ORIGINAL ARTICLE

The immune microenvironment in cutaneous leishmaniasis

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Abstract

Background Cutaneous leishmaniasis is an infection that has spread to non-endemic regions, stimulating recent interest for the enhanced understanding of this disease. Downregulation of the CD1a receptor on Langerhans cells has been described in various cutaneous infections.

Objective In this study, the immune response across different Ridley patterns and parasitic indices is outlined in a case series of cutaneous leishmaniasis.

Methods Skin punch biopsies from the interface of normal and lesional cutaneous leishmaniasis were collected from 33 patients with molecularly confirmed Leishmania tropica or L. major infection. Ridley patterns (2–5) were assessed for various clinicopathological features including age, gender, disease duration, parasitic index and constituents of the inflammatory infiltrate. CD1a, CD68, CD3, CD4, CD8, CD20 and CD138 stains were performed on normal skin tissue, cutaneous leishmaniasis biopsies and cytospin/cell block cytology preparations of cultured leishmania promastigotes. CD1a was quantified per mm² in the epidermis and dermis. The remaining stains were graded according to a 4-tiered grading system [0 (0–4%); 1 (5–24%); 2 (25–49%); 3 (50–74%) and 4 (75–100%)].

Results Total CD1a expression significantly decreased (14-fold) from parasitic indices (0–2) to (5–6); (q < 0.001). CD1a expression in the epidermis was at least 5-fold lower than normal skin (58 vs. 400 cells/mm²), inversely correlating with the parasitic index. There was an increase in dermal CD1a Langerhans cells (33 vs. 0 cells/mm² in the dermis). CD1a and CD68 staining of amastigotes was strong and diffuse, whereas promastigotes were negative. The major inflammatory infiltrate, in all Ridley patterns, consisted of macrophages and double-negative CD3+/CD4−/CD8− T lymphocytes. The double-negative CD3 T cells formed a ring around the parasitic laden macrophages. Apart from CD1a, there was no significant difference in inflammatory markers between the various Ridley patterns and parasitic indices. Disease duration did not correlate with Ridley pattern.

Conclusion The significant decrease in CD1a expression is postulated by two mechanisms; either via direct CD1a receptor uptake by leishmania amastigotes and/or negative feedback inhibition of CD1a Langerhans cells by double-negative CD3 T-regulatory cells. Modulation of the immune microenvironment in cutaneous leishmaniasis represents a potential therapeutic and prophylactic target.

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Conflicts of Interest

The authors declare no conflict of interest

Funding disclosure

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Introduction

Leishmaniasis infection is mainly divided into three forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis and visceral leishmaniasis (VL).1 Leishmaniasis is caused by a vector-borne protozoan parasite (phlebotomine sandfly) that is present in endemic and non-endemic regions.2 CL is divided into two forms based on the parasite infection: Old World (southern Europe, the Middle East, Asia and Africa) and New World leishmaniasis (Latin America).3 The major endemic countries include Afghanistan (24, 585 cases), Syria (29, 140 cases) and Iran (26, 824 cases).4 Recent surveys indicate an increased incidence of CL reaching 75/100,000 cases around the Jordan River Valley, exceeding the highest incidence rate from Aleppo, Syria.5

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Approximately 90% of VL is detected in the Indian subcontinent and Sudan. In Europe, CL and VL account for almost 700 cases per year. The VL vectors (*Phlebotomus perniciosus* and *P. neglectus*) have been increasingly detected in the Italian Alpine regions reaching as far as northern Germany. Thus, climate changes, environmental alterations, human behaviour and pet travel are factors that might contribute to the spread of leishmaniasis from endemic to non-endemic regions.

CL is clinically divided based on the duration of the lesion. Skin lesions present for <1 year are defined as acute CL, whereas lesions present for >1 year are regarded as chronic. The latter includes persistent partially/inadequately treated, treatment-resistant (chronic lupoid leishmaniasis) and chronic disease that reactivate few weeks or months following healing (recurrent/recidivan lesions).

