

Original Article**Effect of *Leishmania major* Infection on the Expression of TGF Beta in Murine****Al-Muhsin Al-Khayat, F^{1*}, Ahmed Kalef, D², Mohammed Khashman, B³**

1. Department of Basic Sciences, College of Dentistry, University of Baghdad, Baghdad, Iraq

2. Department of Parasitology, College of Veterinary Medicine, University of Baghdad, Iraq

3. National Cancer Research Centre, University of Baghdad, Iraq

Received 13 August 2021; Accepted 2 September 2021

Corresponding Author: twna_2011@yahoo.com

Abstract

Leishmania major is a protozoan parasite that causes cutaneous Leishmaniasis disease in human beings and animals. The disease is prevalent in tropical and semitropical countries and has great health importance. The present study aimed to identify the histological changes in the organs infected with *L. major* and to provide a sophisticated diagnostic method for infection through detecting TGF- β cytokine by immunohistochemistry technique(IHC) from October 2020 to January 2021. A total of 40 samples of paraffin blocks were used for different organs including skin, spleen, liver, kidney, and heart of male and female BALB/c mice, aged 6-8 weeks, which were previously infected subcutaneously with *L. major* promastigotes at a dose of 1×10^7 promastigotes/mouses. The result indicated epidermal hyperplasia with diffuse severe lymphohistiocytic inflammatory cells infiltration in the dermis. Hyperplasia of the lymphoid follicles was observed in infected spleen and scattered polymorphonuclear cells mainly neutrophil masses with a random distribution of microgranulomas foci composed of lymphocytes and macrophages within the liver parenchyma around central veins and portal areas. The infected kidney showed aggregation of perivascular mononuclear cells (lymphocytes and macrophages) in the renal cortex. Mononuclear lymphocytes and macrophages were observed within the heart parenchyma especially around blood vessels. Additionally, evaluation of TGF- β 1 expression was highly strong for skin, spleen, relatively strong for liver, heart, and weak for the kidney. In conclusion, infection was accompanied by clinical and histological changes as well as inflammatory diseases. Furthermore, the determination of TGF- β expression level depends on the diagnosis of infection. A clear understanding of immune mechanisms is essential for preventing, treating, and controlling strategies of this infection.

Keywords: TGF- β , *Leishmania major*, Immunohistochemistry, Infected mice**1. Introduction**

Leishmaniasis is a disease caused by a protozoan parasite called *Leishmania*, an obligatory intracellular parasite that resides in the macrophages of the mammalian hosts as round to oval amastigotes phase. *Leishmania major* is a source of cutaneous leishmaniasis with an infection burden of about 1–1.5 million with mucosal lesions (1). *Leishmania major* transmitted by sand flies which causes various lesions of cutaneous bumps, nodules, and gross tissue damage

(2). The immune responses of *Leishmania* are mostly determined by the expansion of Th1 and Th2 cells of CD4⁺ T cells. Th1 cells offer IFN- γ , IL-2, and TNF- α protection which plays a significant role in innate and adaptive immune responses against leishmaniasis in humans and mice (3-5). Th2 responses are determined by the production of IL-4, IL-5, IL-10, TGF- β , and IL-13, which inhibit some macrophage functions (4). TGF- β was shown to inhibit releasing of IFN- γ by CD4⁺ T cells in BALB/c mice infected with visceral

leishmaniasis and activating of Th2 cells. Clinical diagnosis of cutaneous lesions and microscopic analysis of *Leishmania major* is usually performed in endemic countries to detect the infection.

Supported diagnostic techniques allow confirmative identification of limited studies in non-endemic countries of cutaneous leishmaniasis. Similarly, previous studies, to the best of our knowledge, have focused on improving diagnostic experiments to detect the release of TGF- β by IHC (6). *Leishmania* antigens were detected by histopathological and immunohistochemical techniques to investigate local immune response in liver development of granulomatous lesions (7).

