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Immunopathology of anthroponotic cutaneous leishmaniasis and incidental diagnostic tool of metastatic granuloma: A case-control study

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ABSTRACT

Background: Cutaneous leishmaniasis (CL) is a neglected disease with important public health concerns in many parts of the world including Iran.
Objectives: We aimed to explore the histological changes and immunohistochemical quantification of inflammatory cells and their role in the immunopathology of acute, chronic non-lupoid, and chronic lupoid skin lesions in anthroponotic CL (ACL).
Methods: In this study, skin biopsies of 53 patients with ACL were taken. Samples were studied by light microscopy and immunohistochemistry to quantify the immune and inflammatory cells.
Results: Of the 53 skin lesions, 38 were acute, nine chronic non-lupoid and six chronic lupoid. $CD68^+$ macrophages were the most common cells. $CD3^+$ T-lymphocytes were present as diffuse and focal dermal infiltrates and $CD8^+$ cytotoxic T-lymphocytes were the dominant lymphocyte type, constituting more than 50% of the lymphocyte population. $CD4^+$ T-lymphocytes in chronic non-lupoid ($10.57 \pm 2.37\%$) and chronic lupoid ($14.40 \pm 1.28\%$) lesions were more than those observed in the acute form ($8.61 \pm 1.31\%$), but the differences were not statistically significant. $CD20^+$ B-lymphocytes constituted a small percentage of inflammatory cell infiltrates. CD1a + Langerhans cells showed progressively higher percentages from acute to chronic non-lupoid to chronic lupoid lesions. The differences were statistically significant ($P < 0.05$) between acute and chronic lupoid lesions. $CD68^+$ macrophages were the most common cells and $CD8^+$ T lymphocytes remained the predominant T-lymphocytes in acute, chronic non-lupoid, and chronic lupoid lesions, suggesting their central role in the pathogenesis and possible healing of CL.
Conclusion: Focusing on the deep dermis, periadnexal and/or peripheral margins or even papillary tip of inflammatory sites of sandfly bites, we sometimes find granuloma inside lymphatic vessels (lymphangiectatic metastatic granuloma) or even infected macrophages with engulfed Leishman bodies faraway. Knowledge of the histopathological and immunohistochemical findings for various forms of ACL is essential in improving clinical and medical strategies and crucial for proper prophylactic and therapeutic plans.

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1. Introduction

Leishmania belongs to the trypanosomatidae family, and leishmaniasis is considered a chronic disease of developing countries and one of the major neglected tropical diseases [1,2].

A broad spectrum of clinical manifestations is ascribed to leishmaniasis, ranging in severity from self-healing cutaneous lesions to fatal visceral appearances associated with the interaction between parasite species and host immune response [3,4]. The infection control of *Leishmania* is dependent on cellular and immunological mechanisms because there is a correlation between the clinical outcome of *Leishmania* infection and the cytokine response profile [5].

12 million people are affected by leishmaniasis with an incidence for 0.5 million cases/year of the visceral form (VL) and 1.5–2.0 million cases/year for the cutaneous form (CL) [1,6,7].

According to the reports of the World Health Organization (WHO) more than one billion inhabitants are at risk of leishmaniasis in over 100 countries; more than 400 million people for CL and more than 600 million people for VL [4]. Also, in 2017, more than 95% of the new CL cases occurred in Afghanistan, Brazil, Colombia, Algeria, Iraq, Iran, and the Syrian Arab Republic [8]. Anthroponotic cutaneous leishmaniasis (ACL) represents an important health problem in Iran and is caused by *Leishmania tropica*. In Kerman province, ACL (dry type) is the most common presentation of leishmaniasis, which usually takes one to two years to heal while leaving a scar. The mechanism of parasite elimination is not fully understood. Humoral and cellular immune responses are thought to induce necrosis of macrophages and lyse of the *Leishmania* amastigotes within them, thus leading to the elimination of parasites and healing of the lesions.

The immunological outcome of leishmaniasis in humans is functionally specified by two diverse T-helper (Th) cell populations, namely Th1 and Th2. Commonly, the disease susceptibility in uncontrolled or non-healed infections has been related to Th2 proliferation and production of IL-4, IL-5 and IL-10; while healing responses and unresponsiveness to infection have been related to the expansion of Th1 cells production [9,10]. Histologically, it is characterized by a spectrum of well-organized epithelioid granulomata to diffuse mononuclear cell infiltrates [11,12]. The presence of well-organized granulomas and giant cells is a factor that is related to the immune system's attempt to eliminate the parasites [13,14]. In the present study, we report the histological changes and immunohistochemical quantification of inflammatory cells and their role in the immunopathology of acute, chronic non-lupoid and chronic lupoid forms of ACL. This would help provide a better understanding of the responsible agent causing the disease e.g., *Leishmania* parasite.

The use of immunomadulatory drugs in combination with glucantime activates parasite-containing macrophages and shows parasiticide effects [15–18].

2. Material and methods

2.1. Ethical consideration

The protocol of the study was approved by the joint Ethical Committees of Kerman University of Medical Sciences (ethics no. IR. KMU. REC.1387.122, project no. 87/110). Patients with ACL voluntarily participated in the study. For each participant, a written informed consent form was completed. All data, including sex, age, and clinical status, were recorded confidentially.

