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# Distinct Pattern of Immunophenotypic Features of Innate and Adaptive Immunity as a Putative Signature of Clinical and Laboratorial Status of Patients with Localized Cutaneous Leishmaniasis

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## Abstract

In this study, we have analysed the phenotypic features of innate/adaptive immunity of patients with localized cutaneous leishmaniasis (LCL), categorized according to their clinical/laboratorial status, including number of lesion (L1; L2–4), days of illness duration (≤60;>60) and positivity in the Montenegro skin test ($MT^-$;$MT^+$). Our findings highlighted a range of phenotypic features observed in patients with LCL (↑%HLA-$DR^+$ neutrophils; ↑$CD8^+$ HLA-$DR^+$/$CD4^+$ HLA-$DR^+$ T cell ratio; ↑HLA-DR in B lymphocytes, ↑%$CD23^+$ neutrophils, monocytes and B cells; ↑α-*Leishmania* IgG and ↑serum $NO_2^-$ + $NO_3^-$). Selective changes were observed in L1 (↑%HLA-$DR^+$ neutrophils, ↑$CD8^+$ HLA-$DR^+$/$CD4^+$ HLA-$DR^+$ T cell ratio and ↑serum $NO_2^-$ + $NO_3^-$) as compared to L2–4 (↑%$CD5^-$ B cells; ↑$CD23^+$ B cells and ↑α-*Leishmania* IgG). Whilst ≤60 presented a mixed profile of innate/adaptive immunity (↓%$CD28^+$ neutrophils and ↑%$CD4^+$ T cells), >60 showed a well-known leishmanicidal events (↑$CD8^+$ T cells; ↑serum $NO_2^-$ + $NO_3^-$ and ↑α-*Leishmania* IgG). $MT^+$ patients showed increased putative leishmanicidal capacity (↑%HLA-$DR^+$ neutrophils; ↑%$CD23^+$ monocytes; ↑$CD8^+$ HLA-$DR^+$/$CD4^+$ HLA-$DR^+$ T cell ratio and ↑ serum $NO_2^-$ + $NO_3^-$). Overall, a range of immunological biomarkers illustrates the complex immunological network associated with distinct clinical/laboratorial features of LCL with applicability in clinical studies.

## Introduction

American cutaneous leishmaniasis (ACL) is a parasitic disease that presents a variety of clinical manifestations, ranging from single skin lesions referred as localized cutaneous leishmaniasis (LCL) to mucosal lesions and diffuse leishmaniasis [1]. It is believed that the parasite species and the host immune response are closely related to the variety of clinical manifestations in ACL [2–11].

Considering the general hypothesis that particularities in the host immune response microenvironment may affect the outcome of *Leishmania* infection, several studies have been developed to characterize the innate and adaptive immunity of ACL patients to better understand the relationship between the host immune response and the clinical mapping of endemic areas [12–23].

There is a general consensus that increased proportions of $CD8^+$ T cells and decreased levels of $CD4^+$ T cells are associated with the resolution of cutaneous lesions [12–19]. However, immunophatological findings have reported distinct proportions of $CD4^+$ and $CD8^+$ T cells in the cellular infiltrate in LCL lesions: $CD4^+$>$CD8^+$ [16, 20], $CD4^+$=$CD8^+$ [21, 22] and $CD4^+$<$CD8^+$ [23, 24], demonstrating an overall heterogeneity of T cell subset distributions even amongst patients with LCL lesions.

Little is known about the immune response associated with distinct status of LCL, such as time and number of lesion as well as the laboratorial records regarding the





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positivity in Montenegro skin test (MT). In general, no significant difference has been found in the histopathology, type of infiltrate and the ratio of lymphocyte subsets in lesions from patients with single and multiple lesions [25]. Moreover, most studies demonstrated that the percentage of lymphocytes subsets remained fixed in acute and chronic lesions [19, 21].

It has been proposed that the magnitude of the immune response in cutaneous leishmaniasis depends upon the duration of the illness and that the development of disease may depend on a transient dysregulation of T cell responses during the initial phase of infection. It has been demonstrated that patients with a short duration of illness (<60 days) had a transitory depression of type 1 immune response characterized by absence of delayed-type hypersensitivity, low levels of IFN-$\gamma$ production, high expression of mRNA for IL-10 along with decreased lymphocyte proliferative responses [26].

From the clinical point of view, usually all patients with LCL exhibit positive MT and present clinical evidence of a potential resistance to *Leishmania* infection that culminate in self-healing outcome [27, 28]. In fact, patients displaying positive MT have partial immune resistance against the infection, leading to localized lesions, scarceness of parasites and a tendency to spontaneous healing or good response to antimonial therapy [29].

Aiming to identify immunological biomarkers in the innate and adaptive immunity applicable in LCL clinical investigations, in this study, we have analysed the frequency and activation status of peripheral blood leucocytes from patients with distinct clinical/laboratorial status of LCL, including the number of lesion, time of illness duration and the reactivity in the MT. Overall, our findings point to a complex immunological network connected with distinct patterns of clinical and laboratorial features ACL.

## Materials and methods

*Human subjects.* The study population consisted of 51 volunteers, including patients with active LCL ($n$ = 27) and non-infected individuals (NI; $n$ = 24).

The LCL groups included 17 men and 10 women, with age ranging from 05 to 83 years. The patients with LCL were evaluated and submitted to clinical and laboratory evaluations and the diagnosis of cutaneous leishmaniasis based on dermatological findings with positive parasitological test (finding of amastigotes in direct microscopy of skin scraping) along with negative or positive MT. Additionally, the patients with LCL were subgrouped according to their clinical status and referred as: (1) patients with a single lesion (L-1; $n$ = 20), (2) patients with 2–4 lesions (L2–4; $n$ = 7), (3) patients with recent lesions – evolution within 60 days (≤60; $n$ = 18), (4) patients with late lesions – evolution over 60 days (>60; $n$ = 9), (5) patients with negative Montenegro skin test ($MT^-$; $n$ = 5) and (6) patients with positive Montenegro skin test ($MT^+$; $n$ = 22). EDTA–whole blood and serum samples were collected from 51 patients with active LCL.

As a control group, EDTA–whole blood and serum samples were also collected from non-infected individuals (NI; $n$ = 24), living in the same geographical area. All NI presented negative Montenegro skin test. The NI group included 12 men and 12 women with age ranging from 08 to 64 years.

All biological samples, including EDTA–whole blood and serum, were collect by a trained healthy technician from the Fundação Nacional de Saúde. The biological samples from LCL were collected immediately before the beginning of aetiological treatment.