Therefore, the present study aimed to detect TGF- β production by independently developing Th2-type cytokines in different organs of mice infected by *Leishmaniamajor* using immunohistochemistry.

2. Material and Methods

2.1. Study Samples

A total of 40 samples of paraffin blocks, formalin-fixed paraffin-embedded (FFPE) tissue of different organs (skin, spleen, liver, kidney, and heart) were used in the present study from October 2020 to January 2021. These tissue samples were obtained from the archives of the Faculty of Sciences, University of Kufa. The archival blocks were collected from male and female BALB/c mice (6-8 weeks and weighing 25-30 gm) that had previously been subcutaneously infected with *Leishmania major* promastigotes at adose of 1×10^7 promastigotes/mouse.

2.2. Histopathology

Tissues were sectioned by microtome at 4 μ m thickness and routinely stained with hematoxylin and eosin (H&E) (8). After H&E staining, the slides were dehydrated through a series of 70%, 80%, 95%, and twice in 100% ethanol, then twice in xylene for 2 minutes each. Finally, the tissues sections were covered with Permout Mounting Medium (DPX). Tissue slides were examined under magnification of 10 \times and then

40 \times of the light microscope to evaluate histopathological changes.

2.3. Immunohistochemistry (IHC)

Unstained glass slides from skin, spleen, liver, kidney, and heart were used to perform IHC. Briefly, the slides were deparaffinized twice for 5 min by xylene and then dehydrated with a series of ethanol concentrations (100 %, 95%, 80%, and 70%) for 5 minutes each, then rinsed with distilled water. Endogenous peroxidase activity was eliminated by incubation with hydrogen peroxide (3%) for 5 min at 37 $^{\circ}$ C, and then the slides were washed with phosphate-buffered saline (PBS) (3 times for 5 min each). The slides were incubated with Blocking Reagent (ab64218) for 20 min and then washed 3 times in PBS to block non-specific binding. After removing the blocking solution, the slides were incubated with diluted primary antibody (anti- TGF beta 1 – BSA) 1:200 for 1 h at 37 $^{\circ}$ C in a humidity chamber, then rinsed with PBS (3 times for 5 min each). Biotinylated-secondary antibody (at assay dependent concentration) was applied to the slides for 30 min at room temperature. Sections were washed with PBS, incubated with a streptavidin-HRP solution for 10 minutes at room temperature, and then washed again with PBS (3 times for 5 min each). Diaminobenzidine hydrochloride (DAB) substrate was added to the glass slide until the desired color was achieved (1-10 min) at room temperature. The tissue sections were counterstained with hematoxylinstain for microscopic examination.

3. Results

Histopathological findings of mice infected with *L. major* were characterized by variable degrees of inflammatory cells infiltration, mainly mononuclear cells (lymphocytes and macrophages) to micro granulomatous lesions. In response to Leishmaniasis, histopathological changes from skin biopsy of the mice infected with *L. major* amastigotes indicated epidermal hyperplasia (acanthosis), with diffuse severe lymphohistiocytic

inflammatory cells infiltration in the dermis with the presence of the *L. major* amastigotes within macrophages (Figure 1). Follicular lymphoid hyperplasia (FLH) was observed in the spleen of mice infected with *L. major* (Figure 2). Scattered polymorphonuclear cells mainly neutrophils accumulated with randomly distributed microgranulomas foci composed of lymphocytes and macrophages accumulations were observed in liver parenchyma around central veins and portal areas with individual necrosis of hepatocytes (Figure 3). Aggregation of perivascular mononuclear cells (lymphocytes and macrophages) was also observed in the renal cortex of the kidney (Figure 4). Mononuclear cells mainly lymphocytes and macrophages were observed in the heart parenchyma especially around blood vessels (Figure 5).

Representative images of skin (Figure 1) from BALB/c mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1×10^7 amastigotes /mouse) (upper right panel). The images below are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed epidermal hyperplasia (acanthosis), with diffuse severe lymphohistiocytic inflammatory cells infiltration in the dermis with the presence of the *L. major* amastigotes in macrophages. None of the BALB/c mice inoculated with PBS indicated remarkable lesions.