2.2. Study site and data collection

The study was done in Kerman, the largest province in southeast Iran and 1000 km away from Tehran. Kerman district is located at a latitude of 30.29 and a longitude of 57.06 and is one of the recognized foci of ACL in Iran [19]. This study was performed at the Outpatient Department of Afzalipour Hospital, the main referral hospital for CL treatment and control activities.

2.3. Sampling

Skin biopsies of 53 patients with ACL, who were referred to the hospital during a year period (January 2012 to February 2016), were taken and studied. Age, sex, location, and duration of the lesions were recorded by interviews with the volunteers. Based on clinical features and duration of disease, the skin lesions were classified into acute, chronic non-lupoid, and chronic lupoid.

2.4. Case definition

Acute patients include those who had received one course of treatment with intramuscular administration (systemic) of glucantime or intra-lesional glucantime together with bi-weekly liquid nitrogen cryotherapy. Patients with chronic non-lupoid lesions are those who do not heal and remain with an active skin lesion, despite receiving three courses of intra-lesional glucantim along with bi-weekly cryotherapy or systemic for at least 24 months after the appearance of the lesion. Patients with chronic lupoid (relapse) lesions are those who have healed (clinical cure) but present with the reappearance of specific nodules, plaque, or ulcerative lesions around or in the old scar after months or years [20,21].

2.5. Histopathological study

Skin biopsies were fixed in 10% formalin, and then paraffin blocks were obtained. Afterward, sections with 4 μm thickness were prepared, and Hematoxylin and Eosin (H&E) staining was performed. The histopathological parameters including parakeratosis, hyperkeratosis, acanthosis, ulceration, exocytosis, spongiosis, abscess formation, apoptotic body, melanophages collection, atrophy epidermis, pseudoepitheliomatous hyperplasia, congestion, and inflammatory cell infiltration were studied according to previous CL histopathological studies [22].

2.6. Light microscopy

Biopsies were classified into five histological groups: 1) anergic macrophage reaction, 2) histiocytic reaction with focalized necrosis, 3) diffuse necrotizing reaction, 4) diffuse lympho-histiocytic reaction, and 5) lupoid granulomatous reaction.

2.7. Immunohistochemical test (IHC)

Deparaffinized sections were stained according to standard procedures using the following antibodies (dilution):

CD1a, DAKO M3571 (1/50), CD68, DAKO M0814 (1/100), CD3, DAKO A0452 (1/100), CD8, DAKO M-7103 (1/50); CD4, Biosystem NCL-L-CD4368 (1/20), CD20, DAKO M9755 (1/100).

2.8. Cell count

Inflammatory/Immune cells in H&E and immunohistochemically stained sections were counted using an ocular micrometer. The micrometer field was divided into 100 squares, each square at 400 magnification measures 0.528 mm^2. We counted 25 central squares (0.132 mm^2). For each biopsy, at least five different foci of inflammation were quantified and mean percentages were recorded for statistical analysis.

2.9. Statistical analysis

For statistical analysis SPSS software version 20 was used. The results were recorded based on mean ± SEM. For comparing different groups,

ANOVA test and in special condition non-parametric Kruskal- Wallis test were used. To determine the correlation between the tests, we used Chi-Square and Pearson correlation tests. In special circumstances, we also used non-parametric Spearman correlation test. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Clinical and demographical findings

There were 30 (56.6%) men and 23 (43.4%) women patients. Of the 53 skin lesions, 38 were classified as acute, nine as chronic non-lupoid, and six as chronic lupoid**.** Chronic non-lupoid and chronic lupoid lesions were located mostly on the face. Acute lesions were distributed equally on both hands and face, with the occasional appearance on the shoulder, back, and chest. There was no significant statistical correlation betweenage, sex, site, and type of the lesions ($P > 0.05$) (Table 1).

3.2. Histopathological findings

Histiocytic giant cells and vascular congestion were mostly seen in chronic non-lupoid forms compared with acute and chronic lupoid groups. Granulomata were present in acute (36.8%), chronic non-lupoid (55.6%), and chronic lupoid (66.7%) forms. Fibrosis in chronic non-lupoid forms was statistically more significant than in acute and chronic lupoid forms ($P < 0.05$). There was a positive correlation between multinucleated histiocytic giant cells in the dermis and epidermal acanthosis regardless of the type of lesions ($P < 0.05$). Histiocytes and lymphocytes were the most common inflammatory cells in all lesions regardless of the type (Table 2). The percentage of eosinophils in the chronic non-lupoid form was statistically more significant than the acute form ($P < 0.05$). There was no statistically significant difference in other inflammatory cells in the three groups of lesions ($P > 0.05$).

In the chronic form, in which Leishman bodies are dispersed and rare to find, with the deep dermis, periadenxal and/or peripheral margins or even papillary tip of the insect's bite inflammatory site, can be a good hint for pathologists to look for these sites too We could find granuloma inside lymphatic vessels (lymphangiectatic metastatic granuloma) (Fig. 1) or faraway infected macrophages engulfed in intra-cytoplasmic Leishman bodies. This suggested the need for a new diagnostic tool in the differential diagnosis of difficult cases of infectious dermatitis. Our findings for quiet site with less immune cells to disturb its surveillance were confirmed IHC staining for CD68, CD3, and CD1a, CD34 (Fig. 2). This idea came from leopard hunting behavior and possible octopus's movement of infected macrophages containing Leishman bodies[3] to prepare a safe life.

