Response of CD4+ and CD8+ T lymphocytes in the evolution of *Leishmania (Viannia) shawi* infection

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Abstract *Leishmania (Viannia) shawi* was recently characterized, and there are no studies of immunopathological alterations induced by this parasite. BALB/c and C57BL/6 mice were infected in the footpad with *L. (V.) shawi* promastigotes. The lesions were monitored during 8 weeks post-infection (pi), and at each 2 weeks pi, immunohistochemistry of skin and lymph nodes has been performed to analyze the densities of CD4+ and CD8+ T lymphocytes as well as amastigote forms. In the skin, BALB/c mice presented higher amastigote densities than C57BL/6 mice; concerning lymphocytes, there are no significant differences between CD4+ T lymphocytes densities; however, C57BL/6 mice presented elevation in densities of CD8+ T cells latter in the infection. The elevation of amastigote densities in lymph nodes of BALB/c mice at 8 weeks pi could be a reflection of suppressed density of CD4+ T. On the other hand, elevated density of CD8+ T lymphocytes in lymph nodes of C57BL/6 seems to exert some degree of resistance compared to BALB/c mice. The present work indicates that CD8+ T lymphocyte can be a key component to generation of resistance in *L. (V.) shawi* infections.

Keywords Experimental leishmaniasis · BALB/c mice · C57BL/6 mice · *Leishmania (Viannia) shawi* · Immunopathology

Introduction

Leishmaniasis is a disease caused by parasites belonging to the *Leishmania* genus. There is a diversity of species described in World, which can induce cutaneous or visceral form of disease. In New World, different species of dermatotropic *Leishmania* can infect humans, generating distinct patterns of skin lesions, ranging from single cutaneous to anergic diffuse leishmaniasis (Silveira et al. 2009).

Different works have demonstrated the spectrum of disease performed by *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis* infections as well as the main histopathological alterations in the skin of both murine and human cases. In addition, these findings, highlighting which kind of cells and approaches, can induce amelioration in the course of disease (Aranha et al. 2005; Beattie et al. 2010).

Although growing knowledge have been accumulated on the basic biology, infection profile, immunology, and histopathology of *L. (L.) amazonensis* and *L. (V.) braziliensis*, scarce information of *Leishmania (Viannia) shawi* parasite have been done. It was isolated from different species of wild animals, such as monkeys, sloths, coatimundis, and *Lutzomyia whitmany*. In the mammal reservoirs, the parasites were detected to be restricted to the skin, except for sloths, of which the places of isolation were the spleen and liver (Lainson et al. 1989).

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Two years afterwards, Shaw et al. (1991) demonstrated the capacity of *L. (V.) shawi* to infect humans. In most patients (~60%), a single ulcerated skin lesion has been detected, although in some individuals, many lesions were recorded. Recently, our group researched the biologic, pathologic, and immunologic characters of *L. (V.) shawi* in murine model of infection. The results have demonstrated the potential of this parasite in infecting both BALB/c and C57BL/6 lineage, being the first one highly susceptible to infection, presenting higher parasite load, intense inflammatory reactions, and dissemination of necrosis regions in the skin (Passero et al. 2009). On the immunological point of view, these animals presented upregulation in the expression of TNF- α by CD4+ and CD8+ T and downregulation of IFN- γ by CD4+ T lymphocytes. In addition, upregulation in the expression of IL-4 by CD8+ T and increases in IL-10 and TGF- β by both CD4+ and CD8+ T lymphocytes have been verified, pointing out to the immune modulation exerted by *L. (V.) shawi*. Moreover, elevation in TGF- β and IL-10 mRNAs verified in both CD4+ and CD8+ lymphocyte can be mechanisms by which parasite induces pathological processes in murine model of infection even in the presence of high amounts of Th1 cytokines (Passero et al. 2010).

The CD4+ and CD8+ T lymphocytes have crucial implications in amelioration and progression of leishmaniasis. Moreover, some studies have demonstrated an association between elevated number of lymphocytes and parasite destruction (Reis et al. 2008; Da-Cruz et al. 1994). However, in *L. (V.) shawi* infections, there are no data associating profile of infection and densities of lymphocytes into skin and lymph nodes. Thus, the present work aimed to analyze the relation between the densities of amastigote forms and T lymphocytes.