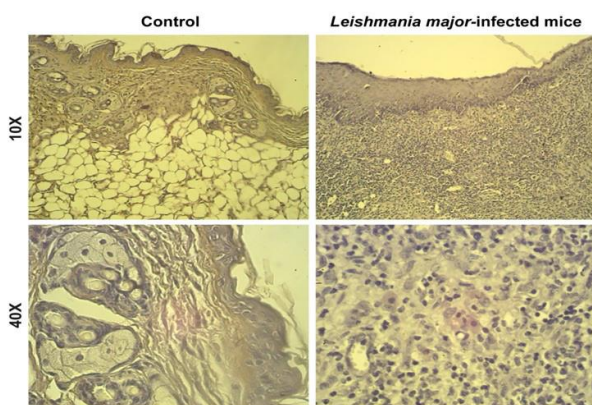


Figure 1. Histopathology of the skin in mice infected with *L. major*

Representative images of spleen (Figure 2) from BALB/c mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1×10^7 amastigotes /mouse) (upper right panel). The images below are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed hyperplasia of the lymphoid follicles. None of the BALB/c mice inoculated with PBS indicated remarkable lesions.

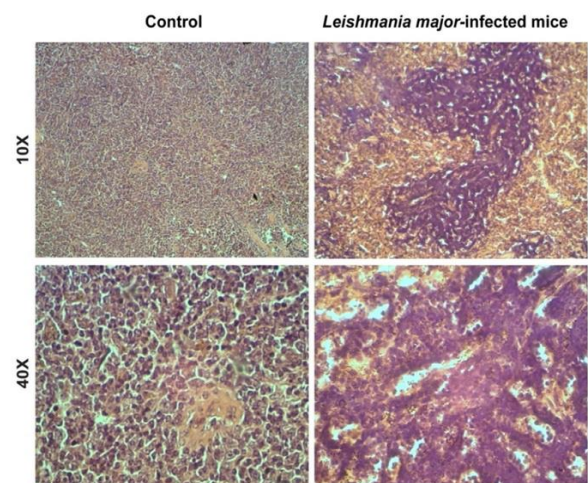


Figure 2. Histopathology of the spleen in mice infected with *L. major*

Representative images of liver (Figure 3) from BALB/c mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1×10^7 amastigotes /mouse) (upper right panel). The images below are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed scattered polymorphonuclear cells, mainly neutrophil masses, with a random distribution of microgranulomas foci composed of lymphocytes and macrophages accumulations within liver parenchyma around central veins and portal areas. None of the BALB/c mice inoculated with PBS showed remarkable lesions.

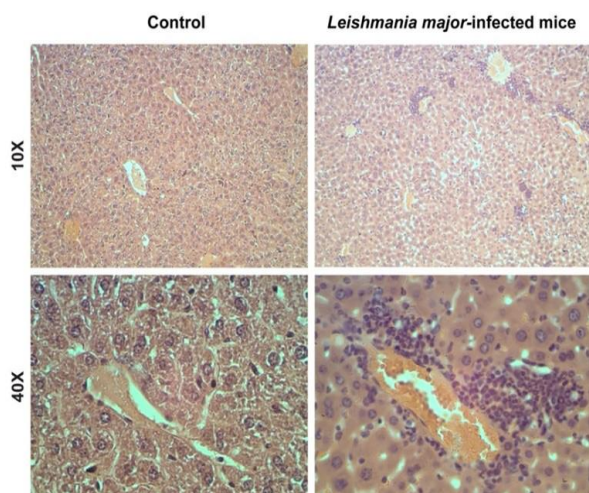


Figure 3. Histopathology of the liver in mice infected with *L. major*

Representative images of kidney (Figure 4) from BALB/C mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1×10^7 amastigotes /mouse) (upper right panel). The images below are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed perivascular mononuclear cells (lymphocytes and macrophages) aggregation in the renal cortex. While the BALB/C mice inoculated with PBS failed to show remarkable lesions.

