Genotypic Differences among Isolates of *L. Braziliensis* and Host Factors in The Pathogenesis of Disseminated Leishmaniasis

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Research Article

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Abstract

Disseminated Leishmaniasis (DL) is an emerging and severe form of *Leishmania braziliensis* infection defined by the presence of 10 and up to more than 1,000 skin lesions. The mechanisms underlying parasite dissemination remain unknown. Genotypic differences among species of *L. braziliensis* have been associated with different clinical forms of disease. The present work compared the function of monocytes obtained from cutaneous leishmaniasis (CL) and DL patients in response to infection with isolates of *L. braziliensis* pertaining to both of these two clinical forms of disease.

Mononuclear cells obtained from DL and CL patients were infected with different *L. braziliensis* isolates. Numbers of infected cells, parasite load, respiratory burst, TLR2 and TLR4 expression and cytokine production were evaluated. DL isolates infected more monocytes, induced greater respiratory burst, higher expression of TLR2 and TLR4 and more cytokine production compared to isolates from CL patients regardless of the origin of monocytes used (DL or CL). However, greater parasite multiplication and higher TLR expression were seen in monocytes from DL patients compared to CL following infection with DL isolates. Our results indicate the participation of both parasite genotype and host factors in the pathogenesis of DL.

Introduction

Disseminated leishmaniasis (DL), a severe form of *Leishmania (Viannia) braziliensis* infection, is defined by the presence of more than 10, and up to 1,000 papular, acneiform and ulcerated lesions across at least two separate parts of the body. [1] DL has also been documented in infection by other *Leishmania* species [2,3,4,5], and differs from classical cutaneous leishmaniasis (CL), which is characterized by the presence of a single or few-well delimited ulcers with raised borders. Often confused with diffuse CL (DCL), DL is considered clinically, immunologically and histopathologically distinct from DCL [6,7,8,9]. While DCL patients exhibit poor lymphocyte proliferation and cellular production of IFN-γ upon exposure to soluble leishmania antigen (SLA) [8,9] an impaired Th1 immune response has not been documented in DL [10]. In fact, cytokine expression at the lesion site is similar between DL and CL, and no comparative histopathological or immunochemical differences have been evidenced on ulcer tissue analysis [10,11]. Recently, emphasis has been placed on the role of $CD8^+$ T cells in the pathogenesis of *L. braziliensis* infection. $CD8^+$ T cells from CL patients were observed to kill leishmania-infected monocytes, yet did not kill parasites [12]. Moreover, transcriptomic analysis of lesions from CL patients revealed the high expression of genes associated with the cytolytic pathway, and $CD8^{(+)}$ T cells obtained from lesions exhibited a cytolytic phenotype [13]. Moreover, disease progression and metastasis in *L. braziliensis*-infected mice was found to occur independently from parasite burden, instead being directly associated with the presence of $CD8^{(+)}$ T cells [13].

In spite of their participation in disease pathology, macrophages are the main cell type responsible for leishmania killing [14,15]. Regarding monocytes, also known to participate in the host inflammatory

response in CL, the enhancement of intermediate monocytes is notable, as these are the most important source of TNF, a cytokine associated with tissue damage in TL [16].

The Toll-like receptor (TLR) signaling pathway is a primary defense mechanism against infectious agents[17,18]. Monocytes from CL patients express more TLR2, TLR4 and TLR9 ex vivo or in soluble leishmania antigen (SLA)-stimulated cultures than cells from healthy subjects (HS) [19,20]. The elevated expression of TLR2 and TLR4 by intermediate monocytes from L. braziliensis-infected CL patients in comparison to HS has been associated with TNF production [21]. On the other hand, the neutralization of TLR4 was found to decrease TNF production by PBMCs infected with L. braziliensis [22]. While monocytes from CL patients produce more reactive oxygen species following exposure to L. braziliensis than HS monocytes [19], less leishmania killing was observed in CL patients compared to subjects with asymptomatic L. braziliensis infection [23]. Parasite survival and proliferation in monocytes are enhanced by molecules such as superoxide dismutase (SOD) and prostaglandin 2 (PGE2), which are both enhanced in CL and visceral leishmaniasis; interestingly, the literature contains no reports on these molecules in DL [24,25,26].

Parasite factors are also known to influence the pathogenesis of leishmaniasis. As L. braziliensis is polymorphic, dissimilarity among strains has been associated with different clinical forms of disease, as well as failure to antimonial therapy [27,28,29]. Supporting the notion that differences among same-species leishmania may influence immune response, SLA prepared using L. braziliensis isolates from DL patients was found to induce higher TNF production in mononuclear cells from both CL and DL patients than SLA similarly prepared using isolates of L. braziliensis from CL patients [30]. Moreover, L. braziliensis isolates obtained from DL patients were less internalized by neutrophils from HS, and induced lower oxidative burst and decreased expression of neutrophil activation markers [31]. Nonetheless, the literature contains scarce data on monocyte function in DL. The present study endeavored to compare the ability of DL and CL isolates to penetrate and survive in monocytes, as well as to induce molecules related to the pathogenesis of DL.

Results

The demographic and clinical features of the 24 DL and 24 CL patients included in the study are shown in Table 1. No differences were detected with regard to age, gender or size of the largest lesion. As expected, higher numbers of lesions were found in DL patients compared to CL, in addition to longer illness duration and a greater occurrence of mucosal disease.

Ability of DL and CL L. braziliensis Isolates to Infect Monocytes from DL and CL Patients

Similar numbers of amastigotes are seen in biopsied ulcer samples from DL and CL patients [11]. However, multiple lesions in DL patients imply greater total numbers of parasites compared to CL. Here we infected monocytes obtained from DL and CL patients with isolates of DL or CL (Fig. 1). Regardless of donor cell origin, we identified higher frequencies of infected cells and greater number of amastigotes per 100 cells

in monocytes infected with isolates from DL compared to CL at both 2 and 48 hours after infection (Fig. 1A). The median frequency of DL monocytes infected with the CL isolate was 46% (30–58 cells) at 2 hours *versus* 58% (45–76 cells) with the DL isolate ($p < 0.01$), compared to monocytes from CL patients: 45% (24–70 cells) for the CL isolate compared to 54% (37–84 cells) for the DL isolate ($p < 0.001$). Similar patterns were observed at 48 hours, yet a higher frequency of DL monocytes infected with the DL isolate observed after 48 hours of infection as compared to the earlier timepoint.

With respect to HS cells, the frequency of monocytes infected with the DL isolate was higher at 2 hours ($p < 0.05$) compared to the CL isolate (Supplementary Fig. 1).

Regarding parasite load after 2 hours of infection, the number of parasites in DL monocytes infected with the CL isolate was 64 (range: 48–101) amastigotes/100 cells *versus* the DL isolate: 107 (range: 66–188) amastigotes/100 cells ($p < 0.05$). The number of amastigotes/100 cells was also higher in CL monocytes infected with the DL isolate compared to CL ($P < 0.05$) at this same timepoint. Again, higher numbers of intracellular parasites were seen in DL monocytes after 48 hours of infection compared to 2 hours.

