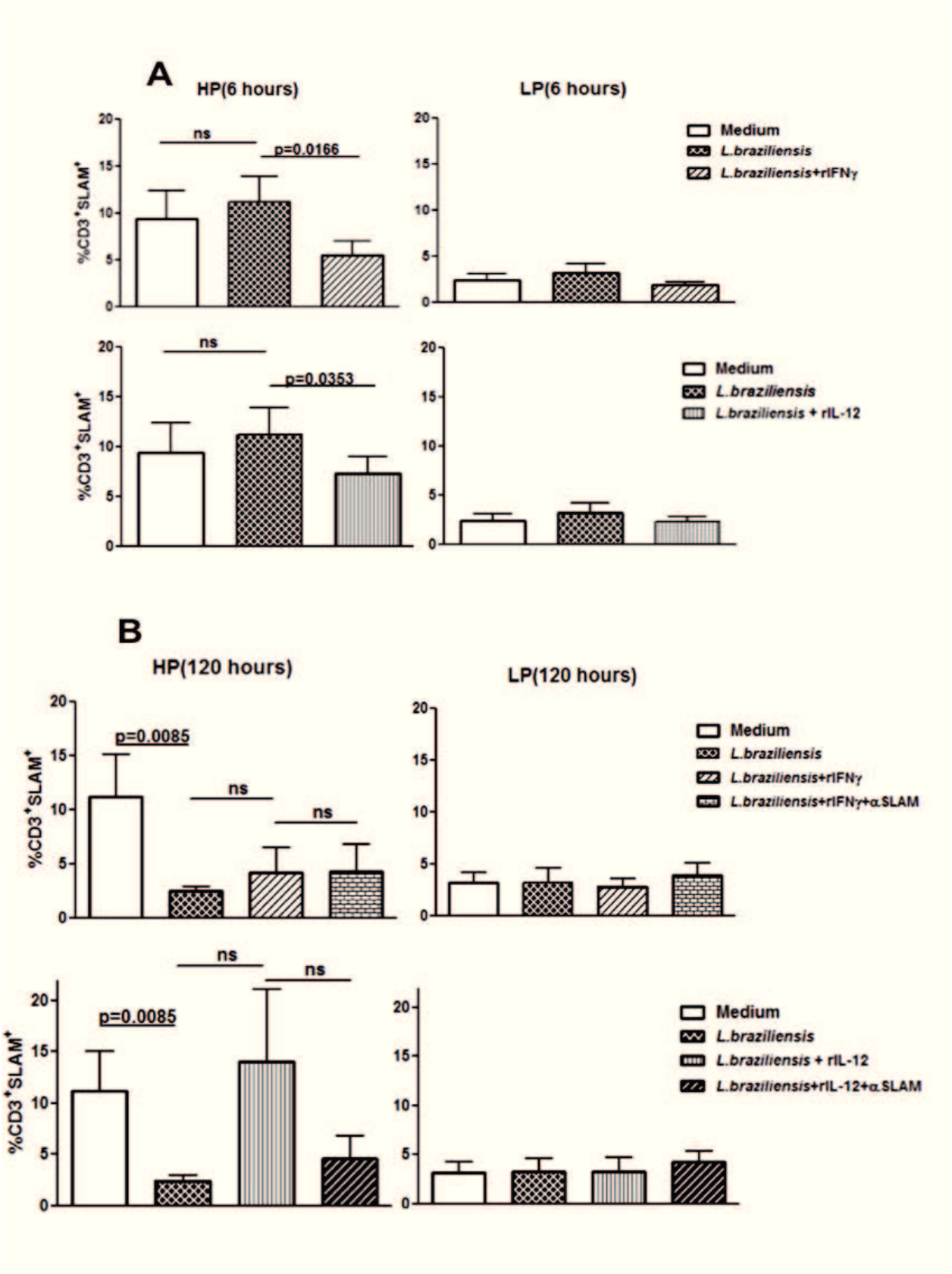