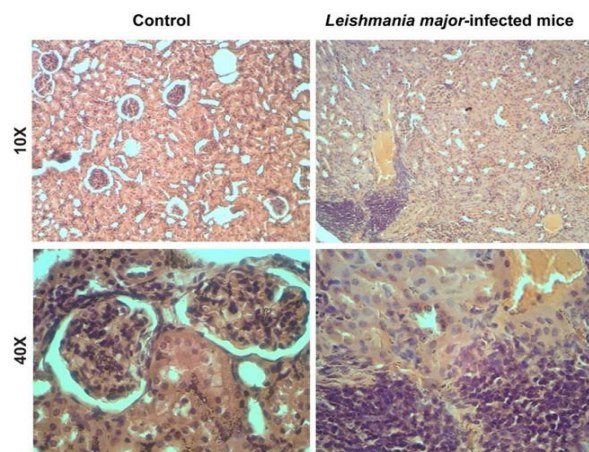


Figure 4. Histopathology of the kidney in mice infected with *L. major*

Representative images of the heart (Figure 5) from BALB/c mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1×10^7 amastigotes

/mouse) (upper right panel). The images below are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed mononuclear cells mainly lymphocytes and macrophages aggregation within the heart parenchyma, especially around blood vessels. Whereas, the BALB/c mice inoculated with PBS failed to show remarkable lesions.

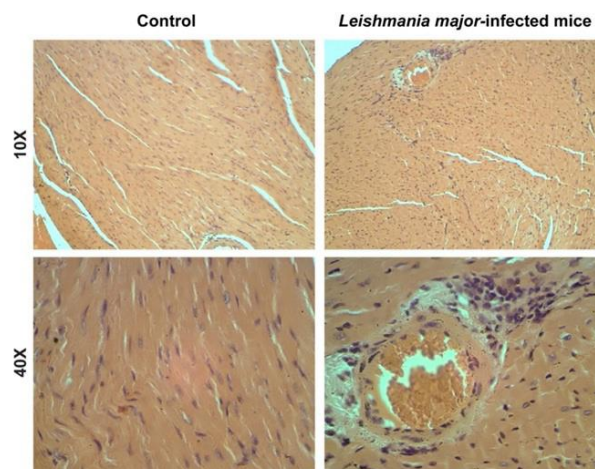


Figure 5. Histopathology of the heart in mice infected with *L. major*

3.1. Immunohistochemistry Detection

Tissue expression of TGF- β 1 was directly accompanied with *L. major* in mice. Immunohistochemistry (IHC) was performed to evaluate the TGF- β 1 expression in the skin, spleen, liver, heart, and kidney. The result of skin activity against the TGF- β 1 biomarker was a very strong signal as shown in table 1 and figure 6. IHC has demonstrated that the spleen and the liver showed moderate to strong immunoreactivity, followed by moderate to weak positive TGF- β 1 signalling in the heart and kidney. Table 1 represents the expression level of TGF- β 1 signalling depending on immunoreactivity and intensity scores in five tissue organs of mice infected with *L. major* amastigotes, and the brown signal was measured according to Rezaee Movassaghi (9).

Table 1. Intensity, immunoactivity, and expression level of TGF-β1 from different tissue organs of mice infected with *L. major* amastigotes.