Langerhans cells are specialized bone marrow-derived antigen-presenting cells distinctly expressing the adenosine triphosphatase and CD1a receptors. CD1a-Langerhans cells represent 2–4% of the epidermal cell population (400–1000 per mm²) with rare Langerhans cells present within the dermis. Alterations in CD1a expression were previously described in CL and various therapeutic interventions. Decreased CD1a receptor expression has also been documented with chronic *Leishmania tropica* and *L. braziliensis guyanensis* infection. Furthermore, leishmania amastigotes located intracytoplasmically within macrophages and Langerhans cells, expressed CD1a. The major inflammatory cell populations in CL were reported to consist of T cells (CD4:CD8 ratio of ~1.05), macrophages, a minute B-cell fraction, natural killer cells and granulocytes. Meymandi et al. identified a significant gradual decrease in epidermal CD1a-Langerhans cell pool from 31.6 cells/mm length of epidermis in acute CL (<2 years) to 5.0 cells/mm in chronic CL (>2 years).

There is no defined correlation between the distribution of CD1a expression among the different parasitic indices and Ridley patterns in CL. The aim of this study was to evaluate CD1a expression and the various inflammatory infiltrates within acute CL lesions, in association with the different parasitic indices and across the various Ridley patterns. An additional aim was to determine whether CD1a immunoexpression by leishmania amastigotes is a unique *in vivo* finding or persistently present within leishmania promastigote collected from cultures.

### Materials and methods

#### Case selection and histopathological classification

Previous biopsy diagnosed patients with CL at the American University of Beirut Medical Center between 1992 and 2013 were selected based on the availability of paraffin-embedded tissue for each corresponding Ridley pattern. The study was approved by the Institutional Review Board (IRB) at the American University. A total of 33 skin punch biopsies of the interface between normal and lesional tissue were included. Clinicopathological features including age, gender, disease duration, Ridley pattern, parasitic index and morphological constituents of the inflammatory infiltrate were documented. A total of 28 cases of Ridley pattern = 2, 9 of Ridley pattern = 3, 8 of Ridley pattern = 4 and 8 of Ridley pattern = 5 were selected. None of the previewed cases were identified as Ridley pattern = 1. The parasitic indices were divided as follows: Parasitic index = 0 (*n* = 8), Parasitic index = 1 (*n* = 3), Parasitic index = 2 (*n* = 3), Parasitic index = 3 (*n* = 3), Parasitic index = 4 (*n* = 8), Parasitic index = 5 (*n* = 6) and Parasitic index = 6 (*n* = 2). Based on the parasitic index, cases were subcategorized accordingly: Low parasitic index = 0–2 (*n* = 14), intermediate parasitic index = 3–4 (*n* = 11) and high parasitic index = 5–6 (*n* = 8). A total of 31 of 33 cases were molecularly confirmed *Leishmania tropica* infections, whereas only two were *Leishmania major*.

#### Immunohistochemistry

Immunohistochemical tests were performed on 3-μm formalin-fixed paraffin-embedded skin tissue (leishmania lesions and normal breast skin tissue removed during plastic surgery as control) sections and cell block/cytospin preparations of leishmania promastigote cultures using the autostainer, Leica-Bond Max (Leica Microsystems Inc., Buffalo Grove, IL, USA) with manufacturer preset timed reagents. The following antibodies against CD1a, CD68, CD3, CD4, CD8, CD20 and CD138 (Table 1) were utilized. CD1a-Langerhans cells were quantified per mm² in the epidermis and dermis. The remaining stains were graded according to a 4-tiered grading system [0 (0–4%); 1 (5–24%); 2 (25–49%); 3 (50–74%) and 4 (75–100%)].

#### Leishmania parasite culture

*L. major* (MHOM/SU/73/5 ASKH) parasites (provided by the London School of Hygiene and Tropical Medicine) were grown at 22 ± 1°C to 25 ± 1°C in a standard monophasic medium and subcultured weekly. The medium was made of nutrient broth supplemented with 20% fetal bovine serum, 100 IU/mL of penicillin and 100 IU/mL of streptomycin (Sigma, St. Louis, MO, USA). Cytospin preparations were performed via the Centrion Scientific Ltd cell-prep centrifuge (Thermo Scientific, UK) with manufacturer preset timed reagents. The following antibodies against CD1a, CD68, CD3, CD4, CD8, CD20 and CD138 (Table 1) were utilized. CD1a-Langerhans cells were quantified per mm² in the epidermis and dermis. The remaining stains were graded according to a 4-tiered grading system [0 (0–4%); 1 (5–24%); 2 (25–49%); 3 (50–74%) and 4 (75–100%)].

