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## **Diagnostic usefulness of immunohistochemical (IHC) assessment of CD1a and CD68 biomarkers for cutaneous leishmaniasis**

**Fatima Jassim Musa Al-Yasiri**

Biology Department, College of Education for Pure Sciences

**Dhafer A. Alghezi \***

Thi-Qar University, Iraq

Corresponding author email: [Dhafer.a.f.alghezi@bath.edu](mailto:Dhafer.a.f.alghezi@bath.edu)

**Fadhil Abbas Munshid Al-Abadi**

Medical Microbiology Department, college of medicine, University of Thi-Qar, Thi-Qar, Iraq

**Abstract**---Cutaneous Leishmaniasis is the only neglected disease that keeps progressing and spreading in many environmental conditions. Approximately two million new cases in various clinical manifestations occur yearly, putting more than 350 million people at risk of developing the disease. It represents one of the endemic diseases in Iraq. This study aims to use immunohistochemistry to investigate the expression of CD1a and CD68 in 15 healthy individuals and 45 leishmaniasis patients treated at different hospitals in a Thi-Qar Governorate method. CD1a1 and CD68 expression was increased significantly in leishmaniasis patients compared to the control group ( $P=0.0325$  &  $0.0022$ ), respectively. The results suggest that both proteins might encourage the development or aggressiveness of leishmaniasis. A further study is also required to clarify the role of these proteins and determine if they could serve as biomarkers for leishmaniasis.

**Keywords**---Cutaneous Leishmaniasis, CD1a, CD68, IHC.

### **Introduction**

An Obligate unicellular protozoan called *Leishmania* is a main cause of a widespread epidemic disease known as Cutaneous Leishmaniasis in Iraq (Akhoundi et al., 2016) This parasite belongs to the a hemoflagellate, which is a member of the Trypanosomatidea family (Modabber, 1996). The name of the

disease depends on the location of the infection. For example, cutaneous leishmaniasis occurs when the parasite affects the skin, whereas, mucocutaneous leishmaniasis (MCL) affects the mucous membranes of the host. In addition, visceral leishmaniasis (VL), is called when the parasite affects the internal organs (lymph nodes, liver, spleen, bone marrow) (Sabzevari *et al.*, 2020).

The parasite life cycle is complicated and has two main hosts; invertebrate and vertebrate hosts. The promastigote form which is found in invertebrate host takes 4-25 days and reproduces by binary fission in the middle of the alimentary canal (Jamal *et al.*, 2020). The amastigote in the vertebral host has an oval or circular shape and measures around 4 microns in size (Eddleston, 2000). The parasite is inoculated into the host's (mammal's) skin by the sand fly (the vector), which then transmits the parasite throughout the body of the final host (human) (Garrido-Jareno *et al.*, 2020).

The parasite can cause the disease by attacking the defence mechanisms in the human body. For example, the outer skin barrier may be damaged by a sand fly bite. In addition, the protein released by the insect during the bite may protect the parasite from phagocytosis when it enters the human blood (Martinez-Lopez *et al.*, 2018).

The parasite's outer surface composition varies depending on the types of *Leishmania* and the evolutionary stage in the life cycle of the parasite, hence it can be utilized as a biomarker for the various life cycle stages (Gerald *et al.*, 2000). It has a significant role in enhancing the defence mechanisms through which the parasite can survive inside the macrophage cells and prevent the phagocytic cells from being eliminated (Wolday *et al.*, 2004). It also has a role in complement system resistance, where it can prevent natural killer T-cells from recognizing the phagocytes infected with the *Leishmania* parasite. (Wolday *et al.*, 2004). In addition, the outer surface of the *Leishmania* cell contains lipophosphoglycan (lpg) that stimulates the immune response in mammals (Robert *et al.*, 2011).

CD1a and CD68 proteins, which are known as anti-glucolipids play a significant role in the intracellular immune response to *Leishmaniasis* infections (Gregory&Olivier, 2015). CD1a is a protein which is found in the cytoplasmic granules of a group of blood cells and muscle cells and may be used as a biomarker for identifying macrophages and monocytes that contains *Leishmania* parasites Where CD1a-restricted T cells are exposed to lipid antigens rather than peptide antigens (de jong & Ogg, 2021)

CD68 is a glycoprotein that binds to low-density lipid proteins that appear on monocytes and phagocytes. Therefore, these cells can present exogenous antigens to T cells that control the outcome of infection after the first uptake of mastigote by macrophages into the phagocyte (Taheri *et al.*, 2021). It was decided to study the expression level of these proteins in skin tissue samples taken from leishmaniasis and non- leishmaniasis people.

