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ORIGINAL INVESTIGATION

American tegumentary leishmaniasis: T-cell differentiation profile of cutaneous and mucosal forms—co-infection with *Trypanosoma cruzi*

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Abstract American tegumentary leishmaniasis displays two main clinical forms: cutaneous (CL) and mucosal (ML). ML is more resistant to treatment and displays a more severe and longer evolution. Since both forms are caused by the same *Leishmania* species, the immunological response of the host may be an important factor determining the evolution of the disease. Herein, we analyzed the differentiation and memory profile of peripheral $CD4^+$ and $CD8^+$ T lymphocytes of patients with CL and ML and their *Leishmania–T. cruzi* co-infected counterparts. We measured the expression of CD27, CD28, CD45RO, CD127, PD-1 and CD57, together with interferon-γ and perforin. A highly differentiated phenotype was reflected on both T subsets in ML and preferentially on $CD8^+$ T cells in CL. A positive trend toward a higher T differentiation profile was found in *T. cruzi*-infected CL and ML patients as compared with *Leishmania* single infections. Association between $CD8^+$ T-cell differentiation and illness duration was found within the first year of infection, with progressive increase of highly differentiated markers over time. Follow-up of patients with good response to therapy showed predominance of early differentiated $CD8^+$ T cells and decrease of highly differentiated cells, while patients with frequent relapses presented the opposite pattern. $CD8^+$ T cells showed the most striking changes in their phenotype during leishmaniasis. Patients with long-term infections showed the highest differentiated degree implying a relation between T differentiation and parasite persistence. Distinct patterns of $CD8^+$ T differentiation during follow-up of different clinical outcomes suggest the usefulness of this analysis in the characterization of *Leishmania*-infected patients.

Keywords Cutaneous leishmaniasis · Mucosal leishmaniasis · Peripheral T lymphocytes · Differentiation and memory phenotype · *Leishmania–T. cruzi* co-infection

Introduction

Leishmaniasis is a parasitic infection caused by different *Leishmania* species (spp.), an obligated intracellular protozoon transmitted by Phlebotomine sandflies. It is endemic in 88 countries [1], and 350 million people are thought to be at risk of infection. Tegumentary leishmaniasis is prevalent in 82 countries of Africa, the Middle East and Latin America [2]. The clinical features of American tegumentary leishmaniasis (ATL) are diverse depending on the *Leishmania* (*L.*) spp. involved and on host factors, including the immune status. Cutaneous leishmaniasis (CL) generally manifests as a chronic, painless ulcer and is the most prevalent form, while mucocutaneous and mucosal leishmaniasis (ML) characterized by the destruction of oral-nasal-pharyngeal cavities are more aggressive forms occurring years after CL and are frequently associated with the spread of *L.* (*V.*) *braziliensis* [3–5].

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As with experimental *Leishmania* infection, results of studies about the role of cell-mediated immune responses in human leishmaniasis depend on the infecting *Leishmania* spp. and the clinical manifestations of infection. Antigens from *L.* (*V.*) *braziliensis* stimulate higher proliferative responses, interferon-γ (IFN-γ) production and increased numbers of multifunctional $CD4^+$ T cells than antigens from *L.* (*L.*) *amazonensis* [6, 7]. In relation to the clinical manifestations, ML and CL were associated with up-regulation of Th1 responses, whereas no pro-inflammatory responses have been found during diffuse leishmaniasis, the anergic form of ATL [6, 8, 9].

The question whether the phenotypic profile of T cells in patients with CL differs from the profile in patients with ML remains unknown. Herein, we analyzed differentiation receptors, memory markers and effector molecules associated with early differentiated or terminally differentiated T lymphocytes in patients with CL or ML during the symptomatic phase of the disease and different disease outcomes. We also evaluated patients harboring both *Leishmania* and *Trypanosoma* (*T.*) *cruzi* infections, as this co-infection is frequent in endemic areas of Argentina [10, 11]. Our results indicate that during the symptomatic phase of leishmaniasis, preferentially in ML but also in CL, the phenotype of $CD4^+$ and $CD8^+$ T lymphocytes is enriched in highly differentiated effector memory cells. In addition, a higher differentiated and memory T profile in CL and ML groups co-infected with *T. cruzi*, as compared with single *Leishmania* infections, was described. We found association between $CD8^+$ T-cell differentiation and illness duration within the first year of CL infection, with progressive increase of highly differentiated markers over time. The follow-up study of ML patients with good response to therapy showed predominance of early differentiated $CD8^+$ T cells and decrease of highly differentiated cells, while patients with frequent relapses had the opposite pattern of differentiation.

Materials and methods

Study groups

The study population comprised patients living in Salta province, an ATL endemic area from the Northwest of Argentina. Diagnosis of CL and ML was made on the basis of clinical evaluation; finding of amastigotes in Giemsa-stained lesion-scraping smears; parasite culture from lesion aspirates in blood agar base; detection of *Leishmania* DNA by using k-DNA based polymerase chain reaction (PCR) [12] and/or Montenegro skin reactivity test (MST). Briefly, for MST, 0.1 ml of the antigen (suspension of 6.25×10^6 promastigotes/ml of autoclaved *Leishmania mexicana pifanoi*) was intradermally injected into the anterior surface of the forearm and the induration diameter was measured 48 h later using a millimeter-graduated ruler and a ball point pen. Of 21 CL patients, 17 cases were diagnosed by positive visualization of parasites in Giemsa-stained lesion-scraping smears and 4 cases were diagnosed by positive *Leishmania* DNA detection by PCR. In the majority of those samples, diagnosis was accompanied by positive results using other diagnostic tests. Regarding to 27 ML patients, visualization of parasites plus positivity for other diagnostic assays accounted for 12 cases; diagnosis of 9 cases was achieved by positive PCR results alone or accompanied by positive cultures and/or MST, and the rest 6 cases were diagnosed by reactive MST (induration diameters over the cut-off value, ≥10 mm) supported by epidemiological and clinical characteristics compatible with leishmaniasis. Differential diagnose entities were also evaluated and ruled out. The control group included 5 women and 8 men, with a median age of 34 years (range 22–65 years). Clinical characteristics of CL and ML patients are shown in Tables 1 and 2.

Ethics statement

The present study was approved by the Ethics Committee of the Facultad de Ciencias de la Salud, Universidad Nacional de Salta (UNSA) and the Ethics Committee of the Academia Nacional de Medicina. Written informed consent was obtained from each participant.

Leishmania spp. identification

Samples obtained from skin/mucosal lesions were placed in tubes containing 300 µl TE, boiled for 10 min and stored at –20 °C until use. DNA extraction from culture isolated parasites, obtained by aspiration of cutaneous/mucosal lesions, was performed with the Wizard Genomic DNA purification kit (Promega, Wisconsin, USA). DNA extraction from lesion-scrapings was performed by lysis with 1 % SDS buffer at 60 °C, 100 ug/ml proteinase K; 100 ug/ml ARNase followed by phenol–chloroform-ethanol DNA extraction.

Polymorphism-specific PCR (PS-PCR) was performed first using the primers V1–V2 and L1–L2 for the subgenera identification, *V.* (*Viannia*) and *L.* (*Leishmania*); and then, using specific primers for the identification of *Leishmania* spp., as reported elsewhere [13].

Blood sampling procedure

Plasma samples were obtained by centrifugation of 20 ml EDTA anticoagulated blood, and aliquots were maintained at −80 °C for anti-*T. cruzi* antibodies analysis. Then, saline solution (0.9 % v/w of NaCl) was added to dilute the EDTA anticoagulated blood sample, and peripheral

Table 1 Characteristics of cutaneous leishmaniasis patients

Patient ID	Gender	Age	*Leishmania* (*L.*) sp.	Anti-*T. cruzi* antibodies	Clinical ATL presentation Cutaneous form	Illness duration[a]	Previous etiological treatment
1	M	27	*L. braziliensis*	−	Localized lesions (2), arm	1 month	No
2	M	26	*L. braziliensis*	−	Localized lesions (2), arm, back	2 months	No
3	M	26	*L. braziliensis*	−	Localized lesion (1), ankle	3 months	No
4	F	33	*L. braziliensis*	−	Localized lesions (2), neck	3 months	No
5	M	49	*L. braziliensis*	−	Localized lesion (1), arm	4 months	No
6	F	15	*L. braziliensis*	−	Localized lesion (1), leg	5 months	No
7	M	60	*L. braziliensis*	−	Localized lesions (2), leg	4 years	No
8	M	35	*L. amazonensis*	−	Localized lesion (1), foot	15 days	No
9	M	40	*L. amazonensis*	−	Localized lesion (1), leg	8 months	No
10	M	37	Undefined *L.* sp.	−	Localized lesions (2), arm, leg	2 months	No
11	F	12	Undefined *L.* sp.	−	Localized lesion (1), leg	2 months	No
12	F	31	Undefined *L.* sp.	−	Localized lesion (1), leg	4 months	No
13	F	10	Undefined *L.* sp.	−	Localized lesion (1), leg	6 months	No
14	M	47	Undefined *L.* sp.	−	Multiple lesions	3 months	No
15	M	22	Undefined *L.* sp.	−	Multiple lesions. Reactivation	3 years	MA
16	M	50	Undefined *L.* sp.	−	Multiple lesions. Reactivation	6 years	MA
17	M	46	*L. braziliensis*	+	Localized lesion (1), hand	2 months	No
18	F	40	*L. braziliensis*	+	Localized lesion (1), forearm	8 months	No
19	M	39	*L. braziliensis*	+	Multiple lesions	4 months	No
20	M	80	*L. amazonensis*	+	Localized lesions (2), hand, foot	6 years	No
21	M	30	Undefined *L.* sp.	+	Localized lesion (1), neck	6 months	No