Viability of extracellular promastigotes

Parasite viability was evaluated in the supernatants of cell cultures 48 hours after infection to determine whether the higher frequency of infected cells and elevated number of parasites detected in monocytes infected with DL isolates could lead to cell lysis and consequently the release of promastigotes in supernatants of monocyte cultures (Fig. 2). Higher numbers of parasites were quantified in the supernatants of DL and CL monocyte cultures ($p < 0.05$) when isolates obtained from DL patients were used for infection (Figs. 2A, 2B). The median number of viable promastigotes in supernatants of DL monocyte cultures infected with CL was 64 (22–205) versus 129 (55–265) in supernatants of DL monocyte cultures infected with DL ($p < 0.05$). Similar behavior was observed in HS monocytes (Fig. 2C). However, in cells from DL this effect was higher. This indicates that upon *L. braziliensis* infection monocytes from DL had a lower ability to kill leishmania than CL monocytes.

Serum SOD levels and PGE2 production by DL monocytes

Increased SOD and PGE2 production in patients with CL has been associated with higher parasite burden[32,33]. While no differences were seen in systemic SOD production between DL (184 pg/ml; range: 130–263 pg/ml) and CL patients (256 pg/ml; range: 139–411 pg/ml), SOD levels were higher ($P < 0.001$) than those in HS (26 pg/ml; range: 7–77 pg/ml). DL monocytes were also observed to produce higher levels of PGE2 (461 pg/mL; range: 0-1443) in the absence of SLA stimulation compared to CL monocytes 0 pg/mL; 0-620 pg/mL). Furthermore, under SLA stimulation DL monocytes produced higher levels of PGE2 (747 pg/mL; range: 0-2186 pg/mL) than monocytes from CL patients (0 pg/mL; range: 0-5313 pg/mL).

Oxidative burst

To determine whether the induction of respiratory burst differed in parasites obtained from DL and CL patients, and to determine the impairment of this capability of DL monocytes, DL, CL and HS monocytes were infected with isolates from DL and CL patients. Median DHR expression, represented by mean fluorescence intensity (MFI) values, in monocytes from DL patients infected with a CL isolate was 3715 (730–5310) *versus* 4790 (4000–8430) when infected with a DL isolate ($P < 0.05$). DHR expression in CL monocytes infected with a CL isolate was 2870 (330–4400) versus 4140 (3180–6700) with DL $P < 0.05$ (Fig. 3). No differences in DHR expression were seen between DL and CL monocytes infected with either type of isolate. In HS cells, infection with the DL isolate induced higher oxidative burst compared to CL (Supplementary Fig. 2)

Expression of TLR2 and TLR4 in monocytes infected with DL and CL isolates

We found no differences in the *ex vivo* expression of TLR2 and TLR4 when comparing monocytes from DL and CL patients (data not shown). Figure 4 illustrates TLR2 and TLR4 expression after infection with each *L. braziliensis* isolate. While monocytes from DL patients expressed elevated levels of TLR2 and TLR4 following infection with a DL isolate compared to CL: 1425 (274–2350) and 2384 (270–3542) *versus* 853 (91-1633) and 1544 (235–3028), respectively ($P < 0.05$), similar expression for both receptors was observed when DL or CL isolates were used to infect CL monocytes ($P > 0.05$). Additionally, TLR2 and TLR4 expression was higher in DL monocytes compared to CL monocytes after infection with a DL isolate ($p < 0.01$ and $p < 0.05$). In HS cells, infection with a DL isolate induced higher expression of TLR compared to CL (Supplementary Fig. 3)

Expression of Proinflammatory Cytokines

Figure 5 shows the median MFI values obtained from DL and CL monocytes expressing TNF, CXCL9 and CXCL10 following infection with isolates of *L. braziliensis* from DL or CL patients.

Median TNF expression (MFI) in DL monocytes infected with a DL isolate was 94 (82–224) *versus* 74 (20–246) using a CL isolate ($p < 001$) (Fig. 5B), yet similar results were not seen in CL monocytes. With regard to CXCL9 expression, higher median MFI was observed in DL cells infected with a DL isolate compared to CL: 76 (30–401) *versus* 60 (19–207) ($p < 0.01$). In CL monocytes, median CXCL9 expression following infection with a DL isolate was 71 (30–199) versus 45 (20–136) for CL ($p < 0.05$) (Fig. 5C). Again, median of CXCL10 production in DL monocytes was also higher after infection with a DL isolate compared to CL: 103 (28–268) versus 95 (53–398) MFI, respectively ($p < 0.01$). Similar results were obtained in monocytes from CL patients: infection with the CL isolate induced a median MFI of 99 (52–331) for CXCL10 production versus 108 (27–161) for DL ($p < 0.05$) (Fig. 5D).

When comparing the monocytes of patients with the different clinical forms, infected with the same *L.braziliensis* isolate, we did not observe any significant differences regarding the expression of CXCL9 and CXCL10.

We found no differences in IL-10 production regardless of monocyte origin (CL or DL) or the isolate used for infection (DL vs. CL) (data not shown).

Discussion

DL is an emergent and severe form of *L. braziliensis* infection [1,10,37]. While the mechanism underlying parasite dissemination has yet to be identified, it is clear that this does not occur at the time of infection. Indeed, multiple acneiform and papular lesions suddenly appear only days or weeks after a primary ulcerated lesion; dissemination has been associated with fever and chills lasting 1–2 days.[10] As *L. braziliensis* is polymorphic, distinct genotypic characteristics among isolates have been linked to different clinical forms of disease, e.g. cutaneous, mucosal, DL or atypical CL [27,28,33]. Here we attempted to evaluate whether infection with DL isolates provokes different behavior compared to CL in terms of parasite internalization and multiplication, as well as monocyte activation and the production of inflammatory molecules. The present results indicate that *L. braziliensis* isolates from DL patients exhibit a greater ability to penetrate and multiply in monocytes compared to CL, in spite of higher respiratory burst induction and enhanced proinflammatory cytokine production. Additionally, host factors also play a role in the parasite dissemination, as monocytes from DL is more permissive to the infection and allow a greater parasite multiplication than CL cells upon infection with a DL isolate. Moreover, cells from DL expressed more TLR-2 and TLR-4 than cells from CL patients infected with a DL isolate, and produce higher levels of PGE2 than CL cells upon stimulation with SLA.

In the first 48 hours after *in vitro* infection with *L. braziliensis*, the percentage of infected monocytes/macrophages and the number of intracellular parasites increases, reaches a plateau and then begins to decrease [19,38]. Parasite internalization is observed via the quantification of these parameters in the first two hours of infection. After this time, the percentage of infected cells and numbers of parasites reflect the ability of leishmania to multiply inside host cells, as well the capacity of these infected cells to kill parasites. Here, we demonstrate that experimental infection using isolate from DL patient enables greater monocyte penetration and survival compared to isolates from CL, regardless of the origin of source cells, i.e. DL or CL monocytes, providing evidence of the capability of genotypic differences among *L. braziliensis* to interfere in parasite internalization and multiplication.

