

Analysis of the protective potential of antigens released by *Leishmania (Viannia) shawi* promastigotes

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Abstract *Leishmania (Viannia) shawi* causes cutaneous lesions in humans. Parasite antigens conferring significant protection against American tegumentar leishmaniasis (ATL) might be important for the development of effective vaccine. Therefore, this work evaluates the protective effect of antigenic fractions released by *L. shawi*. Antigens released by promastigotes to culture medium were concentrated and isolated by SDS-PAGE. The three main fractions *LsPass1* (>75 kDa), *LsPass2* (75–50 kDa) and *LsPass3* (<50 kDa) were electro-eluted according with their molecular mass. Immunized BALB/c mice were challenged with *L. shawi* promastigotes and the course of infection monitored during 5 weeks. *LsPass1*-challenged mice showed no protection, however, a strong degree of protection associated to smaller lesions and high expression of IFN- γ and TNF- α by CD4⁺ T, CD8⁺ T and double negative CD4CD8 cells was achieved in *LsPass3*-challenged mice. Furthermore, *LsPass2*-challenged mice showed an intermediated degree of protection associated to high levels of IFN- γ , IL-4

and IL-10 mRNA. In spite of increased expression of IFN- γ and TNF- α , high amounts of IL-4 and IL-10 mRNA were also detected in *LsPass3*-challenged mice indicating a possible contribution of these cytokines for the persistence of a residual number of parasites that may be important in inducing long-lasting immunity. Therefore, *LsPass3* seems to be an interesting alternative that should be considered in the development of an effective vaccine against ATL.

Keywords *Leishmania shawi* · Released antigens · Immunization · Immune response

Introduction

Leishmaniasis are diseases caused by the parasite of the genus *Leishmania*. This parasite infects a broad range of mammalian species, generating tegumentar or visceral leishmaniasis. Although over 27 species of *Leishmania* have been identified worldwide, there are some species recently identified in New World. Studies able to understand the dynamics of epidemiology and the immune-pathogenesis of these newly identified species are scarce [11, 13, 14, 37, 38].

Leishmania (Viannia) shawi was described by Lainson and collaborators in 1989 [13] in non-human primates from Pará State, Brazil, and recognized as pathogenic to humans 2 years after [37]. Furthermore, Brito and collaborators [2] described five new *L. shawi* isolates obtained from clinical cases identified in Pernambuco State suggesting that this parasite may have a non-negligible epidemiological importance in Brazil. Recently, our group has characterized the murine model of *L. shawi* infection [27], evaluating cellular and humoral immune responses in infected BALB/c mice to better clarify the main immunological alterations induced

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by this parasite, and consequently to establish a standardized susceptible animal model for further studies [28].

Murine models of tegumentar leishmaniosis have been used to assay a variety of experimental vaccines, ranging from whole parasites lysate antigens to DNA vaccines. Formulations using whole parasite lysates have been demonstrating different degrees of protection in both cutaneous and visceral experimental leishmaniosis associated with increasing of cellular immune response [18]. Beside these classical vaccines, a relatively new concept was introduced by Wolff et al. [43]. These researchers used plasmidial DNA to drive the production of several immunizing proteins. This model was immediately transferred to infectious diseases [8] and different *Leishmania* genes, such as gp63 or LACK, have been linked to bacterial plasmid to produce vaccine candidates [32]. Although important levels of protection have been obtained in animal models, few works were able to demonstrate the mode of action of DNA vaccines [6], which can be a limitation for widely application.

Another interesting source of antigens that can be used to immunize against parasitic diseases are the antigen released/excreted by pathogenic agents [35]. Due to their location or because they are released into the host environment, these antigens are the first to establish a close contact with the immune system of the host modulating its function. Previous studies have demonstrated that antigens secreted by microorganisms can quickly bind to class I and II molecules of major histocompatibility complex priming CD4⁺ and CD8⁺ T lymphocytes, while somatic antigens stay encapsulated by cellular membranes, protected from the antigen processing machinery of eukaryotic cells. Only after pathogen destruction the somatic antigens can be processed for cell presentation. Thus, a delay occurs in the stimulation of T cell by this class of antigens [10].

Immunization of mice with *L. major* secreted antigens decrease skin parasite load and induce a mixed Th1/Th2 immune response [42]. Furthermore, the protective potential of released antigens was also demonstrated in experimental infections caused by *L. infantum* in both canine and murine leishmaniosis [15, 33] indicating that the pathogenic evolution of *Leishmania* infection might be profoundly affected by vaccination with this class of antigens. In addition, excreted/secreted antigens are less complex than total leishmanial antigens and can be easily produced and purified, which can favor the identification of their constitution and production of recombinant proteins. For these reasons, released antigens seem to be an interesting target to produce vaccine candidates.