ID identification, *ATL* American tegumentary leishmaniasis, *T. cruzi Trypanosoma cruzi*, *F* female, *M* male, *MA* meglumine antimoniate

[a] Since the appearance of primary cutaneous lesions to time of sample collection

blood mononuclear cells (PBMC) were obtained by Ficoll Paque Plus (GE Healthcare, Umea, Sweden) density gradient. After washing the samples with saline solution, cells were maintained on ice and suspended to 5×10^6 cells/ml in a solution containing 50 % RPMI 1640 tissue culture medium (Gibco, Grand Island, NY, USA) plus 50 % FCS (PAA Laboratories GmbH, Austria) and 10 % DMSO (Sigma-Aldrich, Missouri, USA). PBMC were cryopreserved in aliquots of 5×10^6 cells/ml and maintained at −140 °C until use.

Anti-*T. cruzi* antibodies analysis

Testing for anti-*T. cruzi* antibodies was carried out using a commercial enzyme-linked immunosorbent assay (ELISA) based on the use of recombinant *T. cruzi*-derived proteins as antigens (recombinant ELISA *v.3.0*, Wiener, Argentina). As described by the suppliers, antigens of the assay are obtained by recombinant DNA technologies from specific proteins of epimastigote and trypomastigote stages of *T. cruzi*, related to highly conserved areas between different strains. In a general population, including healthy individuals, donors, patients with Chagas disease and other pathologies, this method present a correlation of 99.6 % with respect to confirmatory tests. A result was considered positive if the absorbance of the tested sample resulted higher than the absorbance obtained for the negative control plus 0.300.

Leukocyte phenotype studies

T-cell markers were determined by flow cytometry. Fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin chlorophyll protein (PerCP) labeled, anti-CD27, anti-CD28, anti-CD127, anti-PD-1, anti-CD45RO, anti-CD57, anti-CD4, anti-CD8, anti-CD3 monoclonal antibodies (Becton–Dickinson, San José, CA, USA) were used at the concentrations recommended by the suppliers. Intracellular staining with FITC-anti-perforin (Becton–Dickinson) was carried out in PBMC previously permeated with Fix and Perm solution (Caltag Laboratories, Burlingame, CA). Cells were evaluated using a FACScan cytometer and FCS 3 Express, De Novo Software (Los Angeles, CA, USA).

Intracellular IFN-γ determination

Short time cell cultures were carried out in a 5 % CO_2 incubator. One million PBMC per ml of RPMI 1640

Table 2 Characteristics of mucosal leishmaniasis patients

Patient ID	Gender	Age	*Leishmania* (*L.*) sp.	Anti-*T. cruzi* antibodies	Clinical ATL presentation Mucosal form	Onset of ATL[a]	Mucosal illness duration[b]	Previous etiological treatment
22	M	32	*L. braziliensis*	−	NP lesions	Unknown	Unknown	No
23	M	13	*L. braziliensis*	−	NP lesions	No CL	7 years	No
24	M	74	*L. braziliensis*	−	Cutaneous and NP lesions. Relapses	6 years	6 years	MA, AB, MF, MA
25	M	27	Undefined *L.* sp.	−	NP lesions	19 years	7 years	No
26	F	69	Undefined *L.* sp.	−	NP lesions	20 years	2 years	No
27	F	45	Undefined *L.* sp.	−	NP lesions	40 years	30 years	No
28	F	30	Undefined *L.* sp.	−	NP lesions	No CL	2 years	No
29	M	14	Undefined *L.* sp.	−	NP lesions	1 year	6 months	MA
30	M	41	Undefined *L.* sp.	−	NP lesions	No CL	2 months	No
31	M	48	Undefined *L.* sp.	−	NP lesions	No CL	Several months	No
32	M	57	Undefined *L.* sp.	−	Cutaneous and NP lesions	3 years	4 months	No
33	M	71	Undefined *L.* sp.	−	Perforation of the septum, NP lesions	62 years	Unknown	No
34	M	66	Undefined *L.* sp.	−	Perforation of the septum, NP lesions. Relapses	40 years	Unknown	MA, MA
35	M	23	Undefined *L.* sp.	−	Palate and NP lesions	Unknown	Unknown	No
36	M	52	Undefined *L.* sp.	−	Palate and NP lesions. Relapses	Unknown	10 years	MA
37	M	53	Undefined *L.* sp.	−	NP lesions. Relapses	13 years	13 years	MA, MA, MF
38	M	39	*L. braziliensis*	+	Nasal lesions	25 years	1 year	No
39	M	44	*L. braziliensis*	+	NP lesions	13 years	5 years	No
40	F	54	*L. amazonensis*	+	NP lesions	40 years	1 year	No
41	M	52	*L. amazonensis*	+	NP lesions. Relapses	13 years	1 year	MA, MA
42	F	12	Undefined *L.* sp.	+	NP lesions	1 year	6 months	MA
43	F	35	Undefined *L.* sp.	+	NP lesions	1 year	6 months	MA
44	M	Unknown	Undefined *L.* sp.	+	NP lesions	13 years	3 years	No
45	M	39	Undefined *L.* sp.	+	Left nasal cavity lesions	15 years	Unknown	No
46	M	56	Undefined *L.* sp.	+	Perforation of the septum, NP lesions	30 years	Unknown	No
47	M	45	Undefined *L.* sp.	+	NP lesions. Relapses	35 years	11 years	AB, MF
48	M	42	Undefined *L.* sp.	+	NP lesions. Relapses	5 years	10 months	MA, MF, AB

ID identification, *ATL* American tegumentary leishmaniasis, *T. cruzi Trypanosoma cruzi*, *F* female, *M* male, *NP* nasopharyngeal lesions, *MA* meglumine antimoniate, *MF* miltefosine, *AB* amphotericin B

[a] Since the appearance of primary cutaneous lesions to time of sample collection

[b] Since the appearance of mucosal lesions to time of sample collection

culture medium plus 10 % FCS and 1 % antibiotics (penicillin/streptomycin) were placed into 24-well plates. PMA (Phorbol 12-Myristate 13-Acetate, Sigma-Aldrich, Missouri, USA) 5 ng/ml plus 1 µg/ml of ionomycin calcium salt (Sigma-Aldrich) and 1 µl/ml of Brefeldin (Golgi Plug, Becton–Dickinson) were added for 4 h. Cells were washed with PBS 1× and surface marker staining was carried out using FITC-anti-CD8 and PerCP-anti-CD3 monoclonal antibodies (Becton–Dickinson). Cells were fixed and permeated with Fix & Perm (Caltag Laboratories, Invitrogen, Carlsbad, CA, USA) following the supplier's instructions. Cells were washed and PE-anti-IFN-γ monoclonal antibodies or PE-IgG1 (Becton–Dickinson) were added at 4 °C for 1 h. The cells were evaluated using a FACScan cytometer and FCS 3 Express, De Novo Software (Los Angeles, CA, USA).

Statistical analysis

Analysis of qualitative data was done with chi-square test or Fisher's exact test. A level of $p < 0.05$ was accepted as being statistically significant. Continuous variables between two groups were compared with Mann–Whitney *U* test for unpaired values. Kruskal–Wallis test and Dunn's post-test were used to compare differences between three groups. One-way ANOVA with post-test for linear trend was used for trend analysis. Differences were considered to be statistically significant at $p < 0.05$. All bivariate continuous correlations were performed using standard linear regression. Data were analyzed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

Results

Characteristics of CL and ML patients

We tested peripheral blood samples of 48 patients with ATL from the Northwest of Argentina. Twenty-one patients presented CL while ML was found in 27 cases (Tables 1, 2).