Parasite proliferation inside phagocytic cells leads to cell lysis and the release of leishmania [36]. Our results document the greater viability of DL isolate compared to CL in the supernatants of DL, CL and HS monocyte cultures. Interestingly, and perhaps more important than genotypic differences among parasites, monocytes from DL patients were observed to allow enhanced leishmania multiplication, as higher numbers of viable promastigotes were observed in the supernatants of cultured monocytes from DL patients, in comparison to CL, following infection with a DL isolate. This results provides evidence that DL monocytes were more permissive to leishmania survival, regardless of the source of *L. braziliensis*, i.e. isolates from DL or CL patients.

SOD enhances parasite multiplication in macrophages and SOD is highly expressed in host tissue during CL infection by *L. braziliensis* [32]. In addition, in CL caused by *L. amazonensis*, SOD plasma levels constitute a predictor of failure to meglumine antimoniate therapy [24]. More recently, PGE-2 has been associated with leishmania proliferation and survival [25,26,39]. Here we found no differences in SOD serum levels but observed that, upon stimulation with SLA, monocytes from DL patients produced higher levels of PGE-2 than cells from CL patients. This finding lends support to the role of monocytes, since origin, i.e. DL vs. CL, as was shown to influence parasite survival.

Monocytes from CL patients present higher oxidative burst and also produce more reactive oxygen species (ROS) and nitric oxide (NO) than cells from HS; still, these phenomena are not sufficient to control parasite multiplication and may still lead to pathology [19]. The importance of NO in the killing of *L. infantum* and *L. amazonen*sis has been documented, as well as that of ROS in the control of *L. braziliensis* proliferation [15,40,41]. Ávila et al. showed that *L. braziliensis* promastigotes and amastigotes isolated from CL and ML patients produced similar amounts of NO in culture. However, promastigotes from ML isolates were found to be more resistant to NO and H_2O_2 than CL parasites [42]. Our results document that infection with DL isolates induces higher respiratory burst in monocytes from both DL and CL patients compared to isolates from CL, yet the observed enhancement in oxidative burst did not inhibit parasite multiplication, which suggests that DL isolates are less susceptible to monocyte killing than CL.

While TLRs are known to participate in host defense mechanisms, the expression of TLRs has also been associated with inflammatory and autoimmune diseases [43]. Our results show that monocytes from DL patients infected with DL isolates express more TLR2 and TLR4 compared to CL isolates. However, we did not similarly document this in CL monocytes. As cells from DL expressed more TLRs upon infection with DL isolates, but also exhibited less capability to kill leishmania, we suggest that the exaggerated inflammatory response observed in DL may likely be more closely related to pathology than protection.

Proinflammatory cytokines, such as TNF, CXCL9 and CXCL10, are mainly produced by monocytes/macrophages. In CL patients, these cells produce higher levels of cytokines than healthy subjects [38]. Elevated systemic production of CXCL9 has been documented in sera from DL patients compared to CL [10]. Additionally, upon stimulation in PBMCs of patients with both forms of disease, SLA obtained from isolates of DL patients induced higher TNF and IFN-γ expression than SLA from CL [30]. The present work expanded on these observations by demonstrating that DL isolate induce higher levels of TNF, CXCL9 and CXCL10 expression than CL isolate in cultured monocytes from both CL and DL patients. Indeed, it appears controversial that infection using DL isolate induces a pronounced proinflammatory response in monocytes, yet at the same time permits parasite survival inside phagocytic cells. However, it is important to consider that proinflammatory cytokine production by monocytes has not been definitively linked to parasite killing in CL caused by *L. braziliensis* [38].

Our data indicate that parasite dissemination in DL occurs due to parasite multiplication, cell death and the release of amastigotes that infect monocytes at different sites of the skin. However, we cannot rule

out the possibility that parasite dissemination may also occur through the metastasis of infected monocytes from the original lesion site to other areas of the body. In cancer, inflammation influences metastasis. More specifically, the production of CXCL9 and CXCL10, among other proinflammatory cytokines, has been associated with the severity of melanoma and metastasis [44,45]. Here we expanded on a previous report that documented higher pro-inflammatory cytokine production induced by DL isolates compared to CL isolates [30]. In light of the lack of evidence that inflammation correlates with leishmania killing in *L. braziliensis* infection, it is important to consider the possibility that inflammation may hold influence over parasite dissemination.

DL, a severe and emergent form of *L. braziliensis* infection, has been associated with high rates of failure to antimony therapy. Our results show that parasite dissemination can be influenced by host and parasite factors, and that parasite multiplication in macrophages is highly linked to parasite dissemination. As inflammation has been associated with the pathology of *L. braziliensis* infection, emphasis has been placed on a combined regimen of chemotherapy with immunomodulatory agents in the treatment of TL [46,47]. In addition to offering insight into the pathogenesis of DL, the present results point to the necessity of identifying novel therapeutic agents capable of enhancing leishmania killing by macrophages in order to enable the control leishmaniasis.

Material And Methods

Patients

A total of 24 patients with DL and 24 with CL were included; all individuals sought medical attention at the Corte de Pedra Health Clinic, located in the municipality of Presidente Tancredo Neves (Bahia-Brazil), between September 2017 and May 2019. For comparison purposes, we included 12 healthy subjects (HS) without exposure to leishmania from a non-endemic area. DL patients presented more than 10 acneiform, popular, and ulcerated lesions, in two or more non-contiguous parts of the body (head, trunk, arms or legs). CL patients presented 1–3 ulcers with raised borders. Infection was diagnosed via detection of *L. braziliensis* DNA by PCR in biopsied ulcer tissue samples [34] in conjunction with parasite isolation in culture or identification by histopathologic analysis. All experiments were performed prior to the administration of therapy.

Ethics Statement

The present research protocol received approval from the Institutional Review Board of the Federal University of Bahia, and was approved by the National Commission for Ethics in Research (CONEP) in Brazil (protocol number 2.114.874). All subjects agreed to voluntarily participate and provided a written term of informed consent. All methods were performed in accordance with the guidelines and regulations stipulated by CONEP.

Parasites

Species determination was based upon HSP70 PCR-restriction fragment length polymorphism and later confirmed by serial real-time quantitative PCR [34]. The genotyping of *L.* (*V.*) *braziliensis* recovered from CL and DL lesions was performed as previously described [27,33]. The isolates from CL and DL used to infect cells were genotyped according to the haplotypes of polymorphic nucleotides in the locus CHR28/455451, previously shown to distinguish *L. braziliensis* strains [27]. The CL and DL *L.* (*V.*) *braziliensis* isolates were cultured in biphasic Novy-MacNeal-Nicolle medium with liver infusion tryptose at 26 °C for 1–2 weeks. The suspension was then transferred to Schneider's medium containing 10% heat-inactivated fetal calf serum and 2 mM l-glutamine, and re-incubated at 26 °C for up to two weeks. Parasites were frozen in liquid nitrogen without any further subculturing in 10% dimethyl sulfoxide with 90% growth medium, and thawed prior to use in experimentation.

Isolation of Human Peripheral Blood Cells and Infection with L. braziliensis Isolates

Peripheral blood mononuclear cells (PBMC) were isolated from DL and CL patients and HS. Heparinized venous blood was layered [35] and resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum and antibiotics (GIBCO BRL, Grand Island, NY USA). PBMCs (1×10^6 cells/tube) were infected with stationary-phase *L. braziliensis* isolates at a ratio of 5:1. After 2 hours of infection at 37 °C under 5% CO_2, extracellular parasites were removed following centrifugation. Cells were placed in complete RPMI 1640 medium and incubated for an additional 48 hours. Finally, the numbers of infected cells and intracellular parasites were determined by microscopic evaluation of 100 monocytes following Romanowsky staining of cytocentrifuge preparations.