3.3. Immunohistochemistry

$CD68^+$ macrophages were the most common cells in the dermis and were also scattered in the epidermis. There was no significant statistical difference in the percentage of macrophages in the three groups ($P > 0.05$, Table 3).

$CD3^+$ T-lymphocytes were present as diffuse and focal dermal infiltrates, also scattered in post-capillary venules and along the basal zone. There was, however, no significant statistical difference in T-lymphocytes in the three groups ($P > 0.05$).

$CD8^+$ cytotoxic T-lymphocytes were the dominant lymphocyte type, constituting about 50% of the lymphocyte population in all three types of lesions, but exhibited a patchy dermal distribution. They were also present along the basal zone and around the necrotic areas.

$CD4^+$ helper T-lymphocytes had a uniform distribution in the dermis, in close proximity of the epidermis, and in the basal layer. $CD4^+$ T-lymphocytes in chronic non-lupoid ($10.57 \pm 2.37\%$), and chronic lupoid ($14.40 \pm 1.28\%$) forms were observed more than in the acute form ($8.61 \pm 1.31\%$), but the differences were not statistically significant ($P > 0.05$, Fig. 3).

$CD20^+$ B-lymphocytes constitute a small percentage of inflammatory cell infiltrates; they were higher in the chronic non-lupoid ($5.22 \pm 1.98\%$) than the chronic lupoid ($6.41 \pm 2.84\%$), and the acute ($4.52 \pm 0.72\%$) forms, but the differences were not statistically significant ($P > 0.05$). B-lymphocytes had a patchy distribution in the dermis, mostly around the skin adnexa, and were rarely seen around macrophages containing Leishman bodies.

CD1a + Langerhans cells, in varying numbers, were present in the epidermis and in the dermis among the dermal inflammatory cell infiltrates and sometimes obscured the epidermal dermal junction or formed collarets around epithelioid granulomata. They were also present in the hair follicle infundibula and in or around lymphatic vessels. They were surprisingly absent in ulcerated epidermis. In chronic lupoid and non-lupoid lesions, interlinking dendritic processes produced net-like appearances. Epidermal and dermal Langerhans cells (Fig. 4) showed progressively higher percentages from acute to chronic non-lupoid to chronic lupoid lesions (Fig. 5). However, the differences between acute, and lupoid lesions were statistically significant ($P < 0.05$) but not between other groups.

There was an inverse relationship between Langerhans cells and parasite loads. Epidermal Langerhans cells in parasite load 0 ($18.66 \pm 1.70\%$) were more frequent than in parasite load 4+ ($7.62 \pm 1.37\%$), and the difference was statistically significant ($P < 0.05$). Also, dermal Langerhans cells in parasite load 0 ($11.16 \pm 2.02\%$) were more frequent than in parasite load 4 ($1.25 \pm 0.40\%$), and the difference was statistically significant ($P < 0.05$, Fig. 6).

Table 1
Baseline characteristics of eligible patients in acute cutaneous leishmaniasis, chronic non-lupoid leishmaniasis and chronic lupoid leishmaniasis groups.

Groups	Number	Mean of duration of lesion (month)	Mean of age	Sex in each group (%)		Location of lesion
				Female	Male	
Acute	38	6.13 ± 0.65	34.51 ± 3.01	36.8	63.2	Face (26.5%) Hand (26.3%)
Chronic non-lupoid	9	40.00 ± 4.89	25.77 ± 5.87	55.6	44.4	Face (66.7%)
Chronic lupoid	6	1.33 ± 0.21	23.33 ± 9.26	66.7	33.3	Face (83.3%)

Table 2
Frequency of cell types (Mean ± SEM) (%) in acute cutaneous leishmaniasis (ACL), chronic non-lupoid leishmaniasis (CNLL) and chronic lupoid leishmaniasis (CLL) in H&E stained sections.

Cell type	Acute	Chronic non-lupoid	Chronic lupoid
Histiocyte	65.31 ± 2.64	66.50 ± 4.04	64.83 ± 4.42
Lymphocyte	31.19 ± 2.71	29.88 ± 3.90	33.08 ± 4.81
Plasma cell	2.67 ± 0.52	1.38 ± 1.09	0.08 ± 0.08
Eosinophil	0.01 ± 0.01	0.33 ± 0.16	0.16 ± 0.16
Giant cell	0.59 ± 0.12	1.44 ± 0.55	1.00 ± 0.34
Neutrophil	0.44 ± 0.18	0.44 ± 0.24	0.50 ± 0.50

4. Discussion

In Iran, CL is one of the major health issues, particularly in the province of Kerman, especiallyACLform caused by *L. tropica*. Non-healing forms such as chronic non-lupoid and chronic lupoid forms may occur by host factors, including the type of immune response, demographical characteristics, clinical status, and treatment compositions in addition to parasite species [23–25]. We studied some aspects of immunopathology for acute, chronic non-lupoid and chronic lupoid

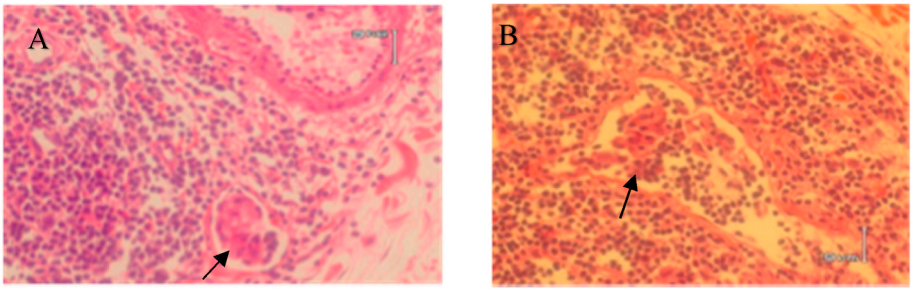

Fig. 1. A and B metastatic granuloma in the lymphatics vessels deep to the primary site.

