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Original Article

Effect of *Leishmania major* Infection on the Expression of TGF Beta in Murine

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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/moues. 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-β1 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-αprotection 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-βwas 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. Materials 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 μm 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 Permount Mounting Medium (DPX). Tissue slides were examined under magnification of 10× and then 40× 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°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°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.

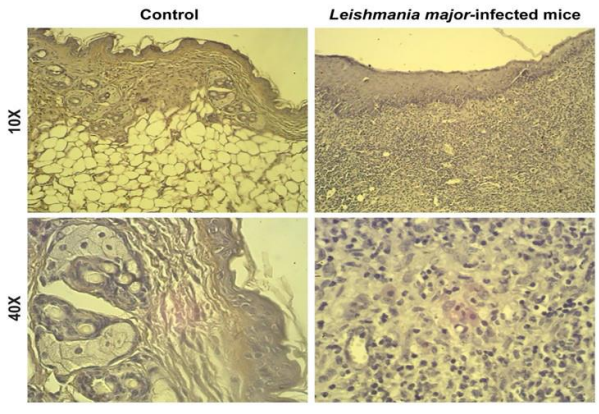

Figure 1. Histopathology of the skin in mice infected with *L. major*

Representative images of spleen (Figure 2) from BALB/c mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1×10^7amastigotes /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.

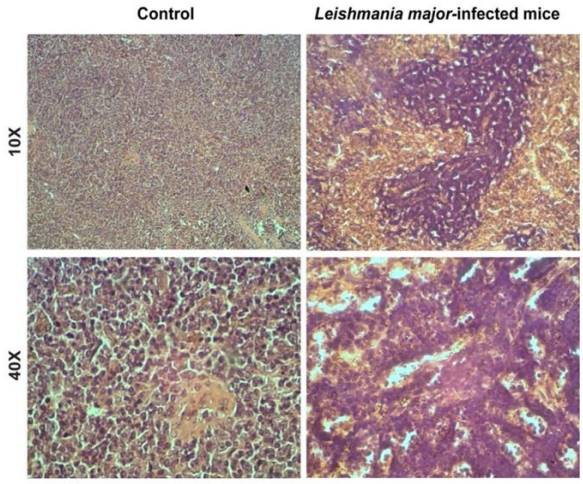

Figure 2. Histopathology of the spleen in mice infected with *L. major*

Representative images of liver (Figure 3) from BALB/c mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1×10^7amastigotes /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.

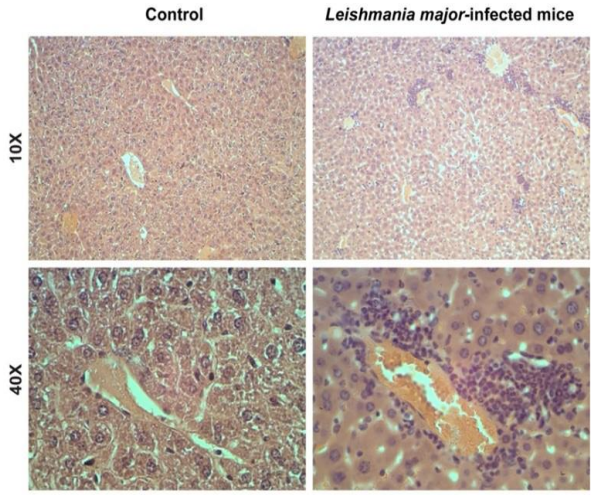

Figure 3. Histopathology of the liver in mice infected with *L. major*

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.

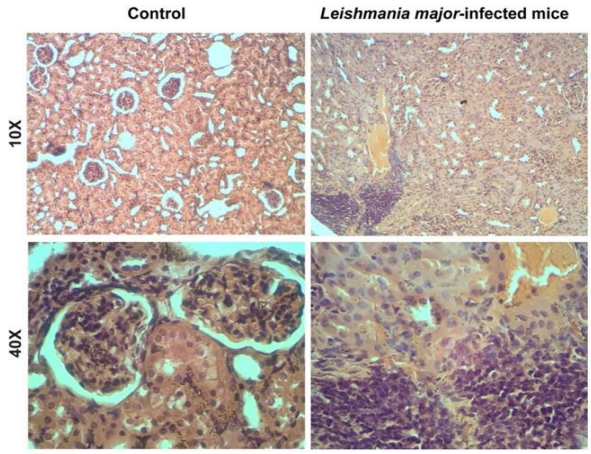

Figure 4. Histopathology of the kidney in mice infected with *L. major*

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.