Evaluation of Parasite Viability

After 48 hours of infection with *L. braziliensis* isolates, PBMCs were washed and the medium was replaced with 0.5 ml of Schneider's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum. Cells were cultured at 26 °C for an additional 48 h. Viable parasites was determined by counting extracellular motile promastigotes using a hemocytometer [36].

Determination of PGE2 and SOD

PGE2 production was evaluated using reagents purchased from R&D Systems (Minneapolis, USA) in the supernatants of PBMCs (3×10^6 cells/ml) stimulated with SLA (5 µg/ml). Results are expressed in pg/ml. SOD serum levels were determined using a Human Cu/Zn Superoxide Dismutase ELISA kit (ABCAM. Cambridge Science Park, UK).

Evaluation of oxidative burst

PBMCs (1×10^6) were stimulated with 10 ng/mL dihydrorhodamine 123 (DHR) (Cayman Chemical Company) for 10 minutes at 37ºC under 5% CO^2. Cells were then exposed to *L. braziliensis* (ratio 5:1) for 25 minutes (37ºC, 5% CO^2). Phorbol 12-myristate 13-acetate (PMA-Invivogen) at 1 µg/mL was used as positive control. Monocytes were stained for anti-CD14 surface markers (APC clone M5E2, BD

Pharmingen) and quantified by nonspecific fluorescence using forward scatter (FSC) and side scatter (SSC) parameters to determine cell size and granularity, respectively. Cells were then gated based on CD14 expression and DHR 123 oxidation (Fig. 3). A total of 200,000 cells per tube were evaluated on a FACS Canto II flow cytometer (BD); data analysis was performed using FlowJo software (Tree Star Inc).

Monocyte Expression of TLR2 and TLR4

Monocyte surface expression of TLR2 and TLR4 was analyzed both *ex vivo* and *in vitro* after infection with *L. braziliensis* for 2 hours. The following antibodies were used: APC-conjugated anti-CD14 (APC clone M5E2, BD Pharmingen); PE-conjugated anti-TLR2 (clone TL2.1); PE-conjugated anti-TLR4 (clone HTA125) (eBioscience, San Diego, CA, USA). A total of 200,000 cells per tube were evaluated on a FACS Canto II flow cytometer (BD); data analysis was performed using FlowJo software (Tree Star Inc).

Evaluation of cytokine production

PBMCs were either infected with different isolates of *L. braziliensis* or left uninfected. To perform flow cytometry, 10^6 PBMCs were stained with a fluorochrome-conjugated CD14 surface marker antibody (APC clone M5E2, BD Pharmingen) and fixed in 2% formaldehyde. For intracellular staining, fixed cells were permeabilized using a cytofix/cytoperm kit (BD-Bioscience) and stained intracellularly with PE-conjugated anti-TNF (clone Mab11, eBioscience), PE-conjugated anti-CXCL9 (clone B8-11, BD Biosciences) and PE-conjugated anti-CXCL10 (clone J034D6, BioLegend) antibodies. A total of 200,000 cells per tube were evaluated on a FACS Canto II flow cytometer (BD); data analysis was performed using FlowJo software (Tree Star Inc).

Statistical Analysis

Categorical variables with normal distribution were analyzed using the Student's T test. Comparisons between different isolates within a single group were performed using Wilcoxon's signed-rank test, while comparisons between groups were performed using the nonparametric Mann-Whitney U test. Differences among three or more groups were assessed by analysis of variance (Kruskal-Wallis) with Dunn´s post-test. Statistical significance was considered when $p < 0.05$. Data analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

Declarations

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Author contributions

WNO, AS, OB and EMC designed the study; WNO, ASD, PPC, MTN performed the experiments. WNO, OB, LPC and AS analyzed and interpreted the data. PRM participated in the diagnosis and treatment of the patients. OB and EMC wrote the manuscript. All authors approved the final version of the manuscript.

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Data Availability

The data used to support the findings of this study have been deposited in the figshare repository (https://doi.org/10.6084/m9.figshare.13393091.v1) and are included within the article.

References

1. Turetz, M. L. *et al.* Disseminated leishmaniasis: a new and emerging form of leishmaniasis observed in northeastern Brazil. *The Journal of infectious diseases.* **186**, 1829–1834 (2002).
2. Alborzi, A. *et al.* Isolation of Leishmania tropica from a patient with visceral leishmaniasis and disseminated cutaneous leishmaniasis, southern Iran. *The American journal of tropical medicine and hygiene.* **79**, 435–437 (2008).
3. Calvopina, M. *et al.* Atypical clinical variants in New World cutaneous leishmaniasis: disseminated, erysipeloid, and recidiva cutis due to Leishmania (V.) panamensis. *The American journal of tropical medicine and hygiene.* **73**, 281–284 (2005).
4. Newlove, T., Robinson, M., Meehan, S. A. & Pomerantz, R. Old World cutaneous leishmaniasis. *Dermatol Online J.* **18**, 32 (2012).
5. Rincon, M. Y., Silva, S. Y., Duenas, R. E. & Lopez-Jaramillo, P. [A report of two cases of disseminated cutaneous leishmaniasis in Santander, Colombia]. *Rev Salud Publica (Bogota).* **11**, 145–150 (2009).

6. Carvalho, E. M., Barral, A., Costa, J. M., Bittencourt, A. & Marsden, P. Clinical and immunopathological aspects of disseminated cutaneous leishmaniasis. *Acta tropica.* **56**, 315–325 (1994).
7. Silveira, F. T., Lainson, R. & Corbett, C. E. Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil: a review. *Memorias do Instituto Oswaldo Cruz.* **99**, 239–251 (2004).
8. Christensen, S. M. *et al.* Host and parasite responses in human diffuse cutaneous leishmaniasis caused by L. amazonensis. *PLoS Negl Trop Dis.* **13**, e0007152 (2019).
9. Petersen, E. A. *et al.* Monocyte suppression of antigen-specific lymphocyte responses in diffuse cutaneous leishmaniasis patients from the Dominican Republic. *Journal of immunology.* **132**, 2603–2606 (1984).
10. Machado, P. R. *et al.* Reappraisal of the immunopathogenesis of disseminated leishmaniasis: in situ and systemic immune response. *Trans R Soc Trop Med Hyg.* **105**, 438–444 (2011).
11. Dantas, M. L. *et al.* Comparative analysis of the tissue inflammatory response in human cutaneous and disseminated leishmaniasis. *Memorias do Instituto Oswaldo Cruz.* **109**, 202–209 (2014).
12. Cardoso, T. M. *et al.* Protective and pathological functions of CD8 + T cells in Leishmania braziliensis infection. *Infection and immunity.* **83**, 898–906 (2015).
13. Novais, F. O. *et al.* Cytotoxic T cells mediate pathology and metastasis in cutaneous leishmaniasis. *PLoS Pathog.* **9**, e1003504 (2013).
14. Scott, P., Natovitz, P., Coffman, R. L., Pearce, E. & Sher, A. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J Exp Med.* **168**, 1675–1684 (1988).
15. Novais, F. O. *et al.* Human classical monocytes control the intracellular stage of Leishmania braziliensis by reactive oxygen species. *The Journal of infectious diseases.* **209**, 1288–1296 (2014).
16. Passos, S. *et al.* Intermediate monocytes contribute to pathologic immune response in Leishmania braziliensis infections. *The Journal of infectious diseases.* **211**, 274–282 (2015).
17. Medzhitov, R. TLR-mediated innate immune recognition. *Seminars in immunology.* **19**, 1–2 (2007).
18. Tuon, F. F. *et al.* Toll-like receptors and leishmaniasis. *Infection and immunity.* **76**, 866–872 (2008).
19. Carneiro, P. P. *et al.* The Role of Nitric Oxide and Reactive Oxygen Species in the Killing of Leishmania braziliensis by Monocytes from Patients with Cutaneous Leishmaniasis. *PloS one.* **11**, e0148084 (2016).
20. Vieira, E. L. *et al.* Immunoregulatory profile of monocytes from cutaneous leishmaniasis patients and association with lesion size. *Parasite immunology.* **35**, 65–72 (2013).
21. Polari, L. P. *et al.* Leishmania braziliensis Infection Enhances Toll-Like Receptors 2 and 4 Expression and Triggers TNF-alpha and IL-10 Production in Human Cutaneous Leishmaniasis. *Front Cell Infect Microbiol.* **9**, 120 (2019).
22. Galdino, H. Jr. *et al.* Leishmania (Viannia) braziliensis amastigotes induces the expression of TNFalpha and IL-10 by human peripheral blood mononuclear cells in vitro in a TLR4-dependent