### Table 1 Antibodies utilized for immunohistochemistry

<table>
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<th>Primary Ab</th>
<th>Company</th>
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<th>Dilution</th>
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<td>MTB1</td>
<td>RTU</td>
</tr>
<tr>
<td>CD68</td>
<td>Dako</td>
<td>KP1</td>
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</tr>
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<td>CD138</td>
<td>Novocastra</td>
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</tr>
</tbody>
</table>

RTU, Ready to use.
Marietta, OH, USA). Cell block cytology slides were prepared by centrifuging the specimen at 200 g for 10 min. The supernatant was discarded, whereas the concentrate was placed between two filter papers, embedded within a cassette and processed to produce a paraffin-embedded cytology cell block.

Statistical analysis
Chi-square and Mann–Whitney rank sum tests were used for univariate analysis in case of categorical and continuous variables respectively. Relationship between CD1a counts (epidermal, dermal and total) and Ridley pattern or parasitic index subcohorts was done using analysis of variance on ranks. A two-tailed P-value less than 0.05 was always used to indicate statistical significance. SPSS version 21.0 (IBM, Armonk, NY, USA) was used for statistical analysis.

Results

Clinicopathological features
The cases were distributed approximately equally between the different Ridley patterns (2–5) (Fig. 1). The average age at diagnosis was 15 years (range 10–20 years). There was a slight

Figure 1  Representative H&E sections of Ridley Pattern = 2/Parasitic Index = 4 (a and b); Ridley Pattern = 3/Parasitic Index = 3 (c and d); Ridley Pattern = 4/Parasitic Index = 6 (e and f); Ridley Pattern = 5/Parasitic Index = 5 (g and h) (40×). Note the leishmania amastigotes (arrows, 400×).
male predominance, with a male-to-female ratio of 2.5 : 1.4. The duration of skin lesions averaged 4 months (range 4–6 months) (Table 2). The size of CL lesions decreased mildly from parasitic index (0–2) to parasitic index (5–6) by a non-significant difference of 1.0 cm.

CD1a and CD68 expression in cutaneous leishmaniasis

Analysis of CD1a-Langerhans cells expression in the various CL Ridley patterns vs. control normal breast skin tissue reveals the following: Epidermal CD1a was markedly decreased in comparison with control skin tissue with the highest epidermal CD1a reaching 25 cells/mm² vs. 400 cells/mm² in control skin. The dermal CD1a was significantly increased reaching 35 cells/mm² vs. 0 to 2 cells/mm² in control skin tissue (P < 0.001). With respect to the various Ridley patterns, there was no significant correlation between Ridley pattern = (2–5) and both epidermal CD1a and dermal CD1a expression (P > 0.05, Fig. 2a). However, an inverse correlation was identified between the parasitic indices and epidermal and dermal CD1a expression (Table 3 and Fig. 2b, P < 0.001). Following an increase in parasitic index from (0–2) to (5–6), total CD1a expression decreased significantly from 60 cells/mm² to 4 cells/mm² (P < 0.001) (Fig. 3). A uniform, intense and diffuse uptake of CD1a and CD68 by leishmania amastigotes was noted in all cases (Fig. 4). To determine whether the stain was innate, i.e. due to antigen epitope homology vs. acquired, cultured leishmania promastigotes were stained with CD1a and CD68 on both cell block cytology and cytospin preparations. The result was a negative stain for both markers, indirectly indicating that leishmania amastigotes acquired CD1a and CD68 epitopes during host infectivity.