Taken together, the above considerations suggest that released antigens can have a strong potential to induce protection. Therefore, the present study aims to evaluate the protective effect of antigens released to culture medium by *L. shawi* promastigotes.

Materials and methods

Parasite

Leishmania shawi (MHOM/BR/96/M15789) parasites were isolated from a patient with cutaneous leishmaniosis from the Buriticupu town, Maranhão State, Brazil and identified by monoclonal antibodies and multilocus enzyme electrophoresis at the Evandro Chagas Institute (Belém, Pará State, Brazil). The parasites maintained in BALB/c mice footpad were isolated and grown in RPMI-1640 medium (Gibco Invitrogen, USA) supplemented with 10% heat-inactivated FCS, 0.25 mM HEPES, 10 mg/ml gentamicin and 100 IU/ml penicillin. On the sixth day of culture promastigote forms were centrifuged (1,200×g, 10 min) with phosphate-buffer saline solution (PBS, pH 7.4) and used for mice infection.

Production of antigens released from *L. shawi*

Stationary phase *L. shawi* promastigotes (~10⁹ promastigotes) from cultures with two passages in vitro were added to 40 ml of RPMI 1640 protein-free. After 24 h, parasites were counted and washed at 1,000×g, 10 min, 4°C, and the supernatant was collected and immediately concentrated by centrifuging using Amicons filters with a pore of 3 kDa (Millipore, USA). The supernatant were submitted to electrophoresis [12] followed by electroelution [32] of the three main fractions: *LsPass1* (>75 kDa), *LsPass2* (75–50 kDa) and *LsPass3* (<50 kDa). The antigenic fractions were further purified from possible LPS contamination using Detoxi-Gel™ Endotoxin Removing Gel (Pierce Biotechnology, USA) according to the manufacturer's instructions. The protein content of each fraction was quantified in a spectrophotometer at 280 nm with correction at 320 nm. The maintenance of *L. shawi* promastigotes in protein-free medium for such a short time did not affect the viability or the concentration of parasites that suffer a slight increase (~2.0 × 10⁹ promastigotes).

Immunization and challenge

Groups of 10 male BALB/c mice were immunized once a week for two consecutive weeks by subcutaneous route in the rump with 25 µg of *LsPass1*, *LsPass2* or *LsPass3* antigenic fraction. Another 10 healthy mice were injected with 50 µl of PBS by the same route, in a similar way. One week after the last immunization, five immunized and five healthy mice were infected intradermally into the hind footpad with 10⁶ stationary phase *L. shawi* promastigotes (designated as challenged and infected groups, respectively). Five immunized and healthy (non-infected) animals were injected with 50 µl of PBS into the footpad. The infection

was monitored weekly for 5 weeks by measuring the size of lesions with a dial micrometer and expressed as the difference in size between the infected and the contra lateral uninfected footpad. Then animals were sacrificed in a CO₂ chamber and skin fragments were obtained for parasite burden determination through limiting-dilution assay. Popliteal lymph nodes were collected for determination of parasite load and isolation of CD4⁺ and CD8⁺ T cells and double negative (dn) CD4CD8 cells.

In previous studies, our group had verified that after 6 weeks of infection BALB/c mice develop ulcerated lesions with extensive necrosis, usually associated to secondary infections (data not shown). Therefore, to avoid the emerging of extreme clinical situations the experiments were design for a maximum of 5 weeks.

Determination of viable parasite load

The number of viable promastigotes was estimated by limiting dilution assay (LDA) [23]. Briefly, skin fragments and lymph nodes were weighed and homogenized individually in a Schneider 10% FCS. An initial homogenized suspension was placed into the first well (200 µl) and serial dilutions (1:4) were distributed in a 96-multiwell plate (Nunc, Germany). For each sample, 12 replicates were made. After 10 days at 25°C, each well was examined by optical microscopy and the final titer was set as the highest dilution for which the well contained at least one parasite. The viable parasitic load per gram of homogenized organ was calculated as follows: (reciprocal titer of the last positive well per total volume of homogenized organ/tissue × dilution factor) divided by the weight (gram) of the homogenized organ/tissue.