All individuals included in this study live in the endemic area situated in Caratinga, Minas Gerais State, Brazil. All participants signed an informed consent prior to their inclusion in the study. This work was approved by the Ethics Committees (#070/99) from Fundação Oswaldo Cruz (FIOCRUZ), Ministério da Saúde, Rio de Janeiro, Brazil.

*Antigen preparation for flow cytometry immunofluorescence.* *Leishmania (Viannia) braziliensis* promastigote forms (MHOM/BR/75/2903) were obtained according to the study by Rocha *et al.* [30]. Briefly, after 10-day growth in LIT media, the promastigote organisms were washed twice with 0,15M phosphate-buffered saline supplemented with 10% foetal bovine serum and parasite suspension adjusted to $10^7$ promastigotes/ml.

*Immunofluorescence assay by flow cytometry.* FC-ALPA-IgG tests were carried out as described by Rocha *et al.* [30]. Briefly, $5.0 \times 10^5$ live promastigotes/well were incubated in the presence of previously diluted serum sample, following by re-incubation in the presence of prediluted (1:400) FITC-labelled anti-human IgG (Sigma Chemical Corp., St Louis, MO, USA) and fixed with 10% paraformaldehyde fix solution. A total of 10,000 promastigotes were acquired in a FACScalibur flow cytometer and the relative FITC fluorescence intensity analysed for each sample, as previously described by Rocha *et al.* [30].

*Conventional indirect immunofluorescence assay.* Conventional indirect immunofluorescence assay (IFA) to detect anti-*Leishmania* IgG antibodies was carried out as described by Camargo [31] using a kit for human leishmaniasis (Biomanguinhos; FIOCRUZ, Rio de Janeiro, Brazil). Briefly, 10-$\mu$l aliquots of fixed *Leishmania* promastigotes suspension were attached to grease-free glass slides and incubated at 37 °C for 30 min in a humid chamber in the presence of 10 $\mu$l of different serum dilutions, ranging from 1:40 to 1:640 in PBS. After incubation, the slides were washed in PBS and in distilled water. After washing, the parasites were re-incubated in the presence of 15 $\mu$l of

anti-human IgG policlonal antibody labelled with FITC diluted 1:100 in PBS–4% of Evans Blue (Biomanguinhos, FIOCRUZ). After being stained, parasites were washed again in PBS and distilled water. The slides were then mounted with coverslips in buffered alkaline glycerol and the fluorescence measurements performed by UV light microscopy. In all batches of the experiments, positive and negative LCL control sera samples were included. The results were considered positive when antibody titre was ≥1:40 [32].

*Montenegro skin test.* Montenegro skin test was performed as previously described [33], using a standardized antigen preparation. Briefly, 4 μg (0.1 ml) of antigen was injected intradermally into the forearm. Readings were performed 48 h after injection. The presence of an enduration with a diameter of equal/more than 5 mm was considered a positive result. MT was performed in individuals of all groups before blood collection. The MT was performed by a trained healthy technician from the Fundação Nacional de Saúde.

*Monoclonal antibodies used for immunophenotypic staining by flow cytometry.* In this study, we evaluated different cell phenotypes using anti-human fluorescein isothiocyanate (FITC), phycoerithrin (PE) monoclonal antibodies (mAbs) at a final concentration of 0.5 mg/ml, including isotypic control (clone X40), anti-CD3 (clone Leu-4), anti-CD4 (clone SK3), anti-CD5 (clone UCHT2), anti-CD8 (clone SK1), anti-CD14 (clone M0P9), anti-CD16 (clone B73.1), anti-CD19 (clone 4G7), anti-CD23 (clone M-L233), anti-CD28 (clone FAB342P) and anti-HLA-DR (clone G.46.6). All the antibodies were purchased from B.D. Pharmingen (San Diego, CA, USA).

*Ex vivo cell immunophenotypic staining.* Fifty microlitres of EDTA–whole blood was incubated in the presence of 5 μl of undiluted anti-human cell surface molecules mAbs for 30 min, in the dark and at room temperature. Following incubation, erythrocytes lysis was performed using 2 ml of FACS brand lysing solution (Becton Dickinson Biosciences Pharmingen, San Diego, CA, USA), followed by incubation for 10 min, at room temperature. Leucocyte suspension was further washed with 2 ml of phosphate-buffered saline containing 0.01% sodium azide. Prior to flow cytometric acquisition, stained cells were fixed in 200 μl of FACS fix solution (10 g/l paraformaldehyde, 10.2 g/l sodium cacodylate, 6.65 g/l sodium chloride; Sigma Chemical Company) for at least 15 min at 4 °C.

Data collection was performed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and acquisition and analysis using CELLQUEST™ (Becton Dickinson, Franklin Lakes, NJ, USA) software provided by the manufacturer. A total of 15,000 events were acquired for each sample preparation. Analysis of immunophenotyping was carried out using distinct gating strategies depending on the phenotype of interest. Thus, using the multiple resources of data analysis available in the program CELLQUEST™ were adopted specific strategies for phenotypic analysis of cells involved in innate and adaptive immunity, as follows: (1) analysis of monocytes was performed by staining immunophenotyping using SSC versus FL-1/anti-CD14-FITC dot plots to select the monocytes as $SSC^{low}$ $CD^{14high+}$ cells versus FL-2 (anti-CD16-PE, anti-HLA-DR-PE and anti-CD23-PE); (2) analysis of neutrophils was performed by establishing a specific scatter gate using the combination of anti-cell surface antigens and laser SSC to discriminate, and the neutrophils were gated as SSC high $CD16^{high+}$ versus FL-2 (anti-HLA-DR-PE, anti-CD23-PE and anti-CD28-PE); (3) identification of T cells and B lymphocytes and their subsets was performed by initially gating the whole lymphocyte population based on their light forward versus side scatter profile (FSC versus SSC), using dot plot distributions, followed by immunophenotyping analysis on dot plot graphic of fluorescence 1 – FITC/FL1 (anti-CD3, anti-CD19, anti-CD4 or anti-CD8) versus fluorescence 2 – phycoerithrin/FL2 (anti-CD4, anti-CD5 and anti-CD8). Additional analysis of activation status was also performed in dot plot graphics using FITC/FL1 (anti-CD3, anti-CD4, anti-CD8 or anti-CD19) versus PE/FL2 (anti-HLA-DR or anti-CD23-PE).

The results were expressed as the percentage of positive cells within the selected gate for cell surface markers presenting bimodal distributions (neutrophils: $CD16^+HLA\text{-}DR^+$, $CD16^+CD23^+$, $CD16^+CD28^+$; monocytes: $CD14^+HLA\text{-}DR^+$, $CD14^+CD23^+$; T cells: $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+HLA\text{-}DR^+$, $CD4^+HLA\text{-}DR^+$, $CD8^+HLA\text{-}DR^+$ and B lymphocytes: $CD19^+$, $CD19^+CD5^-$, $CD19^+CD5^+$, $CD19^+CD23^+$).