## 1. Materials and Methods

This study was approved by the ethical board of Thi- Qar Health Department, Thi-Qar city, Iraq (Thi-Qar 1421 on 2/12/2021). 45 leishmaniasis (36 males and 9 females) and 15 healthy skin (as a control group) tissue samples were taken and then diagnosed by a specialized doctor when they visited Al Hussein Teaching, Al Shatrah, Souq Al Shuyukh and Al Rifai General Hospitals.

### 1.1. Immunohistochemistry

Using anti-CD1a1 (Rabbit monoclonal, PathnSitu Biotechnology, cat. number EP80, 1:100) and anti CD68 antibodies (mouse monoclonal, PathnSitu Biotechnology Biotechnology, cat. number KP1s, 1:100), IHC was used to stain skin tissue samples. Several pre-treatment steps were done before IHC staining. First, xylene was used twice to deparaffinize the tissue sections for 5 minutes each. Then, different grades of alcohol (100%, 95%, and 70%, respectively) were used to rehydrate them for two minute each. The tissue sections were then permeabilized with 0.5% triton X-100 in phosphate buffer saline (PBS), then heated for 30 minutes at 90°C to induce epitope retrieval in a citrate buffer with a pH of 6.0 before cooling for 20 minutes. In addition, drops of H2O2 were then added to these sections, and they were incubated in a humid chamber for 10 minutes at room temperature in order to inhibit endogenous peroxidase activity. Finally, drops of the solution containing PBS, 10% normal goat serum, and 0.05% bovine serum albumin were applied to tissue sections for 30 minutes at room temperature. The primary antibody was then incubated overnight at 4°C.

Next day, after three time washing by PBS for 10 minutes each, a secondary antibody was incubated with these sections for 30 minutes at room temperature in a humid chamber. The reaction products were seen using diaminobenzidine tetrahydrochloride as a chromogen. The sections were then counterstained with haematoxylin.

To assess the expression of these proteins in skin tissue samples, ten randomly chosen pictures were obtained and scored using a semi-quantitative scoring system. The proportion and intensity score were then used for both proteins' expression as follow: 0 (0%), 1 (1-10%), 2 (11-50-%), 3 (51-80%) and 4 (< 80). Negative (0), mild (+1), moderate (+2), or high (+3). The total score was ranged from 0 to 7 (Yuan, et al, 2019).

### 1.2. Statistical analysis

The mean, standard error, standard deviation values, unpaired t-test were determined using GraphPad Prism version 8.00 for Windows, GraphPad Software, La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com). P<0.05 was considered significant.

## Results

IHC was used to evaluate the expression level of CD1a1 and CD68 in leishmaniasis and non- leishmaniasis skin tissues. IHC result showed CD68 staining in both groups with varied level of signal intensity, ranging from strong

(Figure 1 A), Moderate (Figure 1 B), and weak (Figure 1 C). CD1A1 expression was also observed in both group with different level of staining ranging from strong (Figure 2 A), Moderate (Figure 2 B), and weak (Figure 2 C). The negative control (NC) group, which did not use a primary antibody (Figure 1 & 2 D).

Quantification of the IHC staining showed that CD1a and CD68 staining was increased significantly in leishmaniasis tissues compared to control groups ( $p=0.0325$  &  $0.0022$ , respectively) (Figure 3 A&B).