Material and methods

Parasites

The strain of *L. (V.) shawi* (MHOM/BR/96/M15789) was isolated from a patient with cutaneous leishmaniasis in the Buriticupu county, Maranhão State, Brazil. It was classified by monoclonal antibodies and isoenzymes at the Evandro Chagas Institute, Belém, Pará State, Brazil.

The parasites maintained in BALB/c mice footpad were isolated and expanded in RPMI-1640 medium, supplemented with 10% heat-inactivated serum, 0.25 mM HEPES, 10 μ g/ml gentamicin, and 100 IU/ml penicillin, and incubated at 25°C. On the sixth day of culture, the promastigote forms were washed three times with phosphate buffered saline (PBS) pH 7.4 and centrifuged at 1,200 $\times$ *g* for 10 min using a refrigerated centrifuge for infection in mice.

Experimental animals

Eight-week-old BALB/c and C57BL/6 male mice obtained from the Animal Facility of the São Paulo University, Medical School Brazil were maintained in our laboratory during the experiments according to the guidelines of the institutional rules regarding the welfare of experimental animals and with the approval of the Animal Ethics Committee of São Paulo University.

Infection protocol and measurement of lesion size in BALB/c and C57BL/6 mice

The stationary phase promastigote forms were washed three times in PBS, and the concentration of the suspension was adjusted to 2×10^7 promastigotes per milliliter. Aliquot of 50 μ l was injected subcutaneously in the hind footpads of the mice (about 10^6 promastigotes/mouse). Healthy mice (control group) received only PBS in footpads. The infection was monitored for 8 weeks. At 2, 4, 6, and 8 weeks post-infection (pi), one group of five infected mice of both mouse strains were sacrificed, and biopsies from skin and lymph nodes were collected, fixed in buffered 5% formalin for histopathological studies, or frozen using tissue tack for CD4+ and CD8+ T cells detection by immunohistochemistry. The experiment was repeated three times.

Evaluation of parasite density

The evaluation of parasite densities in skin and lymph nodes was performed through immunohistochemistry according to Passero et al. (2008). After the procedure, the slides were mounted, and ten different fields of each section (skin or lymph node) were photographed, and the parasite numbers were evaluated with a microscopy coupled to the computer using the Axion Vision 5.0 software. The parasite densities were expressed as amastigotes per square millimeter using the following expression: parasite load=means of parasite number per group/total area of each picture.

Evaluation of CD4+ and CD8T+ cells

Frozen sections from skin and lymph nodes of BALB/c and C57BL/6 mice were submitted to immunohistochemical reaction for CD4+ and CD8+ T cell detection using rat anti-CD4+ T and anti-CD8+ T monoclonal antibodies (B&D—USA) as primary antibodies in a dilution of 1:20 for 60 min. The color was developed through the addition of anti-Rat Ig HRP detection kit and by addition of DAB, following manufacturer's recommendations (B&D—USA). Quantitative analyses were performed using the Axion

Vision 5.0 software (details in “Evaluation of parasite density”). Lymph nodes of healthy BALB/c and C57BL/6 mice were used as positive controls.

Statistical analysis

The results were expressed as mean±standard deviation of three independent experiments, and the non-parametric test of Mann–Whitney was employed to compare the results found in this work. Differences were considered statistically significant when $p<0.05$.

Results

In vivo infection

BALB/c and C57BL/6 mice presented elevation in lesion size with the evolution of lesion. Moreover, BALB/c mice presented higher lesion size compared to C57BL/6 (Fig. 1).

Analysis of the amastigote density

In the skin, the density of amastigote forms increased significantly with the evolution of lesion in both mice strains ($p<0.05$) in the experimental time analyzed, although BALB/c mice presented higher amastigote density than C57BL/6 ($p<0.05$) from 2 weeks pi (Fig. 2a).

In lymph nodes, amastigote forms were detected at 6 weeks pi by immunohistochemistry (Fig. 2b), and at this experimental point, the density of amastigote forms were similar between mice strain; however, at 8 weeks pi, BALB/c mice presented a significant increasing in amastigote density compared to C57BL/6 ($p<0.05$).