Isolation of lymph node cell subpopulations

CD4⁺ and CD8⁺ T cells and dnCD4CD8 cells were isolated from lymph nodes of all groups by positive selection on a magnetic separation system. Lymph nodes were homogenized in RPMI 1640 and the cell number was counted and adjusted to 10⁷ cells/90 µl of elution buffer [PBS + 0.5% bovine serum albumin (BSA, Boehringer, Germany)] with 10 µl of anti-mouse CD4 (L3T4) and CD8 (CD8α) micro beads. After 20 min of incubation cells were washed (300×g, 10 min, 4°C), resuspended in 500 µl of elution buffer and applied to a LS column on magnetic separator (MACS Separator, Miltenyi Biotec, Germany) followed by three washes (500 µl/each) with elution buffer. After the washes, columns were removed from the magnetic separator and CD4⁺ (purity of 94.1%) or CD8⁺ T cells (purity of 91.7%) were eluted. dnCD4CD8 cells were collected

directly from the LS+column. CD4⁺ and CD8⁺ T cells and dnCD4CD8 cells were used for RNA extraction.

RNA extraction, cDNA synthesis and real-time PCR

To evaluate the ex vivo lymphocyte activity the accumulation of cytokines mRNA in CD4⁺ T, CD8⁺ T and dnCD4CD8 cell subpopulations freshly isolated was quantified by real-time PCR.

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. The target RNA was reverse transcribed into cDNA using 200U M-MLVRT (Promega, USA), at 37°C for 60 min in the presence of 3 mM 5× M-MLV RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl and 15 mM MgCl₂), 10 mM BSA, 1 mM dNTPs (Promega), 40 U rRNasin ribonuclease inhibitor and 10× Oligo (dT)15 (Promega). The samples were then heated 10 min at 95°C for RT inactivation and cooled to 4°C [15]. Quantitative real-time PCR was performed in the ABI GeneAmp 5700-sequence detection system (Perkin-Elmer/Applied Biosystems, USA). Reaction conditions were powered on a Pentium III Dell Opti Plex GX110 linked directly to the sequence detector. PCR amplifications were performed by using SYBR[®] Green as a double-strand DNA-specific binding dye with continuous fluorescence monitorization. Amplification was carried out in a total volume of 20 µl containing 2 µl of cDNA sample, 10 µl of 2× SYBR[®] Green I dye PCR Master Mix [(AmpliTaq Gold[®] DNA polymerase, dNTP mix with dUTP, passive reference I and optimized buffer components) (Perkin-Elmer/Applied Biosystems)] and primers for HPRT, IFN-γ, TNF-α, IL-4, IL-10 and TGF-β as described by Rosa et al. [33] and Rodrigues et al. [30]. PCR amplification was performed in triplicate wells using the following conditions: 10 min at 95°C for AmpliTaq Gold activation followed by a total of 40 cycles (thermal profile for each cycle: 15 s at 95°C, 1 min at 60°C). To quantify the cytokine expression cDNA plasmid standards for each cytokine and HPRT [30] were used to construct a standard curve in each PCR run.

Statistical analysis

The experiments have been repeated three times and results were expressed as mean ± standard deviation and the non-parametric Mann-Whitney *U* test was used to compare lesion size, parasite load and cytokine expressions between the groups of mice. Differences were considered statistically significant with a 5% significance level (*p* < 0.05). Statistical analysis was performed with the SPSS 17.0 for Windows software (SPSS Inc. USA).

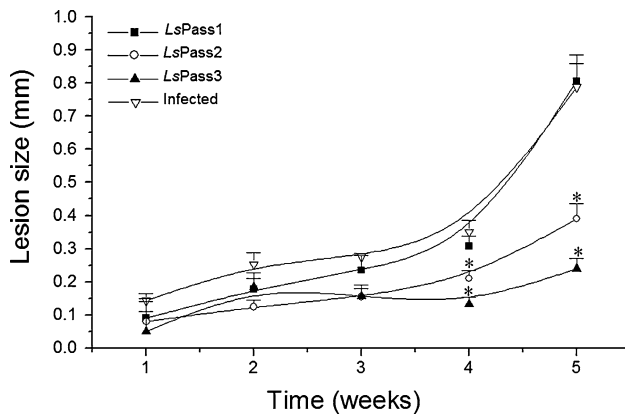


Fig. 1 The course of infection in infected and *LsPass1*-, *LsPass2*- and *LsPass3*-challenged BALB/c mice was accompanied during five weeks. Results are expressed as the mean of lesion sizes determined every week for five consecutive weeks. The experiment was repeated three times. * $p < 0.05$ indicates statistically significant differences when comparing the lesion size of infected and challenged mice

Results

Immunization by *LsPass2* and *LsPass3* confers partial protection to *L. shawi* infection

During the first 3 weeks, the course of infection was identical in all infected animals, however at 4 and 5 weeks post infection (pi) *LsPass2*- ($p = 0.012$; $p = 0.023$, respectively) and *LsPass3*-challenged (immunized and infected) mice showed a significant reduction of lesion size ($p = 0.018$; $p < 0.001$) when compared with infected mice (Fig. 1).