Organ	Average of Intensity grade	Average of Immunoactivity score	TGF-β1 expression level
Skin	4	15	Very strong
Spleen	4	12	Strong
Liver	3	9	Moderate-Strong
Kidney	1	4	weak
Heart	2	6	Moderate

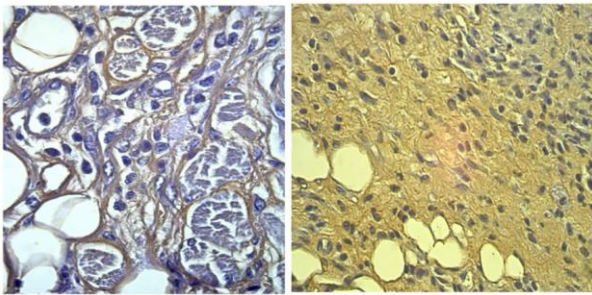


Figure 6. Immunohistochemistry of the skin in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1×10^7 amastigotes /mouse).

Mice skin tissues indicated strong brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4), 40× magnification.

Mice spleen tissues indicated strong brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4), 10× magnification (Figure 7).

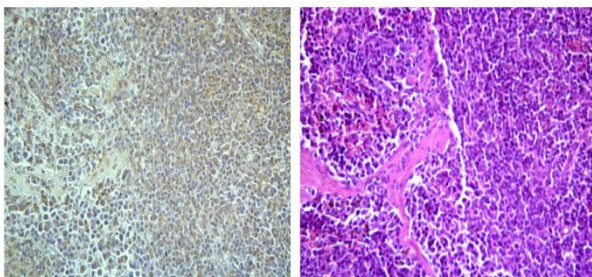


Figure 7. Immunohistochemistry of the spleen in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1×10^7 amastigotes /mouse).

Mice liver tissues indicated strong brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4), 40× magnification (Figure 8).

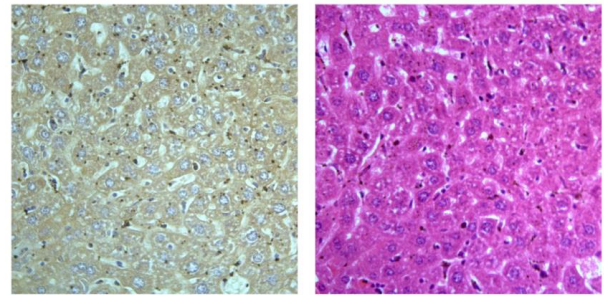


Figure 8. Immunohistochemistry of the liver in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1×10^7 amastigotes /mouse).

Mice heart tissues detected moderate brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4), 40× magnification (Figure 9).

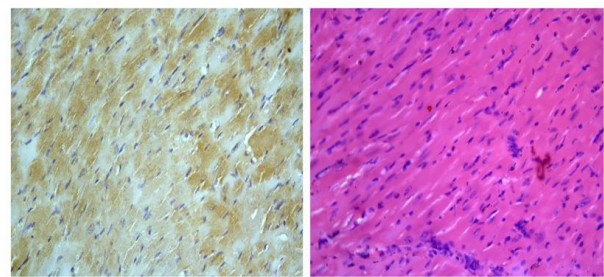


Figure 9. Immunohistochemistry of the heart in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1×10^7 amastigotes /mouse).

Mice kidney tissues indicated weak brown signal staining of positive cells to TGF- β biomarker of BALB/c mice (n=4), 40 \times magnification (Figure 10)

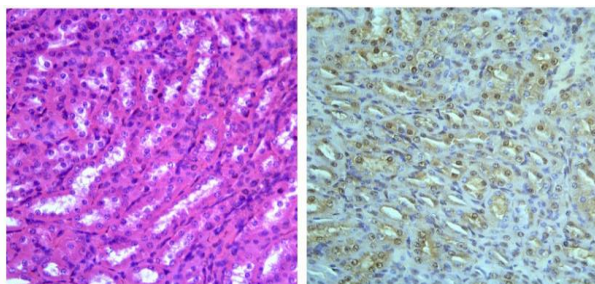


Figure 10. Immunohistochemistry of the kidney in mice infected with *L. major*. TGF- β expression of BALB/c mice injected with *L. major* amastigotes (1×10^7 amastigotes/mouse).

