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Research Article

RESEARCH ARTICLE

Long-term cell culture isolated from lesions of mice infected with *Leishmania amazonensis*: a new approach to study mononuclear phagocyte subpopulations during the infection

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One sentence summary: A new approach to study mononuclear phagocytes: cells isolated from *Leishmania*-infected mice footpad skin lesions were for the first time cultivated *ex vivo* and exhibited an anti-inflammatory/mixed phenotype.

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ABSTRACT

Leishmanioses are neglected diseases and the parasite *Leishmania* survives and proliferates within mononuclear phagocytes, particularly macrophages. *In vitro* studies of the immunology and cell biology of leishmaniosis are performed in murine peritoneum and bone marrow macrophages and immortalized cell lines despite the normal and injured tissue-specific heterogeneity of macrophages. In this work, we established an *ex vivo* methodology to culture lesional cells from BALB/c mice infected with *Leishmania amazonensis*. The cells were successfully isolated from footpad skin lesions and those exhibiting macrophage morphology were maintained in long-term culture (12 days), while the small number of lymphocytes, polymorphonuclear and unidentified cells died after 1 day of culture. The frequency of infected cells decreased over 2 days. Most lesional cells cultivated *ex vivo* were myeloid $CD11b^+$ $CD14^+$ $F4/80^+$ $CD68^+$ cells. Low levels of IFN-γ and IL-4, IL-10 production and low arginase and phagocytic activities were detected in *ex vivo* lesional cell cultures. The *ex vivo* model developed in this study open perspectives for studying the biology of leishmanial lesions in cellular subpopulations and at the single-cell level.

Keywords: *Leishmania amazonensis*; *ex vivo* culture; macrophages; myeloid cells; arginase; infection

INTRODUCTION

Leishmanioses are a group of insect-transmitted diseases, involving the intracellular parasite *Leishmania* as causative agent, and account for about two million cases annually (Okwor and Uzonna 2016). *Leishmania amazonensis* causes localized and diffuse cutaneous diseases and has an epidemiological impact in the Amazon region (McGwire and Satoskar 2014). Leishmaniosis are neglected diseases with few drug options and no available

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human vaccine (No 2016). *Leishmania* survives and proliferates within mononuclear phagocytes, especially macrophages (Kaye and Scott 2011). Based on mainly *in vitro* results, it is evident that resistance to *Leishmania* is due to inflammatory and activating cytokines (interleukin-12; IL-12), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and microbicidal molecules, especially nitric oxide; and susceptibility to the parasite due to immunosuppressive molecules, such as arachidonic acid metabolites, arginase and regulatory cytokines, such as IL-10 (Soong 2012). The historical use of easily accessible murine macrophage populations from peritoneum and bone marrow for these studies ignores the normal and injured tissue-specific heterogeneity of macrophages (Kaye and Scott 2011; Giorgio 2013; Wang *et al.* 2013).

The mouse model of *L. amazonensis* infection has been widely studied and all inbred strains develop lesions at some point during the infection (Pereira and Alves 2008). Although the same mouse models and inoculum size and site were not used, analyses of cells from lesions and proximal lymph node cells confirmed the presence of $CD11b^+$ $F4/80^+$ $LyGC^-$ cells, $CD11b^+CD11c^-GR1^+$ cells, $CD11c^+CD40^+$ cells and $CD3^+CD4^+CD8^+$, $CD3^+CD4^-CD8^+$ and $CD4^+CD25^+$ T cells (Ji, Sun and Soong 2003; Carneiro *et al.* 2015). The most frequently infected cells present in the lesions are myeloid cells, predominantly macrophages (Araujo, Arrais-Silva and Giorgio 2012). However, to our knowledge, analysis of cells isolated from lesions using an *ex vivo* culture system has not been performed previously. In this study, cells isolated from lesions of Balb/c mice infected with *L. amazonensis* were established and maintained in the proposed culture setting ($CD11b^+F4/80^+$ and $CD11b^-F4/80^-$ myeloid cells), and analyses of inflammatory and anti-inflammatory cytokines, leukocyte surface markers and arginase and phagocytic activities were performed.

MATERIALS AND METHODS

Parasites and mice

The promastigotes of *Leishmania amazonensis* (MHOM/BR/73/M2269) were cultured in RPMI 1640 supplemented with 10% inactivated fetal bovine serum (FBS) and 0.1% gentamicin at 25°C. The amastigotes were isolated from active skin lesions from mice and used immediately after isolation (Barbieri *et al.* 1993). BALB/c mice were subcutaneously infected in the right hind footpad with 1×10^7 promastigotes. After the inoculum, the lesion size was determined as the difference between the length of the infected and the hind left footpad measured by a paquimeter once a week (Arrais-Silva *et al.* 2006). Mice were euthanized at determined intervals to obtain cells from the lesions. All animal experiments were performed following the approved protocol of the Institute of Biology/CEUA-UNICAMP Ethical Committee for Animal Research.