Semiquantitative analyses were carried out for the cell surface markers, exhibiting unimodal distributions (CD16 in neutrophils, HLA-DR in monocytes and HLA-DR in B lymphocytes), besides the expression of CD16 on macrophage-like cells (CD16 in monocytes) to evaluate differential expression, and the results were expressed as mean fluorescence channel (MFI) on a log scale.

*Analysis of serum nitric oxide levels.* The concentration of $NO_2^- + NO_3^-$ existing in serum samples was measured using the Griess reaction [34]. Briefly, a 100-μl aliquot of serum was mixed with 100 μl of Griess reagent (1% sulphanylamide, 0.1% naphthylethylenediamide-dihydrochloride and 2.5% phosphoric acid, all from Sigma, St. Louis, MO, USA). Following 10 min of incubation at room temperature, in the dark, the absorbance was measured at 540 nm, using a microplate reader. Each sample was assayed in duplicate and the concentration of nitrite was determined by interpolation from a standard curve constructed using sodium nitrite solutions of known concentration in the range 0–100 μM. To discount the interference of nitrites already present in the culture medium, data were calculated taking into

account the blank for each experiment. The results were expressed as nitrite concentration ($\mu$M).

*Statistical analysis.* Statistical analyses were performed with the aid of PRISM 5.0 software package (Prism Software, Irvine, CA, USA). Student's *t*-tests were employed to evaluate differences in values determined between non-infected individuals (NI) versus patients with active LCL groups. One-way analysis of variance (ANOVA) and Tukey post-tests were used to investigate differences between NI and LCL subgroups (L1, L2–4, ≤60, >60, $MT^-$, $MT^+$). In all cases, differences were considered significant when *P* values were <0.05.

## Results

### Regardless the overall activation status, the neutrophils from patients with LCL displayed low expression of CD28.

Data analysis demonstrated that patients with LCL displayed an overall activated profile characterized by decreased expression of CD16 along with increased frequency of HLA-$DR^+$ and $CD23^+$ in comparison with NI. On the other hand, decreased frequency of $CD28^+$ neutrophils was observed in LCL as compared to NI (Fig. 1, top panel). Analysis of eosinophil phenotypic features (HLA-DR, CD23 and CD28) did not show any significant differences between LCL and NI (data not shown).

### Despite the low expression of HLA-DR in circulating monocytes, patients with LCL displayed increased frequency of $CD23^+$ monocytes and increased levels of serum $NO_2^-$ + $NO_3^-$

Our results showed that although a decreased expression of HLA-DR was observed in monocytes from LCL, increased frequency of $CD23^+$ monocytes was found in LCL as compared to NI (Fig. 1, middle panel). Data analysis demonstrated that LCL displayed increased levels of serum $NO_2^-$ + $NO_3^-$ as compared to NI (Fig. 1, bottom panel).

### The outstanding activation status of T cell subsets and B lymphocytes along with increased levels of anti-*Leishmania* IgG is the hallmark of the adaptive immunity in patients with LCL

Our findings revealed increased frequencies of the $CD3^+$ cells in LCL compared to NI group, including both $CD4^+$ and $CD8^+$ T lymphocytes. No differences in the frequency of B cells and the major subsets ($CD5^-$ and $CD5^+$) were observed in LCL as compared to NI (Fig. 2, top panel).

Our results showed that LCL displayed an outstanding increase in the frequency of activated $CD3^+$HLA-$DR^+$, including both subsets $CD4^+$ and $CD8^+$ T lymphocytes as compared to NI. Moreover, increased $CD8^+$HLA-$DR^+$/$CD4^+$HLA-$DR^+$ ratio is observed in LCL as compared to NI. Interestingly, increased expression of HLA-DR on B cells and increased frequency of $CD23^+$ B cells demonstrated the prominent increase in the activation status of the humoral immune compartment in LCL as compared to NI (Fig. 2, middle panel).

Analysis of anti-*Leishmania* IgG reactivity, assessed by conventional (IFA) and non-conventional (FC-ALPA) methods, revealed that patients with LCL presented significant higher levels of specific antibodies as compared to non-infected subjects (Fig. 2, bottom panel).

### Distinct patterns of activation status are observed in the cellular and humoral immunity according to the clinical/laboratorial features of patients with LCL

During the course of *Leishmania* infection, distinct clinical and laboratory aspects can be identified and their use as morbidity markers has been previously reported [35, 36]. Here, we selected two clinical (number and time of lesions) and one laboratorial (MT) parameter considered worthwhile to evaluate their association with the immunological aspects of LCL, including: (1) the number of lesions (single or multiple – L1 or L2–4); (2) the time of lesions (recent or late – ≤60 days or >60 days) and the reactivity in the Montenegro skin test (negative or positive – $MT^-$ or $MT^+$). Using this approach, we have further categorized the LCL into six subgroups referred as: L-1 and L2–4, ≤60 and >60 as well as $MT^-$ and $MT^+$ and compared with the NI control group.

The phenotypic features of neutrophils and monocytes in LCL subgroups are illustrated by Fig. 3. Data analysis demonstrated an overall decrease in the expression of CD16 by neutrophils in all LCL subgroups as compared to NI. However, increased frequency of HLA-$DR^+$ neutrophils was selectively observed in L1, ≤60 and $MT^+$ as compared to NI. The frequency of $CD23^+$ neutrophils was particularly increased in $MT^+$ subgroup in comparison with NI, with analogous increase regardless the number and the time of lesion. Further analysis revealed that the frequency of $CD28^+$ neutrophils was selectively increased in L1 and ≤60 as compared to NI (Fig. 3 – top panel). Analysis of monocytes showed significant decrease in the frequency of HLA-$DR^+$ cells particularly in L1 in comparison with NI. Moreover, the frequency of $CD23^+$ monocytes was particularly increased in $MT^+$ subgroup in comparison with NI, with analogous increase regardless the number and the time of lesion (Fig. 3 – middle panel). The analysis of eosinophil fenotypic features did not show any significant differences (data not shown).

The analyses of serum $NO_2^-$ + $NO_3^-$ in LCL subgroups are shown in the bottom panel of Fig. 3. Increased levels of serum $NO_2^-$ + $NO_3^-$ were observed in L1, >60 and $MT^+$ as compared to NI.

