Effect of Murine *leishmania major* Infection on the Expression of TGF Beta

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Abstract

*Leishmaniamajor* is a protozoan parasite that causes a cutaneous Leishmaniasis disease in human beings and animals. The disease is widely distributed in tropical and semitropical countries and has great health importance. The current study aimed to identify the histological changes in the infected organs with *L. major* in addition to provide a sophisticated diagnostic method for infection through detection of TGF-β cytokine by immunohistochemistry technique (IHC) which lasted from October 2020 to January 2021. Forty samples were used of paraffin blocks for different organs including skin, spleen, liver, kidney and heart of BALB/C mice, aged 6-8 weeks of both sexes that previously infected subcutaneously with *L. major* promastigotes with dose 1×10⁷ promastigotes/moues. For skin, the result showed epidermal hyperplasia with diffuse severe lymphohistocytic inflammatory cells infiltration in the dermis. Hyperplasia of the lymphoid follicles was indicated in infected spleen and scattered polymorphonuclear cells mainly neutrophils aggregate with randomly distributed of microgranulomas foci composed of lymphocytes and macrophages accumulations within liver parenchyma around central veins and portal areas were shown in infected liver. Infected kidney showed perivascular mononuclear cells (lymphocytes and macrophages) aggregation in the renal cortex. Heart showed mononuclear cells mainly lymphocytes and macrophages aggregation were seen within the heart parenchyma especially around blood vessels. On the other hand, evaluation of TGF-β1 expression was very strong
for skin, spleen, and moderate-strong for liver, heart, and weak for kidney. In conclusion, the infection was accompanied by clinical and histological changes and inflammatory diseases additionally, the determination of the level of expression of TGF-β depends on the detection of infection. A clear understanding of immune mechanisms is essential for the prevention, treatment and control 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 parasites that resides in the macrophages of the mammalian hosts as round to oval amastigotes phase. *Leishmania major* sources of cutaneous leishmaniasis that is a public health problem, which the burden of its infection is about 1–1.5 million with mucosal lesions (1). *Leishmania major* transmits by sandfly 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 and it has a significant role in both innate and adaptive immune responses against leishmaniasis in humans and mice (3-5). Whereas, 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 showed to inhibit releasing of IFN-γ by CD4⁺ T cells in BALB/c mice infected with visceral leishmaniasis and activation of Th2 cells. Clinical diagnosis of cutaneous lesions and microscopic analysis of *Leishmania major* is usually made in endemic countries to detect the infection.

The supported diagnostic techniques allow the confirmative identification of restricted studies in non-endemic countries of cutaneous leishmaniasis. Likewise, previous studies focused on the improvement of diagnostic experiments for the finding of TGF-β releasing by IHC (6). *Leishmania* antigens were detected by Histopathology and immunohistochemical techniques to investigate local immune response in liver development of the granulomatous lesions (7).
Therefore, the aim of this work is the detection of TGF-β production independently of development of Th2-type cytokines in different organs of infected mice with Leishmania major by immunohistochemistry.

2. Materials and Methods

2.1. Study Samples

Forty samples of paraffin blocks, formalin fixed paraffin embedded tissue (FFPE) of different organs (skin, spleen, liver, kidney, and heart) were used in the current study during the period from October 2020 till January 2021. These tissue samples were obtained from the archives of the College of Science/ University of Kufa. The archival blocks were collected from BALB/C mice of both sexes (6-8 weeks old and 25-30gm weight) previously infected subcutaneously with Leishmania major promastigotes with dose 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 per mount-mounting medium (DPX). Tissue slides were examined under magnification of 10× 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, slides were deparaffinised by xylene twice for 5 min each, then dehydrated through a series of ethanol concentration (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). To block non-specific binding, the slides were incubated with Blocking Reagent (ab64218) for 20 min, and then washed 3 times in PBS. After removing the blocking solution, the slides were incubated with diluted primary antibody (anti- TGF beta 1 – BSA) 1:200 for 1 hour 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 minutes 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 colour was achieved (1-10 min) at room temperature. The tissue sections were counterstained with hematoxylin stain for microscopic examination.

3. Results

Histopathological findings of mice infected with *L. major* were mainly characterized by variable degrees of inflammatory cells infiltration mainly mononuclear cells (lymphocytes and macrophages) to micro granulomatous lesions. In response to Leishmaniasis, the histopathological changes from the skin biopsies of the infected mice with *L. major* amastigotes showed epidermal hyperplasia (acanthosis), with diffuse severe lymphohistocytic inflammatory cells infiltration in the dermis with the presence of the *L. major* amastigotes within macrophages (Figure1). Hyperplasia of the lymphoid follicles was found within the spleen of infected mice with *L. Major* (Figure 2). While liver showed scattered polymorphonuclear cells mainly neutrophils aggregated with randomly distributed microgranulomas foci composed of lymphocytes and macrophages accumulations within liver parenchyma around central veins and portal areas with individual necrosis of hepatocytes (Figure3). In the renal cortex of the kidney, perivascular mononuclear cells (lymphocytes and macrophages) aggregation were also seen (Figure4). Mononuclear cells mainly lymphocytes and macrophages aggregation was seen within the heart parenchyma especially around blood vessels (Figure5).
Figure 1. Histopathology of the skin in mice infected with *L. Major*

Representative images of skin from BALB/C mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1 X 10^7 amastigotes /mouse) (upper right panel). Images at bottoms are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed epidermal hyperplasia (acanthosis), with diffuse severe lymphohistocytic inflammatory cells infiltration in the dermis with the presence of the *L. major* amastigotes within macrophages. None of the BALB/C mice inoculated with PBS showed remarkable lesions.