Peritoneal macrophages

Peritoneal macrophages were obtained from normal BALB/c mice and cultured in RPMI 1640 medium supplemented with 10% SFB and 0.1% gentamicin. Cells were maintained under standardized culture conditions (37°C, 21% O_2, 5% CO_2). Macrophages were isolated according the method of peritoneal wash described by Barbiéri *et al.* (1993).

Isolation of lesional cells from mice infected with *Leishmania amazonensis*

Groups of three BALB/c mice were euthanized on different days after promastigote inoculation. The lesion and adjacent regions were disinfected with 70% antiseptic alcohol. When ulceration or secondary bacterial infection was observed, the crusts were removed with sterile scissors. Small pieces of lesions were collected in polystyrene tubes with RPMI 1640 medium supplemented with 10% FBS and 0.3% gentamicin. The tissue samples were vigorously macerated with a plastic, sterile serological pipette. After maceration, ice-cold supplemented RPMI 1640 medium was added and the suspension was filtrated through sterile gaze. The cells were incubated without stirring at 4°C for 30 min for debris decantation and to avoid cell adhesion to the tube surface. The supernatant was collected and the cells were counted using a hemocytometer chamber.

Magnetic separation of lesional cells

The cells were sorted through magnetic separation using Miltenyi technology. Single-cell suspensions (from peritoneal wash or lesion suspension) were maintained at 4°C until purification. Cell suspensions were passed through a 30-μm nylon mesh to remove cell clumps. The resultant flowthrough was centrifuged (300 g, 10 min, 4°C), and the supernatant was discarded. All the magnetic purification stages were performed at 4°C using separation buffer (phosphate-buffered saline pH 7.2, 2 mM EDTA, 0.5% bovine serum albumin). The label of cells with magnetic $CD11b^+$ microbeads (MiltenyiBiotec, USA) and all the magnetic purification stages were performed in accordance with the manufacturer's instructions. The number of unlabeled cells, i.e. those that passed through the column, and magnetically labeled cells, i.e. those that remained attached to the column, were determined by a hemocytometer counter and cells were cultured in 24-well culture plates with RPMI 1640 supplemented with 10% FBS and 0.3% gentamicin.

Leishmania amazonensis in vitro infection

The $CD11b^+$ peritoneal macrophages were cultured for 24 h in 24-well plates (5×10^5 cells/mL) with sterile 13-mm round cover slips. The culture medium was discarded and replaced by 1 mL of *L. amazonensis* amastigote suspension in RPMI 1640 medium at a 3:1 parasite to macrophage ratio. After 1 h of incubation under standard culture conditions, the amastigote suspension was discarded and the infected macrophages were washed with PBS, pH 7.4. Fresh RPMI 1640 was added and the plates were incubated for 24 h under standard culture conditions. Lesional $CD11b^+$ cells were cultured for 4 days (until no parasites or low number of parasites could be detected), and then infected with amastigotes as described for peritoneal cells. Only cell cultures with 60%–70% of infected macrophages were used for lipopolysaccharide (LPS) and IFN-γ treatment. The percentage of infected macrophages and the number of amastigotes per infected cell were determined by counting Giemsa stained slides (at least 200 cells counted) at ×100 magnification.

Immunofluorescence and flow cytometry

The phenotypic markers used in immunofluorescence and flow cytometry experiments were CD11b, CD14 and F4/80 and CD68. For immunofluorescence experiments, lesional cell and

peritoneal macrophage cultured in 24-wells plates (2×10^5 cells/mL) with sterile 13-mm round cover slips were washed with PBS pH 7.2 and fixed with 4% formalin for 10 min at 4°C. After washes with PBS, cells were incubated with ammonium chloride (50 mM) for 5 min at room temperature. For CD68 marker, cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature followed by washes with PBS, and blocked with PBS and 3% w/v of bovine serum albumin. For CD14 and CD68, cells were incubated for 2 h at room temperature with primary antibodies (rat IgG2a, κ purified anti-mouse CD14, rat IgG2a, κ purified anti-mouse CD68 (BioLegend, San Diego, CA). After washes with PBS, cells were incubated with secondary conjugated antibody (Alexa Fluor 488 goat anti-rat IgG H+L (Life Technologies, Waltham, MA), washed with PBS and incubated with DAPI (Sigma) in PBS. Dry-air slides were prepared with mounting solution and analyzed under an epifluorescence microscope (Leica DM LB). For F4/80 marker, after blocking treatment, cells were incubated for 2 h at room temperature with anti-F4/80 conjugated antibody (PE anti-mouse F4/80, Biolegend).

For flow cytometry analysis, cells (10^6) were incubated with the antibody to block unspecific ligations (purified rat anti-mouse CD16/CD3 Fc block FcyIII/II receptor, BD Pharmigen) and then with the conjugated antibodies or isotype controls (ctrl) diluted in staining buffer to detect the surface markers (PerCP/Cy5.5 rat IgG2b, κ, anti-mouse CD11b, PerCP/Cy5.5 rat IgG2b, κ isotype ctrl, PE rat IgG2a, κ anti-mouse F4/80, PE rat IgG2a, κ isotype control or fibroblast marker ER-TR7 PerCP) (Biolegend, Santa Cruz). Next, cells were washed with staining buffer and homogenized in the fixation and permeabilization buffer from the Cytofix/Cytoperm Fixation/Permeabilization kit. Fixed and permeabilized cells were homogenized with BD Perm/Wash buffer and incubated with anti-CD68 antibody or isotype control anti-mouse CD68 (Alexa Fluor 488 rat IgG2a, κ anti-mouse CD68 or Alexa Fluor 488 rat IgG2a, κ isotype ctrl) (BioLegend). Cells were washed with BD Perm/Wash buffer and the analyses were performed in FACS Canto II (BD Biosciences) (10 000 events for tube). Data analysis was performed with the software BD FACS Diva.