manner. *Cytokine.* **88**, 184–192 (2016).

23. Muniz, A. C. *et al.* Immunological markers of protection in Leishmania (Viannia) braziliensis infection: A five years cohort study.*The Journal of infectious diseases*(2016).
24. Khouri, R. *et al.* SOD1 plasma level as a biomarker for therapeutic failure in cutaneous leishmaniasis. *The Journal of infectious diseases.* **210**, 306–310 (2014).
25. Saha, A. *et al.* Prostaglandin E2 negatively regulates the production of inflammatory cytokines/chemokines and IL-17 in visceral leishmaniasis. *Journal of immunology.* **193**, 2330–2339 (2014).
26. Araujo-Santos, T. *et al.* Prostaglandin E2/leukotriene B4 balance induced by Lutzomyia longipalpis saliva favors Leishmania infantum infection. *Parasites & vectors.* **7**, 601 (2014).
27. Queiroz, A. *et al.* Association between an emerging disseminated form of leishmaniasis and Leishmania (Viannia) braziliensis strain polymorphisms. *Journal of clinical microbiology.* **50**, 4028–4034 (2012).
28. Guimaraes, L. H. *et al.* Atypical Manifestations of Cutaneous Leishmaniasis in a Region Endemic for Leishmania braziliensis: Clinical, Immunological and Parasitological Aspects. *PLoS Negl Trop Dis.* **10**, e0005100 (2016).
29. Silva, R. E. D. *et al.* Towards a standard protocol for antimony intralesional infiltration technique for cutaneous leishmaniasis treatment. *Memorias do Instituto Oswaldo Cruz.* **113**, 71–79 (2018).
30. Leopoldo, P. T. *et al.* Differential effects of antigens from L. braziliensis isolates from disseminated and cutaneous leishmaniasis on in vitro cytokine production. *BMC Infect Dis.* **6**, 75 (2006).
31. Cardoso, T. *et al.* Leishmania braziliensis isolated from disseminated leishmaniasis patients downmodulate neutrophil function. *Parasite immunology.* **41**, e12620 (2019).
32. Khouri, R. *et al.* IFN-beta impairs superoxide-dependent parasite killing in human macrophages: evidence for a deleterious role of SOD1 in cutaneous leishmaniasis. *Journal of immunology.* **182**, 2525–2531 (2009).
33. Schriefer, A. *et al.* Multiclonal Leishmania braziliensis population structure and its clinical implication in a region of endemicity for American tegumentary leishmaniasis. *Infection and immunity.* **72**, 508–514 (2004).
34. Weirather, J. L. *et al.* Serial quantitative PCR assay for detection, species discrimination, and quantification of Leishmania spp. in human samples. *Journal of clinical microbiology.* **49**, 3892–3904 (2011).
35. Bacellar, O. *et al.* Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infection and immunity.* **70**, 6734–6740 (2002).
36. Novais, F. O. *et al.* Neutrophils and macrophages cooperate in host resistance against Leishmania braziliensis infection. *Journal of immunology.* **183**, 8088–8098 (2009).
37. Vernal, S., De Paula, N. A., Gomes, C. M. & Roselino, A. M. Disseminated Leishmaniasis by Leishmania viannia Subgenus: A Series of 18 Cases in Southeastern Brazil. *Open forum infectious*

diseases. **3**, ofv184 (2016).

38. Giudice, A. *et al.* Macrophages participate in host protection and the disease pathology associated with Leishmania braziliensis infection. *BMC Infect Dis.* **12**, 75 (2012).
39. Lonardoni, M. V., Barbieri, C. L., Russo, M. & Jancar, S. Modulation of Leishmania (L.) amazonensis Growth in Cultured Mouse Macrophages by Prostaglandins and Platelet Activating Factor. *Mediators Inflamm.* **3**, 137–141 (1994).
40. Liew, F. Y., Millott, S., Parkinson, C., Palmer, R. M. & Moncada, S. Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. *Journal of immunology.* **144**, 4794–4797 (1990).
41. Diaz, N. L. *et al.* Nitric oxide and cellular immunity in experimental cutaneous leishmaniasis. *Clin Exp Dermatol.* **28**, 288–293 (2003).
42. Avila, L. R. *et al.* Promastigote parasites cultured from the lesions of patients with mucosal leishmaniasis are more resistant to oxidative stress than promastigotes from a cutaneous lesion. *Free radical biology & medicine.* **129**, 35–45 (2018).
43. Patra, M. C. *et al.* A Novel Small-Molecule Inhibitor of Endosomal TLRs Reduces Inflammation and Alleviates Autoimmune Disease Symptoms in Murine Models.*Cells*9 (2020).
44. Lok, E., Chung, A. S., Swanson, K. D. & Wong, E. T. Melanoma brain metastasis globally reconfigures chemokine and cytokine profiles in patient cerebrospinal fluid. *Melanoma Res.* **24**, 120–130 (2014).
45. Harlin, H. *et al.* Chemokine expression in melanoma metastases associated with CD8 + T-cell recruitment. *Cancer Res.* **69**, 3077–3085 (2009).
46. Lessa, H. A. *et al.* Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. *The American journal of tropical medicine and hygiene.* **65**, 87–89 (2001).
47. Almeida, R. P. *et al.* Successful treatment of refractory cutaneous leishmaniasis with GM-CSF and antimonials. *The American journal of tropical medicine and hygiene.* **73**, 79–81 (2005).

Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

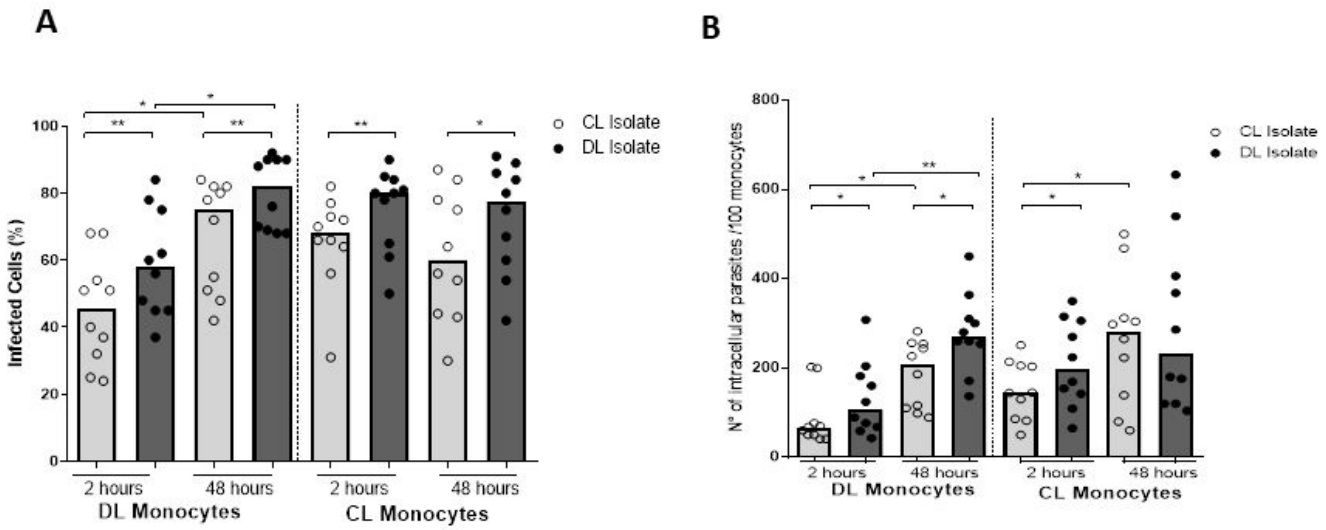

Figure 1

Frequency of infected monocytes and parasite load after infection with different isolates of L. braziliensis: PBMC-derived monocytes from patients with DL (n=12) or CL (n=12) were infected with different isolates of L. braziliensis at a ratio of 5:1 for 2 h or 48 h. The number of infected cells (A) and the number of intracellular parasites (B) were evaluated by optical microscopy in 100 monocytes following Romanowsky staining. P values were calculated using Wilcoxon's signed-rank testing for analyses within the same clinical form, while comparisons between different clinical forms were assessed using the Mann-Whitney test (*p<0.05) (**p<0.01).

Figure 2

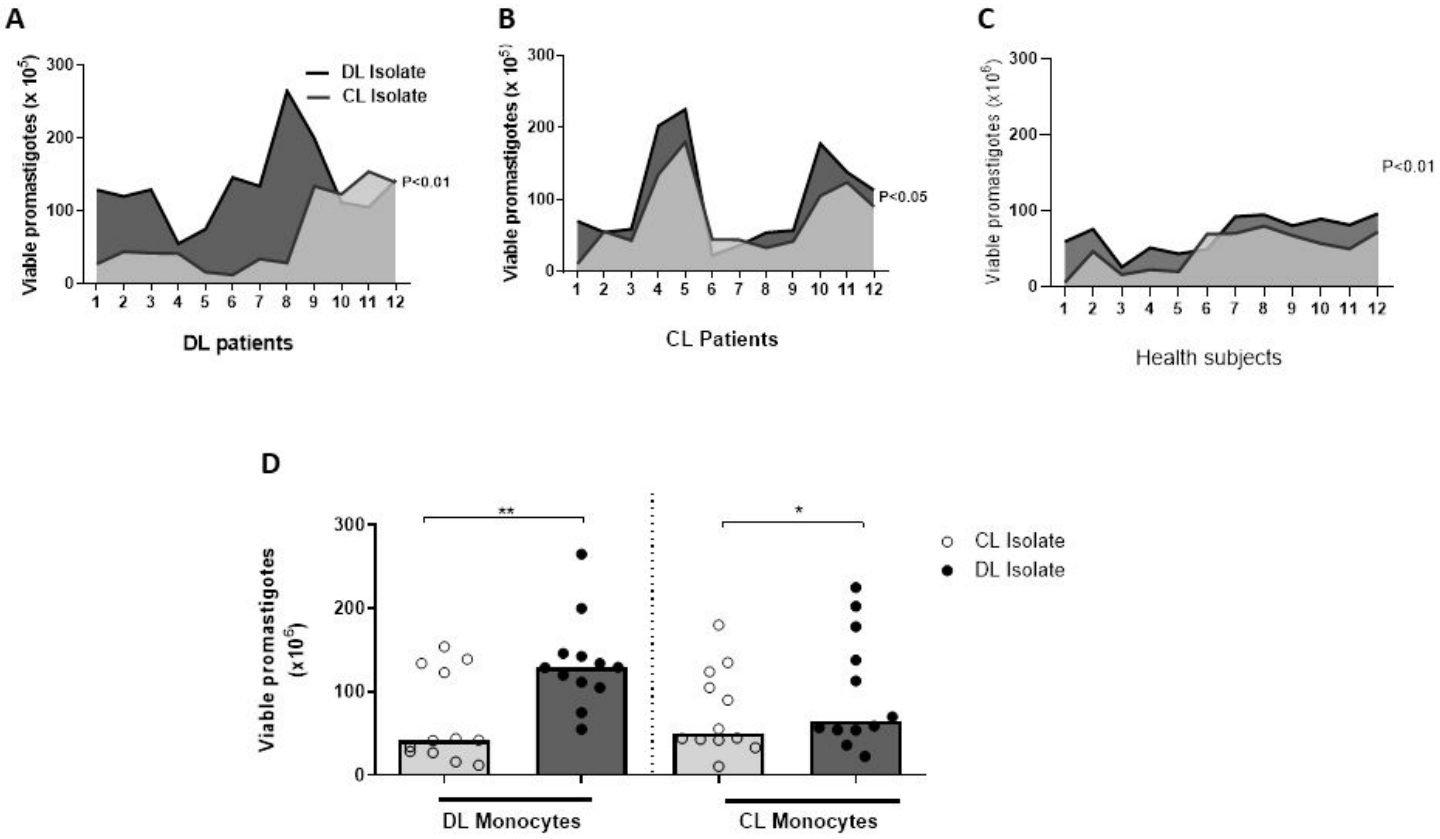

Figure 2

Viability of extracellular promastigotes in the supernatants of cultured monocytes from patients with DL, CL or healthy subjects following infection with different L. braziliensis isolates: PBMC-derived monocytes from patients with DL (n=12), CL (n=12) or HS (n=12) were infected with different isolates of L. braziliensis (DL or CL) for 48 hours. After this period RPMI medium was replaced by Schneider culture medium for another 48 hours. The number of viable promastigotes was evaluated by optical microscopy. A) Monocytes from patients with DL; B) Monocytes from patients with CL; C) Monocytes from HS. D) Viability of promastigotes in CL and DL monocytes following infection with different isolates. Results are expressed as median values calculated using the Wilcoxon's signed-rank test. Comparisons between groups were performed using the Mann-Whitney statistical test (*p<0.05) (**p<0.01).