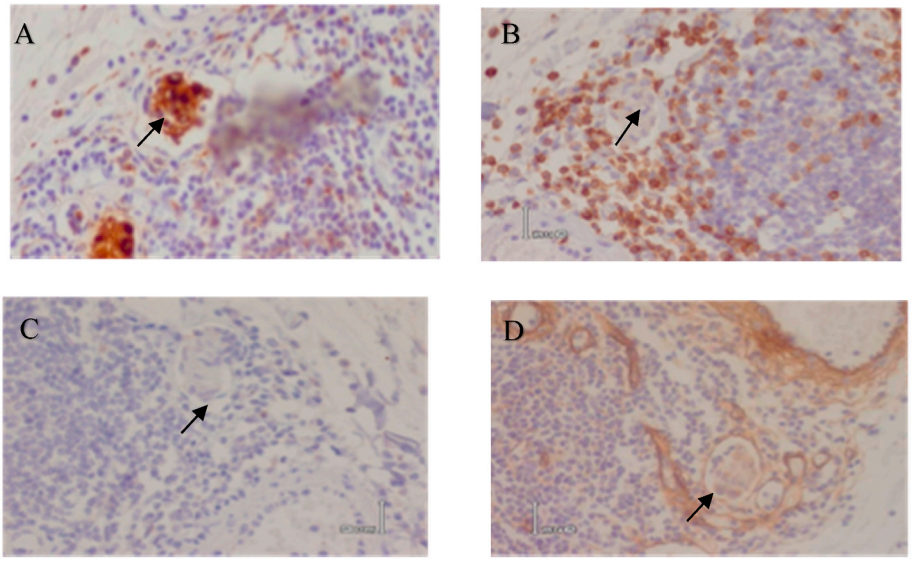

Fig. 2. Immunohistochemical details of metastatic granuloma; A) CD68 strong positive for histiocytes aggregate, B) CD3 T lymphocytes, rare, C) CD1a Langerhans cells negative in granuloma, D) CD34 showed lymphangiectatic vessels containing granuloma.

Table 3
Cell markers % (Mean ± SEM) in cutaneous leishmaniasis immunohistochemically stained sections in acute cutaneous leishmaniasis, chronic non-lupoid leishmaniasis and chronic lupoid leishmaniasis groups.

Cell markers	Acute (Mean ± SEM)	Chronic nonlupoid (Mean ± SE	Chronic lupoid (Mean ± SEM)
CD 68	62.89 ± 2.52	58.00 ± 3.78	54.83 ± 5.16
CD3	29.88 ± 2.12	29.77 ± 1.43	29.00 ± 3.53
Epidermal CD1a	10.57 ± 0.89	14.77 ± 2.47	18.66 ± 1.70
Dermal CD1a	2.72 ± 0.46	7.88 ± 2.58	11.16 ± 2.02
CD20	4.52 ± 0.72	5.22 ± 1.98	6.41 ± 2.84
Others	0.50 ± 0.16	0.55 ± 0.55	0.25 ± 0.25

forms.

The difference in the rate of chronic non-lupoid leishmaniasis and chronic lupoid leishmaniasis as a chronic form in females can be due to the women have severe stress after receiving infection of CL especially on the face due to the importance of their beauty, and this stress can cause a weakening immune system and creating a chronic form [26]. Although the women have a stronger immune system, they are more potentially prone to chronic immune-based diseases such as lupus erythematosus and other autoimmune disorders comparing to male patients. The reason for this may be the hypersensitivity of the women's immune system [27–30].

The histological spectrum of ACL includes an anergic response, focal or diffuse necrosis, and epithelioid granulomata. The anergic response is characterized by a brisk proliferation of macrophages and abundant

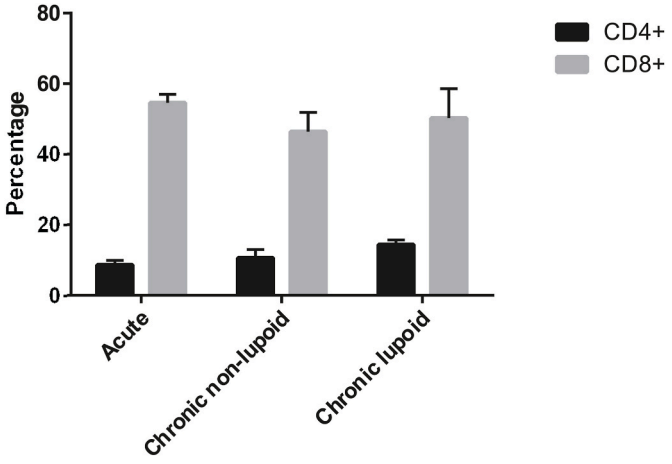

Fig. 3. The mean percentages of $CD4^+$ and $CD8^+$ lymphocytes in acute, chronic non-lupoid and chronic lupoid leishmaniasis as several clinical forms (Cell/0.0132 mm2 in x 400), ($P > 0.05$).