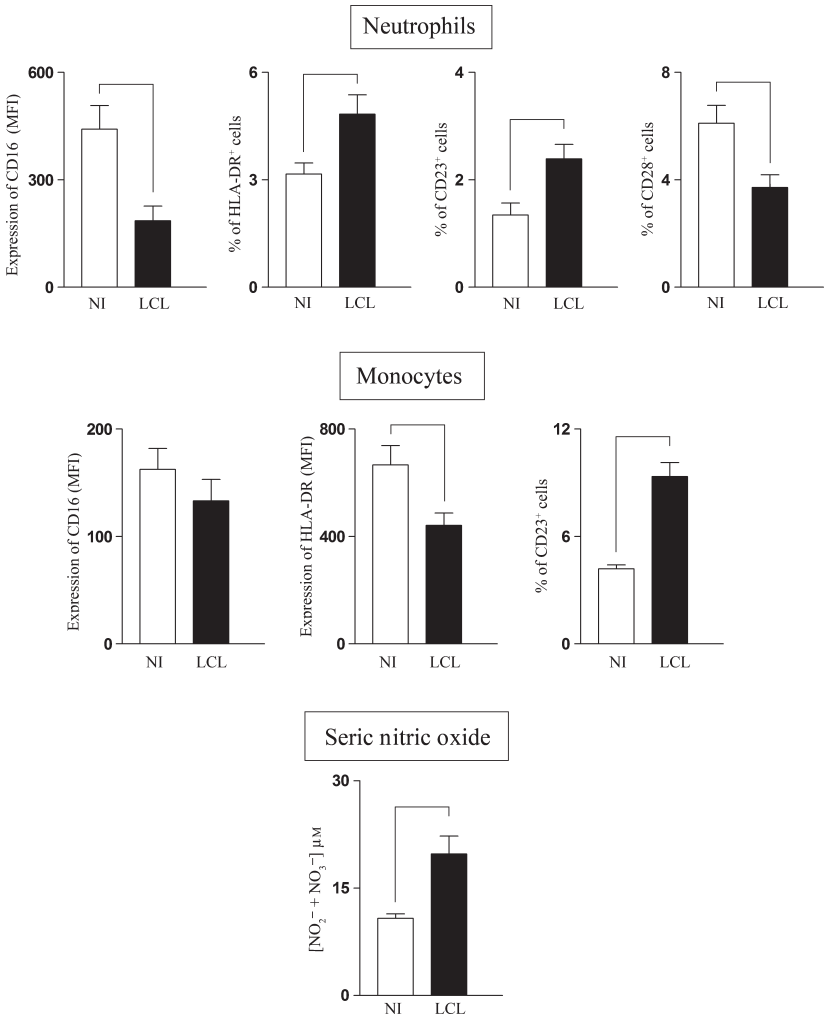


**Figure** 1 Phenotypic features peripheral blood phagocytes (neutrophils, top panels and monocytes, middle panels) and serum nitric oxide levels (bottom panels) in patients with localized cutaneous leishmaniasis (LCL = ■) and non-infected individuals (NI = □). Phenotypic studies were performed by a double-labelling protocol as described in material and methods. The results are expressed as mean percentage (%) ± standard error of neutrophils (HLA-DR$^+$, CD23$^+$ and CD28$^+$) and monocytes (CD23$^+$). Phenotypic features presenting unimodal distribution were expressed as the average of the mean fluorescence intensity (MFI) ± standard error for CD16 in neutrophils and macrophage-like cells and HLA-DR by monocytes. Serum levels of nitric oxide were expressed as mean concentration ($\mu$M) ± standard error of $NO_2^-$ + $NO_3^-$. Significant differences at $P < 0.05$ are highlighted by connecting lines.

The phenotypic features of the adaptive immunity in LCL subgroups are shown in Fig. 4. Data analysis demonstrated increased frequency of CD3$^+$ T cells in L1, ≤60 and MT$^+$ in comparison with NI. Significant increase of CD4$^+$ T cell subset was observed selectively in L1 and ≤60, whereas CD8$^+$ T cells was increased in L1, >60 and MT$^+$ as compared to NI (Fig. 4 – top panels).

The analysis of the activation status of T cells demonstrated an increased frequency of CD3$^+$HLA-DR$^+$, both CD4$^+$ and CD8$^+$ subsets in all LCL subgroups in comparison with NI (Fig. 4 – top panels). Furthermore, the analysis of CD8$^+$HLA-DR$^+$/CD4$^+$HLA-DR$^+$ ratio showed an increased levels selectively in the L1, >60 and MT$^+$ as compared to NI (Fig. 4 – top panels).

Increased levels of B cells, particularly CD5$^-$ B cells were observed in L2–4 in comparison with L1 and NI, with no differences amongst the other LCL subgroups and the NI control group (Fig. 4 – left bottom panels). The activation status of B lymphocytes was increased in all LCL subgroups as compared to NI (Fig. 4 – left bottom panels).

The analysis of anti-*Leishmania* IgG reactivity, assessed by conventional (IFA) and non-conventional (FC-ALPA) methods, revealed that although all LCL subgroups presented higher levels of specific antibodies as compared to non-infected subjects, L2–4 and >60 subgroups showed significant higher levels of antilive *Leishmania* IgG detected by the non-conventional (FC-ALPA) method (Fig. 4 – right bottom panels).

The Fig. 5 displays a representative diagram that summarizes all significant differences observed in the LCL subgroups. This assembled analysis allows the identification of multiple changes in phenotypic features of cellular and humoral immune compartments observed in all patients with LCL (Fig. 5 – left panel) and also those selectively found in LCL subgroups, categorized as L1 and L2–4, ≤60 and >60, and MT$^-$ and MT$^+$. Our findings highlighted that several phenotypic changes were intrinsic to the LCL infection (intersection), whereas other were selectively associated with distinct clinical and laboratorial parameters of the LCL subgroups (outer section). Although the activation of neutrophils, monocytes,