Figure 3

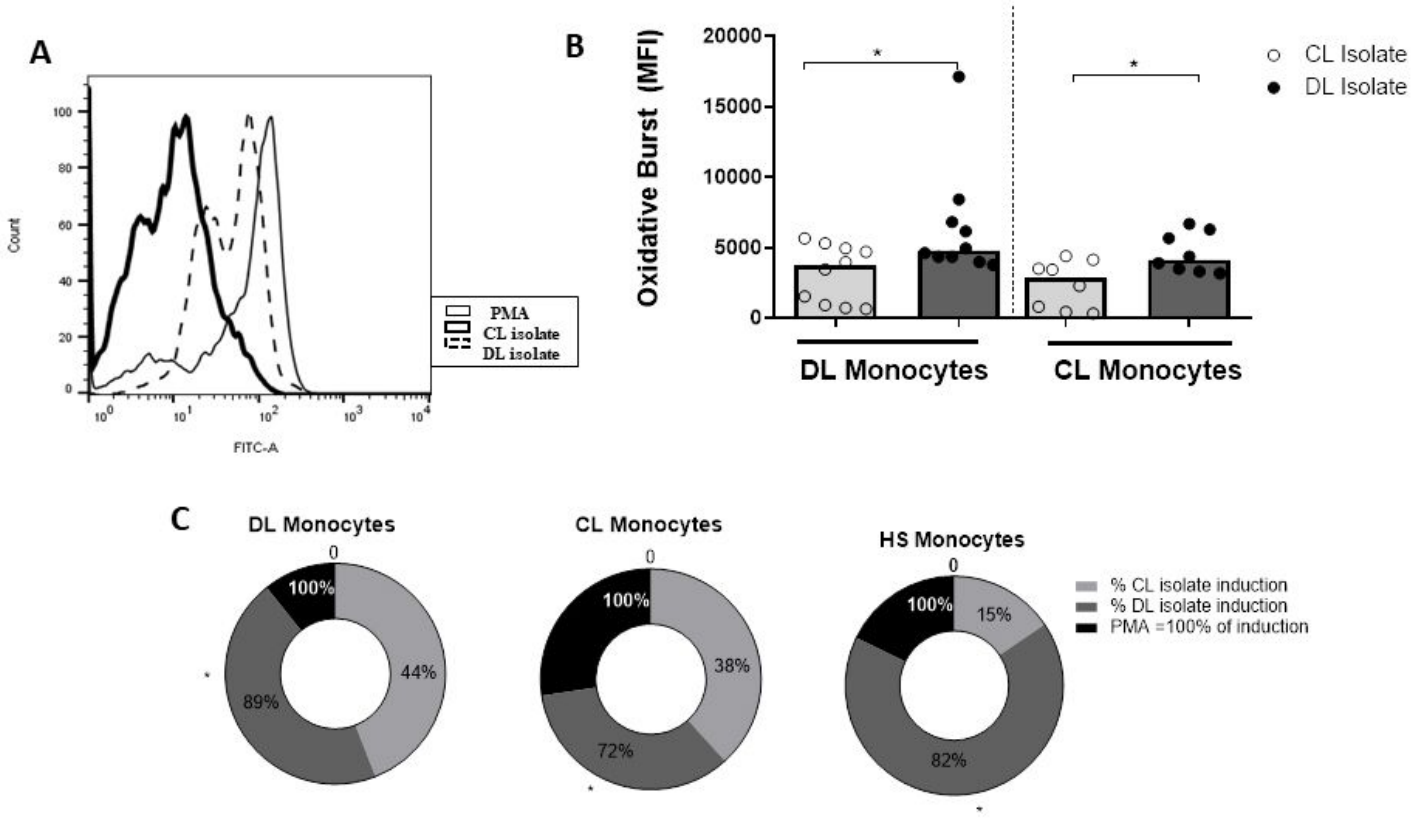

Figure 3

Induction of oxidative burst by monocytes from patients with DL, CL or healthy subjects following infection with different L. braziliensis isolates: PBMC-derived monocytes from patients with DL (n=12), CL (n=12) or HS (n=8) were treated with DHR (10ng/mL; 10 min) and infected with L. braziliensis isolates from DL or CL patients for 25 minutes at a ratio of 5:1 cells. PMA (1ug/ml) was used as positive control. Cells were stained with anti-CD14 for flow cytometric evaluation. (A) Representative gating strategy detailing CD14+ and DHR expression in monocytes from a CL patient. (B) Data representative of median mean fluorescence intensity (MFI) (MIF) of oxidative burst induction in DL and CL monocytes infected with different isolates. (C) Representative oxidative burst expression in monocytes from different clinical forms compared to PMA (100% induction). All p values obtained using Wilcoxon's signed-rank testing. Comparisons made between groups using Mann- Whitney statistical testing. (*p<0.05)