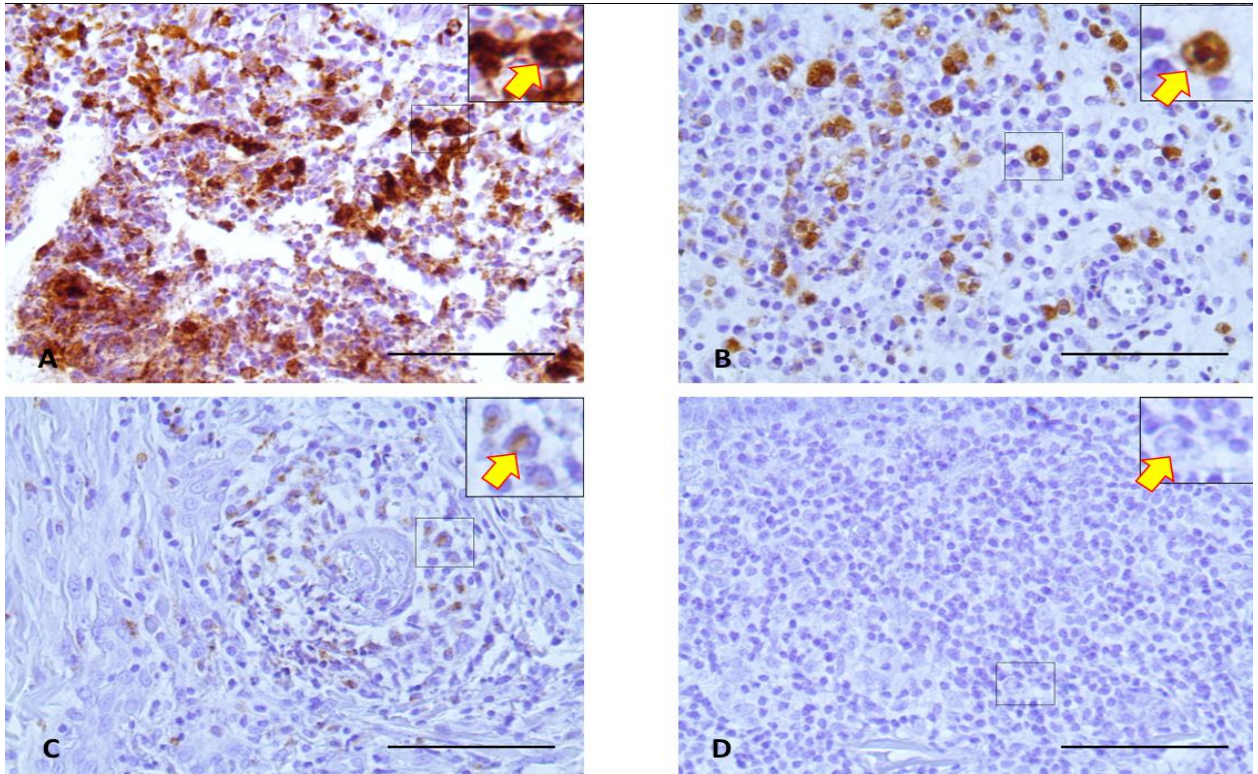


Figure 1: CD1a IHC result in leishmaniasis and non- leishmaniasis skin tissues. A) Strong CD1a immunostaining (yellow arrow) was observed in skin tissue samples. B) Moderate CD1a immunostaining (yellow arrow) was observed in skin tissue samples. C) Weak CD1a immunostaining (yellow arrow) was observed in skin tissue samples. D) Negative control showed no CD1a staining. Scale bars=100 $\mu$ m.