Arginase and phagocytosis activities

Peritoneal macrophages (infected with *L. amazonensis* amastigotes or not) were incubated for 24 or 48 h, and viability, infection intensity, arginase activity and phagocytosis rate were registered. The same treatments were performed on lesional $CD11b^+$ and $CD11b^-$ cells. Infection intensity was evaluated by counting Giemsa stained slides as described previously (Barbiéri *et al*. 1993). Intracellular arginase activity was measured using cell lysates with a QuantiChrom arginase assay kit (BioAssay Systems), following the manufacturer's instructions. The kit measures the conversion of arginine to urea by arginase. After total protein quantification, each sample was incubated with arginine buffer, the urea detection reagent containing antiisonitrosopropiophenone and the optical density was read using a 430 nm filter. In phagocytosis assays, cells were exposed to latex beads coated with fluorescently labeled rabbit IgG, following the manufacturer's instructions (Phagocytosis Assay Kit IgG-FITC, Cayman Chemical, Ann Arbor, MI, USA). Phagocytosis was examined by fluorescent microscopy and fluorescence intensity (485 nm Ex/535 nm Em) was measured using a multi-mode microplate reader (Synergy HT; Biotek, Winooski, VT, USA). Samples were analyzed by duplicate in at least two independent experiments.

Cytokine profile

Samples of cell culture supernatants (5×10^5 cells/mL/well) were taken daily and stored at –80°C to quantify IFN-γ, IL-4 and IL-10 contents by ELISA Kits (sensitivity 4, 1 and 16 pg/mL, respectively) (ELISA MAX Set Deluxe, BioLegend). Samples were analyzed by triplicate in three independent experiments. The microplates were read in 450/570 nm and the difference between the absorbance measured at both wavelengths was used to determine the concentration of cytokines by the five-parameter logistic curve fit interpolation method compared with a standard curve in each experiment.

LPS and IFN-γ treatment

Peritoneal macrophages infected with *L. amazonensis* and lesional cells were incubated with 50–100 pg/mL LPS from *Escherichia coli* (Sigma) and 5 U/mL recombinant mouse IFN-γ (Sigma) for 1 or 2 days (Corradin, Buchmüller-Rouiller and Mauël 1991), and viability and infection intensity were evaluated as described above.

Statistical analyses

Two-way ANOVA tests were used to determine significant differences between samples in most experiments, except the cytokine production assays, which were analyzed by Mann-Whitney *U* tests. A value of $P < 0.05$ was used as the cut-off for statistical significance.

RESULTS

Ex vivo culture of lesions from BALB/c mice infected with *Leishmania amazonensis*

First, we evaluated the cutaneous lesion progression, microscopic characteristics, number and viability of cells obtained from lesions of Balb/c mice at different times after *L. amazonensis* inoculum in order to choose an adequate tissue lesion for cell isolation (Fig. 1). Based on these results, we choose to work with lesions from mice at 8 weeks after inoculum, since this period yielded adequate cell numbers (Fig. 1D) and there were no macroscopic ulcerations and no secondary bacterial infections (Fig. 1A–C) (Giorgio *et al*. 1998).

Next, cells isolated from mouse lesions were cultured *ex vivo*, and the morphological features (Fig. 2) and infection rate were evaluated over several days. It should be noted that the cellular composition of *ex vivo* cell cultures was largely composed of cells exhibiting macrophage morphology (Fig. 2 and Table 1), together with more than three types of lymphocytes, polymorphonuclear cells and morphologically unidentified cells, which were only observed during the first 24 h (Table 1). Cells exhibiting macrophage morphology retained viability of higher than 85% for at least 1 week after isolation from lesions. On day 1, cell cultures showed 95%–100% of infected cells and 18.4 ± 6.6 intracellular amastigotes per cell. On day 2, the number of intracellular parasites was still high (13.9 ± 6.1), but the percentage of infected cells greatly decreased. On days 3 and 4, cell cultures were characterized by sporadic visualization of infected cells and a sharp decrease in intracellular parasites (5.1 ± 2.9 and 1.7 ± 0.6, respectively), there were no infected cells recorded on days 5 and 7. Morphologically, cells with vacuolated cytoplasm were observed on days 2 and 3 (Fig. 2). From then on, these cells were less frequently observed and cells with uniform cytoplasm and