intracellular organisms, but it does not constitute a clinico-pathological entity. It is simply a stage in the chronological development of ACL lesions. Chronic lupoid leishmaniasis resembles lupus vulgaris. The lesions are characterized by non-caseating epithelioid granulomata, and organisms are absent or extremely scanty [11]. Extensive histological examination revealed occasional macrophages containing a few

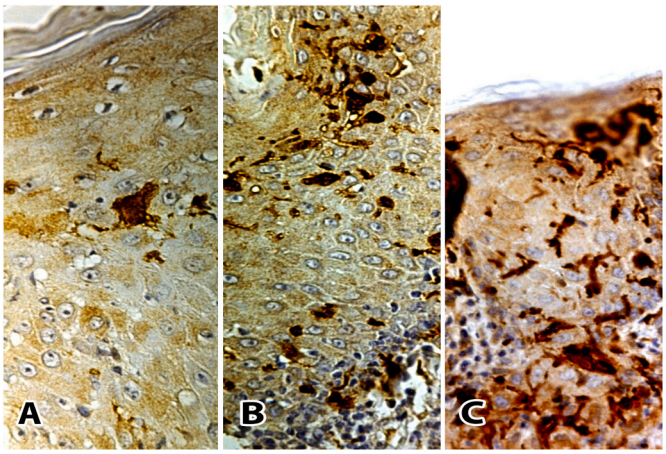

Fig. 4. Increasing the number of the epidermal Langerhans cells along with dermal migration of them from acute group (A) to chronic non-lupoid group (B) and chronic lupoid group (C) leishmaniasis were noticed. S100 immunostaining.

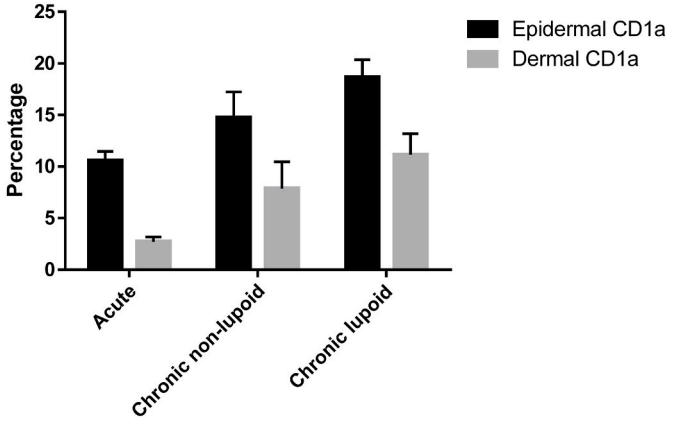

Fig. 5. The mean percentage of CD1a + Langerhans cells in acute, chronic non-lupoid and chronic lupoid leishmaniasis (between acute and lupoid lesion $P < 0.05$ and between other groups $P > 0.05$).

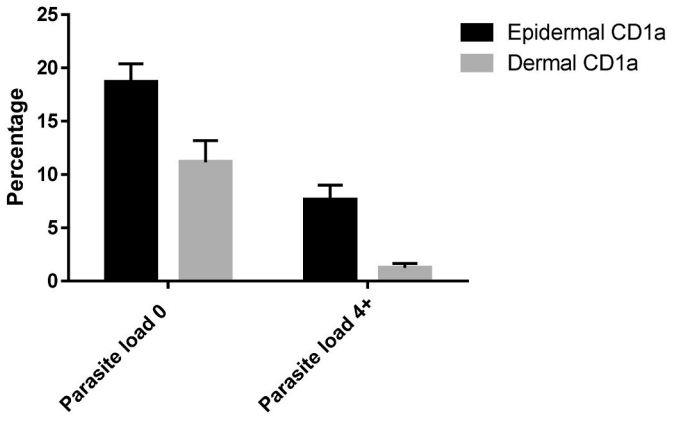

Fig. 6. The mean percentage of CD1a + Langerhans cells in parasite load 0 and parasite load 4+ in epidermis and dermis ($P < 0.05$).

amastigotes in only 12 cultures yielded *Leishmania* promastigotes in 20 and polymerase chain reaction identified *Leishmania* DNA in 30 (47.6%) of the 63 cases studied [12].

In our series, all skin lesions contained $CD68^+$ macrophages, $CD4^+$ helper cells, $CD8^+$ cytotoxic cells and a few $CD20^+$ B lymphocytes. $CD8^+$ T lymphocytes remained the predominant T-lymphocytes in acute, chronic non-lupoid, and chronic lupoid lesions. Their number and topographic distribution around granulomata and necrotic zones suggest a significant role in the pathogenesis of CL. Clinicopathological and experimental studies have underlined the critical roles of helper (Th1) and cytotoxic T lymphocytes for CL healing in humans and mice [31]. Castellano and colleagues have suggested that cure was associated with a sustained Th1 response with elevated IFN-gamma levels and down-modulation of IL-4 and IL-10 production [10,32]. Resistance and susceptibility to experimental infection with *L. major* was associated with the development of Th1 and Th2 immune responses, respectively [33]. However, studies from India have suggested that an unfavorable clinical outcome in CL was not related to an inadequate Th1 cell response, but rather to impairment in multiple immune functions [34].