Furthermore, *LsPass2*- ($p = 0.014$) and *LsPass3*-challenged mice ($p = 0.021$) presented 72 and 92% lesser parasites, respectively, compared with infected mice. Moreover, *LsPass3* antigen was four times more effective than *LsPass2* in the induction of protection against *L. shawi* infection ($p < 0.016$). The immunization with *LsPass1* showed no protection (Fig. 2).

Parasites were not detected in lymph nodes of infected and challenged animals.

Immunization induced the expression of pro-inflammatory cytokines

Mice immunized with *LsPass1* fraction presented elevated expression of IFN- γ by CD4⁺ ($p = 0.039$) and CD8⁺ T ($p = 0.035$) cells, however, dnCD4CD8 ($p = 0.031$) cells showed minimal expression compared to healthy mice. *LsPass2*-immunized mice had decreased expression of IFN- γ by CD4⁺ T ($p = 0.002$) and dnCD4CD8 ($p = 0.041$) cells. However, CD8⁺ T cells showed high accumulation of IFN- γ mRNA ($p = 0.005$). In the group of mice immunized

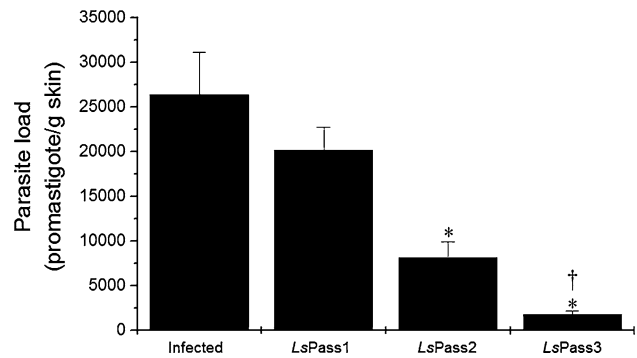


Fig. 2 Determination of parasite burden in skin of infected *LsPass1*-, *LsPass2*- and *LsPass3*-challenged BALB/c mice at 5 weeks pi. Parasite load was determined by limiting dilution assay and results are expressed as the mean value of parasites per gram (g) of homogenized skin \pm SEM of three independent experiments. * $p < 0.05$ indicates statistically significant differences when comparing the parasite load of infected and challenged mice

with *LsPass3*, an elevated expression of this cytokine was only observed in CD4⁺ T cells ($p = 0.023$) (Fig. 3a).

CD4⁺ T cells isolated from *LsPass1*- ($p = 0.002$) and *LsPass3*-immunized mice ($p = 0.013$) expressed high amounts of TNF- α . CD8⁺ T cells of *LsPass2*- ($p = 0.005$) and *LsPass3*-immunized mice ($p = 0.029$) revealed increased levels of TNF- α mRNA. On the other hand, dnCD4CD8 cells isolated from lymph nodes of *LsPass2*-immunized mice showed a reduced expression of TNF- α gene ($p = 0.041$) (Fig. 3b).

Increases of IL-4 expression was detected in CD4⁺ ($p = 0.008$) and CD8⁺ T cells ($p = 0.034$) of *LsPass1*-immunized mice. CD4⁺ T cells from *LsPass2*-immunized mice presented elevated expression of IL-4 ($p = 0.009$) while CD8⁺ T cells ($p = 0.012$) expressed reduced amounts of this cytokine. High levels of IL-4 mRNA were accumulated by CD8⁺ T ($p = 0.019$) and dnCD4CD8 cells ($p = 0.002$) of *LsPass3*-immunized mice whereas CD4⁺ T cells revealed a significant reduction of this cytokine ($p = 0.012$) (Fig. 3c).

In *LsPass1*-immunized mice, CD4⁺ T cells exhibited a decreased expression of IL-10 ($p = 0.023$) while CD8⁺ T cells ($p = 0.031$) presented high mRNA accumulation. CD8⁺ T ($p = 0.012$) and dnCD4CD8 cells ($p = 0.023$) of *LsPass2*-immunized mice showed decreased expression of IL-10. CD8⁺ T cells of *LsPass3*-immunized mice expressed low amounts of IL-10 mRNA ($p = 0.014$) whereas dnCD4CD8 cells presented significant high levels of this cytokine ($p = 0.032$) (Fig. 3d).

In both *LsPass1*- and *LsPass3*-immunized mice elevated expression of TGF- β was verified in CD4⁺ ($p = 0.004$) and CD8⁺ T cells ($p = 0.027$). CD8⁺ T cells of *LsPass2*-immunized mice also presented high levels of TGF- β mRNA ($p = 0.003$), while dnCD4CD8 cells showed minimal expression ($p = 0.023$) (Fig. 3e).