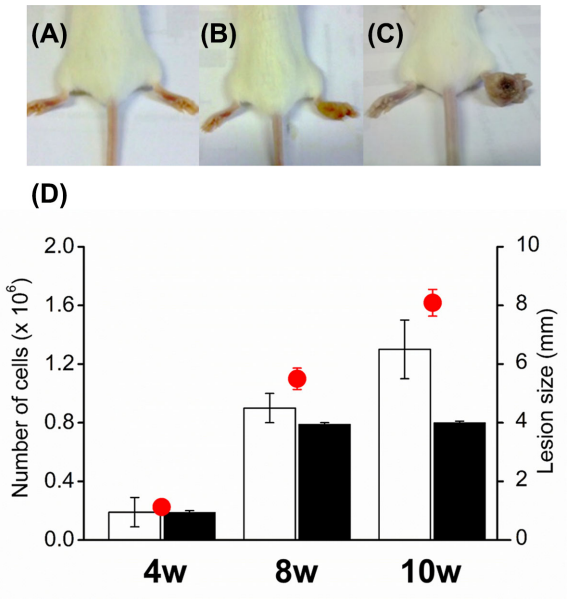

Figure 1. (A–C) Progression of skin lesions. (D) Evaluation of cutaneous lesions in BALB/c mice at different weeks (w) after *L. amazonensis* inoculum. The left axis represents the number of cells (white bars) and viable cells (black bars) obtained from the lesions of three mice per time point. The right axis represents lesion size (•). One representative experiment of three independent experiments is shown.

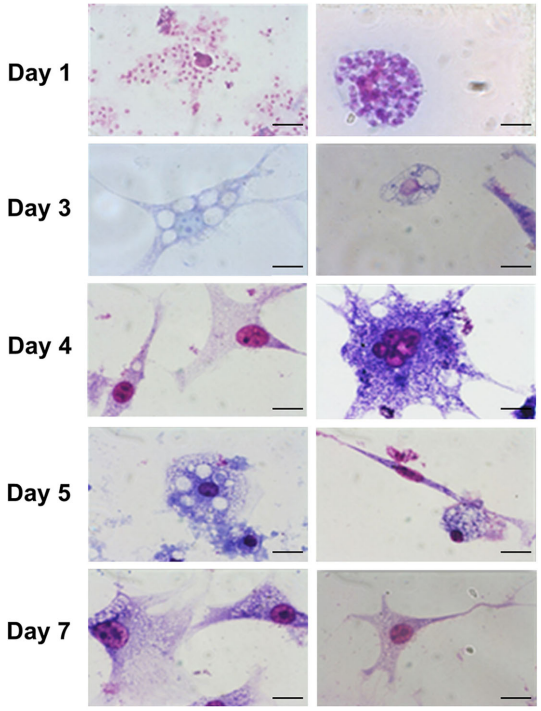

Figure 2. Morphology of lesional cells cultivated *ex vivo*. Cells obtained from lesions from mice were cultivated for different periods in RPMI medium and stained with Giemsa. Note parasitized cell on day 1 and large and spread stellate cells after 3 days of culture. Bar = 10 μm (micrometers).

diverse morphology were observed in the plates (Fig. 2). On day 7, when no signal of infection was detected, we tested whether *ex vivo* cells were susceptible to a challenge with *L. amazonensis* infection. The results were variable with infection susceptibility ranging between 0.5% and 75% of the cell cultures.

Analysis of leukocyte markers in lesional cells cultivated *ex vivo*

Flow cytometry and immunofluorescence phenotypic characterization of lesional cells were performed for 2 and 4 days on *ex vivo* cell cultures. The representative results are shown in Fig. 3 and gating strategies for flow cytometry analyses are shown in Fig. S1–S3 (Supporting Information). The results indicate that the percentage of lesional cells expressing C11b$^+$ was 47.2% on day 2 and 74.8% on day 4 and, the expression of CD68$^+$ was relatively unchanged at both time points (Fig. 3A). Meanwhile lesional cells expressing three markers (CD11b, F4/80 and CD68) showed variation mainly in the expression intensity of CD11b$^+$ and F4/80$^+$, but all the samples tested positive for immunofluorescence to CD14 and F4/80 on days 2, 4 and 8 (Fig. 3B and data not shown), confirming their identity as macrophages.

Since the majority of lesional cells cultivated *ex vivo* were CD11b$^+$ cells (Fig. 3A), we decided to perform magnetic separation based on CD11b$^+$ microbeads recognition. In fact, ~80% of cells from the lesions were retained on the separation column (CD11b$^+$ cells) and showed 100% viability. The cell fraction CD11b$^+$ was cultured, and phenotypical analyses confirmed that these cells expressed CD11b on days 2 and 4 (around 60% of cells display high intensity of CD11b$^+$). The percentage of CD11b$^+$ F4/80$^+$ cells was 18.6% on day 2 and 33.9% on day 4 (Fig. 3A). In the case of CD68$^+$, around 25% of cells were positive only for this marker (Fig. 3). These cells are positive for immunofluorescence to CD14 and F4/80 (Fig. 3B and data not shown).