Fig. 1 Evolution of footpad swelling in BALB/c and C57BL/6 mice infected with 10^6 promastigotes/animal. * and ** $p<0.05$ in comparison with infected BALB/c and C57BL/6 mice at 2 weeks pi, respectively. † $p<0.05$ in comparison with infected BALB/c mice

Fig. 2 Parasite density in skin (a) and lymph nodes (b) of BALB/c and C57BL/6 mice in paraffin section stained by immunohistochemistry reaction. Each value represents the mean±standard deviation of amastigote per square millimeter of tissue of ten sections from five mice. * and ** $p<0.05$ in comparison with infected BALB/c and C57BL/6 mice at 2 weeks pi, respectively. † $p<0.05$ in comparison with infected BALB/c mice

Density of CD4+ T and CD8+ T cells in skin and lymph nodes

In the skin, BALB/c and C57BL/6 mice presented similar densities of CD4+ T cells in all experimental time ($p>0.05$); however, there was an increase in CD4+ T cell density in comparison with the evolution of lesion ($p<0.05$) in both mouse strains (Fig. 3a). The density of CD8+ T lymphocyte were similar in BALB/c mice with the evolution of lesion (Fig. 3b); however, C57BL/6 mice presented an increase in CD8+ T cell density in skin at 6 and 8 weeks pi ($p<0.05$). Control groups presented neither CD4+ nor CD8+ T lymphocytes.

In the lymph nodes of BALB/c mice, there was a significant suppression in CD4+ T cell density (Fig. 4a) at 8 weeks pi ($p<0.05$); in contrast, at same experimental point, a significant increase in the CD4+ T cell density has been verified in

Fig. 3 Densities of CD4⁺ and CD8⁺ T cells in the skin. **a** CD4⁺ T lymphocyte in the skin of BALB/c and C57BL/6 mice. **b** CD8⁺ T lymphocyte in the skin of BALB/c and C57BL/6 mice. * and ** $p < 0.05$ in comparison with infected BALB/c and C57BL/6 mice at 2 weeks pi, respectively. † $p < 0.05$ compared with infected BALB/c mice

C57BL/6 mice ($p < 0.05$). Moreover, C57BL/6 mice presented elevated density of CD4⁺ T cells compared to BALB/c mice ($p < 0.05$) for all experimental time. Regarding CD8⁺ T cells of lymph nodes (Fig. 4b), BALB/c presented similar densities with the lesion evolution. C57BL/6 mice presented a tendency to decreasing CD8⁺ T cells. In addition, C57BL/6 mice presented higher CD8⁺ T cell density compared to BALB/c mice ($p < 0.05$). Noteworthy, healthy BALB/c and C57BL/6 mice presented higher CD4⁺ and CD8⁺ T lymphocytes compared to both infected groups, indicating a suppressive action induced by parasite (data not shown).

Discussion

BALB/c mice are the classical models of susceptibility in cutaneous leishmaniasis caused by *Leishmania major*,

while C57BL/6, the model of resistance, presented self-healing lesions (Gumy et al. 2004). In contrast, New World *Leishmania* sp. shows different profile of infection in murine model of infection. For example, *L. (L.) amazonensis* is able to infect and exert pathology in both BALB/c and C57BL/6 (Cupolilo et al. 2003), *L. (V.) shawi* induces exacerbated disease in BALB/c mice and intermediate in C57BL/6 (Passero et al. 2009); in contrast, *L. (V.) braziliensis* generates self-healing lesions in these mice strains (Rocha et al. 2007). Besides the broad spectrum of diseases generated by *Leishmania* from New World, these facts demonstrate that infections can differ significantly in terms of immunopathology.

The present work demonstrated that BALB/c mice presented higher parasite densities compared to C57BL/6 mice, and it was associated with increased densities of CD4⁺ T lymphocytes in the skin of both mouse strains. Studies concerning natural and experimental infections have postu-