Inflammatory cell distribution in cutaneous leishmaniasis

Different inflammatory markers were utilized to determine the immune microenvironment during the acute stage of infection. In all cases, there was diffuse expression of CD3 and CD68 (Fig. 5a–c). The latter was elevated in Ridley patterns 4 and 5, a finding consistent with the observed granulomas in such patterns (Fig. 5c). CD3 T lymphocytes were noted to form a ring around amastigote-laden macrophages (Fig. 5b). In Ridley pattern = 3, the expression of CD138 plasma cells was noted (Fig. 5d). Subtyping of the immune process with CD4 and CD8 markers revealed a weak staining pattern (<25% of all cells) across all Ridley patterns indicating that the minority of CD3+ lymphocytes were CD4 and CD8 T cells. The remaining major population was double-negative CD3 (+) CD4 (−) CD8 (−) T cells. A minute fraction of scattered CD20 (+) B cells was noted. Overall, there was no significant difference in CD3, CD68, CD4, CD8, CD20 and CD138 expression across Ridley patterns = (2–5)(Table 4; P > 0.05).

Table 2 Clinicopathological characteristics of cutaneous leishmaniasis cases

<table>
<thead>
<tr>
<th>Ridley Pattern</th>
<th>Average Age (years)</th>
<th>Gender (M : F)</th>
<th>Average Duration of Skin Lesion(s) (months)</th>
<th>Parasitic Index</th>
<th>Molecular Subtype</th>
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<td>5</td>
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<tr>
<td>5 (n = 8)</td>
<td>12</td>
<td>1 : 1.7</td>
<td>5</td>
<td>0 (n = 3); 1; 2; 4; 5 (n = 2)</td>
<td>Leishmania tropica (n = 8)</td>
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</tbody>
</table>

Figure 2 Box-plot graph showing CD1a expression in Langerhans cells vs. Ridley Pattern (a) and Parasitic Index (b).
Discussion

Langerhans cells in cutaneous leishmaniasis

The current report is unique in demonstrating an inverse correlation between the parasitic index and CD1a expression in the acute phase of CL. During the cellular interaction between the Human Leukocyte Antigen Class I molecule of the antigen-presenting cell and the CD8 molecule of the mature T cell, CD1a plays a pivotal role by interacting with the HLA Class I molecule on the antigen-presenting cell. Therefore, CD1a may exert a positive or negative stimulatory effect on the HLA Class I-CD8 intermolecular complex. In murine models of CL, epidermal Langerhans cells, along with macrophages, ingest L. major parasite, transport the antigen, migrate and differentiate into antigen-presenting cells within the lymph nodes resulting in the induction of a delayed-hypersensitivity reaction by CD8 T cells. The parasite antigen persists within Langerhans cells allowing for continuous stimulation of T cells, providing immunity against recurrent infection. Two potential mechanisms postulate the observed decreased expression of CD1a on Langerhans cells: either via downregulation of CD1a receptors or through a decrease in the residing population of antigen-presenting epidermal and dermal Langerhans cells secondary to regional lymph node migration for antigen presentation. The former hypothesis is supported by the observed coexpression of CD1a antigen by leishmania amastigotes, internalized within macrophages, as compared to the absent expression within

<table>
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<th>Parasitic Index</th>
<th>CD1a (E)</th>
<th>CD1a (D)</th>
<th>CD1a (T)</th>
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<td>7</td>
<td>21</td>
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<tr>
<td>(5–6)</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>&lt;0.001</td>
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<tr>
<td>Control Skin</td>
<td>~400*</td>
<td>Rare</td>
<td>400</td>
<td></td>
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</table>

CD1a (E): Epidermis (cells/mm2); CD1a (D): Dermis (cells/mm2) & CD1a (T): Total (cells/mm2). *Range (350–480)

Figure 3  A decrease in Langerhans cells expressing CD1a proceeding from highest to lowest: (a) Control skin tissue, (b) PI = 1, (c) PI = 3, and (d) PI = 6. Of note, the amastigotes in (d) show uniform, intense and diffuse staining by CD1a antibody (100×).