In both groups, men predominated over women (i.e. 71 % of CL cases and 78 % of ML cases). CL patients were younger than ML patients ($p = 0.0394$).

The determination of the infecting *Leishmania* spp. could be assessed in 13 samples from the CL group and in 7 samples from the ML group. In the rest of the tested samples, negative PS-PCR results were obtained after DNA extraction from clinical samples due to scant number of parasites in chronic lesions (Sensitivity of PS-PCR: 10^4 parasites per 300 ml. Unpublished observations, Gonzalez Prieto AG). *L.* (*V.*) *braziliensis* was found in the majority of the studied samples followed by *L.* (*L.*) *amazonensis* in the rest of the samples.

As the Northwest of Argentina is also endemic for Chagas disease, the presence of *T. cruzi*-specific antibodies was also evaluated. Twenty-four and forty-one percent of plasma samples from CL patients and ML patients, respectively, were positive for anti-*T. cruzi*-specific antibodies. In the majority of CL patients, cases with localized CL (one or two lesions) predominated (81 %). In the ML group, more aggressive lesions included perforation of the septum in three cases, and palate lesions in two cases.

The duration of active cutaneous lesions, since their appearance until time of sample collection, could be confirmed in the 21 cases of CL with a median of 4 months (range 15 days–6 years). The duration of active mucosal lesions in ML could be established in 20 out of the 27 cases, and it was longer than the duration in active CL (median 2 years; range 2 months–30 years).

At the time of sample collection, 2/21 CL patients had received etiological treatment in the past, but showed reactivation of their lesions later on. Ten out of 27 patients of the ML group received therapy in the past either for primary cutaneous lesions and/or mucosal lesions, while seven of them suffered subsequent reactivation of their mucosal lesions.

Differentiation profile of T-cell subsets from patients with active CL and ML disease

Expression of CD27 and CD28 defines distinct populations of T cells: $CD27^+CD28^+$ early differentiated cells (ED); $CD27^+CD28^-$ or $CD27^-CD28^+$ intermediately differentiated cells (ID1 or ID2, respectively) and $CD27^-CD28^-$ late differentiated cells (LD). Herein, we analyzed the degree of differentiation of $CD4^+$ and $CD8^+$ T cells in active localized (1–2 lesions) CL patients and in ML patients, with no detectable antibodies for *T. cruzi* infection in comparison to healthy subjects (N). We performed the analysis of the entire CL and ML populations after verifying that no significant differences existed between *L.* (*V.*) *braziliensis*; *L.* (*L.*) *amazonensis* and undefined *L. spp.* CL and ML groups (Supplementary Tables 1–4).

We found statistical differences among the three studied groups (N, CL, ML) analyzing ED, ID2 and LD within $CD4^+$ and $CD8^+$ T cells (Fig. 1a, b). Within both T lymphocyte subsets, ML showed diminished percentages of ED and elevated numbers of LD compared with N (Fig. 1a, b), whereas ED $CD4^+$ and ED $CD8^+$ T cells tended to be lower in CL versus N and LD $CD8^+$ T cells were significantly higher in CL compared to N. ML showed higher numbers of ID2 $CD4^+$ and ID2 $CD8^+$ T cells compared with CL and N (Fig. 1a, b). These data are in accordance with the differences found between the three studied groups analyzing the expression of $CD127^+PD\text{-}1^-$ and the senescence marker CD57 (Fig. 1c, d). We found lower expression of $CD127^+PD\text{-}1^-$ T

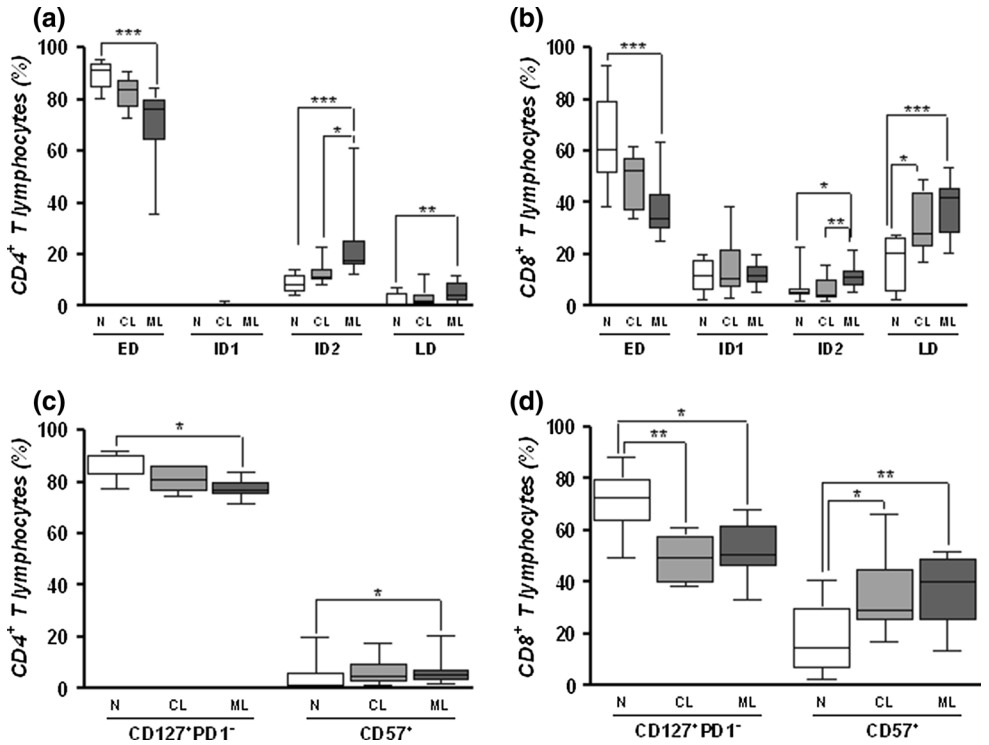


Fig. 1 Differentiation profile of total $CD4^+$ and $CD8^+$ T cells in individuals with American tegumentary leishmaniasis. Three clinical groups were compared for each set of markers: healthy subjects (N, *white boxes*, $n = 9$–13); patients with localized cutaneous leishmaniasis (CL, *light grey boxes*, $n = 7$–13); patients with mucosal leishmaniasis (ML, *dark grey boxes*, $n = 13$–16). **a** $CD4^+$ and **b** $CD8^+$ T-cell populations were gated and analyzed for CD27 versus CD28. Early differentiated cells (ED, $CD27^+CD28^+$); intermediately differentiated cells (ID1, $CD27^+CD28^-$; ID2, $CD27^-CD28^+$) and late differentiated cells (LD, $CD27^-CD28^-$). **c** $CD4^+$ and **d** $CD8^+$ T-cell populations were gated and analyzed for $CD127^+PD\text{-}1^-$ and $CD57^+$ expression. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ using Kruskal–Wallis test and Dunn's post-test. *Boxes* represent values between the 25th and 75th percentiles and medians; *bars* indicate 10th and 90th percentiles

cells and higher expression of the senescence marker CD57 on $CD4^+$ T cells in ML versus N (Fig. 1c). The same pattern of expression was observed on $CD8^+$ T cells in both CL and ML compared with N (Fig. 1d).

In order to better characterize highly differentiated T cells, we measured the effector molecules perforin and IFN-γ in $CD8^+$ T lymphocytes (Fig. 2). CL and ML showed increased percentages of the cytolytic molecule perforin in comparison to N (Fig. 2). Moreover, intracellular IFN-γ production was also increased after mitogen stimulation of PBMC from ML in contrast to the low IFN-γ production of N (Fig. 2).

As a whole, T cells in patients with ATL are characterized by a high degree of differentiation and effector capacity. Representative dot plots of CD27 and CD28 expression on gated $CD4^+$ and $CD8^+$ T cells are shown in Fig. 3a, b.

Memory profiles of T-cell subsets from patients with active localized CL and ML disease

The expression of CD27 in combination with CD45RO was also assessed to characterize the memory profile of T lymphocytes in patients with ATL (Fig. 4). CL and ML showed lower percentage of naïve cells ($CD27^+CD45RO^-$) and higher percentage of effector memory (EM) $CD27^-CD45RO^+$ and terminal effector (TE) $CD27^-CD45RO^-$ $CD8^+$ T cells than N (Fig. 4b). In the $CD4^+$ T-cell compartment, naïve cells from ML were also lower than N but, whereas EM was significantly increased in both groups of patients, TE remained unaltered in both CL and ML patients compared with N (Fig. 4a). Representative dot plots of CD27 and CD45RO expression on gated $CD4^+$ and $CD8^+$ T cells are shown in Fig. 3c, d.