We found an inverse relationship between the number of CD1a + Langerhans cells (LCs) and parasite load. Epidermal and dermal Langerhans cells were more frequent in chronic lupoid and chronic non-lupoid than in acute forms. Langerhans cells were absent in ulcerated epidermis in our series. Geiger and co-workers noted that epidermal LCs were absent above the center of the lesions, but normally distributed in the surrounding tissue [31]. Other investigators have suggested that, dermal dendritic cells (Langerin negative) are important in priming of $CD4^+$ T cells, whereas dermal LCs (Langerin + dermal dendritic cells) are involved in the early priming of $CD8^+$ T cells [35].

Leishmania parasites, unlike viruses, do not render infected myeloid cells susceptible to the cytotoxicity of NK cells. Instead, soluble products of NK cells trigger the leishmanicidal activity of macrophages [36]. Dermal dendritic cells may mediate protection against *L. major* by cross-activation with NK cells and NK-cell-derived interferon-γ [37].

We found a statistically significant correlation between acanthosis and multinucleated histiocytic giant cells. Keratinocyte-derived IL-6 and IL-4 are important for resistance against *Leishmania*. It has been suggested that the epidermis controls Th1-differentiation and keratinocyte-derived IL-6 and IL-4 are important for resistance against *Leishmania* [38].

In this study and other studies, we noticed IgE antibodies and increased eosinophils. Mast cells have been said to be important in CL and related to the healing of lesions [39–41]; however, their exact roles in the immunopathology of ACL require further systematic investigations in the future.

We found no report on lymphangiectatic metastatic granuloma. One study showed that the predominance of one type of cell leads to an inadequate immune reaction as a result of insufficient control from other components of the immune system [42]. For example, during diffuse CL, the excess of immature non-activated macrophages becomes a source for non-controlled amastigote replication and leads to dissemination of the infection which is specific to ACL [42]. It is good to compare these findings with infectious dermatitis-like lupus vulgaris, leprosy, and deep fungal infection for an exact diagnosis.

Focusing on the deep dermis, periadnexal and/or peripheral margins, or even papillary tip of insect bite inflammatory sites, we sometimes find granuloma inside lymphatic vessels (lymphangiectatic metastatic granuloma) or even infected macrophages with engulfed Leishman bodies faraway. Regular monitoring of chronic forms and recognition of causing factors linked with non-healing and relapsed forms of ACL is crucial for proper prophylactic and therapeutic strategic plans. A better understanding of the histopathological and immuno histopathological changes for several clinical forms of ACL is vital in improving therapeutic and medical strategies and the prevention of chronic forms in the future. Since humans are the main reservoir hosts, early detection and efficient, complete, and timely treatment could play a major role in the control and prevention of the non-healed forms of the disease.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M. den Boer, the W.L.C. Team, Leishmaniasis worldwide and global estimates of its incidence, PloS One 7 (2012), e35671, https://doi.org/10.1371/journal.pone.0035671.

[2] M. Bamorovat, I. Sharifi, M.R. Aflatoonian, H. Sharifi, A. Karamoozian, F. Sharifi, A. Khosravi, S. Hassanzadeh, Risk factors for anthroponotic cutaneous leishmaniasis in unresponsive and responsive patients in a major focus, southeast of Iran, PloS One 13 (2018) 1–13, https://doi.org/10.1371/journal.pone.0192236.

[3] F. Bruschi, L. Gradoni, The Leishmaniases: Old Neglected Tropical Diseases, Springer International Publishing, 2018, https://doi.org/10.1007/978-3-319-72386-0.

[4] H. Goto, J.A. Lauletta Lindoso, Cutaneous and mucocutaneous leishmaniasis, Infect. Dis. Clin. 26 (2012) 293–307, https://doi.org/10.1016/j.idc.2012.03.001.

[5] G. Natarajan, S. Oghumu, S. Varikuti, A. Thomas, A. Satoskar, Mechanisms of immunopathology of leishmaniasis, in: Pathog. Leishmaniasis New Dev. Res., Springer, New York, 2013, pp. 1–13, https://doi.org/10.1007/978-1-4614-9108-8_1.

[6] P. Desjeux, The increase in risk factors for leishmaniasis worldwide., Trans. R. Soc. Trop. Med. Hyg. 95 (n.d.) 239–243. http://www.ncbi.nlm.nih.gov/pubmed/11490989.

[7] WHO, WHO to implement online epidemiological surveillance for leishmaniasis, 2016. Available [Http://Www.Who.Int/Neglected_diseases/News/WHO_implement_epidemiological_surveillance_leishmaniasis/En].

[8] World Health Organization(WHO), GHO | Global Health Observatory Data Repository (Region of the Americas) | Number of Cases of Cutaneous Leishmaniasis Reported - Data by Country, WHO, 2017.

[9] M. Bamorovat, I. Sharifi, M.R. Aflatoonian, B. Sadeghi, A. Shafiian, R.T. Oliaee, A. Keyhani, A.A. Afshar, A. Khosravi, M. Mostafavi, Host's immune response in unresponsive and responsive patients with anthroponotic cutaneous leishmaniasis treated by meglumine antimoniate: a case-control study of Th1 and Th2 pathways, Int. Immunopharm. 69 (2019) 321–327.