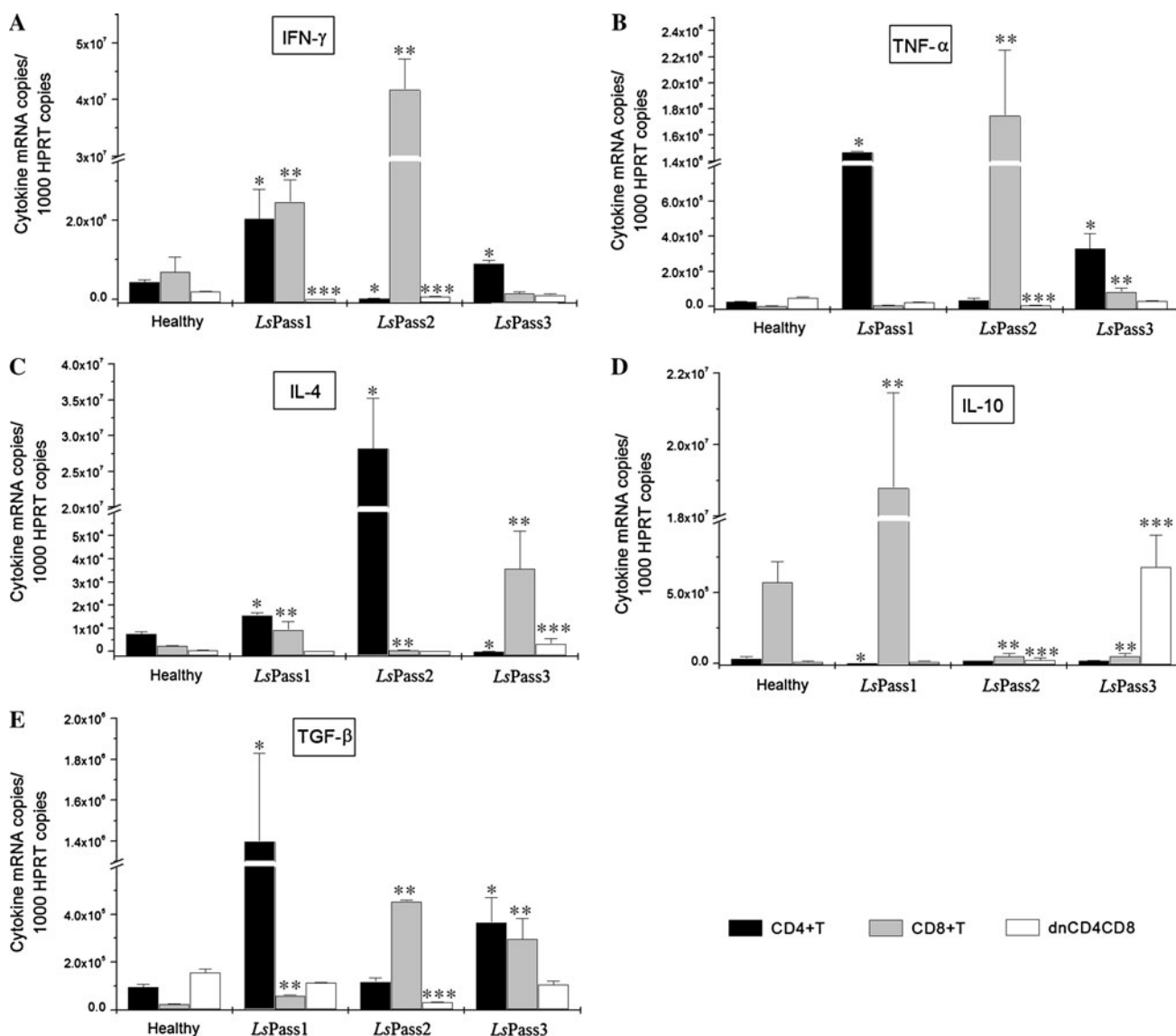


Fig. 3 Ex vivo mRNA expression of IFN- γ (a), TNF- α (b), IL-4 (c), IL-10 (d) and TGF- β (e) by CD4⁺ T, CD8⁺ T and dnCD4CD8 cells of healthy and *LsPass1*-, *LsPass2*- and *LsPass3*-immunized BALB/c mice. The levels of cytokines mRNA were obtained by quantitative real-time PCR and data are mean values \pm SEM of three independent

experiments expressed as the number of copies per 1,000 copies of housekeeping gene HPRT. *, ** and *** $p < 0.05$ indicate statistically significant differences when comparing samples from healthy and immunized mice

LsPass3-challenged mice develop a mild pro-inflammatory response

CD4⁺ T cells of challenged animals presented elevated expression of IFN- γ when compared with infected mice ($p_{LsPass1}$ and $LsPass3 = 0.008$; $p_{LsPass2} = 0.009$). CD8⁺ T cells from *LsPass2*-challenged mice also presented a strong accumulation of IFN- γ mRNA ($p = 0.003$), although in *LsPass1*- and *LsPass3*-challenged mice a significant decrease in the expression of this cytokine was observed ($p_{LsPass1} = 0.006$; $p_{LsPass3} = 0.008$). dnCD4CD8 cells from both *LsPass2*- and *LsPass3*-challenged mice presented a

significant high expression of IFN- γ in comparison to infected mice ($p_{LsPass2} = 0.001$; $p_{LsPass3} = 0.007$) (Fig. 4a).