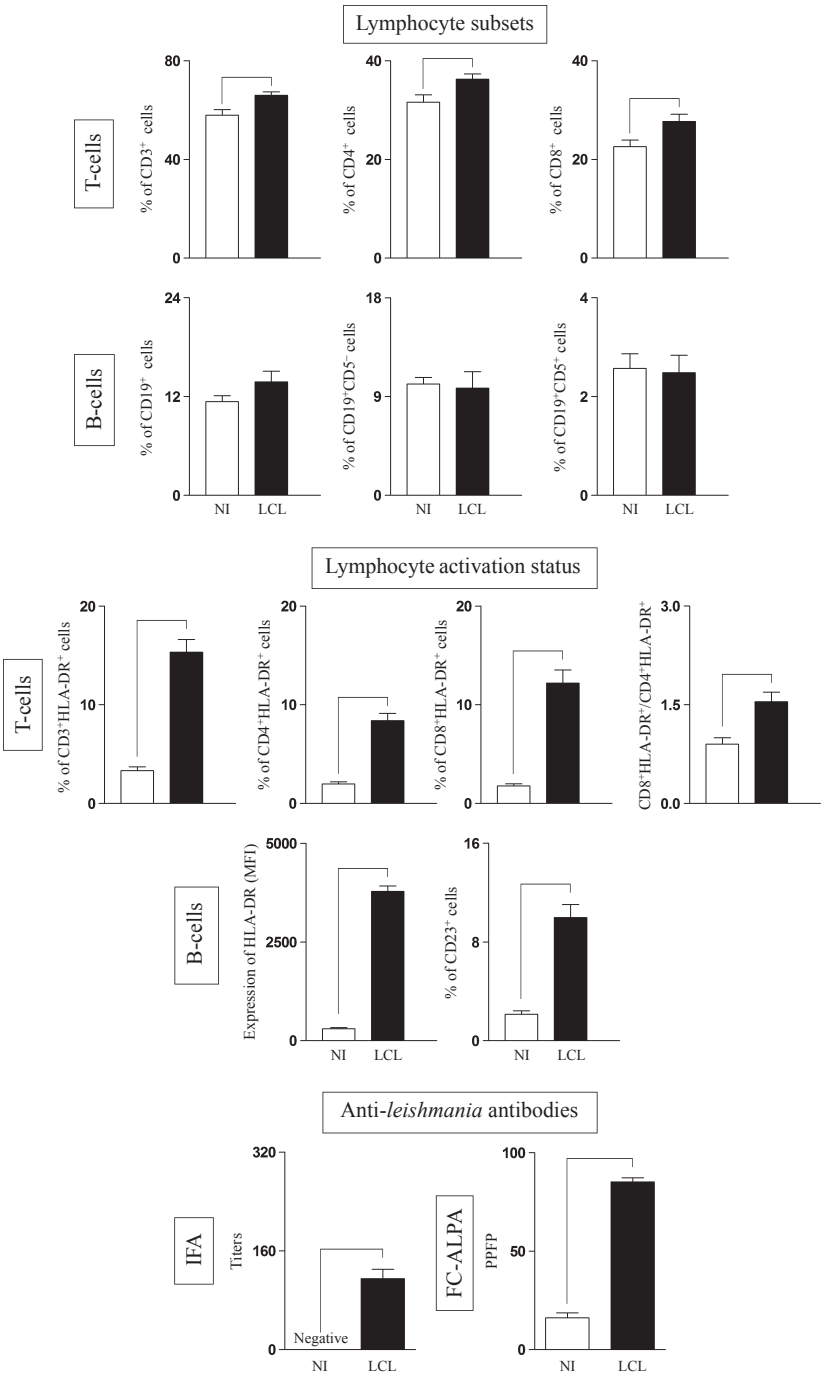


**Figure 2** Analysis of T and B cell subsets (top panels), lymphocyte activation status (middle panels) and anti-*Leishmania* IgG reactivity (bottom panel) in the peripheral blood of patients with localized cutaneous leishmaniasis (LCL = ■) and non-infected individuals (NI = □). Phenotypic studies were performed by a double-labelling protocol as described in material and methods. The results are expressed as mean percentage (%) ± standard error of T cells ($CD3^+$), T cell subsets ($CD4^+$ and $CD8^+$), B cells ($CD19^+$) and B cell subsets ($CD5^-$ and $CD5^+$). Activation status was expressed as mean percentage (%) ± standard error for HLA-$DR^+$ T cell subsets, $CD4^+$HLA-$DR^+$/$CD8^+$HLA-$DR^+$ T cell ratio and $CD23^+$ B lymphocyte subsets and as the average of mean fluorescence intensity (MFI) ± standard error for HLA-DR expression by B cells. Anti-*Leishmania* IgG reactivity was measured by conventional immunofluorescence assay (IFA) and by flow cytometric analysis of antilive *L. braziliensis* promastigote antibodies (FC-ALPA) as described in material and methods. Significant differences at $P < 0.05$ are highlighted by connecting lines.

T cells and B lymphocytes is a common feature in all LCL subgroups, particularities could be found as follows: (1) L1 was essentially characterized by phenotypic features associated with enhanced cellular immunity (innate and adaptive), whilst L2–4 was mostly marked by increased humoral immune response (Fig. 5 – top rings); (2) recent lesions (≤60) was characterized by mixed profile of innate and adaptive immunity, whereas late lesion (>60) was featured by changes in adaptive immunity with prominent activation of $CD8^+$ T cells (Fig. 5 – middle rings); and (3) $MT^+$ patients displayed increased putative leishmanicidal capacity, mainly related to phenotypic changes in neutrophils, monocytes and $CD8^+$ T cells (Fig. 5 – bottom rings).

## Discussion

The mechanisms linking the immune response to distinct clinical/laboratorial status of LCL are incompletely understood. The aim of the present work was to assess