4. Discussion

Cutaneous Leishmaniasis caused by *L. major* manifested clinical lesions in patients ranged from weeks to months (10). In the present study, histopathological and immunohistochemical changes of different tissue sections of mice infected with *L. major* were reported to better understand the tissue damage of different sections during infection.

In response to Leishmaniasis, hyperkeratosis and parakeratosis of the dermis were observed from the skin biopsies of mice infected with *L. major*. Variable hallmarks were observed in skin lesions of patients with cutaneous Leishmaniasis (11) and in mice (12) to discover the structural component of the tissue against *L. major*.

Obvious granuloma lesions were shown in liver and spleen biopsies with aggregation of lymphocytes, plasma cells, and macrophages. These results are usually present in the chronic inflammatory response and are associated with the development of a Th1 response initiated by the IL-12 response to control the disease (13, 14). Previous studies have reported that macrophages activated polarised Th1 cells to eradicate *L. major* in IL-4 $^{-/-}$ BALB/c mice (15, 16). The granulomatous lesions were less common in the kidney and heart as these organs were not similar to lymphatic

tissues and less important in the immune response of the mice (17).

Five organs (skin, spleen, liver, heart, and kidney) were selected and bioactivity scoring was investigated to screen and quantify the expression of TGF- β intensity and their effects. The immunohistochemical staining method was described as one of the methods for measuring TGF- β activation and quantitation of TGF- β synthesis levels in different experimental situations (18). Also, this method has previously been used for measuring the growth of cancer cells and tumorigenesis where there are substantial changes in TGF- β synthesis, secretion, or activation (19). In the present study, samples stained with anti-TGF- β biomarkers would provide information on the presence of active TGF- β that elicit intracellular signalling and develop an immune response in BLAB/c mice (20, 21).

In mice, successful primary immunity against *L. major* includes IL-12 dependent IFN- γ production from CD4 $^{+}$ and CD8 $^{+}$ T cells (Th1 response) which mediates macrophage killing mechanism (20). In addition, the dominance of an IL-4 induces a strong Th2 response in BALB/c that subcutaneously inoculated with a high dose of promastigotes of *L. major* resulted in rapidly evolving cutaneous lesions (22). A significant increase was detected in the expression of TGF- β in skin tissues as a result of the cutaneous infection effect. BALB/c mice are vulnerable to *L. major* infection and fail to develop cellular mediated immunity to produce self-healing lesions compared to other breeds of mice (such as the C3H, C57BL/6, and B10.D2) (14). Subsequently, a strong signal (brown staining) was observed in the spleen and liver as a consequence of immune infiltration and the binding of active TGF- β to its cell surface receptors (19).

The elucidation of TGF- β from the matrix in the heart and kidney which is slightly activated in the tissues that displayed the ability of *L. major* parasites might display mild visceralization incomparable to the cutaneous manifestation (23).

Leishmania major is a parasitic disease that can cause cutaneous Leishmaniasis. It appears with a wide range of clinical, histological, and inflammatory manifestations. In tissue sections indicating histopathological infiltration of a granulomatous reaction that is included in the diagnosis of the disease. Immunohistochemical detection of *Leishmania major* depends on identifying the expression level of TGF-beta in lesion samples.

Authors' Contribution

Study concept and design: D. A. K.

Acquisition of data: F. A. A.

Analysis and interpretation of data: B. M. K.

Drafting of the manuscript: D. A. K.

Critical revision of the manuscript for important intellectual content: D. A. K.

Statistical analysis: F. A. A.

Administrative, technical, and material support: D. A. K.

Ethics

All the procedures were approved by the Ethics Committee at the University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The authors would like to express their sincere appreciation to Dr. Omar H. Khalaf, Department of Veterinary Pathology & Poultry Diseases, Faculty of Veterinary Medicine, University of Baghdad, Baghdad, Iraq to assist in reading histopathological results.