[10] S. Meymandi, S. Dabiri, M. Shamsi-Meymandi Msc, H. Nikpour, A. Kharazmi, Immunophenotypic pattern and cytokine profiles of dry type cutaneous leishmaniasis, Arch. Iran. Med. 12 (2009) 371–376.

[11] B. Azadeh, A. Samad, S. Ardehali, Histological spectrum of cutaneous leishmaniasis due to *Leishmania tropica*, Trans. R. Soc. Trop. Med. Hyg. 79 (1985) 631–636, https://doi.org/10.1016/0035-9203(85)90174-9.

[12] A.Z. Momeni, S. Yotsumoto, D.R. Mehregan, Chronic lupoid leishmaniasis: evaluation by polymerase chain reaction, Arch. Dermatol. 132 (1996) 198–202, 1996.

[13] K. González, R. Diaz, A.F. Ferreira, V. García, H. Paz, J.E. Calzada, M. Ruíz, M. Laurenti, A. Saldaña, Histopathological characteristics of cutaneous lesions caused by *Leishmania Viannia panamensis* in Panama, Rev. Inst. Med. Trop. Sao Paulo 60 (2018) e8, https://doi.org/10.1590/s1678-9946201860008.

[14] F.T. Silveira, R. Lainson, C.E.P. Corbett, Clinical and immunopathological spectrum of american cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil - a review, Mem. Inst. Oswaldo Cruz 99 (2004) 239–251, https://doi.org/10.1590/S0074-02762004000300001.

[15] M. Hakimi Parizi, A. Pardakhty, I. sharifi, S. Farajzadeh, M.H. Daie Parizi, H. Sharifi, A.R. Keyhani, M. Mostafavi, M. Bamorovat, D. Ghaffari, Antileishmanial activity and immune modulatory effects of benzoxonium chloride and its entrapped forms in niosome on *Leishmania tropica*, J. Parasit. Dis. 43 (2019) 406–415, https://doi.org/10.1007/s12639-019-01105-7.

[16] M. Hakimi Parizi, I. Sharifi, S. Farajzadeh, A. Pardakhty, M. Parizi, H. Sharifi, A. Keyhani, M. Mostafavi, M. Bamorovat, A. Khosravi, D. Ghaffari, Tioxolone niosomes exert antileishmanial effects on *Leishmania tropica* by promoting promastigote apoptosis and immunomodulation, Asian Pac. J. Trop. Med. 12 (2019) 365–374, https://doi.org/10.4103/1995-7645.262566.

[17] M.H. Parizi, S. Farajzadeh, I. Sharifi, A. Pardakhty, M.H.D. Parizi, H. Sharifi, E. Salarkia, S. Hassanzadeh, Antileishmanial activity of niosomal combination forms of tioxolone along with benzoxonium chloride against *Leishmania tropica*, Kor. J. Parasitol. 57 (2019) 359–368, https://doi.org/10.3347/kjp.2019.57.4.359.

[18] M. Bamorovat, I. Sharifi, A. Fekri, A. Keyhani, M.R. Aflatoonian, A. Heshmatkhah, R.T. Oliaee, A. Khosravi, A. Naderi, M.H. Parizi, A single-group trial of end-stage patients with anthroponotic cutaneous leishmaniasis: levamisole in combination with Glucantime in field and laboratory models, Microb. Pathog. 128 (2019) 162–170.

[19] I. Sharifi, M.R. Aflatoonian, A.R. Fekri, M. Hakimi Parizi, A. Aghaei Afshar, A. Khosravi, F. Sharifi, B. Aflatoonian, A. Khamesipour, Y. Dowlati, F. Modabber, A. Nadim, A comprehensive review of cutaneous leishmaniasis in kerman province, southeastern Iran-narrative review article, Iran. J. Public Health 44 (2015) 299–307. http://www.ncbi.nlm.nih.gov/pubmed/25905072.

[20] Y. Dowlati, Cutaneous leishmaniasis: clinical aspect, Clin. Dermatol. 14 (1996) 425–431.

[21] W.H. Organization, Manual for Case Management of Cutaneous Leishmaniasis in the WHO Eastern Mediterranean Region, 2014.

[22] S. Shamsi Meymandi, A. Javadi, S. Dabiri, M. Shamsi Meymandi, M. Nadji, Comparative histological and immunohistochemical changes of dry type cutaneous leishmaniasis after administration of meglumine antimoniate, imiquimod or combination therapy, Arch. Iran. Med. 14 (2011) 238–243, https://doi.org/0011144/aim.003.

[23] R. Hadighi, M. Mohebali, P. Boucher, H. Hajjaran, A. Khamesipour, M. Ouellette, Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites, PLoS Med. 3 (2006) e162, https://doi.org/10.1371/journal.pmed.0030162.

[24] A. Maurer-Cecchini, S. Decuypere, F. Chappuis, C. Alexandrenne, S. De Doncker, M. Boelaert, J.-C. Dujardin, L. Loutan, J.-M. Dayer, G. Tulliano, Immunological determinants of clinical outcome in Peruvian patients with tegumentary leishmaniasis treated with pentavalent antimonials, Infect. Immun. 77 (2009) 2022–2029.