Cells that were not retained in the column were also cultivated. Comparative flow cytometry analyses showed, as expected, a lower percentage of cells expressing CD11b on day 2 among these cells compared with lesional cells retained on the separation column. These cells (CD11b$^-$ cells) are negative

Table 1. Cellular composition of *ex vivo* cultures from lesions of BALB/c mice infected with *L. amazonensis*.[a]

Cell type	Lesional *ex vivo* culture (time)						
	0 h	1 h	9 h	1 day	2 days	6 days	12 days
				Cells (%)			
Macrophages[b]	55.5 ± 2.5	78 ± 5.6	91 ± 9.8	72.1 ± 6	98.1 ± 2	100	100
PMN	12.1 ± 3.1	12.1 ± 2.7	0	0	0	0	0
Lymphocytes	13 ± 0.9	4.0 ± 0.5	5.5 ± 0.5	2 ± 0.2	1.9 ± 0.5	0	0
Unidentified cells	19.4 ± 4.7	5.9 ± 2.4	3.5 ± 0.6	25.9 ± 3	0	0	0

[a]Cells were obtained from lesions of BALB/c mice after 8 weeks of *L. amazonensis* inoculation. Cells were stained with Giemsa after *ex vivo* culture at different *time* points and the percentage of different cell types were determined by optical microscopy. Data are the median values ± SD of three independent experiments.
[b]Include macrophages and monocytes.

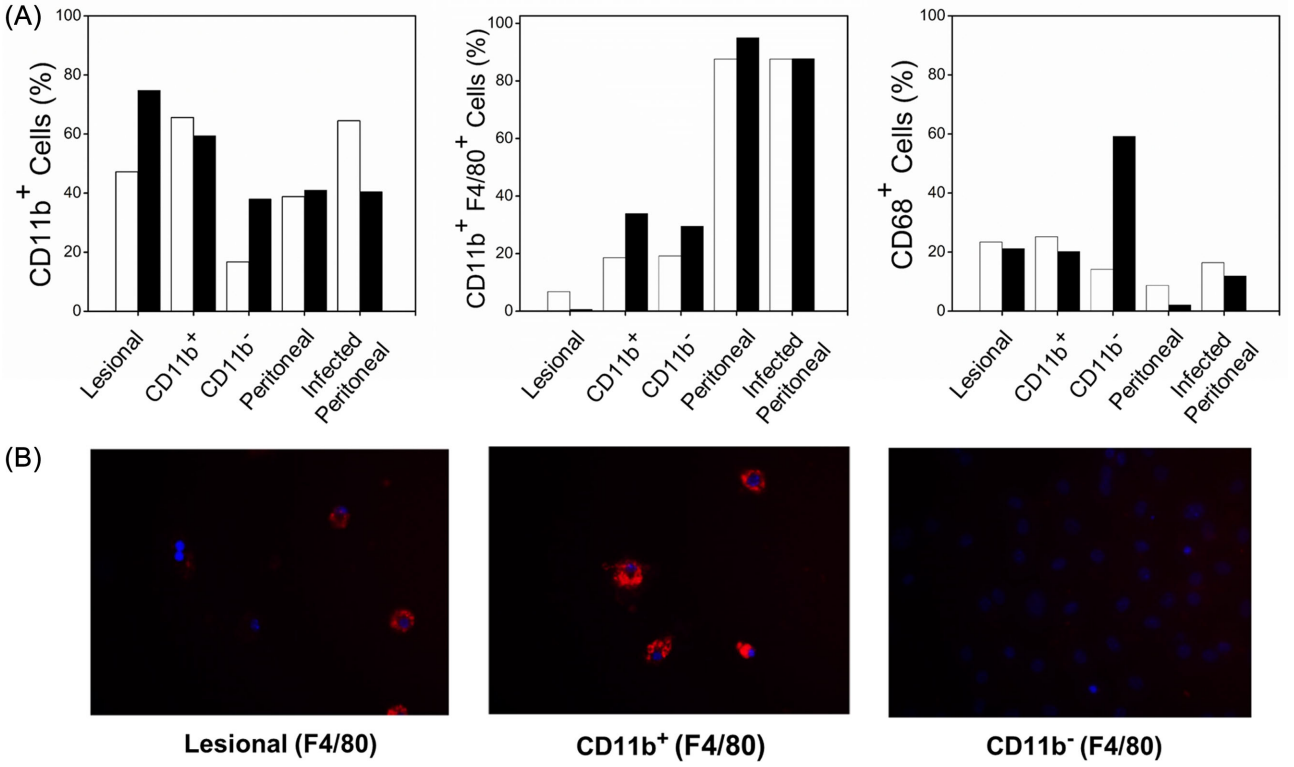

Figure 3. Leukocyte surface markers in cells cultivated *ex vivo*. Lesional cells (lesional), lesional CD11b$^+$ cells (CD11b$^+$); lesional CD11b$^-$microbeads cells (CD11b$^-$); peritoneal CD11$^+$ cells (peritoneal); peritoneal CD11$^+$ cells infected with *L. amazonensis* (infected peritoneal). (**A**) Two days of culture (white boxes), 4 days of culture (black boxes). One representative experiment of three independent experiments is shown. Epifluorescence microscope images of cells obtained from lesions on day 4 of culture (B).

for immunofluorescence to CD14 and F4/80 (Fig. 3B and data not shown). In all cases, concomitant experiments with CD11b$^+$ cells isolated from peritoneal fluid obtained from healthy mice using the same methodology were infected *in vitro* or not with *L. amazonensis* and conducted as controls of immunofluorescence and flow cytometry reproducibility, CD11b+ was expressed frequently in these cells and F4/80 was expressed by both uninfected and infected peritoneal cells (Fig. 3 and data not shown).

