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BRIEF DEFINITIVE REPORT

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Leishmania amazonensis induces modulation of costimulatory and surface marker molecules in human macrophages

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Summary

Manipulation of costimulatory and surface molecules that shape the extent of immune responses by *Leishmania* is suggested as one of the mechanisms of evading the host's defences. The experiments reported here were designed to evaluate the expressions of CD11b, CD11c, CD14, CD18, CD54, CD80, CD86, CD206, MHC class II and TLR-2 (Toll-like receptor 2) in human macrophages infected with *L. amazonensis*. Phenotypic evaluation revealed a negative modulation in CD11b, CD11c, CD14, CD18, CD54 and MHC class II molecules, depending on the level of infection. The results showed that as early as 1 hour after infection no reduction in marker expression occurs, whereas after 24 hours, downregulation of these molecules was observed in macrophages. No significant changes were observed in the expressions of CD80, CD86, CD206 and TLR2. Evidence of the differential modulation of markers expression and that after parasite uptake no reduction in surface marker expression occurs indicates that parasite internalization is not involved in the phenomena of down-modulation.

KEYWORDS

costimulatory molecules, *Leishmania*, leishmaniosis, macrophage

1 | INTRODUCTION

Intracellular pathogens, such as *Leishmania*, *Toxoplasma gondii* and Mycobacterium *tuberculosis*, infect mononuclear phagocytes.[1,2] Manipulation of costimulatory molecules that shape the extent of immune responses by these microorganisms is suggested as one of the mechanisms of evading the host's defences.[1,2]

Leishmaniosis is a neglected disease caused by several species of *Leishmania*.[3] The protective immune response against *Leishmania* is associated with a Th1 phenotype and the activation of leishmanicidal activities by macrophages, while a disease Th2 phenotype prevents leishmanicidal macrophages.[4] Costimulatory molecules may influence the development of Th1/Th2 cell responses and stimulate or inhibit macrophage activation.[1]

Data in the literature support the modulation of expression patterns of costimulatory molecules and surface markers in macrophage by *Leishmania*.[5-14] However, the majority of studies examined 3 or 4 surface molecules in macrophages and only one level of infection. While human macrophages downregulate CD86 when infected with *L. chagasi*, these cells infected with *L. major* showed increased CD86[6,8], suggesting that different *Leishmania* species induce distinct modulations of costimulatory molecules in macrophages; these analyses have not been performed on *L. amazonensis*.[15] The experiments reported in this study were designed to evaluate the expression of integrins CD11b, CD11c and CD18, CD14 cell marker, adhesion molecule CD54, costimulatory molecules CD80 and CD86, mannose receptor CD206, MHC class II and TLR-2 (Toll-like receptor 2) in human macrophages infected with *L. amazonensis* amastigotes at variable infection rates.

2 | METHODS

2.1 | Parasite and cells

Leishmania amazonensis (MHOM/BR/73/M2269) amastigotes were isolated from skin lesions of BALB/c mice, as previously described.[16]

The experimental protocols were approved by the Ethics Committee for Animal Research of the Campinas State University (certificate number 4048-1). Peripheral blood mononuclear cells isolated from heparinized blood obtained of consenting healthy human adults and submitted to centrifugation using a Histopaque gradient (Sigma) were cultured in 24-well plates (5×10^5 cells/mL/well) for 7 days, with RPMI medium supplemented with gentamicin and 10% foetal bovine serum. Medium changes were performed after 2, 24 and 96 hours to achieve differentiation into macrophages.[17] The study protocol was approved by the Medical Ethical Committee of Campinas State University (certificate number 15620).

2.2 | Macrophage infection and assessment of intracellular parasites

The cells cultured on 24-well plates containing 13-mm diameter glass coverslips were infected with *L. amazonensis* amastigotes suspension in RPMI 1640 medium at a 1:1, 3:1 and 8:1 parasite-to-macrophage ratio for 1 or 24 hours at 37°C in 5% CO_2, 5% O_2 and balanced N_2. After the interaction period (1 or 24 hours), the cultures were washed to remove extracellular parasites. To evaluate the percentage of infected macrophages and the number of intracellular amastigotes, at least 600 macrophages were counted on triplicate coverslips stained with Giemsa and examined microscopically.[18] The experiments were performed in triplicate and were repeated independently at least 5 times.