CD4⁺ T cells isolated from *LsPass2*- and *LsPass3*-challenged animals showed a decrease of TNF- α expression when compared with infected mice ($p_{LsPass2} = 0.003$; $p_{LsPass3} = 0.046$). High expression of this cytokine was also observed in CD8⁺ T cells of *LsPass1*- ($p = 0.021$) and *LsPass3*-challenged mice ($p = 0.019$). dnCD4CD8 cells of *LsPass3*-challenged mice also exhibited a significant accumulation of TNF- α mRNA ($p = 0.034$) (Fig. 4b)

CD4⁺ and CD8⁺ T cells from *LsPass2*-challenged mice presented high levels of IL-4 mRNA when compared with

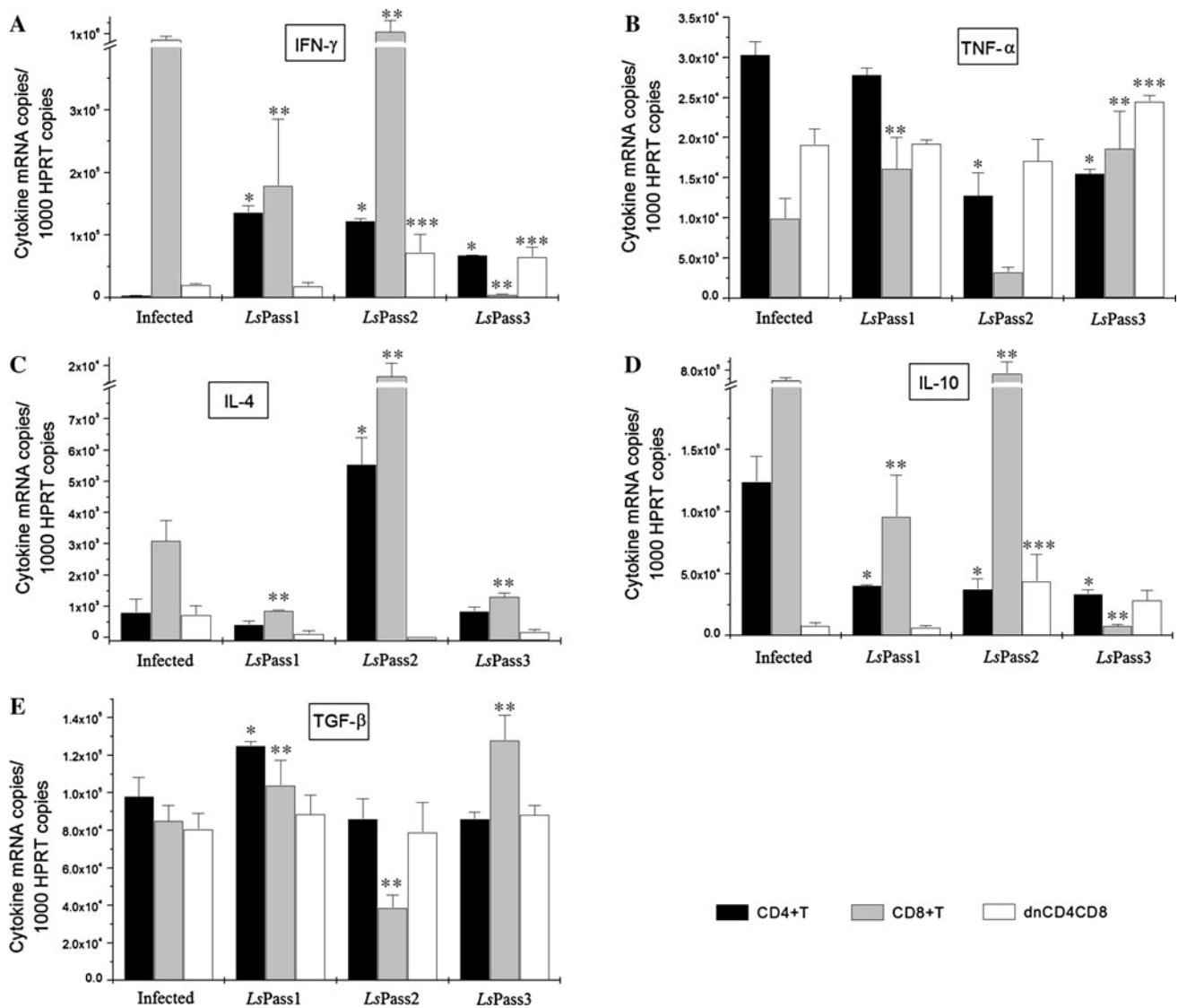


Fig. 4 Ex vivo mRNA expression of IFN- γ (a), TNF- α (b), IL-4 (c), IL-10 (d) and TGF- β (e) by CD4⁺ T, CD8⁺ T and dnCD4CD8 cells of infected and challenged BALB/c mice. The levels of cytokines mRNA were obtained by quantitative real-time PCR and data are mean values

\pm SEM of three independent experiments expressed as the number of copies per 1,000 copies of housekeeping gene HPRT. *, ** and *** p < 0.05 indicate statistically significant differences when comparing samples from healthy and immunized mice

infected mice ($p = 0.008$). On the other hand, CD8⁺ T cells from *LsPass1*- and *LsPass3*-challenged animals showed low levels of IL-4 ($p = 0.006$) (Fig. 4c)

CD4⁺ T cells of challenged animals presented reduced expression of IL-10 ($p = 0.001$). However, high levels of IL-10 were observed in CD8⁺ T and dnCD4CD8 cells of *LsPass2*-challenged mice. CD8⁺ T cells of both *LsPass1*- ($p = 0.007$) and *LsPass3*-challenged animals ($p = 0.003$) also revealed low accumulation of IL-10 mRNA (Fig. 4d).

In *LsPass1*-challenged mice an increasing expression of TGF- β was detected in both CD4⁺ ($p = 0.002$) and CD8⁺ T cells ($p = 0.032$). *LsPass2*-challenged mice showed low levels of TGF- β expressed by CD8⁺ T cells ($p = 0.004$), while elevated accumulation of this cytokine were observed

in CD8⁺ T cells from *LsPass3*-challenged mice ($p = 0.032$) (Fig. 4e).

Discussion

Antigens released from promastigote forms have been identified as potent modulators of the immune system, having a crucial role in promoting the establishment and maintenance of the infection by interfering in the activation of effector mechanisms, such as the microbicidal activity of macrophages and cytokine production. Moreover, interesting studies using released antigens from *Leishmania* sp. as a model for the development of affordable vaccines have