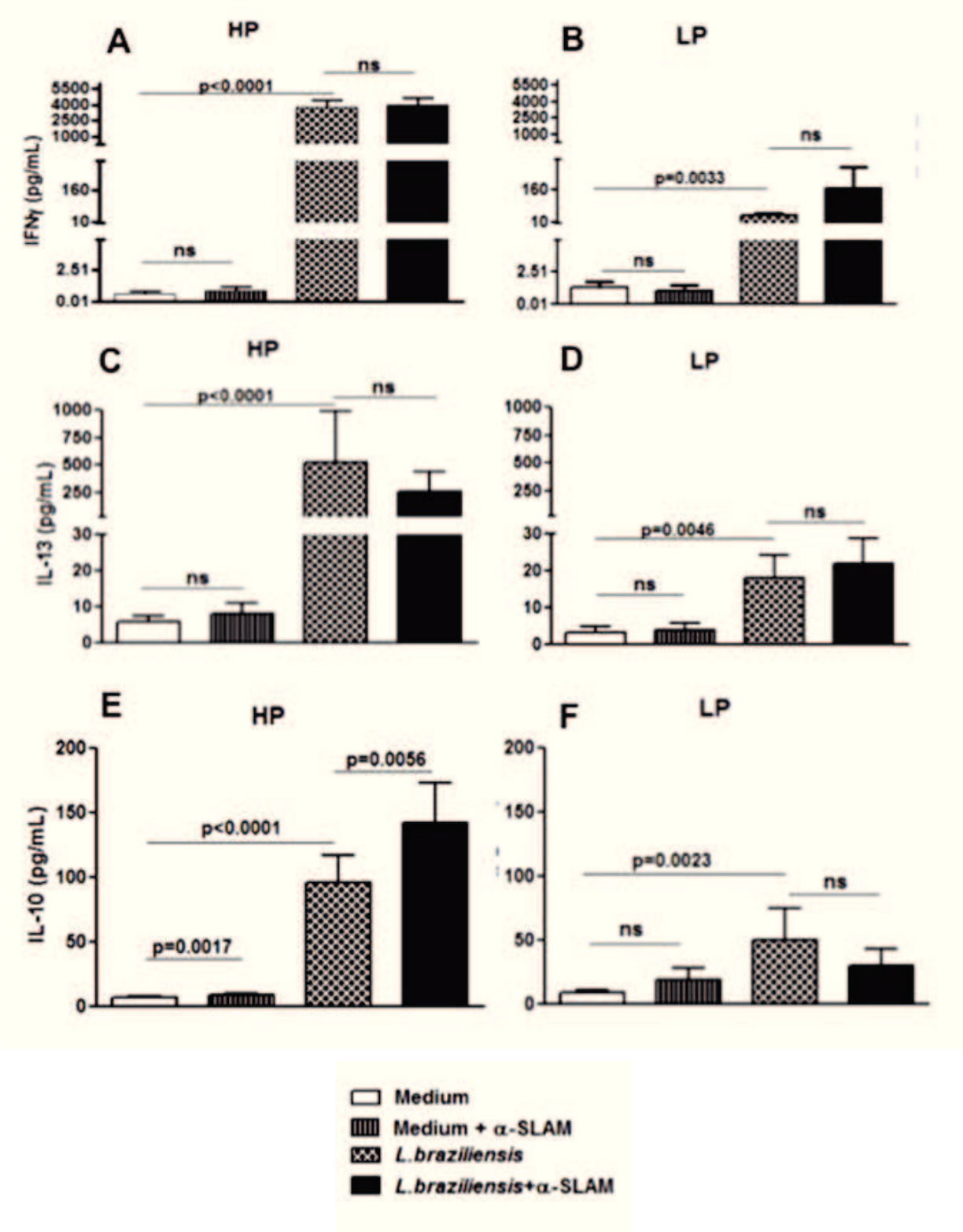