2.3 | Flow cytometric analyses

Macrophages cultured in medium alone or infected with *L. amazonensis* were detached from the plates, washed and stained with anti-CD3–FITC (fluorescein), anti-CD4–FITC, anti-CD8–APC (allophycocyanin), anti-CD11c–APC Cy7 (cyanine dye Cy7), anti-CD11c–APC, anti-CD18–FITC, anti-CD54–PE (phycoerythrin), anti-MHC II–PE, anti-HLA-DR–PE, anti-CD80–PE, anti-CD86–APC, anti-CD206–PE, anti-TLR-2–Alexa Fluor 647 antibodies for 20 min at 4°C. All antibodies and IgG isotypes were purchased from BD Pharmingen. The cells were washed and fixed with 2% paraformaldehyde, as previously described.[19] Flow cytometry was performed in a FACSCanto flow cytometer, and the analyses were performed using FCS Express Software (Denovo Software). The data shown are representative of at least 3 independent experiments with similar results.

2.4 | Statistical analyses

All quantified data are presented as mean ± standard deviation or as indicated. A two-way analysis of variance (ANOVA) was used to analyse differences in the experimental means for flow cytometric values.

3 | RESULTS

The identity of human peripheral blood mononuclear-derived macrophages was confirmed by their large cell size, elongated or amoeboid shape with the majority expressing the phenotype $CD11b^{high}$ $CD14^{int}$ MHC-II^{high} $CD3^{neg}$ $CD8^{neg}$ (Figure 1 and data not shown).[20] As shown in Figure 1A with the 8:1 infection ratio (amastigote:macrophage), the percentage of infected cells and intracellular parasites is high compared to the level of infection intensity for macrophages inoculated with 1:1 and 3:1. The macrophages showed amastigotes inside large vacuole characteristic of infection with *L. amazonensis* (Figure 1B).

To determine whether *L. amazonensis* infection influences the costimulatory and marker molecules on macrophages, cells were infected and analysed by flow cytometer after 24 hours. The gating strategy is shown in Figure 1E. The data (ie, the percentage of cells expressing the cell markers) indicate that downregulation of some of the cell surface molecules was directly related to the intensity of infection (CD11b, CD11c, CD14 and MHC class II), while the expression of CD206 remained unaltered (Figure 1C). Although CD80 and CD86 showed a tendency towards downregulation in macrophages with high parasite burden no significant differences were observed in comparison with uninfected macrophages (Figure 1C). Results of MFI (median fluorescence intensity) indicated a decrease in expressions of CD11b, CD11c, CD14 and MHC class II of macrophages infected with *L. amazonensis* compared with uninfected cells (Figure 1E). Macrophages with high parasite burden also showed differences in CD18 and CD54 expressions but not TLR-2 (data not shown). It should be noted that the viability of infected and uninfected macrophages was similar for 24-30 hours.

Next, we investigated whether *L. amazonensis* adhesion and internalization trigger modulation of cell surface markers in human macrophages. The overall parasite burden was similar at 1 hour and 24 hours post-infection and significant changes in macrophage surface molecules only occurred at 24 hours (Figure 2).

4 | DISCUSSION

Phenotypic evaluation of the expression of human macrophage surface markers after *L. amazonensis* infection revealed no significant changes in the expressions of CD80, CD86, CD206 and TLR2 and negative modulation in CD11b, CD11c, CD14, CD18, CD54 and MHC class II molecules that was dependent on the level of infection level. The parasite internalization was not the mechanism responsible for the decreased expression of surface markers, since as early as 1 hour after the uptake of parasites no reduction in marker expression occurred, whereas after 24 hours, downregulation of surface molecules in macrophages was observed. The fact that only some of the markers are downregulated in *L. amazonensis* infected macrophages, that is differential modulation of marker expression occurs, is additional evidence that parasite internalization is not involved in this phenomena.

For the first time, this work presents data that MHC class II molecules responsible for presenting peptides derived from parasites to $CD4^+$ T cells, show diminished expression in human macrophages infected with *L. amazonensis*, in agreement with previous