Differentiation and memory profile in the context of two protozoan infections

Our cohort comprises patients with leishmaniasis, and patients with co-infection of *Leishmania* and *T. cruzi* (Tables 1, 2). As infection with *T. cruzi* is known to strongly modulate the differentiation profile of T cells, we compared the differentiation profile of T cells of patients with leishmaniasis according to the presence or absence of anti-*T. cruzi* antibodies in plasma.

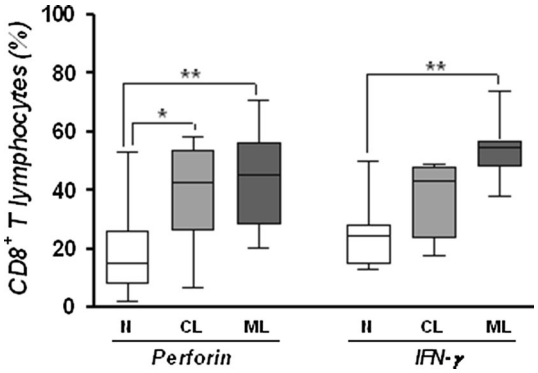


Fig. 2 Expression of effector molecules in $CD8^+$ T lymphocytes from patients with cutaneous and mucosal leishmaniasis. Three clinical groups were compared: healthy subjects (N, *white boxes*, $n = 10–12$); patients with localized cutaneous leishmaniasis (CL, *light grey boxes*, $n = 5–10$); patients with mucosal leishmaniasis (ML, *dark grey boxes*, $n = 7–10$). $*p < 0.05$; $**p < 0.01$ using Kruskal–Wallis test and Dunn's post-test. *Boxes* represent values between the 25th and 75th percentiles and medians; *bars* indicate 10th and 90th percentiles

Trypanosoma cruzi-infected CL patients (with localized cutaneous lesions) showed statistically significant reduction of ED $CD4^+$ T cells accompanied by higher numbers of ID2 $CD4^+$ T cells compared with localized CL patients (Table 3).

A positive trend toward a higher T differentiation and memory profile and a negative trend in the percentages of naïve and $CD127^+$ T cells in the $CD4^+$ and $CD8^+$ T-cell compartments was found in CL and ML co-infected (*Leishmania–T. cruzi*) groups regarding to their mono-infected (*Leishmania*) counterparts, and N was also recorded (Tables 3, 4). In accordance to the latter, the frequency of senescent $CD4^+$ and $CD8^+$ $CD57^+$ T cells was also increased in *T. cruzi*-infected CL patients and $CD8^+CD57^+$ T cells in *T. cruzi*- infected ML patients (Tables 3, 4, Trend analysis).

Association of T-cell differentiation degree, clinical manifestations and illness duration

We compared $CD8^+$ T-cell subsets from CL patients with localized lesions and CL patients with multiple (≥ 3) lesions (Table 1). The group with localized lesions showed significantly higher values of ED [49.45 % (20.92–75.80) vs. 18.24 % (8.09–53.53); $p = 0.0184$] and lower percentages of LD [29.37 % (10.40–55.72) vs. 51.77 % (36.17–77.28); $p = 0.0232$] and TE [21.68 % (6.15–62.68) vs. 42.45 % (29.61–67.15); $p = 0.0232$] than CL patients with multiple lesions. Analysis of $CD8^+$ T-cell subsets from ML patients with nasopharyngeal lesions and ML patients with more aggressive mucosal lesions and/or relapses (Table 2) demonstrated a significantly higher percentage of ID2 in the latter group [18.05 % (2.19–25.03) vs. 4.87 % (1.67–16.35); $p = 0.0024$]. We did not find statistical differences analyzing $CD4^+$ T cells from these CL and ML groups [data are expressed as median (range)].

We investigated the evolution of T-cell differentiation in regard to illness duration within the first year of localized CL infection, period which comprised the majority of the studied samples (88 %) from this group (Table 1). We found that ED $CD8^+$ T cells (Fig. 5a) inversely correlated with illness duration, while LD and TE $CD8^+$ T cells positively correlated with duration of infection (Fig. 5b, c). We did not find any correlation between $CD4^+$ T-cell subsets and illness duration. Then, we examined the correlation of $CD4^+$ and $CD8^+$ T-cell differentiation phenotype of ML patients, with time since the appearance of primary cutaneous lesions (Fig. 5d–f), and with mucosal illness duration. Both situations comprised much longer periods of time (Table 2), and no associations were found between variables in these cases.

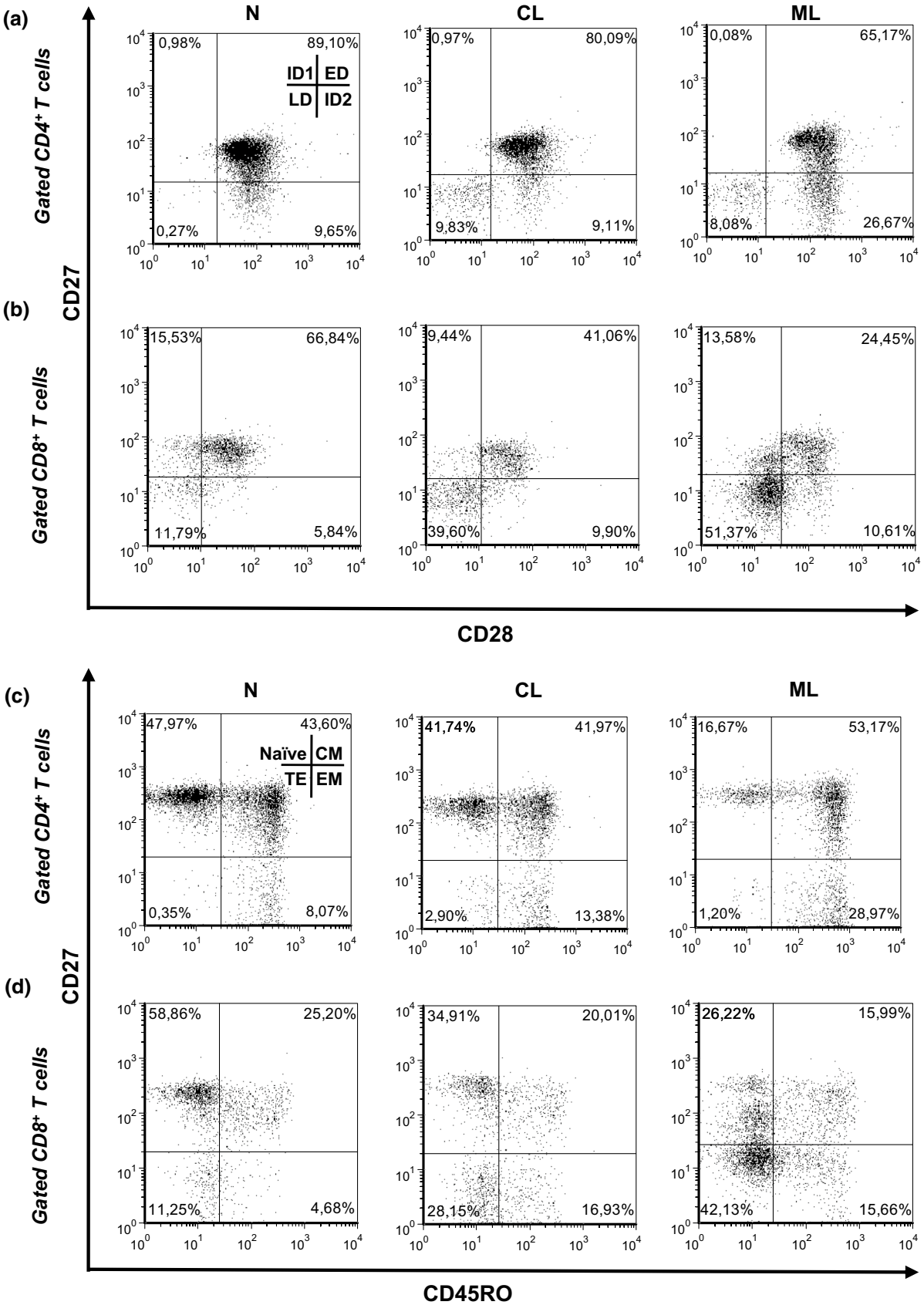
Longitudinal analysis of the T differentiation and memory profiles of patients with different ML disease outcome

We performed a follow-up of six patients (P) whose clinical characteristics are described in Table 2. P29, P40 and P43 showed good response to therapy and recovery of their mucosal lesions. P47, P48 and P24 did not respond to different therapeutic regimens and subsequently suffered reactivation of their mucosal lesions.