Fig. 4 Densities of CD4⁺ and CD8⁺ T cells in lymph nodes. **a** CD4⁺ T lymphocyte in lymph nodes of BALB/c and C57BL/6 mice. **b** CD8⁺ T lymphocyte in lymph nodes of BALB/c and C57BL/6 mice. * and ** $p < 0.05$ in comparison with infected BALB/c and C57BL/6 mice at 2 weeks pi, respectively. † $p < 0.05$ compared to infected BALB/c mice

lated that CD4⁺ T lymphocytes can direct the infection toward progression or resolution of lesion based only on their cytokine secretion profile (Ajadary et al. 2000). In *L. (V.) shawi* infection, these cells can be associated to the pathogenesis of disease once their densities in both BALB/c and C57BL/6 mice increase in conformity with the amastigote numbers, indicating that these cells are not restraining amastigote spreading. Concerning CD8⁺ T cells, BALB/c mice present similar CD8⁺ T cell densities with the evolution of infection, but C57BL/6 showed an increase in the densities of CD8⁺ T lymphocytes at latter stages of infection. CD8⁺ T lymphocytes present an important role in elimination of infected cells through cytotoxic effects and activating infected cells (Da-Cruz et al. 2005; Ruiz and Becker 2007), therefore exerting anti-leishmania immunity. Thus, the intermediate control of amastigote replication in skin of C57BL/6 mice, in comparison with BALB/c mice, can be accounted to the increase of CD8⁺ T lymphocyte in the skin.

In lymph nodes, amastigote forms have been detected in both mice strains at 6 and 8 weeks pi. At these experimental points, the density of CD4⁺ T lymphocytes decreased in BALB/c, which can facilitate the infection spreading in this lymphoid organ, while in C57BL/6, a significant increase occurred; in addition, the density of CD4⁺ T lymphocytes were higher than BALB/c mice, which explains the lesser parasite density observed at 8 weeks pi. In cutaneous leishmaniasis, high rate of apoptosis and cell death in CD4⁺ T lymphocytes have been verified, which can modulate the ratio of immune cells and consequently the outcome of disease (Bertho et al. 2000). Similarly, in infected BALB/c mice, suppression in the density of CD4⁺ T lymphocyte has been verified, which can allow parasite growth and persistence. In the lymph nodes of BALB/c mice, there was relatively constancy in the CD8⁺ T lymphocyte densities with the lesion progression, C57BL/6 already presented tendency to decreasing CD8⁺ T lymphocyte (without significance), but these mice still retained higher cell densities than BALB/c mice. Some works have demonstrated the importance of elevated number of CD8⁺ T cells in comparison with CD4⁺ T cell number (CD4⁺/CD8⁺>1) to reach resistance in natural infections by *Leishmania* sp. (Da-Cruz et al. 2002, 2005; Morgado et al. 2008). In fact, C57BL/6 mice infected with *L. (V.) shawi* presented, at the time of amastigote detection in lymph nodes, higher density of CD4⁺ than CD8⁺ T lymphocytes, indicating a decreased potential to contain the amastigote growth.

The present work demonstrated that both BALB/c and C57BL/6 mice presented susceptibility to *L. (V.) shawi* infection; however, the immunological features associated to the number of lymphocytes can differ significantly in these mice; thus, in BALB/c mice, the parasite seems to

modulate mainly CD4⁺ T lymphocytes. In C57BL/6 mice, which have lesser parasite numbers in both skin and lymph nodes, the densities of CD4⁺ and CD8⁺ T lymphocytes in skin and lymph nodes were modulated for *L. (V.) shawi* parasites. This study indicates that CD8⁺ T lymphocytes can be a key element for resistance to the *L. (V.) shawi* infection, and their modulation should be important to the vaccine development.