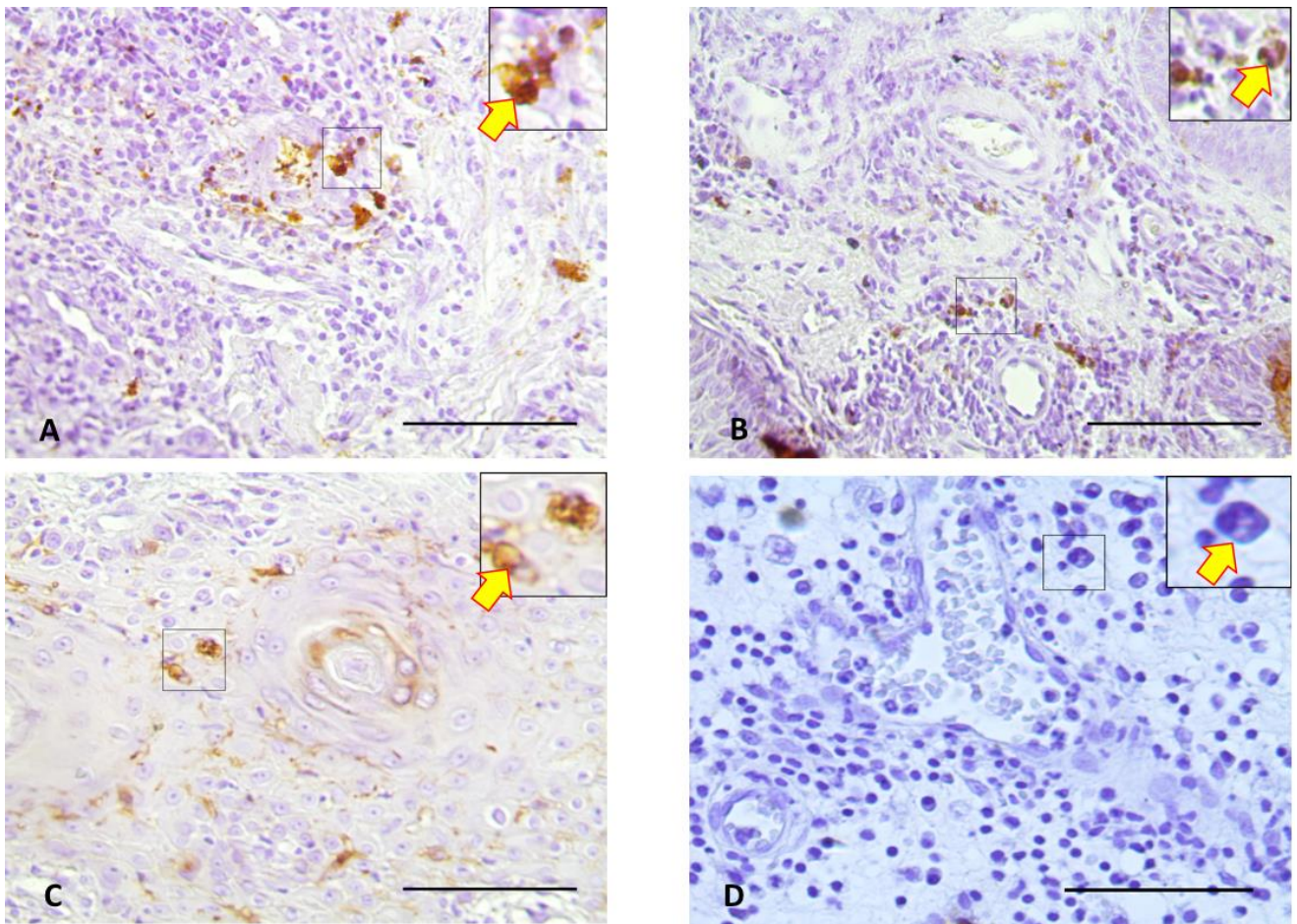


Figure 2: CD68 IHC result in leishmaniasis and non- leishmaniasis skin tissues. A) Strong CD68 immunostaining (yellow arrow) was observed in skin tissue samples. B) Moderate CD68 immunostaining (yellow arrow) was observed in skin tissue samples. C) Weak CD68 immunostaining (yellow arrow) was observed in skin tissue samples. D) Negative control showed no CD68 staining. Scale bars=100 $\mu$ m.