Patients who could achieve recovery showed a predominance of ED $CD8^+$ T cells prior to treatment and during the post-treatment follow-up (i.e. P29 and P40 in Fig. 6a) or increased after therapy administration (i.e. P43 in Fig. 6a). In contrast, LD were diminished prior and after treatment (i.e. P29 and P40 in Fig. 6a) or decreased after recovery (i.e. P43 in Fig. 6a).Besides, diminished percentages of ID2 were observed in these three cases (Fig. 6a).

Patients who suffered frequent relapses showed lower percentages of ED $CD8^+$ T cells prior and after treatment, and they were markedly diminished in P47 (Fig. 6b). LD presented intermediate or increased values, mainly in P47, while ID2 were low in the studied period. Interestingly, P47 could heal his lesions at 39 weeks of follow-up, after the administration of two etiological treatments: Amphotericin B and Miltefosine, but at that point a $CD8^+$ T highly differentiated profile still persisted (Fig. 6b).

Analyzing memory receptors (Fig. 7), variable percentages of naïve $CD8^+$ T cells were found in the six cases. Recovered ML patients showed low EM and TE $CD8^+$ T cells prior to treatment and even tended to further decrease post-treatment (Fig. 7a). In contrast, patients with frequent relapses showed predominance of EM or TE cells (i.e. P24 and P47 Fig. 7b).


(a)
N
CL
ML
Gated CD4+ T cells
0,98%
89,10%
ID1
ED
LD
ID2
0,27%
9,65%
0,97%
80,09%
9,83%
9,11%
0,08%
65,17%
8,08%
26,67%
(b)
Gated CD8+ T cells
15,53%
66,84%
11,79%
5,84%
9,44%
41,06%
39,60%
9,90%
13,58%
24,45%
51,37%
10,61%
CD27
CD28
(c)
N
CL
ML
Gated CD4+ T cells
47,97%
43,60%
Naïve
CM
TE
EM
0,35%
8,07%
41,74%
41,97%
2,90%
13,38%
16,67%
53,17%
1,20%
28,97%
(d)
Gated CD8+ T cells
58,86%
25,20%
11,25%
4,68%
34,91%
20,01%
28,15%
16,93%
26,22%
15,99%
42,13%
15,66%
CD27
CD45RO

◀ **Fig. 3** Degree of expression of T differentiation and memory markers in cutaneous and mucosal leishmaniasis. Representative dot plots from a healthy subject (N); a localized cutaneous leishmaniasis patient (CL, P9, Table 1) and a mucosal leishmaniasis patient (ML, P26, Table 2) are shown. Lymphocytes were gated by forward and side light scatter and subsequently $CD4^+$ or $CD8^+$ T cells were selected. The upper graphs show CD27 and CD28 staining of gated $CD4^+$ T cells (**a**) and $CD8^+$ T cells (**b**). The *lower graphs* show CD27 and CD45RO staining of gated $CD4^+$ T cells (**c**) and gated $CD8^+$ T cells (**d**). Early differentiated (ED), intermediately differentiated (ID1; ID2), late differentiated (LD), central memory (CM), effector memory (EM), terminal effector (TE) T cells

We did not find differences between both groups of patients in the follow-up of $CD4^+$ T-cell compartment. We found predominance of ED accompanied with diminished numbers of ID2 and LD, variable percentages of naïve cells and low counts of TE and EM in the follow-up of patients who recovered and those who did not.

Discussion

Leishmaniasis is considered a re-emergent disease with increased incidence in various regions of the world, mostly in the last two decades, due to the migration of people to urban areas, disturbances in microenvironment including climate change and human intervention [14]. In the northern provinces of Argentina, emergence of leishmaniasis has become a growing health concern [15–17]. Deforestation has a great impact and is associated with an increased risk of transmission, not only for leishmaniasis, but also for Chagas disease and other infections [17, 18].

The higher prevalence of ML in our cohort is likely explained by the fact that many of these patients come from distant areas seeking for a diagnosis in order to cure their mucosal and sometimes disfiguring lesions. The predominance of infected men over women was expected as field activities related to the risk of transmission are preferentially performed by adult men. The older age in ML over CL patients and the substantially longer duration of mucosal lesions with respect to cutaneous ulcers might be accounted for the fact that the majority of ML cases develop over months or years after the cure of cutaneous ulcers [19]. Moreover, in the majority (22/27) of our ML cases, cutaneous scars related to the primary infection were found.

L. (*V.*) *braziliensis*, followed by *L.* (*L.*) *amazonensis,* are the predominant spp. found in the Northwest of Argentina [2]. In this geographical area, *L.* (*L.*) *amazonensis* was first reported by Frank et al. [11], related to cases of cutaneous leishmaniasis. Its association with the cutaneous form of the disease was also demonstrated by our group before [13, 20]. In the present study, *L.* (*V.*) *braziliensis* was found in the majority of CL and ML cases. However, *L.*

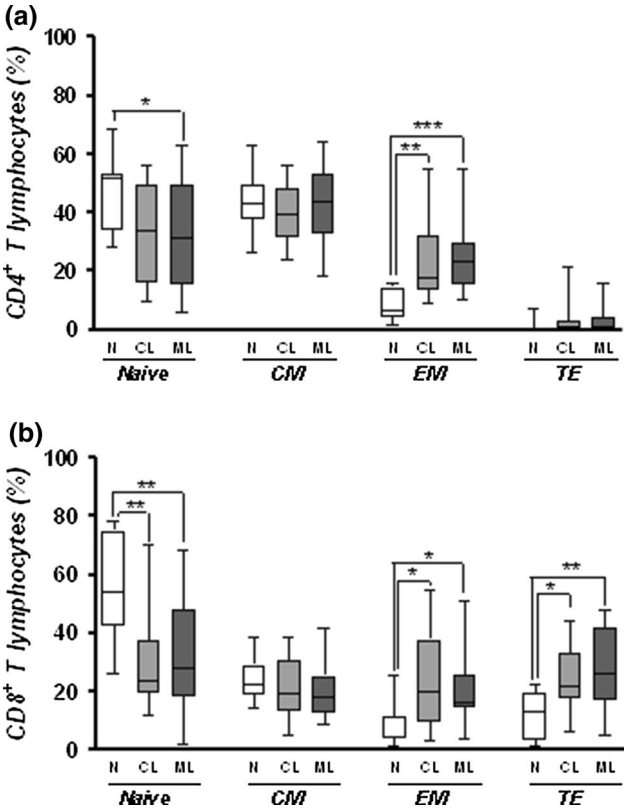


Fig. 4 Expression of memory markers on peripheral T cells from patients with American tegumentary leishmaniasis. Three clinical groups were compared for each co-expression of markers: healthy subjects (N, *white boxes*, $n = 13$); patients with localized cutaneous leishmaniasis (CL, *light grey boxes*, $n = 13$); patients with mucosal leishmaniasis (ML, *dark grey boxes*, $n = 15$–16). **a** $CD4^+$ and **b** $CD8^+$ T-cell populations were gated and analyzed for CD27 versus CD45RO. Naïve ($CD27^+CD45RO^-$); central memory (CM, $CD27^+CD45RO^+$); Effector memory (EM, $CD27^-CD45RO^+$); terminal effector (TE, $CD27^-CD45RO^-$) T cells. $^*p < 0.05$; $^{**}p < 0.01$ using Kruskal–Wallis test and Dunn's post-test. *Boxes* represent values between the 25th and 75th percentiles and medians; *bars* indicate 10th and 90th percentiles

(*L.*) *amazonensis* was not only found in cutaneous ulcers but also in the mucosal form of the disease. Recently, we reported mucosal involvement in two patients infected with *L.* (*L.*) *amazonensis* [21], and its association with the mucosal form was also reported in Brazil [22]. In our experience, no cases of diffuse cutaneous leishmaniasis have been observed [21], and previous reports agree with this observation [11].