Figure 4  Leishmania amastigotes express CD1a (a) and CD68 (b) antigens (400×).
cultured leishmania promastigotes. Note that the CD1a cross-reactivity identified in leishmania amastigotes within Langerhans cells appears to be antibody clone specific with reactivity against clone MTB1 as opposed to clone 01035; the absence of promastigote staining implies that the amastigotes internalized the receptor protein homology of clone MTB1. Intracytoplasmic CD1a expression by leishmania amastigotes was observed in most of the current case series corroborating the results by Karram et al.; therefore, there is a potential diagnostic utility of the CD1a antibody for detecting leishmania amastigotes on skin biopsies. What remains to be determined is whether the reactivity is species specific or perhaps even genus specific? A decrease in the residing Langerhans cell population is recognized in a variety of situations, most particularly secondary to skin UV irradiation, whereby initially there is internalization of the receptors and, with chronic UV exposure, a consequent reduction in Langerhans cell numbers; a finding postulated to underlie the pathophysiology of post-Kala-azar dermatosis, whereby chronic UV exposure leads to decreased Langerhans cells and subsequent evolution to chronic cutaneous lesions.

**T-regulatory cells in cutaneous leishmaniasis**

Selective alteration of the anti-leishmania response was achieved by in vivo time-dependent depletion of Langerhans cells producing smaller volume lesions. Double-negative (CD3+CD4+CD8−) T-regulatory cells have been described in

<table>
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<tr>
<th>PI</th>
<th>RP</th>
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<th>CD1a (T)*</th>
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<th>CD3†</th>
<th>CD4†</th>
<th>CD8†</th>
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<td>4</td>
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</tbody>
</table>

*CD1a (E): Epidermis (cells/mm²); CD1a (D): Dermis (cells/mm²); CD1a (T): Total (cells/mm²).
†CD68, CD3, CD4, CD8, CD20 and CD138 are scored according to four grades: Grade 0: 0–4%; Grade 1: 5–24%; Grade 2: 25–49%; Grade 3: 50–74%; Grade 4: 75–100%.
PI, Parasitic index; RP, Ridley pattern.
various entities including graft-versus-host disease of the skin. In CL, the pool of double-negative T cells is composed of 75% double-negative $\gamma\delta$ T cells and 25% $\gamma\delta$ T cells involved in the production of both proinflammatory cytokines, such as Interferon-$\gamma$ and tumour necrosis factor-$\alpha$, and anti-inflammatory cytokines including interleukin 10. The current observation of a significant population of CD3$^+$CD4$^+$CD8$^+$ T cells raises the question about the role of double-negative T-regulatory cells in CL.

**Immune response at a low parasitic index**

Initially, at a low parasitic index, Langerhans cells assume the role of antigen-presenting cells concomitantly secreting cytokines, such as interleukin 12, that result in activation of CD4 T cells and the Th1 response. Activated CD4 T cells produce various lymphokines including interferon-$\gamma$, interleukin 17 and interleukin 4. Interferon-$\gamma$ induces expression of the costimulatory molecules CD80 and CD86 on Langerhans cells, resulting in inhibition of double-negative T-regulatory cells. Double-negative T-regulatory cells abrogate the Th1 response via Fas/FasL pathway. Additional molecular markers expressed by double-negative T-regulatory cells responsible for modulating effector T lymphocytes include interferon-$\gamma$, tumour necrosis factor-$\alpha$, chemokine receptor 5 (CXCR5) and FcR$\gamma$. Alternative T-regulatory subsets including CD4$^+$CD25$^+$ T-regulatory cells release interleukin 10, inhibiting a Th1 response in *L. major* cutaneous infection. The upregulated CD80/86 receptors on Langerhans cells secondary to interferon-$\gamma$ bind the cytotoxic T-cell lymphocyte antigen-4 (CTLA4) receptor expressed on double-negative T-regulatory cells, resulting in attenuation of the T-regulatory response. Therefore, at a low parasitic index, the immune system favours a Th1 response. Thus, host control of infection in the early acute phase is through the persistence of parasites within dendritic cells (Fig. 6). However, several reports have observed, with different leishmania species, a relative increase in CD1a expressing Langerhans cells in the acute as compared to the chronic phase of infection; this is associated with a Th1 response through an increase in proinflammatory cytokines such as interferon-$\gamma$, tumour necrosis factor-$\alpha$ and interleukin 12. However, the Langerhans cell counts rarely exceeded the reported lower limit (400 cells/mm$^2$) of normal skin tissue residing epidermal Langerhans cells. The variation may be leishmania species specific.