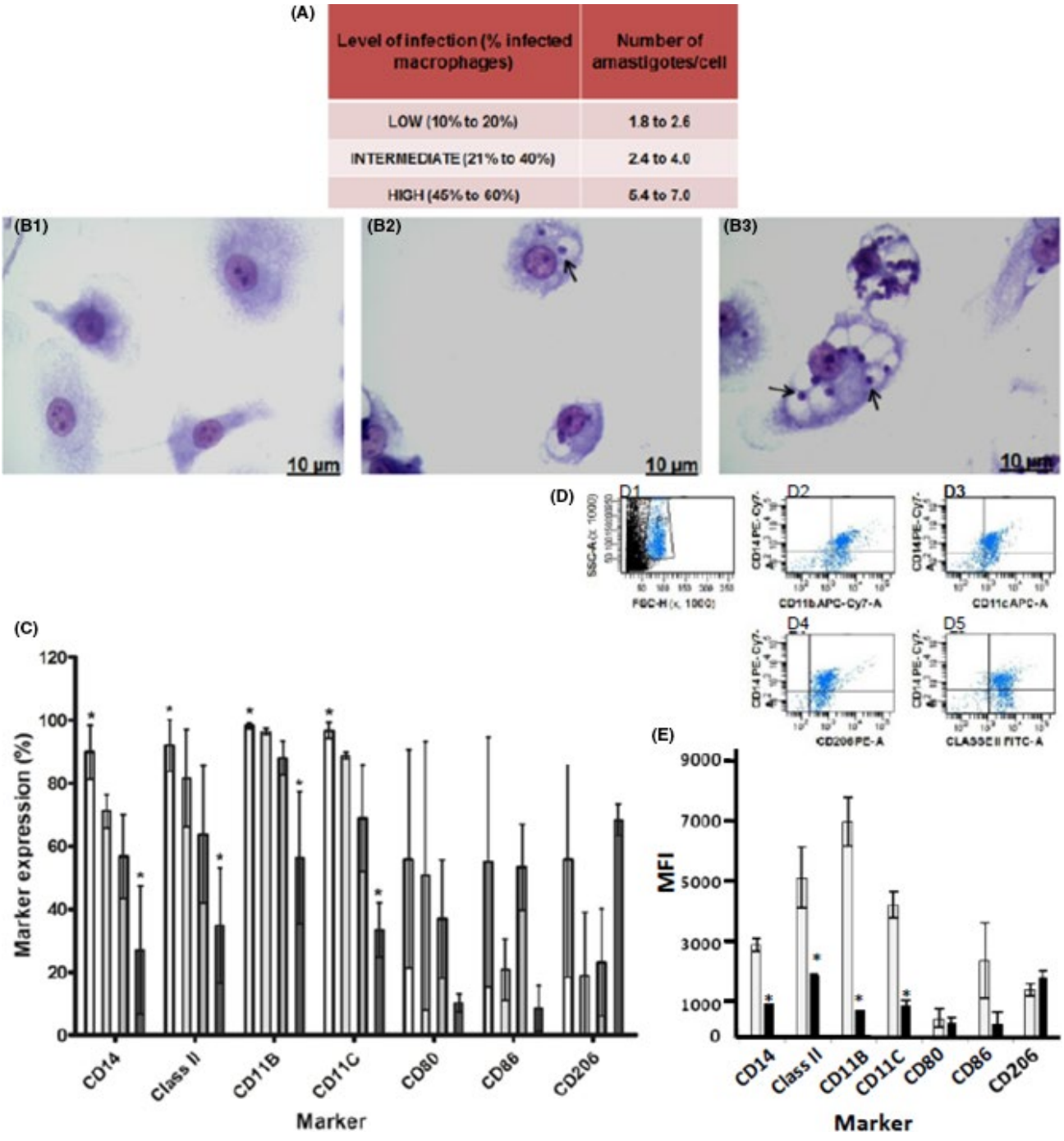

FIGURE 1 The level of *Leishmania amazonensis* infection intensity and expression of surface markers by human macrophages. (A) The level of infection was based on percentage of infected cells and number of amastigotes/cell for macrophage cultures infected with parasite: macrophage ratios 1:1 (low), 3:1 (intermediate) and 8:1 (high) after 24 h; (B) Micrographs of uninfected macrophages (B1), macrophages with low (B2) and high (B3) levels of infection, arrows indicate amastigotes within vacuoles; (C) The percentages of positive cells for surface markers in uninfected macrophages (white bars), macrophages with low (grey bars) intermediate (grey-black bars) and high (black bars) levels of infection after 24 h. * $P < .05$; (D) Gating strategy for flow cytometry; macrophages were gated using FSC/SSS-A profile (P1) (D1). Cells were stained with different cocktails of antibodies; 4 representative dot plots of some surface markers in uninfected macrophages (D2-D5); (E) The MFI of cell markers on uninfected macrophages (white bars) and infected macrophages with high levels of infection after 24 h (black bars). *$P < .05$

studies demonstrating that macrophages infected with *L. donovani*, *L. major* and *L. mexicana* are downregulated for MHC class II molecules.[10,13]

The CD11b, CD11c and CD18 leukointegrins play a role in phagocytosis, cell-to-cell and cell-to-extracellular matrix.[21] Our data indicate similarity with those in the literature, where reports have shown