been conducted. Antigens released by *L. infantum* showed a strong potential to modulate host immunity through a Th1 immune response and induce protection in murine and canine models of zoonotic visceral leishmaniasis [16, 34]. The above considerations support the hypothesis that antigens released by *Leishmania* species that cause American tegumentar leishmaniasis (ATL) may have potential to induce host protection and be used for vaccine development against these diseases.

The immunization of BALB/c mice with *LsPass1* induced overexpression of IFN- γ , IL-4 and TGF- β by both CD4⁺ and CD8⁺ T cells. In addition, this immunization increased the levels of TNF- α mRNA by CD4⁺ T cells and IL-10 by CD8⁺ T lymphocytes. Therefore, *LsPass1* immunized mice presented strong potential to develop cellular immune response and both CD4⁺ and CD8⁺ T lymphocytes expressed Th1 cytokines, indicating beneficial response. However, the presence of regulatory mechanism, as measured by IL-10 and TGF- β expression, could be associated to generation of susceptibility since these cytokines induce unresponsive effector cells [7, 19] leading to low protection. In fact, challenged animals presented lesion size and parasite load similar to infected mice. The absence of protection has been associated to high expression of TGF- β by CD4⁺ and CD8⁺ T cells. TGF- β has the capacity to convert CD4⁺ T lymphocytes to a regulatory phenotype [36, 41], which in turn can depress the effector functions of phagocyte cells [3]. Since TGF- β controls several essential cellular functions a finely tuned regulation of its activity is required. Therefore, it is important to underline that the accumulation levels of TGF- β mRNA does not always correlate with the amount of active protein [41].

Interestingly, in both immunized and challenged groups, the dnCD4CD8 cells (group which contain macrophage, dendritic cell, neutrophil, plasma cells and, natural killer cells) presented an anergic state. Thus, the absence of protection in this group can be associated to both elevation of TGF- β mRNA and suppression of dnCD4CD8.