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

Representative images of spleen from BALB/C mice inoculated with PBS (upper left panel)
and *L. major* amastigotes (1 X 10^7 amastigotes /mouse) (upper right panel). Images at bottoms 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 showed remarkable lesions.

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

Representative images of liver from BALB/C mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1 X 10^7 amastigotes /mouse) (upper right panel). Images at bottoms are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed scattered polymorphonuclear cells mainly neutrophils aggregate with randomly distributed 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 4. Histopathology of the kidney in mice infected with *L. major*

Representative images of kidney from BALB/C mice inoculated with PBS (upper left panel) and *L. major* amastigotes (1 X 10^7 amastigotes/mouse) (upper right panel). Images at bottoms 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 did not show remarkable lesions.

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

Representative images of the heart from BALB/C mice inoculated with PBS (upper left panel)
panel) and *L. major* amastigotes (1 X 10^7 amastigotes /mouse) (upper right panel). Images at bottoms are magnifications of the upper sections. Animals infected with *L. major* amastigotes showed mononuclear cells mainly lymphocytes and macrophages aggregation were seen within the heart parenchyma especially around blood vessels. Whereas, the BALB/C mice inoculated with PBS did not show remarkable lesions.

### 3.1. Immunohistochemistry Detection

To confirm the expression of tissue TGF-β1 expression 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 results of skin activity against 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 signal of the heart and the kidney. Table 1 showed the expression level of TGF-β1 signal depending on immunoreactivity and intensity scores in five tissue organs of infected mice with *L. major* amastigotes and the brown colour signal was measured according to Rezaee, Movassaghi (9).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Average of Intensity grade</th>
<th>Average of Immunoreactivity score</th>
<th>TGF-β1 expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>4</td>
<td>15</td>
<td>Very strong</td>
</tr>
<tr>
<td>Spleen</td>
<td>4</td>
<td>12</td>
<td>Strong</td>
</tr>
<tr>
<td>Liver</td>
<td>3</td>
<td>9</td>
<td>Moderate-Strong</td>
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<tr>
<td>Kidney</td>
<td>1</td>
<td>4</td>
<td>weak</td>
</tr>
<tr>
<td>Heart</td>
<td>2</td>
<td>6</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

Table 1. intensity, immunoactivity and expression level of TGF-β1 from different tissue organs of infected mice with *L. major* amastigotes.
Figure 6. Immunohistochemistry of the skin in mice infected with \textit{L. major}. TGF-β expression of BALB/c mice injected with \textit{L. major} amastigotes (1 X 10^7 amastigotes /mouse).

Skin mice tissues indicate strong brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4). 40x magnification.

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

Spleen mice tissues indicate strong brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4). 10x magnification.

Figure 8. Immunohistochemistry of the liver in mice infected with \textit{L. major}. TGF-β expression of BALB/c mice injected with \textit{L. major} amastigotes (1 X 10^7 amastigotes /mouse).
Livermice tissues speculate strong brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4). 40x magnification.

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 X 10^7 amastigotes /mouse).

Heartmice tissues detect moderate brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4). 40x magnification

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 x 10^7 amastigotes /mouse).

Kidneymice tissues show weak brown signal staining of positive cells to TGF-β biomarker of BALB/c mice (n=4). 40× magnification

4. Discussion

Cutaneous Leishmaniasis caused by *L. major* manifested clinical lesions in patients ranged from weeks to months (10). In the current study, we report the histopathological and immunohistochemistry changes of different tissue sections of mice infected with *L. major* for better understanding the tissue damage of different sections during infection.

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

Obvious granulomas lesions were showed in liver and spleen biopsies accompanied by aggregation of lymphocytes, plasma cells and macrophages. These results are usually present in the chronic inflammatory response and associated with the development of a Th1 response initiated by the IL-12 response to control the disease (13, 14). Previous studies reported that macrophage activated polarised Th1 cells for eradication of the *L. major* in IL-4/- BALB/c mice (15, 16). Whereas, the granulomatous lesion was less in the kidney and heart because these organs were not as lymphatic tissues and have less importance in the immune response of the mice (17).

In order to screen and quantify the expression of TGF-β intensity and their effects, five organs were selected (skin, spleen, liver, heart and kidney) and bioactivity scoring, were investigated. The immunohistochemistry staining method was described as one of the methods for measuring the active TGF-β and Quantitation of the levels of TGF-β synthesis in different experimental situations (18). Also, this method was previously used in measuring of cancer cells growth and tumorigenesis where there are substantial changes in TGF-β synthesis, secretion, or activation (19). In this study, stained samples with anti-TGF-β biomarker would provide information on the presence of active TGF-β that elicit intracellular signalling and development of the 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 [20]. On the other hand, the dominance of an IL-4 drive strongly Th2 response in BALB/c that inoculated subcutaneously with a high dose of promastigotes of *L. major* resulted in rapidly evolving cutaneous lesions (22). A significant increase in the expression of TGF-β was detected in skin tissues as a result of the cutaneous infection effect. BALB/c mice are vulnerable to *L. major* infection do not develop cellular mediated immunity to produce self-healing lesions compared with other mouse strains (such as the C3H, C57BL/6, and B10.D2) (14). Subsequently, the 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 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 presents with a wide range of manifestation clinically, histologically and inflammatory diseases. In tissue sections showing histopathological infiltration of a granulomatous reaction that be included in the diagnosis of the disease. Immunohistochemistry detection of *Leishmania major* relies on the identification of the expression level of TGF-beta in lesion samples.

Acknowledgement

The authors would like to thank doctor Omar H. Khalaf Pathology (Department of Veterinary Pathology & Poultry Diseases, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq) for his helping in the reading of the Histopathology results.

References