Figure 3. Effect of anti-SLAM on IFN-γ, IL13 and IL-10 production induced by L. braziliensis in (A, C and E) individuals high (HP) and (B, D and F) low producers (LP) of IFN-γ. PBMC were stimulated with live promastigotes of L. braziliensis associated with anti-SLAM mAb (10 µg/mL). Supernatants were analyzed for IFN-γ, IL-13 and IL-10 production by ELISA. Mean values ± SEM are shown. The Wilcoxon test was used for comparison within groups and Mann-Whitney test for comparison between groups. P values <0.05 were considered significant. ns = no statistical significance.



Effect of anti-SLAM on IFN-γ, IL-13 and IL-10 productions induced by L. braziliensis and proinflammatory cytokines in individuals high or low IFN-γ producers

To evaluate the effect of anti-SLAM on IFN-γ, IL-13 and IL-10 productions, PBMC from individuals HP or LP were cultured with live promastigotes of L. braziliensis in the absence or presence of anti-SLAM and proinflammatory cytokines (rIL-12 or rIFN-γ) and after 5 days, the supernatant was collected and evaluated cytokines productions. The blockade of SLAM signaling pathway in response to stimulation with L. braziliensis and rIFN-γ simultaneously did not modify the IFN-γ, IL-13 and IL-10 productions in both groups (Fig. 4). There was no IL-10 production in both groups when the cells were stimulated with rIFN-γ only. We also observed a significant reduction (p = 0.0382) of IL-13 production in HP group after stimulation with rIFN-γ associated with L. braziliensis simultaneously (mean = 56.64) when compared to stimulation with antigen alone (mean = 524.3) (Fig. 4). L. braziliensis associated with rIL-12 did not modify the IFN-γ production in both groups, when compared to sensitization with antigen alone. L. braziliensis associated with rIL-12 and anti-SLAM was able to induce 2-fold higher IFN-γ in LP group (mean = 302.3; p = 0.0161) than L. braziliensis associated only with rIL-12 (Fig. 5). Regarding production of IL-13 by both groups, no change was observed after treatment with rIL-12 and sensitization by L. braziliensis. However, anti-SLAM induced a significant increase in IL-13 production (p=0.0043) in PBMC from HP stimulated by L. braziliensis and rIL-12 simultaneously when compared to L. braziliensis associated only with rIL-12 (Fig. 5). Treatment with rIL-12 did not affect IL-10 production, even when associated with anti-SLAM in both groups (Fig. 5).

Figure 4. Effect of anti-SLAM on IFN-γ, IL-13 and IL-10 production induced by L. braziliensis and rIFN-γ stimulation in individuals high (HP) and low (LP) producers of IFN-γ. PBMC were stimulated with live promastigotes of L. braziliensis, anti-SLAM (10 µg/ml) and rIFN-γ (7.5 ng/ml). Supernatants were analyzed for IFN-γ, IL-13 and IL-10 production by ELISA 5 days later. Mean values ± SEM are shown. Wilcoxon test was used to compare groups. P values <0.05 were considered significant. ns = no statistical significance.