References

- Desjeux P. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis.* 2004;27(5):305-18.
- Reithinger R, Dujardin J-C, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. *Lancet Infect Dis.* 2007;7(9):581-96.
- Breitling R, Klingner S, Callewaert N, Pietrucha R, Geyer A, Ehrlich G, et al. Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expr Purif.* 2002;25(2):209-18.
- Liew F, O'donnell C. Immunology of leishmaniasis. *Adv Parasitol.* 1993;32:161-259.
- Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today.* 1996;17(3):138-46.
- Ji J, Sun J, Soong L. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with *Leishmania amazonensis*. *Infect Immun.* 2003;71(8):4278-88.
- Salguero FJ, Garcia-Jimenez WL, Lima I, Seifert K. Histopathological and immunohistochemical characterization of hepatic granulomas in *Leishmania donovani*-infected BALB/c mice: a time-course study. *Parasit Vectors.* 2018;11(1):1-9.
- Yong L. The Theory and Practice of Histological Techniques. *Pathology.* 1992;24(4):320.
- Rezaee M, Movassaghi AR, Maleki M. Immunohistochemical expression of transforming growth factor Beta-1 in canine mammary carcinomas: its relationships with histologic grading, survival rate, and recurrence. *Comp Clin Path.* 2017;26(3):519-24.
- Sundharkrishnan L, North JP. Histopathologic features of cutaneous leishmaniasis and use of CD1a staining for amastigotes in Old World and New World leishmaniasis. *J Cutan Pathol.* 2017;44(12):1005-11.
- González K, Diaz R, Ferreira AF, García V, Paz H, Calzada JE, et al. Histopathological characteristics of cutaneous lesions caused by *Leishmania Viannia panamensis* in Panama. *Rev Inst Med Trop Sao Paulo.* 2018;60.
- Cangussú SD, Souza CCd, Campos CF, Vieira LQ, Afonso LCC, Arantes RME. Histopathology of *Leishmania major* infection: revisiting *L. major* histopathology in the ear dermis infection model. *Mem Inst Oswaldo Cruz.* 2009;104(6):918-22.
- Gaafar A, El Kadar A, Theander T, Permin H, Ismail A, Kharazmi A, et al. The pathology of cutaneous leishmaniasis due to *Leishmania major* in Sudan. *Am J Trop Med Hyg.* 1995;52(5):438-42.

14. Scott P. Development and regulation of cell-mediated immunity in experimental leishmaniasis. *Immunol Res.* 2003;27(2):489-98.
15. Heinzel FP, Sadick MD, Holaday BJ, Coffman R, Locksley RM. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *Exp Med.* 1989;169(1):59-72.
16. Noben-Trauth N, Kropf P, Müller I. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science.* 1996;271(5251):987-90.
17. Lohrberg M, Wilting J. The lymphatic vascular system of the mouse head. *Cell Tissue Res.* 2016;366(3):667-77.
18. Jurukovski V, Dabovic B, Todorovic V, Chen Y, Rifkin DB. Methods for measuring TGF- β using antibodies, cells, and mice. *Fibrosis Research: Springer*; 2005. p. 161-75.
19. Taipale J, Saharinen J, Keski-Oja J. Extracellular matrix-associated transforming growth factor- β : role in cancer cell growth and invasion. *Adv Cancer Res.* 1998;75:87-134.
20. Alexander J, Bryson K. T helper (h) 1/Th2 and *Leishmania*: paradox rather than paradigm. *Immunol Lett.* 2005;99(1):17-23.
21. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol.* 2002;2(11):845-58.
22. Rogers KA, DeKrey GK, Mbow ML, Gillespie RD, Brodskyn CI, Titus RG. Type 1 and type 2 responses to *Leishmania major*. *FEMS Microbiol Lett.* 2002;209(1):1-7.
23. Wege AK, Florian C, Ernst W, Zimara N, Schleicher U, Hanses F, et al. *Leishmania major* infection in humanized mice induces systemic infection and provokes a nonprotective human immune response. *PLOS Negl Trop Dis.* 2012;6(7):e1741.