The *LsPass2*-immunized mice stimulated mainly CD8⁺ T cells to express high levels of IFN- γ and TNF- α cytokines. On the other hand, CD4⁺ T and dnCD4CD8 cells decreased the expression of these cytokines. Increases in the expression of IL-4 by CD4⁺ and TGF- β by CD8⁺ T and dnCD4CD8 cells have been observed. In addition, CD8⁺ T and dnCD4CD8 cells expressed lesser IL-10 than healthy mice. Therefore, the immunization with *LsPass2* induced the expression of pro inflammatory cytokines by cytotoxic T cells, which seem to be an important factor of protection in leishmaniasis. In addition, this immunization also induced the expression of regulatory cytokine TGF- β , probably to regulate the exacerbated immune response developed by these animals and to achieve immune homeostasis [4, 9]. After challenge, this group was able to restrain the

lesion and parasite load in skin associated to elevated expression of IFN- γ in all cell subsets, indicating that different cells could be activated by immunization such macrophage and natural killer cells [17]. In contrast, CD8⁺ T cells of these animals have reduced ability to express TNF- α gene, thus the synchronization of different cells subsets to express IFN- γ can favor the protection verified in this group. On the other hand, the level of protection observed in *LsPass2*-challenged mice can be associated to high accumulation of IL-4 mRNA by CD4⁺ and CD8⁺ T cells and of IL-10 mRNA by CD8⁺ T and dnCD4CD8 cells after vaccination. Therefore, after challenge this group exhibited a mixed Th1/Th2 and Tc1/Tc2 immune response associated to the activation of CD8⁺ T cell subset with regulator phenotype.

LsPass3-challenged mice were able to enhance the expression of IFN- γ by CD4⁺ T and TNF- α by CD4⁺ and CD8⁺ T lymphocytes, which demonstrates the degree of lymphocyte activation. In comparison with *LsPass1*- and *LsPass2*-challenged mice, this group presented lower expression of Th1 and Th2 cytokines possible due to the elevated expression of IL-10 and TGF- β by dnCD4CD8 cells and CD4⁺/CD8⁺ T lymphocytes, respectively. This profile of mild expression of both inflammatory and anti-inflammatory cytokines could facilitate the induction of immunity, since this group presented high rate of protection after inoculation of *L. shawi* promastigotes, associated to decreased lesion size and parasite numbers in skin. Furthermore, after challenged mice still presented a mild Th1 immune response with increased expression of IFN- γ by CD4⁺ T lymphocytes and dnCD4CD8 cells. The high expression of TNF- α by CD8⁺ T lymphocytes and dnCD4CD8 cells may be related to phagocyte cells activation and higher levels of protection. Moreover, this group also presented low expression of IL-4 by CD8⁺T cells and IL-10 was expressed at low levels by both CD4⁺ and CD8⁺ T lymphocytes. Beside the impairment of Th2 response and reduced expression of IL-10, the immunization induced the expression of TGF- β by CD8⁺T cells. Recently, it was also demonstrated that TGF- β restrained the protection induced by immunization with soluble antigen from *L. shawi* promastigotes even when in presence of a favorable immunological response [29]. Although IL-10 and TGF- β abrogate protection in experimental immunization allowing the persistence of few parasites in the infection site [1], these cytokines avoid immune pathological injuries generated by excessive inflammatory response [31]. In addition, the presence of residual parasites could account for the establishment of a long-lasting immunity conferring a concomitant protection [21].

Infection by parasites of *Leishmania* subgenus promotes elevated parasitism and low cellular immune response in patients and animals. On the contrary, *Leishmania* parasites

belonging to *Viannia* subgenus induce low parasitism and high cellular immune response in rodent models and patients [20, 39, 40] suggesting that *Viannia* antigens are highly immunogenic. In fact, BALB/c mice immunized with antigens derived from *L. shawi* plus the adjuvant monophosphoryl lipid (MPL) induced IFN- γ associated to a dramatic increase of IL-4 and IL-10 (data not shown). Thereby, additional amplification of cellular immune responses due to the use of adjuvants should also elicit a regulatory and Th2 immune response [5] that may be unfavorable to ATL protection.

Taken together, the results obtained in the present study indicate that antigens release by *L. shawi* promastigotes to culture medium have stimulatory effects on the host immune system and their pre-exposure to naïve host can prime competent cells to activate macrophages to eliminate intracellular parasites. In spite of conferring high levels of protection, *LsPass2* and *LsPass3* fractions can be constituted by several molecules, some of which may be counter protective favoring the evasion of the host immune system by the parasite, thus prolonging its survival in host cells due to immunosuppressive antigens [1, 21–26].

Although this study open promising perspectives for the development of a vaccine against ATL further studies are needed to elucidate the role of persistent parasites and regulatory immune response in the maintenance of anti-*Leishmania* pool of central and effector memory cells. The complete understanding of these factors is crucial for the designing of vaccination strategies against ATL.

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