Production of IFN-γ, IL-4 and IL-10 in lesional cells cultivated *ex vivo*

Next, cytokine production (IFN-γ, IL-4 and IL-10) was examined in the culture supernatants of the lesional, CD11b$^+$ and CD11b$^-$ cells. The supernatant of lesional CD11b$^+$ cells during the culture period (1–7 days) showed low levels of IFN-γ (Fig. 4A) ranging between 13 and 29 pg/mL. Similar IFN-γ concentrations were verified in the supernatant of lesional CD11b$^-$ cells and cells from unpurified lesions (Fig. 4A). IFN-γ production from infected or uninfected peritoneal CD11b$^+$ cells were similar, and the registered values were close to the limit of ELISA detection (4 pg/mL) (data not shown).

The unpurified and CD11b$^+$ lesional cells cultures showed the presence of IL-4 during the first days (21–53 pg/mL up to day 4) (Fig. 4B), which continuously decreased up to day 12 (data not shown). The supernatants of lesional CD11b$^-$ cells showed low IL-4 concentrations during the observation window ($\leq$20 pg/mL) (Fig. 4B). It should be noted that on days 5 and 6, unpurified lesional cells produced significantly greater concentration of IL-4 (around 80 pg/mL) than CD11b$^+$ cells (around 50 pg/mL) (Fig. 4B). IL-4 production was not detectable in the supernatant of uninfected CD11b$^+$ peritoneal cells, and *L. amazonensis*-infected CD11b$^+$ peritoneal cells produced low IL-4 concentration in two out of three experiments (5–10 pg/mL).

The IL-10 concentration in the supernatants of lesional CD11b$^+$ cells between 78 and 143 pg/mL was detected during the seven days (Fig. 4C) and decreased up to day 12 (31 pg/mL) (data not shown). Registered values of IL-10 in lesional CD11b$^+$ cell supernatants were greater than the values verified in the supernatants of lesional CD11b$^-$ cells ($\leq$25 pg/mL) and unpurified lesional cells (50–60 pg/mL) (Fig. 4C). IL-10 concentration in the supernatants of uninfected peritoneal CD11b$^+$ cells was 233.9 pg/mL, higher than those of infected peritoneal CD11b$^+$ cells (13.93 pg/mL) (data not shown).

Activities of arginase and phagocytosis in lesional cells cultivated *ex vivo*

Figure 5A shows the arginase activity of cells cultivated on days 1 and 2. Arginase activity in lesional CD11b$^+$ and CD11b$^-$ cells was lower than those of infected and uninfected CD11b$^+$ peritoneal cells (Fig. 5A). A slight increase (20%–30%) in arginase activity was detected in lesional CD11b$^+$ cells after LPS stimulus (0.2 ± 0.05 and 0.15 ± 0.09 A.V./L/cell on days 1 and 2 versus 0.7 ± 0.05 and 1.1 ± 0.08 A.V./L/cell on days 1 and 2 after LPS stimulation) and lesional CD11b$^-$ cells were unresponsive to LPS (data not shown).

The phagocytic rate was lower in cells derived from lesions (CD11b$^+$ and CD11b$^-$ cells) than in peritoneal CD11b$^+$ cells