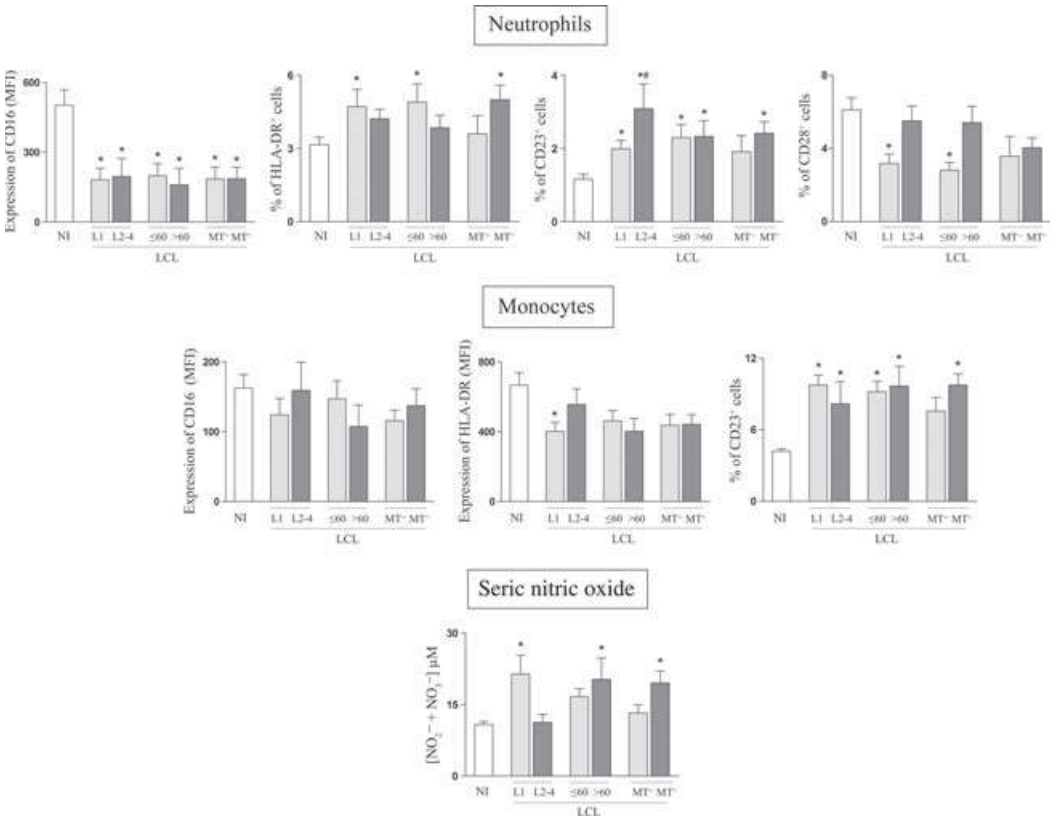


**Figure 3** Differential phenotypic patterns of peripheral blood phagocytes (neutrophils, top panel and monocytes, middle panel) and serum nitric oxide levels (botton panel) in patients with localized cutaneous leishmaniasis according to their clinical status (L1, ≤60 and $MT^-$ = ▭ and L2–4, >60 and $MT^+$ = ▬) as compared to non-infected individuals (NI = ▭). Phenotypic studies were performed by a double-labelling protocol as described in material and methods. The results are expressed as mean percentage (%) ± standard error of neutrophils ($HLA\text{-}DR^+$, $CD23^+$ and $CD28^+$) and monocytes ($CD23^+$). Phenotypic features presenting unimodal distribution were expressed as the average of the mean fluorescence intensity (MFI) ± standard error for CD16 in neutrophils and macrophage-like cells and HLA-DR by monocytes. Serum levels of nitric oxide were expressed as mean concentration ($\mu$M) ± standard error of $NO_2^- + rmNO_3^-$. Significant differences at $P < 0.05$ are highlighted by * or # for comparisons with NI and L1, respectively.

whether the pattern of immune response detected in the peripheral blood of patients with LCL could be associated with the clinical status (number of lesions and duration of infection) and the positivity in the MT amongst Brazilian patients with cutaneous leishmaniasis.

The analysis of the systemic immune response during infection is a useful strategy for the study of the immunological mechanisms associated with parasitic/infectious diseases. Although particularities can be found at specific immunological sites suggesting the occurrence of compartmentalized immune response, the analysis of dermal inflammatory infiltrate in cutaneous leishmaniasis usually contains unrelated elements of immune response triggered by secondary infections. In the present investigation, we have tested the hypothesis that the analysis of phenotypic features of peripheral blood leucocytes represents a good strategy to decode the clinical status of LCL. Our findings suggested that the immunological events observed in the peripheral blood could represent relevant biomarkers associated with the clinical status of patients with LCL, because several immunological features were closely related to the putative microenvironment observed in the LCL clinical subgroups.

The broad clinical spectrum observed amongst ACL patients has been considered one of most interesting features in viewing of the complex interaction process between human immune response and *Leishmania* parasites. In this context, three major clinical manifestations have been considered, including LCL, mucocutaneous leishmaniasis and anergic diffuse cutaneous leishmaniasis. Although the LCL is defined as one clinical unit, it has been observed that LCL comprises a range of distinct clinical presentations as we considered acute/chronic [37], single/multiple [25] and typical/atypical lesions [38]. Little is known about the heterogeneity of immune response associated with distinct status of LCL, such as time and number of lesion as well as the laboratorial records regarding the positivity in MT.

In this study, we have evaluated the immunological status of patients with LCL categorized according to their