The geographical distribution of Chagas disease and leishmaniasis overlaps in the north of Argentina. In order to meet the problem of cross-reactivity between both parasitic infections, testing for *T. cruzi* infection was carried out using commercial kits based on the use of recombinant *T. cruzi*-derived proteins, since the use of parasite homogenate led to false positive diagnosis of *T. cruzi* infections [10,

Table 3 $CD4^+$ T-cell profile in cutaneous and mucosal leishmaniasis with or without *Trypanosoma cruzi* infection

$CD4^+$ T-cell subset (%)	N ($n = 12–13$)	CL ($n = 11–13$)	*T. cruzi*-infected CL ($n = 4$)	p (*) (**)	ML ($n = 13–16$)	*T. cruzi*-infected ML ($n = 9–11$)	p (**)
ED							
$CD27^+CD28^+$	91.04 (80.24–95.66)	83.85 (72.69–90.67)	64.87 (43.37–75.70)	0.0108* <0.0001**	76.28 (35.64–84.66)	75.04 (32.91–87.44)	0.0015**
ID2							
$CD27^-CD28^+$	8.16 (4.06–14.12)	11.23 (8.07–22.84)	29.03 (15.26–40.20)	0.0109* <0.0001**	17.88 (12.56–60.97)	21.22 (10.62–50.02)	0.0009**
LD							
$CD27^-CD28^-$	0.44 (0.07–7.09)	1.83 (0.74–12.60)	8.63 (3.47–16.07)	0.0038**	4.48 (0.77–12.02)	2.79 (1.03–16.72)	NS
IL7 α chain receptor							
CD127	95.38 (89.63–98.55)	90.15 (84.73–96)	90.02 (84.22–92.58)	0.0086**	90.48 (83.77–94.26)	89.34 (71.61–94.56)	<0.0001**
Senescence marker							
CD57	0.84 (0.20–19.60)	4.71 (1.54–17.70)	12.25 (5.69–18.79)	0.0074**	5.46 (1.73–20.50)	5.98 (2.74–17.94)	NS
Naive							
$CD27^+CD45RO^-$	51.61 (28.12–68.64)	33.79 (9.36–56.33)	16.37 (8.46–26.98)	0.0009**	31.07 (5.70–62.70)	33.65 (3.96–47.66)	0.0062**
CM							
$CD27^+CD45RO^+$	43.15 (26.54–62.99)	39.66 (23.90–55.84)	47.41 (39.42–54.94)	NS	43.89 (18.40–75.63)	45.77 (27.43–61.47)	NS
EM							
$CD27^-CD45RO^+$	6.49 (1.66–15.59)	17.34 (8.76–54.83)	35.35 (17.81–49.30)	0.0002**	22.95 (10.45–54.80)	25.39 (7.76–61.39)	0.0004**
TE							
$CD27^-CD45RO^-$	0.33 (0–6.92)	0.77 (0.26–21.68)	0.87 (0.27–2.70)	NS	1.09 (0–15.81)	0.50 (0.04–2.37)	NS

N healthy subjects, *CL* localized cutaneous leishmaniasis, *ML* mucosal leishmaniasis, *T. cruzi Trypanosoma cruzi*, *ED* early differentiated cells, *ID2* intermediately differentiated cells, *LD* late differentiated cells, *CM* central memory cells, *EM* effector memory cells, *TE* terminal effector cells, *NS* no significant

Data are expressed as median (range). Differences between CL and *T. cruzi*-infected CL groups were considered to be statistically significant at $p < 0.05$ (*) using Mann–Whitney *U* test. One-way ANOVA with post-test for lineal trend was used for trend analysis between N, CL and *T. cruzi*-infected CL; or N, ML and *T. cruzi*-infected ML, differences were considered to be statistically significant at $p < 0.05$ (**)

Table 4 $CD8^+$ T-cell profile in cutaneous and mucosal leishmaniasis with or without *Trypanosoma cruzi* infection

$CD8^+$ T-cell subset (%)	N ($n = 10$–13)	CL ($n = 10$–13)	*T. cruzi*-infected CL ($n = 4$)	p (*) (**)	ML ($n = 6$–16)	*T. cruzi*-infected ML ($n = 5$–11)	p (**)
ED							
$CD27^+CD28^+$	60.69 (38.24–93.00)	52.15 (33.76–61.62)	26.09 (17.56–75.80)	0.0052**	34.09 (24.9–63.66)	30.66 (19.52–72.09)	0.0002**
ID2							
$CD27^-CD28^+$	5.27 (1.64–22.50)	4.46 (1.67–15.66)	15.94 (6.06–19.76)	0.0249* 0.0137**	11.40 (5.62–21.71)	10.66 (2.33–13.56)	NS
LD							
$CD27^-CD28^-$	20.51 (2.37–27.30)	28.33 (17.05–48.91)	50.26 (10.40–55.72)	0.0017**	41.87 (20.41–53.75)	48.43 (14.91–60.53)	<0.0001**
IL-7 α chain receptor							
CD127	82.45 (61.52–97.62)	57.38 (38.24–73.20)	47.70 (41.76–79.77)	0.0014**	54.84 (42.16–74.25)	48.02 (31.04–64.30)	<0.0001**
Senescence marker							
CD57	14.37 (2.18–40.60)	29.06 (16.93–66.41)	44.71 (14.38–57.97)	0.0095**	39.88 (13.48–51.94)	48.49 (17.36–57.57)	<0.0001**
Naive							
$CD27^+CD45RO^-$	54.14 (26.36–78.45)	23.91 (12.02–69.95)	13.51 (7.16–59.37)	0.0241**	28.29 (1.82–68.56)	22.46 (8.53–63.18)	0.0003**
CM							
$CD27^+CD45RO^+$	22.35 (14.63–38.46)	19.43 (4.92–38.45)	18.20 (16.89–20.59)	NS	18.02 (9.00–41.80)	18.77 (7.83–61.40)	NS
EM							
$CD27^-CD45RO^+$	4.36 (1.55–25.65)	19.66 (3.41–54.76)	11.95 (6.96–27.15)	NS	15.92 (4.05–51.15)	20.06 (4.95–46.93)	0.0010**
TE							
$CD27^-CD45RO^-$	12.84 (1.21–22.71)	21.68 (6.15–44.37)	42.45 (13.18–62.68)	0.0009**	25.91 (5.25–47.59)	21.43 (13.18–46.04)	0.0078**

N healthy subjects, *CL* localized cutaneous leishmaniasis, *ML* mucosal leishmaniasis, *T. cruzi Trypanosoma cruzi*, *ED* early differentiated cells, *ID2* intermediately differentiated cells, *LD* late differentiated cells, *CM* central memory cells, *EM* effector memory cells, *TE* terminal effector cells, *NS* no significant

Data are expressed as median (range). Differences between CL and *T. cruzi*-infected CL groups were considered to be statistically significant at $p < 0.05$ (*) using Mann–Whitney *U* test. One-way ANOVA with post-test for lineal trend was used for trend analysis between N, CL and *T. cruzi*-infected CL; or N, ML and *T. cruzi*-infected ML, differences were considered to be statistically significant at $p < 0.05$ (**)