[25] A. Ponte-Sucre, E. Diaz, M. Padrón-Nieves, Drug Resistance in *Leishmania* Parasites: Consequences, Molecular Mechanisms and Possible Treatments, Springer Science & Business Media, 2012.

[26] M.R. Aflatoonian, I. Sharifi, B. Aflatoonian, M. Bamorovat, A. Heshmatkhah, Z. Babaei, P.G.N. Almani, M.A. Mohammadi, E. Salarkia, A.A. Afshar, Associated-risk determinants for anthroponotic cutaneous leishmaniasis treated with meglumine antimoniate: a cohort study in Iran, PLoS Neglected Trop. Dis. 13 (2019), e0007423.

[27] J.L. Turabian, Gender differences in prevalence of chronic diseases: facts and hypothesis; melody and harmony, J. Public Heal. Gen. Med. 1 (2018) 1–3.

[28] D. Fairweather, S. Frisancho-Kiss, N.R. Rose, Sex differences in autoimmune disease from a pathological perspective, Am. J. Pathol. 173 (2008) 600–609, https://doi.org/10.2353/ajpath.2008.071008.

[29] F. Angum, T. Khan, J. Kaler, L. Siddiqui, A. Hussain, The prevalence of autoimmune disorders in women: a narrative review, Cureus 12 (2020), e8094, https://doi.org/10.7759/cureus.8094.

[30] J. Lucke, B. Waters, R. Hockey, M. Spallek, R. Gibson, J. Byles, A. Dobson, Trends in women's risk factors and chronic conditions: findings from the Australian longitudinal study on women's health, Women's Heal. 3 (2007) 423–432, https://doi.org/10.2217/17455057.3.4.423.

[31] B. Geiger, J. Wenzel, M. Hantschke, I. Haase, S. Ständer, E. Von Stebut, Resolving lesions in human cutaneous leishmaniasis predominantly harbour chemokine receptor CXCR3-positive T helper 1/T cytotoxic type 1 cells, Br. J. Dermatol. 162 (2010) 870–874, https://doi.org/10.1111/j.1365-2133.2009.09573.x.

[32] L.R. Castellano, D.C. Filho, L. Argiro, H. Dessein, A. Prata, A. Dessein, V. Rodrigues, Th1/Th2 immune responses are associated with active cutaneous leishmaniasis and clinical cure is associated with strong interferon-γ production, Hum. Immunol. 70 (2009) 383–390, https://doi.org/10.1016/j.humimm.2009.01.007.

[33] E. Mougneau, F. Bihl, N. Glaichenhaus, Cell biology and immunology of *Leishmania*, Immunol. Rev. 240 (2011) 286–296, https://doi.org/10.1111/j.1600-065X.2010.00983.x.

[34] R. Kumar, R.A. Bumb, P. Salotra, Evaluation of localized and systemic immune responses in cutaneous leishmaniasis caused by *Leishmania tropica*: interleukin-8, monocyte chemotactic protein-1 and nitric oxide are major regulatory factors, Immunology 130 (2010) 193–201, https://doi.org/10.1111/j.1365-2567.2009.03223.x.

[35] N. Brewig, A. Kissenpfennig, B. Malissen, A. Veit, T. Bickert, B. Fleischer, E. Al, Priming of CD8+ and CD4+ T cells in experimental leishmaniasis is initiated by different dendritic cell subtypes, J. Immunol. 182 (2009) 774–783.

[36] C.K. Prajeeth, S. Haeberlein, H. Sebald, U. Schleicher, C. Bogdan, Leishmania-infected macrophages are targets of NK cell-derived cytokines but not of NK cell cytotoxicity, Infect. Immun. 79 (2011) 2699–2708, https://doi.org/10.1128/IAI.00079-11.

[37] K.A. Remer, B. Roeger, C. Hambrecht, H. Moll, Natural killer cells support the induction of protective immunity during dendritic cell-mediated vaccination against *Leishmania major*, Immunology 131 (2010) 570–582, https://doi.org/10.1111/j.1365-2567.2010.03329.x.

[38] J. Ehrchen, K. Roebrock, D. Foell, N. Nippe, E. von Stebut, W. JM, E. Al, Keratinocytes determine Th1 immunity during early experimental leishmaniasis, PLoS Pathog. 6 (2010), e1000871.

[39] B. Azadeh, P. Sells, G.C. Ejeckam, D. Rampling, Localized *Leishmania* lymphadenitis: immunohistochemical studies, Am. J. Clin. Pathol. 102 (1994) 11–15.

[40] K. Katakura, S. Saito, A. Hamada, H. Matsuda, N. Watanabe, Cutaneous leishmaniasis in mast cell-deficient W/Wv mice, Infect. Immun. 61 (1993) 2242–2244.

[41] F.F. Tuon, M.I.S. Duarte, V.S. Amato, A quantitative and morphometric study of mast cells in cutaneous leishmaniasis, Parasite Immunol. 30 (2008) 641–645, https://doi.org/10.1111/j.1365-3024.2008.01057.x.

[42] O.E. Akilov, A. Khachemoune, T. Hasan, Clinical manifestations and classification of Old World cutaneous leishmaniasis, Int. J. Dermatol. 46 (2007) 132–142, https://doi.org/10.1111/j.1365-4632.2007.03154.x.