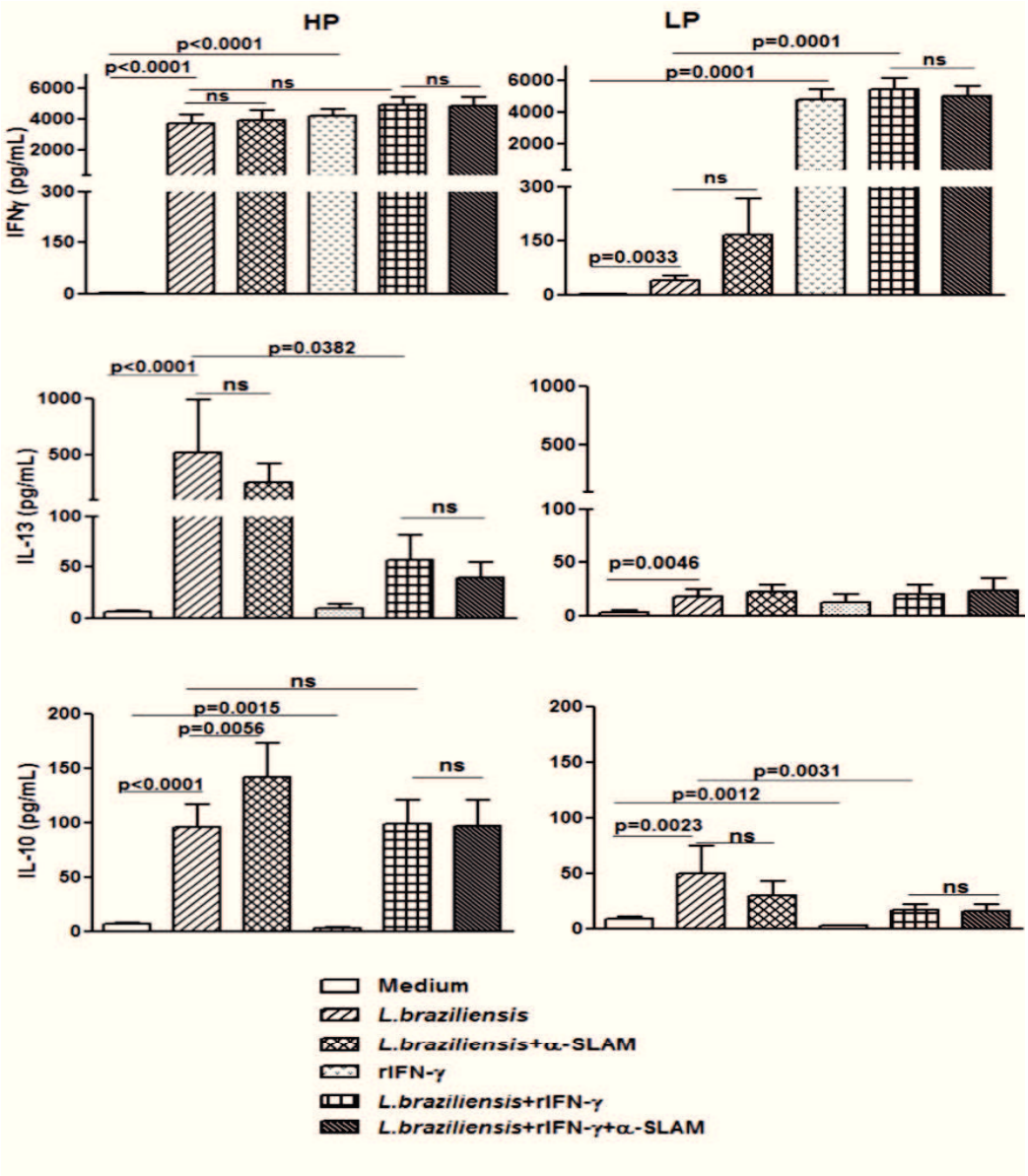
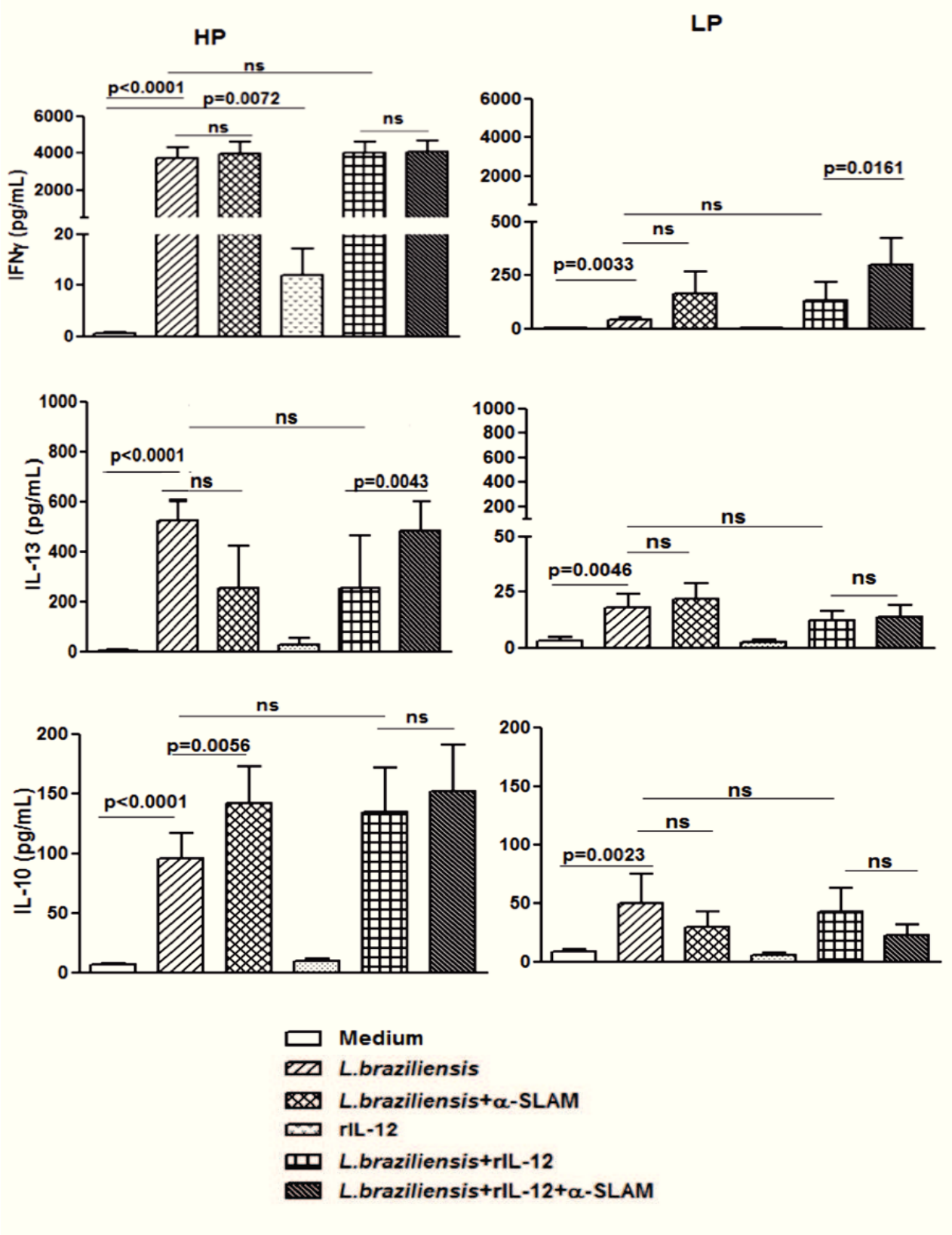