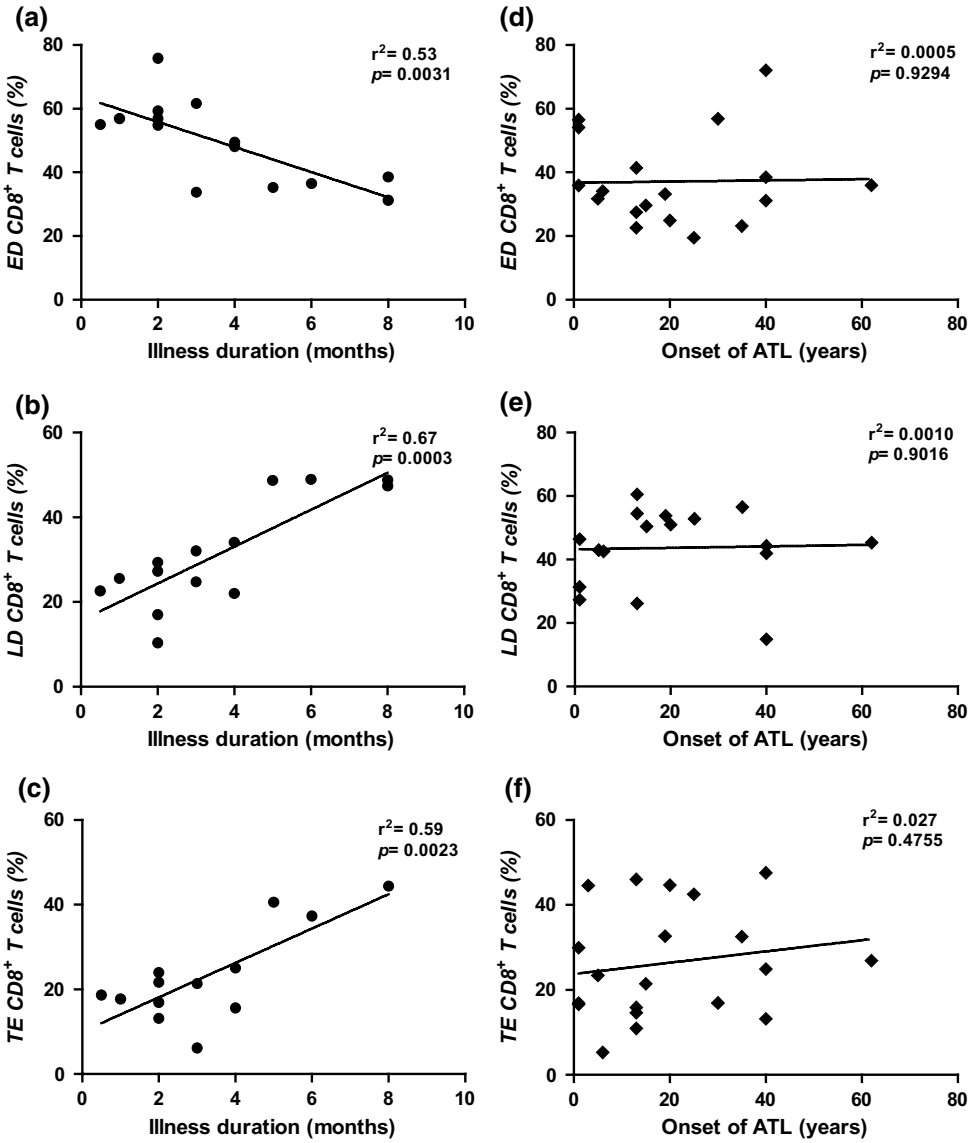


Fig. 5 Association of $CD8^+$ T-cell differentiation degree and illness duration. Correlation between **a** early differentiated cells (ED; $CD27^+CD28^+$); **b** late differentiated cells (LD; $CD27^-CD28^-$); **c** terminal effector cells (TE; $CD27^-CD45RO^-$) and illness duration of CL patients with localized cutaneous lesions (*black circles*, $n = 13–14$). Absence of correlation between: **d** ED; **e** LD; **f** TE and time since the appearance of primary cutaneous lesions (onset of ATL) of ML patients (*black diamond*, $n = 17–19$). Linear regression analysis

11, 23]. We found 24 % of positive *T. cruzi*-infected CL patients and 41 % in ML patients. Diagnosis of co-infections is not only useful to better understand the origin of the immunological findings, but also with regard to treatment options. Pentavalent antimonials used to treat leishmaniasis demand careful monitoring of renal, liver and also cardiac evaluation [24, 25] and many patients with Chagas disease might develop cardiomyopathy overtime.

The nature of the immune mechanisms involved in the control of *Leishmania* spp. and its role in the pathogenesis of this disease are not fully understood. We focused this work on the differentiation and memory phenotype of peripheral T cells from patients infected with *Leishmania* suffering from CL or ML forms of ATL. In the context of persistent human viral infections, T cells have been separated into distinct populations based on the modulation of CD27 and CD28 receptors [26, 27]. These studies showed that early differentiated cells, which possess potent proliferative capacity, conferred best protective immunity than late differentiated T cells. Meanwhile, highly differentiated T cells present signs of senescence during chronic infection, presumably due to exhaustion after persistent activation [26, 27]. Analyzing the expression of CD27 and CD28, we showed that ML patients presented the highest differentiated profile in both $CD4^+$ and $CD8^+$ T cells. A less pronounced differentiated phenotype was observed in localized CL, and significant differences were preferentially demonstrated analyzing $CD8^+$ T cells. The more marked differentiated profile of T cells in ML might be due to the fact that this is a more aggressive form, characterized by the destruction of oral–nasal–pharyngeal cavities [3–5]. In accordance, the most severe forms of Chagas disease were associated with high numbers of late differentiated ($CD27^-CD28^-$) T cells [28, 29].

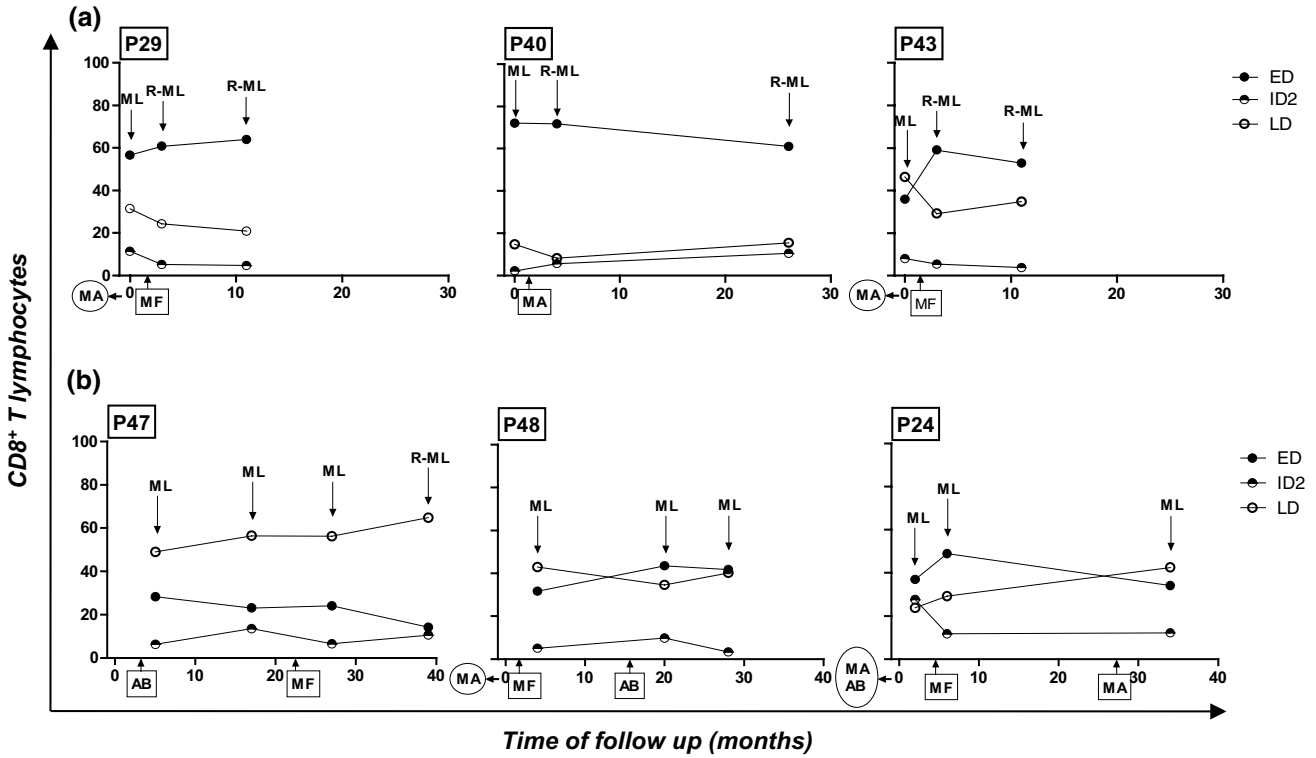


Fig. 6 $CD8^+$ T differentiation profile during follow-up of patients with different mucosal leishmaniasis outcome. $CD8^+$ T-cell population was gated and analyzed for CD27 versus CD28. Early differentiated cells (ED, $CD27^+CD28^+$); intermediately differentiated cells (ID2, $CD27^-CD28^+$) and late differentiated cells (LD, $CD27^-CD28^-$) are shown. Months of follow-up are indicated in the *X* axis. **a** Representative examples of patients who recovered from the disease (P29, P40, P43). **b** Representative examples of patients who presented relapses after different therapeutic regimens (P47, P48, P24). Meglumine antimoniate (MA), miltefosine (MF), amphotericin B (AB). *Short arrows* indicate time of therapy administration. Therapeutic regimens in *circles* indicate treatment received in the past. Therapeutic regimens in *boxes* indicate treatment received during follow-up. Patients with active mucosal leishmaniasis (ML) and patients recovered from mucosal leishmaniasis (R-ML)

ML patients displayed significantly lower values of $CD127^+PD\text{-}1^-$ on $CD4^+$ and $CD8^+$ T cells compared with uninfected subjects, and this profile was also observed in the $CD8^+$ T-cell compartment of CL patients. The up-regulation of CD127, the α chain receptor of interleukin (IL)-7, and the concomitant loss of PD-1 (programmed death 1, receptor associated with functional exhaustion of T cells) correlated with resolution of acute hepatitis B virus infection and clearance of lymphocytic choriomeningitis virus [30, 31]. Besides, down-regulation of CD127 was correlated with disease progression in human immunodeficiency virus (HIV)-infected patients [32–34] and was also demonstrated in the context of experimental models of visceral leishmaniasis [35].

We found increased expression of CD57 on both T-cell subsets in ML and on $CD8^+$ T cells in CL. Higher numbers of $CD57^+$ T cells have been associated with cells characterized by failure to proliferate and replicative senescence resulting from continual stimulation by antigens and/or cytokines [36–38].