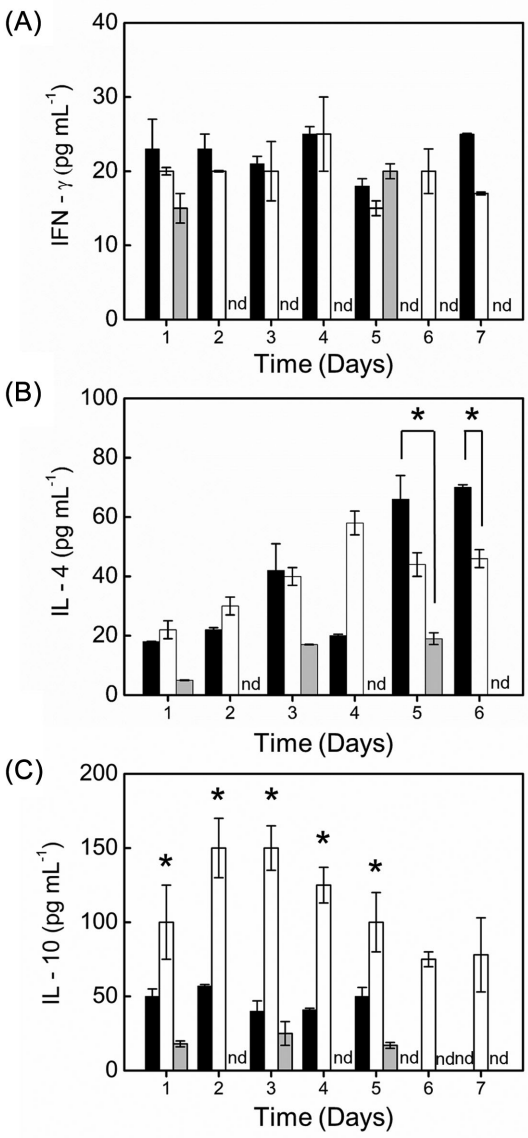

Figure 4. Cytokines production by lesional cells. IFN-γ, IL-4 and IL-10 levels were detected in the supernatants of cell cultures for different periods, as described in Material and Methods section. Lesional cell (black boxes), $CD11^+$ cells (white boxes) and $CD11^-$ cells (gray boxes). One representative experiment of three independent experiments is shown; nd: not done. $^*P < 0.005$.

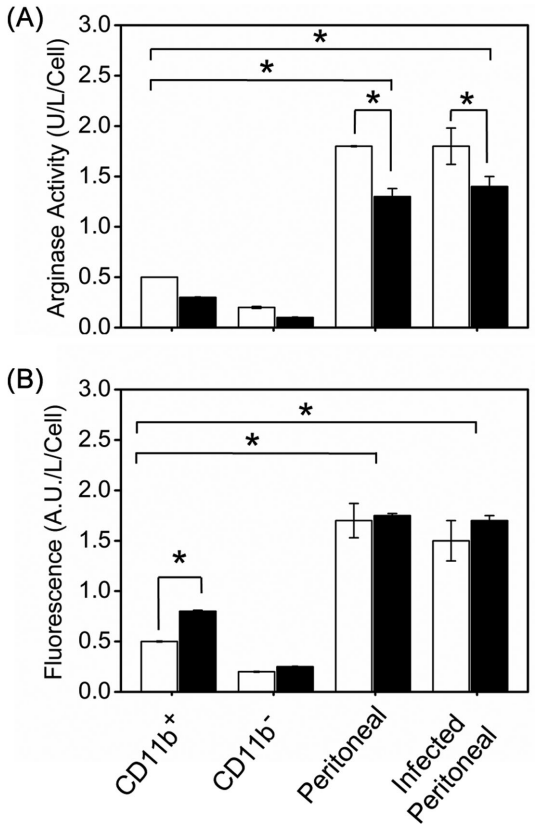

Figure 5. Arginase and phagocytic activities of lesional cells. (**A**) Arginase and (**B**) phagocytic activities after 1 and 2 days of culture. A.U./cells (arbitrary units per cell), U/L/cell (unit/Liter/cell). One day of culture (white boxes), 2 days of culture (black boxes). One representative experiment of three independent experiments is shown. $^*P < 0.001$.

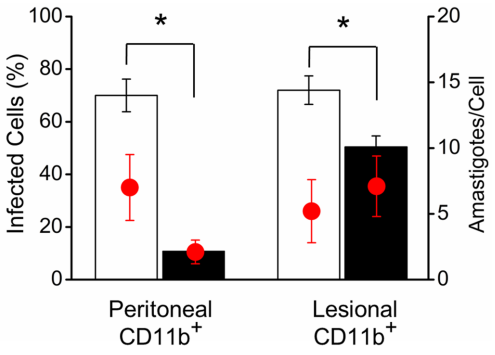

Figure 6. LPS (white bars) and IFN-γ (black bars) treatment on *L. amazonensis*-infected macrophages. Peritoneal and lesional $CD11^+$ cells were infected with amastigotes as described in Materials and methods section and treated with LPS and IFN-γ. The percentage of infected cells (left axis) and intracellular amastigotes (right axis, •) were estimated after 2 days of culture. One representative experiment of three independent experiments is shown. $^*P < 0.05$.

(Fig. 5B), and there was a slight increase in the phagocytic activity of lesion $CD11b^+$ cells after day 2 (Fig. 5B).

Since cultivated peritoneal macrophages and monocyte-macrophage cell lines are able to kill *Leishmania* when stimulated with IFN-γ and LPS (Corradin, Buchmüller-Rouiller and Mauël 1991; Nylén and Gautam 2010; Soong 2012; Kima and Soong 2013; Kima 2014), we tested the ability of lesional cells stimulated *in vitro* to control *L. amazonensis* infection. Assays for standardizing conditions of peritoneal $CD11^+$ cells stimulated with IFN-γ and LPS in order to decrease infection rate were performed. Incubating these cells in the presence of 5 U/mL IFN-γ plus 50 pg/mL LPS for 1 and 2 days generated a decrease of 84.5% in infected cells and diminished the mean number of intracellular amastigotes from 7.0 to 2.1 on day 2 (Fig. 6). The same condition was tested in lesional $CD11b^+$ cells and a decrease of 30% in the infected cells was observed in the cultures, although the number of intracellular amastigotes did not change in the response to stimuli (Fig. 6).