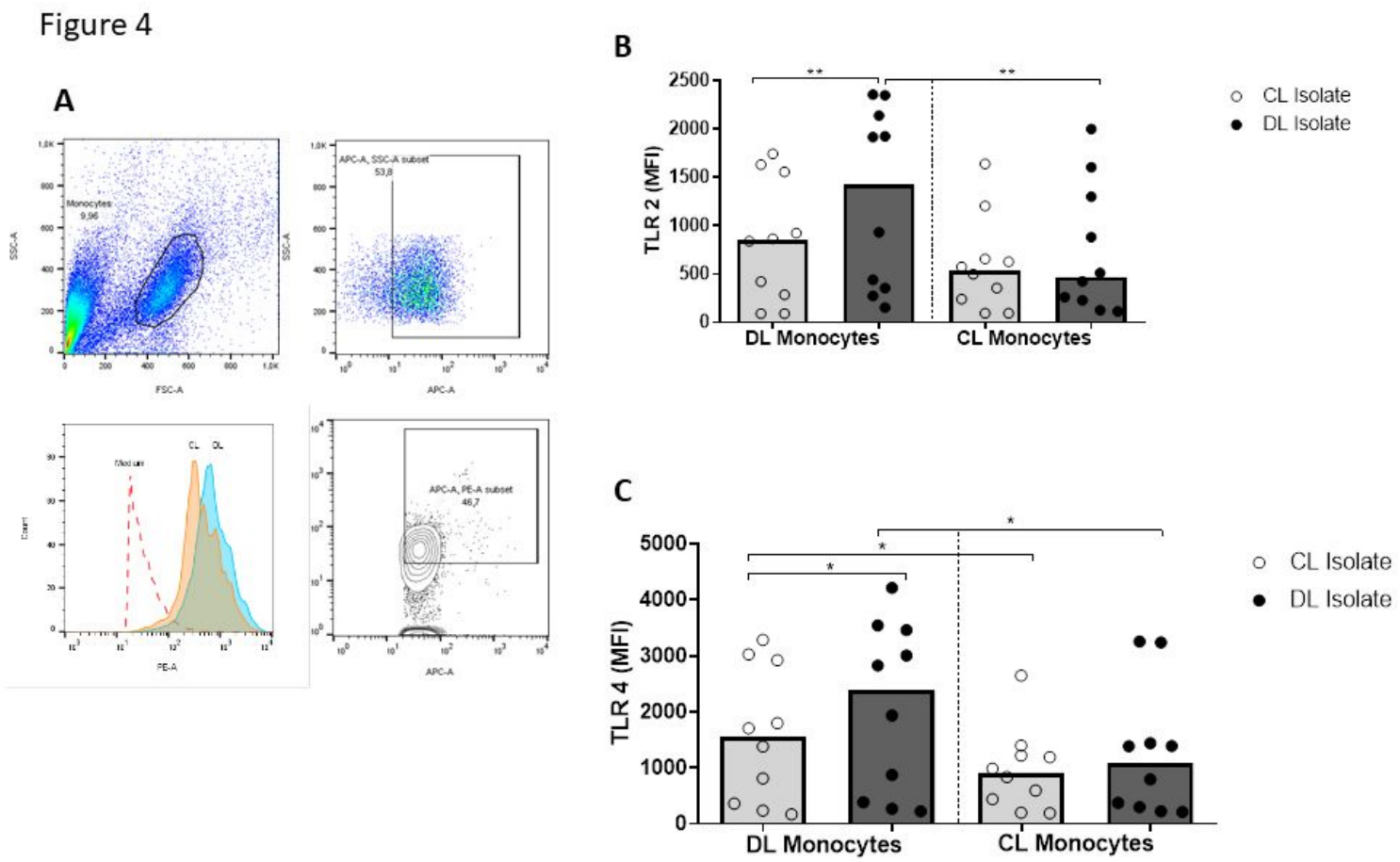

Figure 4

TLR2 and TLR4 expression in monocytes from DL and CL patients. PBMC-derived monocytes from DL (n=12) and CL (n=12) patients were infected with different isolates of L. braziliensis at a ratio of 5:1 for 2 h. Following stimulation, monocytes were marked with anti-CD14 antibodies, and with anti-TLR2 or anti-TLR4 for flow cytometry analysis. (A) Figure representative of flow cytometry gating stragegy; (B) Expression of TLR2 (C) Expression of TLR4. Data representative of median mean fluorescence intensity (MFI) values. All p values obtained using Wilcoxon's signed-rank testing; comparisons between groups made using the Mann- Whitney statistical test. (*p<0.05) (**p<0.01)

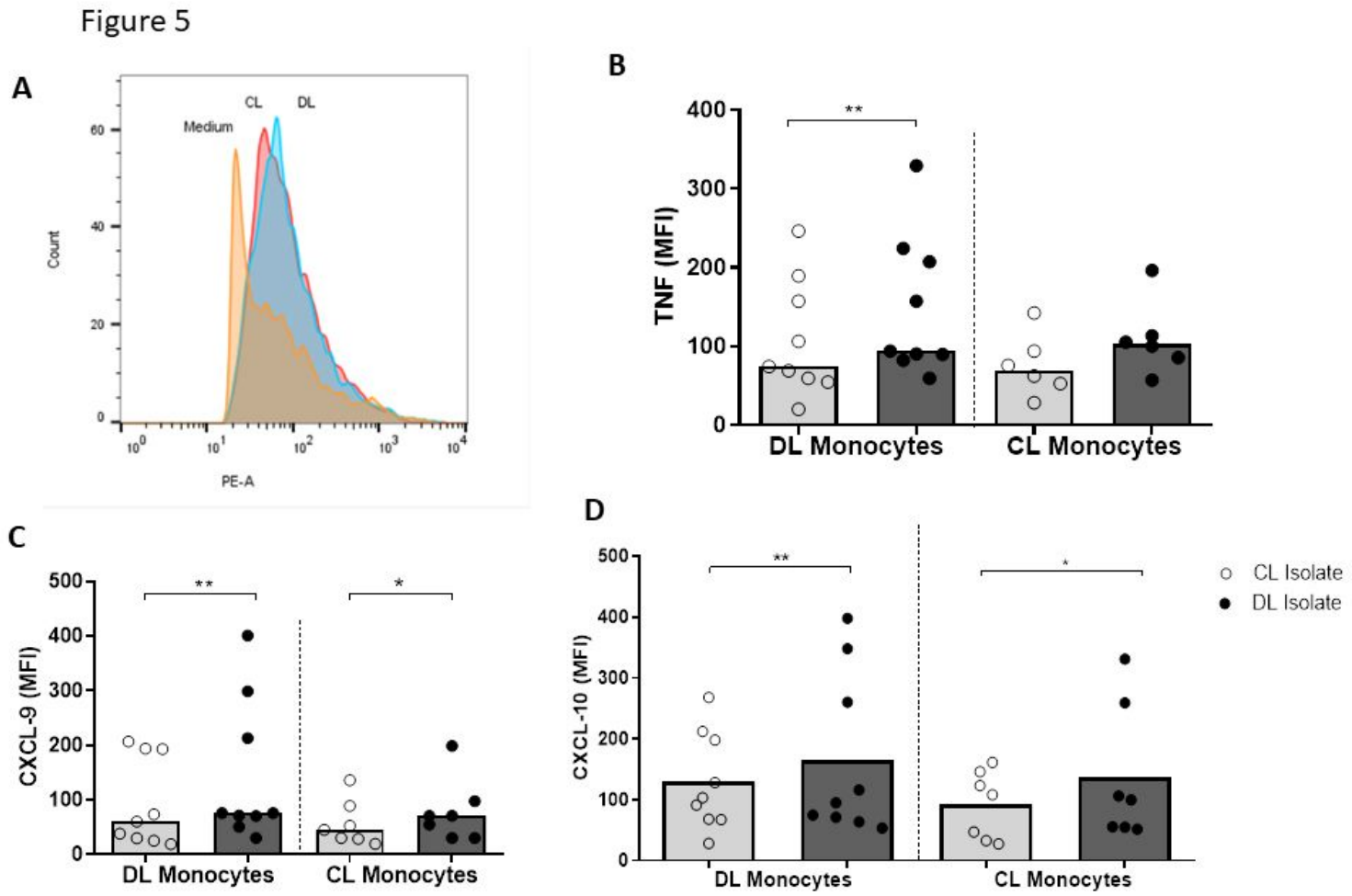

Figure 5

Production of TNF, CXCL9 and CXCL10 by monocytes from patients with DL and CL following infection with different L. braziliensis isolates: PBMC-derived monocytes from patients with DL (n=9) and CL (n=6) were infected with different isolates of L. braziliensis (5:1) for 2 h. Cells were treated for 6 h with Stop Golgi, followed by surface staining with an anti-CD14 antibody for monocyte characterization. After permeative treatment, cells were marked with anti-TNF, anti-CXCL9 and anti-CXCL10 antibodies for flow cytometric analysis. (A) Representative graph illustrating intracellular production by flow cytometry. (B) TNF expression (C) CXCL9 expression. (D) CXCL10 expression. Results are presented as median fluorescence intensity (MFI) values. All p values were obtained using Wilcoxon's signed-rank testing, while comparisons between groups were made using the Mann- Whitney statistical test (* $p<0.05$) (** $p<0.01$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.docx
- Table1.docx