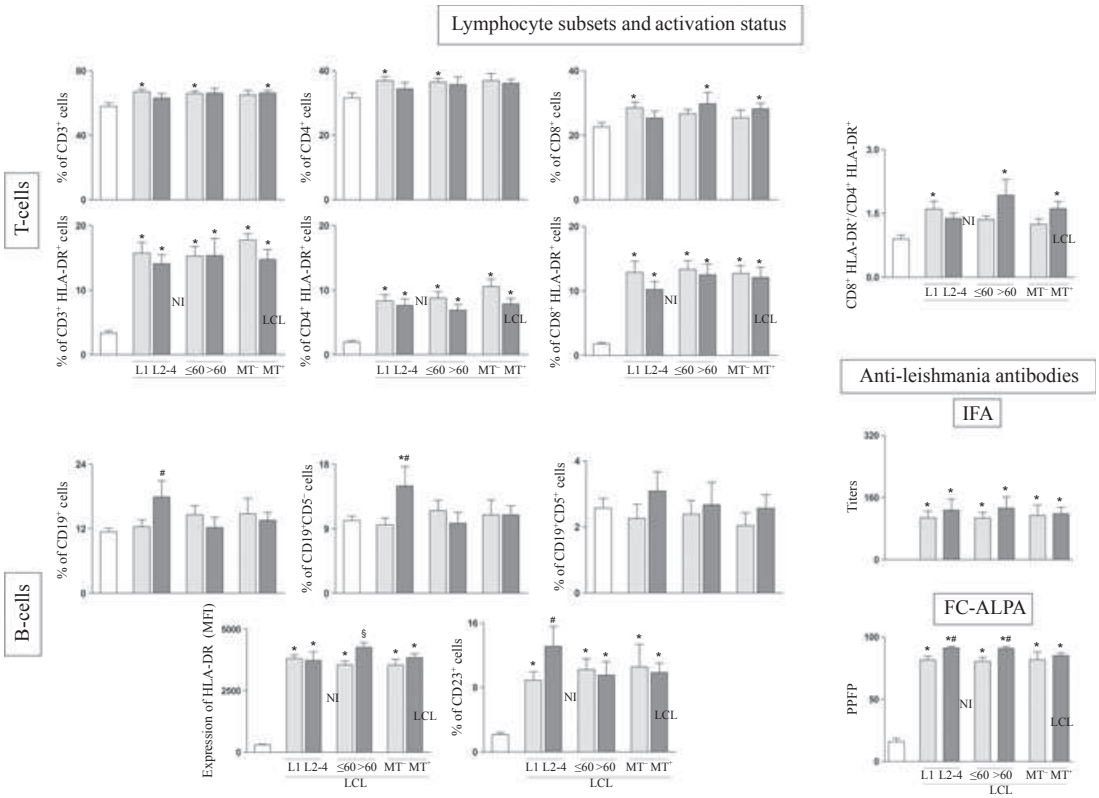


**Figure** 4 Differential frequency/activation status of T cells (top panels) and B cells (left bottom panels) along with anti-*Leishmania* IgG reactivity (right bottom panels) in the peripheral blood of patients with localized cutaneous leishmaniasis according to their clinical status (L1, ≤60, $MT^-$ = ☐ and L2–4, >60, $MT^+$ = ■) as compared to non-infected individuals (NI = ☐). Phenotypic studies were performed by a double-labelling protocol as described in material and methods. The results are expressed as mean percentage (%) ± standard error of T cells ($CD3^+$), T cell subsets ($CD4^+$ and $CD8^+$), B cells ($CD19^+$) and B cell subsets ($CD5^-$ and $CD5^+$). Activation status was expressed as mean percentage (%) ± standard error for HLA-$DR^+$ T cell subsets, $CD4^+$HLA-$DR^+$/$CD8^+$HLA-$DR^+$ T cell ratio and $CD23^+$ B lymphocyte subsets and as the average of mean fluorescence intensity (MFI) ± standard error for HLA-DR expression by B cells. Anti-*Leishmania* IgG reactivity was measured by conventional immunofluorescence assay (IFA) and by flow cytometric analysis of antilive *L. braziliensis* promastigote antibodies (FC-ALPA) as described in material and methods. Significant differences at $P < 0.05$ are highlighted by *, # or § for comparisons with NI, L1 and ≤60, respectively.

clinical/laboratorial status, including the number of lesions (L1 and L2–4), days of illness duration (≤60 and >60) and the positivity in the Montenegro skin test ($MT^-$ and $MT^+$). The general hypothesis is that particularities in the host immune response microenvironment can be observed amongst patients with LCL that are direct associated with their clinical/laboratorial scores. Thus, this investigation may provide novel immunological biomarkers applicable in LCL clinical investigations.

Our data demonstrated that both L1 and L2–4 patients presented an overall increased activation status of neutrophils ($CD23^+$) with concomitant decrease of CD16 expression by these cells along with increased levels of $CD23^+$monocytes, activated T cells ($CD4^+$ and $CD8^+$ expressing HLA-DR) and B cells (CD23 and HLA-DR). Overall, these findings demonstrated that both L1 and L2–4 presented a putative anti-*Leishmania* activity mediated by neutrophils, monocytes and T cells. In general, the phenotypic features of neutrophils show a mixed pattern of cell surface markers expression associated with activation/modulation events. Although the upregulation of HLA-DR by neutrophils has been considered an activation maker because of its ability to mediate T cell antigen presentation [39, 40], the downregulation of CD16 could represent a selective suppression of effector neutrophil function. However, it has been suggested that although the CD16 plays a major role in the secretion of toxic products in response to immune complex, little or no role of CD16 has been demonstrated in the neutrophil phagocytic capacity [41]. Therefore, it seems that the decreased expression of CD16 by neutrophils from patients with LCL does not necessarily refer to impaired anti-*Leishmania* activity.

One important phenotypic features observed between L1 and L2–4 was a dicothomic profile of neutrophils activation with enhanced HLA-DR been observed in L1 and outstanding expression of CD23 observed in L2–4. As previously mentioned, human neutrophils are able to express HLA-DR, molecule involved in antigen presentation to T cells [39, 40]. It has been also reported that

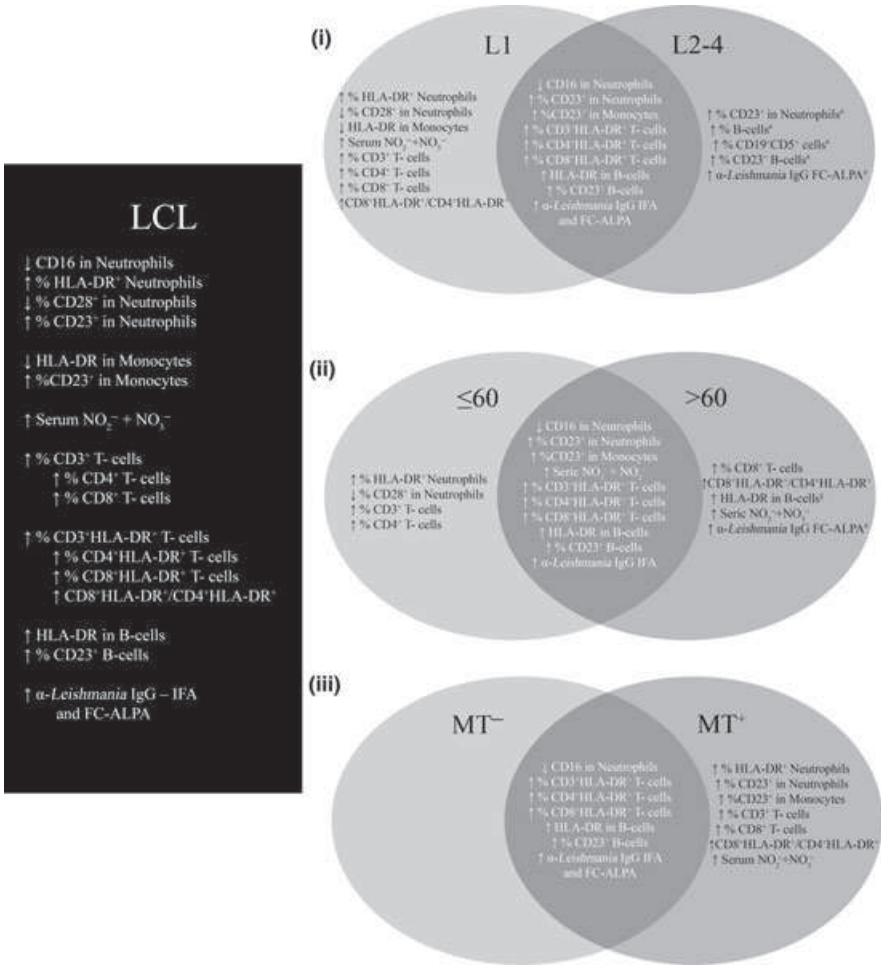


**Figure 5** Cross-sectional analysis of cellular and humoral immune response in the peripheral blood of patients with localized cutaneous leishmaniasis (LCL) (left panel = ■), following categorization according to their clinical status (right ring: L1, ≤60 or $MT^-$ = ◯ and left ring: L2–4, >60 or $MT^+$ = ◯) as compared to NI. This strategy illustrates the intrinsic phenotypic profile of patients with LCL (intersection) and also highlights those selectively associated with distinct clinical/laboratorial parameters of LCL subgroups (outer section), including: (i) the enhanced cellular immunity and nitric oxide in L1 versus increased humoral immune response in L2–4; (ii) mixed profile of innate and adaptive immunity in ≤60 versus increase levels of nitric oxide and prominent activation of $CD8^+$ T cells >60; (iii) increased putative leishmanicidal capacity $MT^+$ mainly related to enhanced nitric oxide levels and activation of neutrophils, monocytes and $CD8^+$ T cells. The arrows represent increase (↑) or decrease (↓) of a given cell phenotype.