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References

- Ajadary S, Alimohammadian MH, Eslami MB, Kemp K, Kharazmi A (2000) Comparison of the immune profile of nonhealing cutaneous leishmaniasis patients with those with active lesions and those who have recovered from infection. *Infect Immun* 68:1760–1764
- Aranha FC, Ribeiro U Jr, Basse P, Corbett CE, Laurenti MD (2005) Interleukin-2-activated natural killer cells may have a direct role in the control of *Leishmania (Leishmania) amazonensis* promastigote and macrophage infection. *Scand J Immunol* 62:334–341
- Beattie L, Peltan A, Maroof A, Kirby A, Brown N, Coles M, Smith DF, Kaye PM (2010) Dynamic imaging of experimental *Leishmania donovani*-induced hepatic granulomas detects Kupffer cell-restricted antigen presentation to antigen-specific CD8 T cells. *PLoS Pathog* 6:e1000805
- Bertho AL, Santiago MA, Da-Cruz AM, Coutinho SG (2000) Detection of early apoptosis and cell death in T CD4⁺ and CD8⁺ cells from lesions of patients with localized cutaneous leishmaniasis. *Braz J Med Biol Res* 33:317–325
- Cupolilo SM, Souza CS, Abreu-Silva AL, Calabrese KS, Goncalves da Costa SC (2003) Biological behavior of *Leishmania (L.) amazonensis* isolated from a human diffuse cutaneous leishmaniasis in inbred strains of mice. *Histol Histopathol* 18:1059–1065
- Da-Cruz AM, Conceição-Silva F, Bertho AL, Coutinho SG (1994) Leishmania-reactive CD4⁺ and CD8⁺ T cells associated with cure of human cutaneous leishmaniasis. *Infect Immun* 62:2614–2618
- Da-Cruz AM, Bittar R, Mattos M, Oliveira-Neto MP, Nogueira R, Pinho-Ribeiro V, Azeredo-Coutinho RB, Coutinho SG (2002) T-cell-mediated immune responses in patients with cutaneous or mucosal leishmaniasis: long-term evaluation after therapy. *Clin Diagn Lab Immunol* 9:251–256
- Da-Cruz AM, Bertho AL, Oliveira-Neto MP, Coutinho SG (2005) Flow cytometric analysis of cellular infiltrate from American tegumentary leishmaniasis lesions. *Br J Dermatol* 153:537–543
- Gumy A, Louis JA, Launois P (2004) The murine model of infection with *Leishmania major* and its importance for the deciphering of mechanisms underlying differences in Th cell differentiation in mice from different genetic backgrounds. *Int J Parasitol* 34:433–444
- Lainson R, Braga RR, de Souza AAA, Pôvoa MM, Ishikawa EAY, Silveira FT (1989) *Leishmania (Viannia) shawi* sp. a parasite of monkeys, sloths and procyonids in Amazonian Brazil. *Annal Par Hum Comp* 64:200–207

- Morgado FN, Schubach A, Rosalino CM, Quintella LP, Santos G, Salgueiro M, Conceição-Silva F (2008) Is the in situ inflammatory reaction an important tool to understand the cellular immune response in American tegumentary leishmaniasis? *Br J Dermatol* 158(1):50–58
- Passero LFD, Laurenti MD, Tomokane TY, Corbett CEP, Toyama MH (2008) The effect of phospholipase A2 from *Crotalus durissus collilineatus* on *Leishmania (Leishmania) amazonensis* infection. *Parasitol Res* 102:1025–1033
- Passero LFD, Sacomori JV, Tomokane TY, Corbett CE, da Silveira FT, Laurenti MD (2009) Ex vivo and in vivo biological behavior of *Leishmania (Viannia) shawi*. *Parasitol Res* 105:1741–1747
- Passero LFD, Marques C, Vale-Gato I, Corbett CE, Laurenti MD, Santos-Gomes G (2010) Histopathology, humoral and cellular immune response in the murine model of *Leishmania (Viannia) shawi*. *Parasitol Int* 59:159–165
- Reis AB, Teixeira-Carvalho A, Giunchetti RC, Guerra LL, Carvalho MG, Mayrink W, Genaro O, Corrêa-Oliveira R, Martins-Filho AO (2008) Phenotypic features of circulating leucocytes as immunological markers for clinical status and bone marrow parasite density in dogs naturally infected by *Leishmania chagasi*. *Clin Exp Immunol* 146:303–311
- Rocha FJ, Schleicher U, Mattner J, Alber G, Bogdan C (2007) Cytokines, signaling pathways, and effector molecules required for the control of *Leishmania (Viannia) braziliensis* in mice. *Infect Immun* 75:3823–3832
- Ruiz JH, Becker I (2007) CD8 cytotoxic T cells in cutaneous leishmaniasis. *Parasite Immunol* 29:671–678
- Shaw J, Ishikawa EAY, Lainson R, Braga RR, Silveira FT (1991) Cutaneous leishmaniasis of man due to *Leishmania (Viannia) shawi* Lainson, de Souza, Póvoa, Ishikawa & Silveira, in Pará state, Brazil. *Annal Par Hum Comp* 66:243–246
- Silveira FT, Lainson R, De Castro Gomes CM, Laurenti MD, Corbett CE (2009) Immunopathogenic competences of *Leishmania (V.) braziliensis* and *L. (L.) amazonensis* in American cutaneous leishmaniasis. *Parasite Immunol* 31(8):423–431