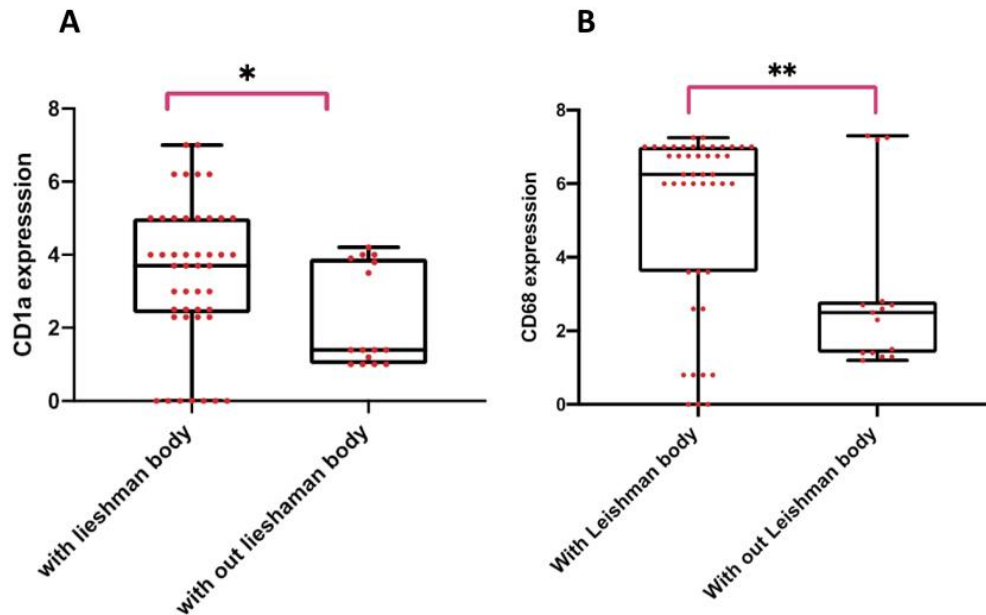


Figure 3: CD1a and CD68 staining in skin tissue samples quantified. (A) Increased CD1a staining significantly in leishmaniasis compared to non-leishmaniasis skin tissues ( $p=0.0325$ ). (B) Increased CD68 staining significantly in leishmaniasis compared to non-leishmaniasis skin tissues ( $p=0.0022$ ). Leishmaniasis skin tissues (n: 45). Non-Leishmaniasis skin tissues (n:15)

## Discussion

Two potential biomarkers (CD1a and CD68) have been selected in this study because of their association to the cutaneous leishmaniasis and/ or their association with the phagocytic cells that engulf the parasite at any stage of its life cycle. This study found that both CD1a and CD68 expression increased significantly in people with cutaneous leishmaniasis compared to those who did not have as well as the morphological changes that occurred in the two layers of the skin: the epidermis and the dermis compared to the control group. This result was in agreement with Farokhpour and his colleagues who found that increased CDa1 immunostaining was observed in patients with cutaneous leishmaniasis compared to the control group (Farokhpour et al., 2021), suggesting that CD1a may have a role in the development of the disease. However, the CD68 results was in consistent with the previous study the showed there was no significant association between the expression of CD68 and the diseases. This difference may be because of using different antibodies, different antigen retrieval and/ or sample size. T-cells which are restricted with CD1a protein and can recognize lipid antigens rather than peptide antigens. (Genardi *et al.*, 2021). In addition, the lipophosphoglycan (LPG) compound that covers the outer surface of the Leishmania cell stimulates the immune response in mammals and turn can be used as a biomarker because its composition varies with different species of Leishmania as well as in the life stage of the parasite (Gerald et al., 2000). CD68 is a glycoprotein that is connected to low-density lipid proteins that appear on

monocytes and phagocytes. So, these cells can present exogenous antigens to T-cells that control the outcome of infection after the first uptake of Amastigote by macrophages in phagocytosis (Taheri, et al., 2017). Taken together, our study suggests that both proteins may have a role in the disease development and progression.

This study showed the presence of the parasite inside the dermis layer, as well as a large infiltration of inflammatory cells such as lymphocytes and phagocytic cells, and this is consistent with the previous study which found that there are abscesses found due to the destruction of cells, especially neutrophil cells because their life span short compared to other immune cells (Sanguenza *et al.*, 1983).

The current study recorded changes in the epidermis, dermis, keratinization, cellular secretion, pseudo-epithelial hyperplasia, ulcers, thinning of the epidermis and extension of inflammation (in the layers of the skin), in addition to the type and amount of inflammatory cells, which include plasma cells, lymphocytes and neutrophils, as well as the presence of necrosis, abscess, and fibrosis, and this is consistent with what was stated by (Taheri et al., 2017), as well as with the study of (Dias-Polak et al., 2017) and with the study of (Farokhpour *et al.*, 2021). As for the control group, there is no change was observed in it. According to our knowledge, there is no local study to compare with our result and this study represents the first IHC study in Iraq on skin tissue samples taken from patients with cutaneous leishmaniasis.

The process of using IHC technology is important to determine the existence of CD1a and CD68 in the cytoplasmic granules of a group of blood cells, which is particularly useful as a marker for the identification of phagocytic and monocytes containing *Leishmania* parasites. The *Leishmania* parasite can survive in difficult conditions and identify these conditions. The manipulation of host cells is important in the design of therapeutic strategies to cure this disease (Gregory & Olivier, 2005).

## Conclusion

CD1a and CD68 immunostaining is increased significantly in patients with cutaneous leishmaniasis compared to healthy people. These proteins may have a role in the development of the disease. A further study is needed to confirm the role of these proteins in skin tissue samples from patients with cutaneous leishmaniasis and non-cutaneous leishmaniasis people using tissue culture.

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