Figure 5. Effect of anti-SLAM on IFN-γ, IL-13 and IL-10 production induced by L. braziliensis and rIL-12 stimulation in individuals high (HP) and low (LP) producers of IFN-γ. PBMC were stimulated with live promastigotes of L. braziliensis, anti-SLAM mAb (10 µg/ml) and rIL-12 (500 pg/ml). Supernatants were analyzed for IFN-γ, IL-13 and IL-10 production by ELISA. Mean values ± SEM are shown. Wilcoxon test was used to compare groups. P values <0.05 were considered significant. ns = no statistical significance.



DISCUSSION

In this study, we investigated the role of SLAM signaling pathway in the context of the human immune response to L. braziliensis in individuals high (HP) or low producers (LP) of IFN-γ. The occurrence of high or low producers of IFN-γ has been documented in some diseases, such as tuberculosis and *Leishmania*sis[5,14,16,17].

Some authors have interpreted the increased SLAM expression as the occurrence of SLAM activation, associated with increased $CD4^+$ T cells proliferation, increased IFN-γ production[13,16,18,19], and IL-4 and IL-13 reduction[28]. Our findings suggest that these events are possibly due to blockage of SLAM signaling pathway by anti-SLAM mAb and not only by SLAM activation. We have found that PBMC from individuals HP reduced significantly SLAM expression after 120 h, demonstrating that SLAM signaling pathway was activated. This fact was confirmed by significant increase of IL-13 production. Such reduced SLAM expression is also an important negative feedback mechanism whose purpose is maintaining a balanced immune response. Differently, SLAM expression in individuals LP remained persistently low, indicating a poor response, with production of IL-13 lower than those of HP. In spite of these individuals presented a low expression of SLAM, they showed activation of SLAM signaling pathway, although it has been of low intensity.

It is possible that individuals LP have defective TCR in inducing the second signal to translate SLAM expression[21,22]. Transduction of this signal would be mediated by SLAM signaling pathway, resulting in IL-13 production[23]. $CD4^+$ T cells from mice deficient in SLAM showed reduced IL-4 and IL-13 productions and a slight increase of IFN-γ production[24]. This differential SLAM expression was also demonstrated in patients with active tuberculosis[14], and leprosy[25].

In our study, anti-SLAM mAb was able to block 30% of SLAM expression on T cells stimulated with L. braziliensis after 6 h of culture. The blockade of SLAM signaling pathway simulates the effects of SLAM and SAP deficiency, since the cascade of tyrosine protein phosphorylation triggered by this pathway, exerts its action only when SLAM and SAP are available[20].

This work also showed that anti-SLAM mAb tends to slightly increase IFN-γ production in PMBC of individuals HP and LP stimulated by L. braziliensis, suggesting that this cytokine is not dependent directly on this pathway. This slight increase may be due to lack of inhibition of GATA-3 on expression of IL-2 receptor[23], and on IFN-γ genomics programs[26]. Similar results were found in patients with active tuberculosis[14]. In tuberculoid leprosy patients was observed increased expression of T-bet, and significant production of IFN-γ after the addition of anti-SLAM[27]. It is possible that this difference is due to the fact that these individuals express large amounts of SLAM caused to inherent characteristics to the antigen, and for this reason the effect of anti-SLAM would be more intense in these individual than in those one that present lower amounts of SLAM. This would lead to an increased release of Th1 cytokines.