**Immune response at a high parasitic index**

The effect of Leishmania organisms on different inflammatory cells involves inhibition of dendritic cell maturation, differentiation and migration. In the presence of a high parasitic index, specifically, *L. major* causes further downregulation of CD1a with inhibition of Langerhans cell interleukin 12 production and resultant attenuation of the Th1 response. Due to the low interferon-$\gamma$, both CD80 and CD86 are downregulated on Langerhans cells causing minimal inhibition of the cytotoxic T-lymphocyte antigen-4 receptor on double-negative T-regulatory cells. The end result of CD1a downregulation is attenuation of the Th1 response and accentuation of T-regulatory cells (Fig. 6). This may explain the persistence of CL infection from the acute to the chronic phase. A decrease in CD1a expression from acute to chronic lesions is associated with a shift from CXCR3 T cells (Th1 response) to CCR4 T cells (Th2 response) in chronic lesions (>6 months). The reason for a shift from a low to a high parasitic index remains elusive. Alternative immunoregulatory factors may be involved in this complex cell–cell interaction. One main argument is that a different host immune response may be initiated secondary to a specific leishmania subspecies. In this study, most cases were *L. tropica* as opposed to previous reports that outlined the immune microenvironment in *L. major* infections. Interestingly, *L. tropica* and *L. major* infections manifest similar clinical symptomatology.

**Targeting the immune response**

The clinical relevance of the proposed immune surveillance mechanism highlights the potential for therapeutic immune targeting either through vaccination and/or immune modulation. Development of *L. major* effector T-cell memory is proposed to occur via the Th1 response, whereas the Th2 response, induced by interleukin 4, interleukin 5 and interleukin 10, lacks a protective role against leishmania infection. The utilization of vaccines in CL provides cellular immunity against the parasite resulting in increased expression of interferon-$\gamma$, interleukin 17, interleukin 2, tumour necrosis factor-$\alpha$ and decreased interleukin 4 secretion. Dendritic cells pulsed with leishmania antigens produce effective immunotherapy against leishmania infection associated with elevated interleukin 12 production. In addition, oligodeoxynucleotides pulsed with leishmania anti-
gen, in the presence of aluminium, can provide immunity against leishmania parasite infection. Upregulation of cytotoxic T-lymphocyte antigen-4 receptor on T-regulatory cells results in a decreased contact time between antigen-presenting cells and T cells with a decrease in the major histocompatibility complex-I and T-cell receptor interaction; hence, a consequent increase in CD8+ T cells with a decrease in the major histocompatibility complex-II expression. Therefore, cytotoxic T-lymphocyte antigen-4 functions as a negative-immune regulator with a dual role either as a T-regulatory cell suppressor and/or a stimulant of the antigen-presenting cell complex-I and T-cell receptor interaction; hence, a consequent negative feedback inhibition of the host immune response.61

In vivo models of delayed hypersensitivity infections secondary to Cryptococcus neoformans infection treated with cytotoxic T-lymphocyte antigen-4 receptor inhibitor also seemed to enhance immunity.62 Blockage of cytotoxic T-lymphocyte antigen-4 receptor has been shown to maintain function and memory of CD8+ T cells and to induce CD4+/CD8+ effector T cells during cancer vaccine therapy.63 Extrapolation of the above models for CL treatment and prevention principally by the concomitant administration of an intradermal vaccine and an immune-modulating agent such as anti-Cytotoxic T-lymphocyte antigen-4 may be beneficial in achieving long-term immunization.

Conclusion

In conclusion, a distinct inverse correlation is identified between CD1a expression by Langerhans cells and the parasitic index during acute CL. This is accompanied by an increase in double-negative T-regulatory cells, thus inhibiting a Th1 response and long-term memory; a morphological finding best explained by the complex cell–cell interaction between Langerhans cells, T-regulatory cells and effector T cells. The above findings represent potential immunotherapeutic targets during treatment-refractory acute CL.

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