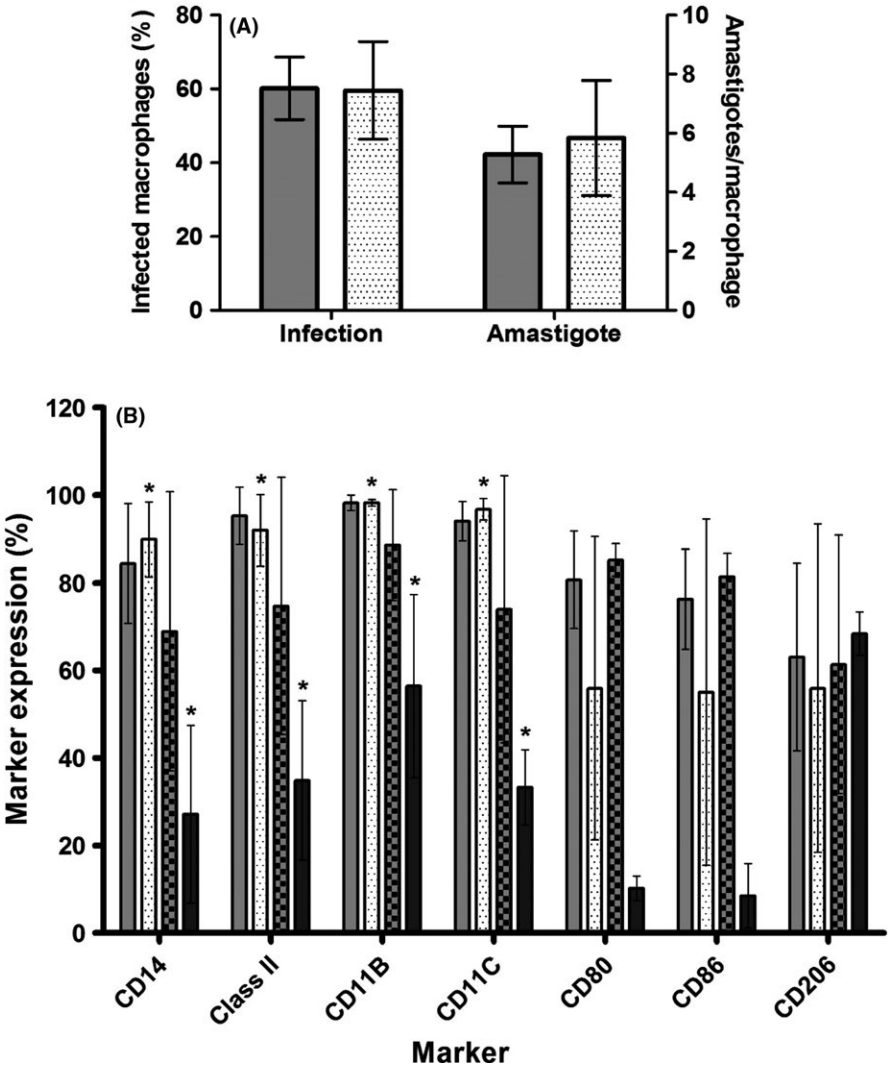

FIGURE 2 The time of *Leishmania amazonensis* infection and expression of surface markers by human macrophages. (A) The percentage of infected cells and number of amastigotes/cell for macrophage cultures infected with parasite: macrophage ratio 8:1 for 1 h (grey bar) and 24 h (doted bar); (B) The percentages of positive cells for surface markers in uninfected macrophages after 1 h (grey bars) and 24 h (doted bars), infected macrophages after 1 h (hatched bars) and 24 h (black bars) *$P < .05$

that *L. chagasi* infection induces decreased expression of CD11b/CD18 in macrophages.[22,23] CD14 a TLR4 and TLR1/2 coreceptors[24] are also low expressed in macrophages infected with *L. amazonensis*.

CD54 is a transmembrane protein of the Ig superfamily and is involved in the adhesion of leucocytes and endothelial cells.[25] Our data indicated decrease in CD54 expression in macrophages infected with *L. amazonensis*. Almeida et al[6] observed a decrease in CD54 expression in human monocytes infected with *L. chagasi*.

CD206, a mannose and pathogen recognition receptor, did not decrease in macrophages during *L. amazonensis* infection. Although there is evidence that *L. donovani* promastigotes use this receptor to enter the macrophage,[12,26] more recently, while using macrophages from mannose receptor knockout and normal mice, Akilov et al[27] observed no differences in infection rates, suggesting that CD206 is not necessary for *Leishmania* uptake. No significant changes were observed in the expressions of CD80 and CD86 costimulatory molecules; these data are similar to those using human macrophages infected with *L. major*, but different from another study using *L. chagasi*.[6,8] These controversial results may due to the different *Leishmania* species and/or parasite forms used in the assays.

Toll-like receptors are important pathogen recognition receptors; in contrast to the upregulation of TLR2 expression detected in human macrophages exposed to *L. panamensis*,[28] this receptor was not modulated by *L. amazonensis* infection.

Based on our results and previous studies, we propose that macrophages that contain numerous parasites show down-modulation of cell surface molecules are not capable of maintaining their immune activities and are responsible for the persistence of parasites in vivo.

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CONFLICT OF INTEREST

The authors affirm no conflict of interest relative to any source of funding, sponsorship or financial benefit.

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