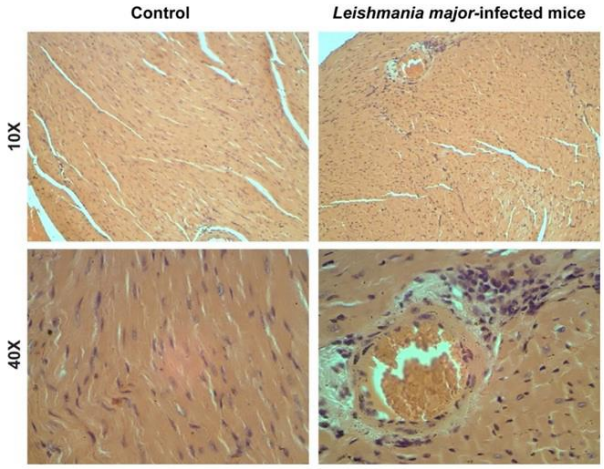

Figure 5. Histopathology of the heart in mice infected with *L. major*

3.1. Immunohistochemistry Detection

Tissue expression of TGF-β1 was directly accompanied with *L. major* in mice. Immunohistochemistry (IHC) was performed to evaluate the TGF-β1 expression in the skin, spleen, liver, heart, and kidney. The result of skin activity against the TGF-β1 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-β1 signalling in the heart and kidney. Table 1 represents the expression level of TGF-β1 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-β1from different tissue organs of mice infected with *L. major* amastigotes.

Organ	Average of Intensity grade	Average of Immunoactivity score	TGF-β1expression level
Skin	4	15	Very strong
Spleen	4	12	Strong
Liver	3	9	Moderate-Strong
Kidney	1	4	weak
Heart	2	6	Moderate

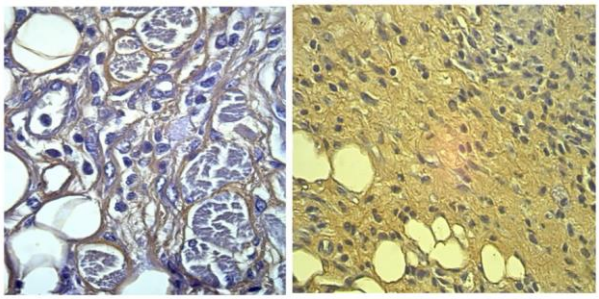

Figure 6. Immunohistochemistry of the skin in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1 X 10^7amastigotes /mouse).

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).

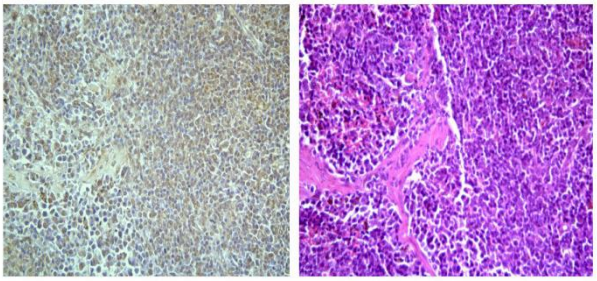

Figure 7. Immunohistochemistry of the spleen in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1 × 10^7 amastigotes /mouse).

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).

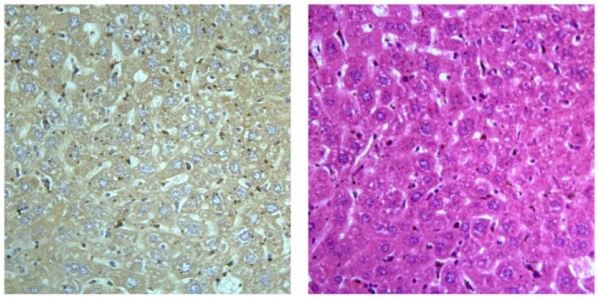

Figure 8. Immunohistochemistry of the liver in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1 × 10^7 amastigotes /mouse).

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).

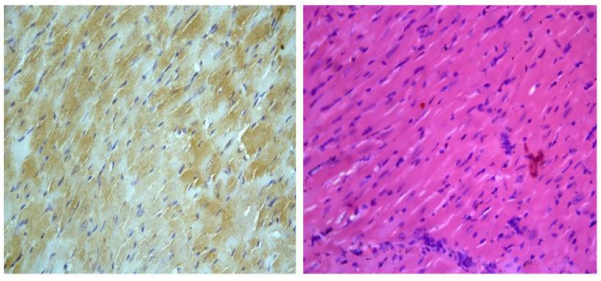

Figure 9. Immunohistochemistry of the heart in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1 × 10^7 amastigotes /mouse).

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

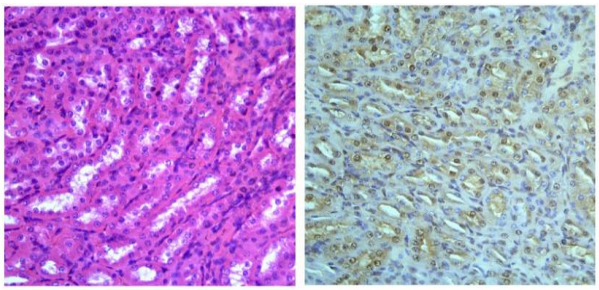

Figure 10. Immunohistochemistry of the kidney in mice infected with *L. major*. TGF-β expression of BALB/c mice injected with *L. major* amastigotes (1 × 10^7amastigotes /mouse).

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* inIL-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-ɣ 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 strongTh2 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 TGIF-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 toother 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 mildvisceralization 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

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