In accordance with the terminal differentiated profile observed in CL and ML, peripheral $CD8^+$ T cells showed high content of the cytolytic molecule perforin and ML displayed higher IFN-γ production after polyclonal in vitro stimulation. Reports from other groups documented a higher expression of IFN-γ in ML analyzing antigen-specific T cells or lesion infiltrates where they also found increased expression of granzyme A [8, 39, 40]. Higher IFN-γ production could be associated not only to chronic immune activation leading to a highly differentiated phenotype, but also to the intense cellular response associated with CL and predominantly with the pathogenesis of ML [41, 42].

We also observed decreased percentages of naïve ($CD27^+CD45RO^-$) $CD4^+$ and $CD8^+$ T cells in ML, whereas CL patients only showed lesser numbers of naïve $CD8^+$ T cells. In the same direction, Hailu et al. [43] only found significantly lower numbers of naïve cells in the $CD8^+$ T-cell compartment during active visceral leishmaniasis. Among CL and ML, the increased numbers of

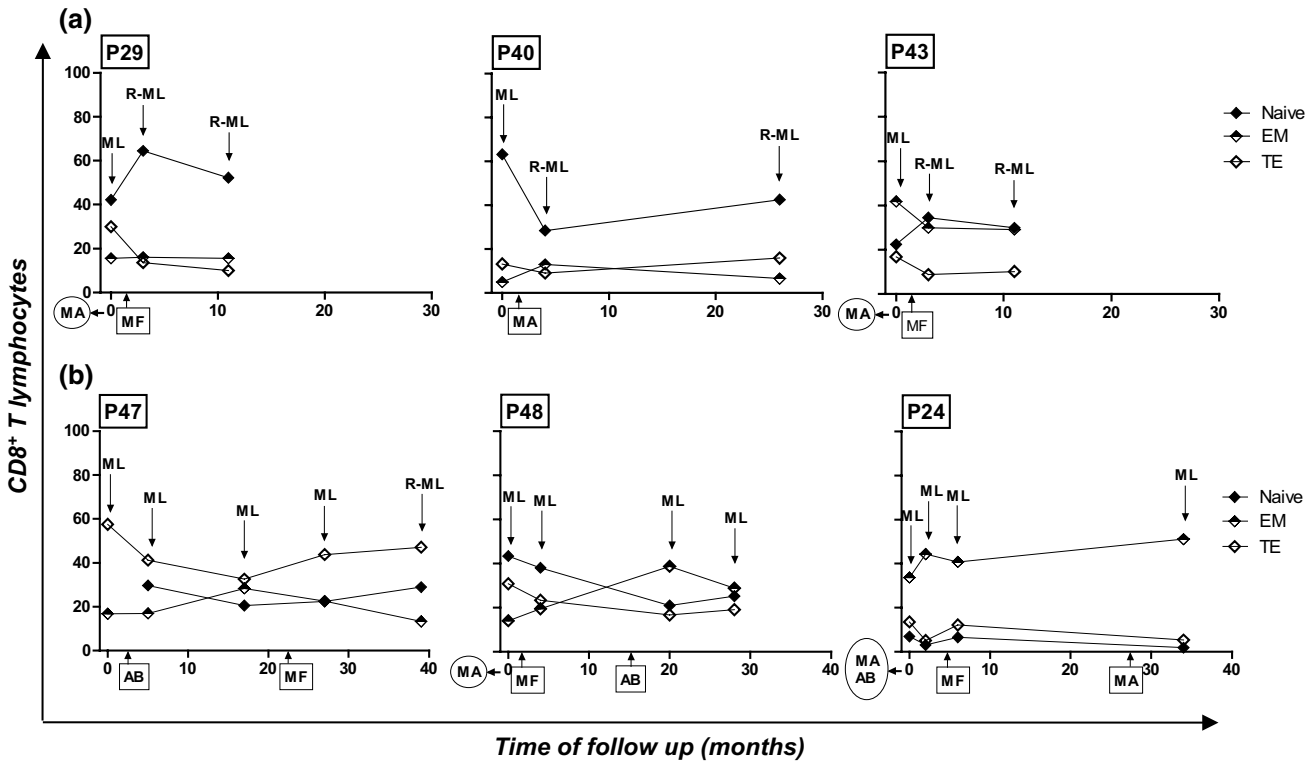


Fig. 7 $CD8^+$ T memory profile during follow-up of patients with different mucosal leishmaniasis outcome. $CD8^+$ T-cell population was gated and analyzed for CD27 versus CD45RO. Naïve ($CD27^+CD45RO^-$); effector memory (EM, $CD27^-CD45RO^+$); terminal effector (TE, $CD27^-CD45RO^-$) T cells. Months of follow-up are indicated in the *X* axis. **a** Representative examples of patients who recovered from the disease (P29, P40, P43). **b** Representative examples of patients who presented relapses after different therapeutic regimens (P47, P48, P24). Meglumine antimoniate (MA), miltefosine (MF), amphotericin B (AB). *Short arrows* indicate time of therapy administration. Therapeutic regimens in *circles* indicate treatment received in the past. Therapeutic regimens in *boxes* indicate treatment received during follow-up. Patients with active mucosal leishmaniasis (ML) and patients recovered from mucosal leishmaniasis (R-ML)

effector memory ($CD27^-CD45RO^+$) cells demonstrated in both T-cell subsets, along with the presence of high terminal effector ($CD27^-CD45RO^-$) cells only on $CD8^+$ T cells indicate that both T subpopulations exhibit different patterns of differentiation.

Analyzing the impact of *Leishmania* and *T. cruzi* co-infection on the overall T-cell compartment, we detected a positive trend toward a higher differentiated T-cell profile in CL and ML groups co-infected with *T. cruzi* compared with single *Leishmania* infections. These findings suggest an enhancement of antigenic stimulation produced by the action of both parasites in the host. Besides, as CL patients presented a short length of infection, *T. cruzi* infection which is a chronic condition in this cohort of patients, might be mainly responsible for the highly differentiated T phenotype.

The positive correlation between highly differentiated markers and the inverse correlation between early differentiated markers on $CD8^+$ T cells support the idea of an association between T-cell differentiation and illness duration within the first year of *Leishmania* infection. ML appears months or years after the onset of ATL. It could be possible that at this point, differences might not be observed in the majority of ML patients, because T lymphocytes have already gained properties of highly differentiated cells due to chronic and continuous stimulation by antigen. According to these observations, during follow-up, we found that ML patients with active lesions and frequent relapses tended to maintain a highly differentiated $CD8^+$ T cell phenotype over time. The predominance of an early differentiated profile was preferentially found among patients who were able to cure their lesions. This is in accordance with preliminary work of our group (Parodi C, Personal communication), showing a similar differentiation profile of recovered CL and ML patients and healthy subjects. According to these observations, recent studies have reported that the most frequent T-cell population in peripheral $CD4^+$ and $CD8^+$ T cells among recovered CL volunteers was consistent with naïve T cells [44, 45].

Although $CD4^+$ T cells have been the major focus of attention in leishmaniasis, since they are an important source of IFN-γ and modulate T responses by regulatory T cells [39, 46, 47]; herein, we demonstrate that during the

course of leishmaniasis, total $CD8^+$ T cells undergo the most significant changes in the differentiation pathway. Thus, indicating that $CD8^+$ T cells might also play a significant role in this infection. Moreover, during follow-up, this T-cell subset showed a modulation in the differentiation profile that differs among distinct clinical outcomes. It is likely that in leishmaniasis, $CD8^+$ T cells could respond earlier to antigen stimulation or the presence of the parasite could impact more strongly in this population than in $CD4^+$ T cells suffering as a consequence, higher numbers of divisions, resulting in the development of $CD8^+$ T cells with signs of replicative senescence and progressive exhaustion, as reflected by the increased expression of CD57 and lesser percentages of $CD127^+PD\text{-}1^-$ cells.

Regardless the *Leishmania* species involved, the results described here show a global effect of *Leishmania* infection on T-cell phenotype as overall peripheral T cells suffered changes in differentiation and memory profiles. This global effect was also observed in other chronic infections, as those caused by hepatitis C virus, HIV, *T. cruzi* [28, 29, 34, 48, 49].

Mucosal forms display scant number of parasites [12, 50, 51], and although more sensitive methods (as PCR) are needed in order to diagnose their presence in mucosal lesions, persistence of amastigotes in CL and ML scars has been reported [52]. Signs of T phenotypic exhaustion were demonstrated in the present work. According to these characteristics of the infection, we point toward the association of the duration of the antigenic stimulus and the intensity of T exhaustion.

This work emphasizes the role of long-term parasite persistence in the modulation of the T-cell differentiation profile. $CD8^+$ T cells showed the most striking changes in their phenotype, suggesting a significant role of this population during leishmaniasis. Distinct patterns of $CD8^+$ T differentiation during follow-up of different clinical outcomes suggest the usefulness of this analysis in the characterization of *Leishmania*-infected patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individuals participants included in the study.

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