HLA-DR expression by neutrophils is related to its ability to release nitric oxide [42, 43]. Our findings lead us to speculate that HLA-DR expressed on human neutrophils from L1 could be involved either in neutrophil activation or in the antigen presentation to T cells and on its ability to produce NO. In contrast, an outstanding frequency of and $CD23^+$ neutrophils was selectively observed in L2–4. The expression of CD23 by neutrophils has been considered a sign of chronic activation [44]. At the moment, we do not know the role of CD23 expression by neutrophils in L2–4 patients and the mechanisms underlying its regulation. Further investigation to define which isoform of CD23 is present on neutrophils from L2 to 4 would help to better understand this phenomenon. It was reported that the two isoforms of CD23 are associated with different signals: isoform B was associated with nitric oxide (NO) release and cytotoxicity [45], whereas isoform A to the antigen presentation to specific T cells after internalization [46, 47].

As far as the adaptive immunity compartment, our data demonstrated that L1 presented a general upregulation of T cell activation and higher levels of NO, whereas L2–4 presented enhanced levels of activated B cells and anti-*Leishmania* IgG reactivity. At the moment, we do not know the cytokine pattern associated with L1 and L2–4, but our findings lead us to speculate that as L1 presented a prominent cellular immune response, it is likely that L1 present a predominant pro-inflammatory [48] response based on the phenotypic profile observed. In contrast, L2–4 showed an outstanding B cell activation, suggestive of an antibody-mediated immune response. Corroborating this hypothesis, our data demonstrated

that L2–4 presented higher levels of antilive promastigote IgG. It has been previously reported that IgG titres are significantly higher in patients with multiple lesions as compared to those with single lesions [49]. Although there is no well-established scoring for LCL morbidity amongst the clinical spectrum of cutaneous leishmaniais, it is plausible to consider the number of lesions, the lesion size and the lesion duration a putative biomarker for debilitating disease. In this context, our findings support the general hypothesis that the humoral immune response plays a negative role in cutaneous leishmaniasis, as showed by the putative relationship between the number of lesions and the higher activation status of B cells and the increasing titres of anti-Leishmania IgG. It is possible that this type 2 immune profile may count to lower response to therapeutic intervention in L2–4. At the moment, there are no differences in the medical guidelines to treat single and multiple lesions. However, it has been proposed that novel guidelines for the treatment of LCL must be drawn, and a worldwide policy should be implemented taking into consideration all factors that affect the outcome of treatment, such as the strain and susceptibility of the parasite, the severity of the disease as indicated by the size and number of lesions, as well as the patients characteristics including their immune system status [50].

An interesting clinical feature that we have also addressed in this study was the time of lesion. Patients with LCL were categorized in ≤60 and >60 as a strategy to investigate early and late immunological events triggered by the *Leishmania* infection. Our data demonstrate that although both subgroups (≤60 and >60) presented evidences for immunological activation at innate and adaptive immunity, those patients with early lesions presented a mixed pattern of activation/modulation features of innate immunity characterized by the upregulation of HLA-DR and downregulation of CD28 in neutrophils, whereas >60 showed a predominance of adaptive immunity illustrated by enhanced T cell and B cell activation along with increased levels of anti-*Leishmania* IgG and NO levels. Evidence has being accumulated, suggesting that innate immune response plays a pivotal role during early events of *Leishmania* infection. In this sense, neutrophils and macrophages mediate either host protective or disease-promoting roles according activation profile during the infection [51]. It has been reported that CD28 play an important role in the regulation of IL-8RA expression and neutrophils chemotactic migration [52] and that CD28-cross-linking on neutrophils may result in the release of IFN-$\gamma$, which restricts *Leishmania* growth and modulates $CD4^+$ T cells cytokine secretion [53]. Together with our findings, these data may suggest that patients with LCL, particularly those with recent lesion (≤60), displayed lower neutrophil-mediated ability to restrict the *Leishmania* infection at least using the CD28 pathway. On the other hand, patients with chronic lesion already presented a well-established adaptive immune response, important to control the parasite growth and to develop protective mechanisms during *Leishmania* infection. In human infection, some differences were observed on the cytokine profile when patients with recent lesions were compared with those with chronic lesions [17, 26]. It has been demonstrated that patients with recent lesions presented an overall modulated cytokine pattern mediated by IL-4 and IL-10 [36]. Together with these studies, our findings demonstrated that neutrophils from patients with early lesions presented a typical susceptibility phenotype (low CD28 expression), consistent with the hypothesis that changes on the immune response early in infection, especially on neutrophils, favour the parasite multiplication and the disease onset [54, 55]. On the other hand, it has been reported that patients with chronic lesions presented a predominant pro-inflammatory profile characterized by enhanced levels of IFN-$\gamma$ and TNF-$\alpha$, consistent with our findings of outstanding cellular activation of $CD8^+$ T cells and enhanced NO secretion.

In general, in LCL, the presence of a strong cellular immune response characterized by a positive MT has been considered a good prognosis [56]. Aiming to investigate whether the positivity in the MT is associated with a particular peripheral blood immunological status, we have compared the phenotypic features of patients with LCL categorized as $MT^-$ and $MT^+$. Our data demonstrated that both $MT^-$ and $MT^+$ displayed increased levels of activated T and B cells. However, the $MT^+$ subgroup showed a predominant $CD8^+HLA\text{-}DR^+$/$CD4^+HLA\text{-}DR^+$ T cells ratio, suggesting an outstanding innate cellular immune response. Moreover, these patients also presented increased levels of NO along with enhanced frequency of $CD23^+$ neutrophils and monocytes. CD23 expression by human macrophages may contribute to the anti-*Leishmania* activity through a previously described NO/TNF-$\alpha$-dependent pathway [57]. It has been also demonstrated that IgE immune complexes binding via CD23 induces *Leishmania* killing by human monocyte/macrophages through the induction of the L-arginine/NO pathway [58].

In conclusion, this study establishes that the complex immunological network observed in patients with LCL can be better understood when analysed under the perspective of distinct patterns of clinical/laboratorial features. We have identified a set of immunological biomarkers in the innate and adaptive immunity applicable in LCL clinical investigations.

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## Conflicts of interest

Three of the authors (V.P.M., A.T.C. and O.A.M.F.) are workers of Fundação Oswaldo Cruz. Bias from competing interest was prevented by participation of two researchers, one from the Federal University of Minas Gerais (W.M.) and other from the Federal University of Ouro Preto (R.C.G.). In addition, other five independent university professionals are working as researchers in the field of infectious diseases. All other authors have no conflicts of interest.

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