Cytokines such as IL-4, IL-13 and IL-10 play an important role in the regulation of proinflammatory cytokines and in the modulation of host resistance to intracellular organisms[6]. Since SLAM pathway is able to induce IL-4 and IL-13 productions, we would expect that blocking this pathway might lead to a reduction of these cytokines and an increase of IFN-γ production. However, we observed a tendency to reduce IL-13 production in response to L. braziliensis in individuals HP after addition of anti-SLAM. When all individuals were evaluated together, we confirmed that anti-SLAM tends to reduce IL-13 production (L. braziliensis mean = 355.5; L. braziliensis + α-SLAM mean = 177.4) (Data not shown). We believe that a more consistent response could have been obtained if we had used a more intense blockage of this pathway. In contrast, in this work, we observed no reduction in IL-13 production in individuals LP. It is likely that the blockade has no effect on this result, due to low SLAM constitutive expression in these individuals.

One aspect that stands out is that in individuals LP the immune response is not being directed to either Th1 or Th2 differentiation. Moreover, LP group showed low IFN-γ and IL-13 production as well as low SLAM expression, suggesting a state of immunosuppression induced by L. braziliensis in these individuals. Immunosuppression has ben associated with L. donovani and L. infantum chagasi in patients with visceral *Leishmania*sis[28,29] but not with L. braziliensis. On the other hand, anergic response has been associated with L. amazonensis in patients with diffuse cutaneous *Leishmania*sis[30]. We also hypothesized that L. braziliensis would induce apoptosis of these cells, as has been described[31].

The role of IL-10 in human cutaneous *Leishmania*sis by L. braziliensis is still poorly understood. It is believed that the impaired IL-10 production is one of the factors that contribute to the pathogenesis of mucocutaneous *Leishmania*sis due to exacerbated inflammation induced by Th1 cytokines[32]. In the present study, L braziliensis induced a significant increase in IL-10 production in HP group. Likely, in these individuals, IL-10 is exerting a regulatory role in the production of IFN-γ, preventing an exaggerated inflammatory response. This was corroborated by a positive correlation ($p = 0.8161$) found between IFN-γ and IL-10 in HP group, after stimulation with antigen. Noteworthy is the fact that individuals LP produced levels of IL-10 significantly lower than those of HP, suggesting that the low production of IFN-γ is not dependent on regulatory role of IL-10. Although it has not yet been demonstrated link between SLAM pathway and IL-10, our data showed increased production of IL-10 associated with the blockade of this pathway in individuals HP, and the same was observed for IL-13.

Resistance to *Leishmania* infection requires Th1 response, which in its initial phase is dependent on IL-12[2]. This study showed that the presence of rIL-12 or rIFN-γ in the microenvironment of PBMC stimulated by L. braziliensis in the first 6 h inhibits SLAM expression. Low SLAM expression in response to antigen

associated with proinflammatory cytokines may be interpreted in two ways: the first it would be that the SLAM signaling pathway was being activated and for this reason its binding sites were occupied; the second it would be that this molecule not would be synthesized and expressed in the T lymphocytes membranes. The data from this study point to the second possibility.

We find a low IL-13 production in the presence of proinflammatory cytokines when compared to production by antigen alone. This indicates that the SLAM pathway possibly was not being activated. In a microenvironment rich in proinflammatory cytokines, it is probably that other costimulatory molecules such as CD28 and B7-1[33], which promotes IFN-γ production, are participating in this scenario rather than SLAM. Based on these data, we can suggest that rIL-12 and rIFN-γ cytokines would be reducing the synthesis of SLAM and providing modulation for Th1 response. However, anti-SLAM associated with L. braziliensis and proinflammatory cytokines simultaneously, caused increased in IL-13 production, when the opposite was expected to happen. We could not find other work in the current literature analyzing these parameters in similar conditions evaluated in this work, which restricts a plausible interpretation for these seemingly contradictory data.

Collectively, the findings presented here suggest that *in vitro* immune response of PBMC of healthy individuals sensitized by L. braziliensis SLAM signaling pathway acts in modulating Th1 response in individual high IFN-γ producers and induces a state of immunosuppression in individuals low IFN-γ producers. This is the first study to evaluate the role of SLAM signaling pathway in the interaction of L. braziliensis with human PBMC *in vitro*. Elucidation of the mechanisms for reduced IFN-γ production in individuals that develop the disease will enhance our knowledge of the pathogenesis of *Leishmania*sis and suggests strategies for developing vaccines.

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