DISCUSSION

In this work, we performed *ex vivo* analysis of cells from lesions of BALB/c mice after 8 weeks of induced infection with *Leishmania amazonensis*. To our knowledge, an *ex vivo*

experimental model has not been developed as a means of clarifying our understanding of the biology of lesional cells. As it well known, BALB/c mice are susceptible to developing cutaneous leishmanial lesions (Pereira and Alves 2008; Kaye and Scott 2011; Mougneau, Bihl and Glaichenhaus 2011). Our data showed that after isolation, cells exhibiting macrophage morphology are the predominant cell in mouse lesion, though three more cell types in low numbers were observed: polymorphonuclear cells, lymphocytes and cells of undefined morphology. The data confirm previous observations (Mcelrath *et al.* 1987; Araujo and Giorgio 2015) that suggested a paucity of lymphocytes and massive numbers of infected macrophages surrounded by eosinophils. The *ex vivo* cultures of leishmanial lesions allowed us to observe cells exhibiting macrophage morphology that maintained their viability during the entire experimental period (12 days), while the other three cell types died within 24 h. Infected macrophages were the predominant cells observed on day 2, and were then replaced by a monolayer of cells with vacuolated cytoplasm and thereafter by cells with vacuoles or uniform cytoplasm. This progressive reduction in the number of intracellular parasites and/or infected macrophages after day 2 suggest either that cells were able to limit the infection or most likely that amastigotes die inside the macrophages, since dermal *Leishmania* species such as *L. amazonensis* is better adapted to temperature lower than 37° C (Scott, Sacks and Sher 1983).

The cells in the lesion are mainly myeloid $CD11b^+$ $CD14b^+$ $F4/80^+$ $CD68^+$ cells, identified on days 2 and 4 of culture, confirming previous studies showing a predominance of $CD11b^+$ cells in footpad lesions (Araujo, Arrais-Silva and Giorgio 2012; Teixeira *et al.* 2015). Comparing the surface molecules' expression, our study revealed that lesional $CD11b^+$ cell cultures showed a relatively low number of $CD11b^+$ $F4/80^+$ cells as compared with peritoneal cell cultures, As F4/80 is a surface molecule related to macrophage maturation (Gordon and Taylor 2005), the data suggest that these cells are less mature than peritoneal macrophages.

Its known that during type 1/type2 cytokine responses polarization is seen in sera and lymph node cells from *L. major* infected resistant/susceptible mice, respectively (McMahon-Pratt and Alexander 2004; Kima and Soong 2013), a mixed cytokine response is suggested in reports using lymph node and spleen cells and lesion homogenates from susceptible mice infected with *L. amazonensis* (Soong 2012). IFN-γ and IL-4 levels are detectable, though relatively low, and IL-10 and tumor growth factor levels are high (Campbell *et al.* 2003; Arrais-Silva *et al.* 2006; Barroso *et al.* 2007; Felizardo *et al.* 2012; de Matos Guedes *et al.* 2014). In addition, $IL\text{-}10^+$ $CD11b^+$ cells were detected in lymph nodes from BALB/c mice (Velasquez *et al.* 2016). It is difficult to compare our data using cultivated lesional cells with data from these reports, since they used draining lymph nodes and spleen cells, and lesion homogenates. In any case, we verified low but detectable levels of both IFN-γ and IL-4 in supernatants of total lesional cells and $CD11b^+$ cells while no production occurred in lesional $CD11b^-$cells and uninfected and infected peritoneal $CD11b^+$ cells. Furthermore, our data confirmed the mixed cytokine profile of lesional microenvironment suggested for *L. amazonensis*-infected human and mice (Campbell *et al.* 2003; Arrais-Silva *et al.* 2006; Barroso *et al.* 2007; Felizardo *et al.* 2012; de Matos Guedes *et al.* 2014). One observation of some interest was that IL-10 levels were high in the supernatant of lesional $CD11b^+$ cells compared with IL-10 levels of supernatants of total lesional cells, lesional $CD11b^-$ cells and infected peritoneal $CD11b^+$ cells, although lower than the IL-10 levels produced by uninfected peritoneal $CD11b^+$ cells. It should be noted that IL-10 production in sorted peritoneal $F4/80^+$ $CD11b^+$ cells was reported by Wang *et al.* (2013). Our results suggest that IL-10 was produced by lesional $CD11b^+$ cells and consumed by other cells in the unpurified lesional cell cultures, since when $CD11b^+$ cells were cultivated alone the IL-10 levels were high. These data also suggest that $CD11b^+$ cells are the source of IL-10 in lesions from *L. amazonensis*-infected BALB/c mice. This is to be expected, since macrophages predominate in these lesions (Mcelrath *et al.* 1987; Araujo and Giorgio 2015). Findings have been reported for *L. major* and *L. mexicana* infection using T-cell-specific and macrophage-specific mutant mice in which T cells are the main source of IL-10. However, cells from lesions were not directly assessed for IL-10 in these studies, rather supernatants from draining lymph node cells and RNA extracted from tissue lesions were analyzed for IL-10 (Schwarz *et al.* 2013; Buxbaum 2015).

Phagocytic capacity and arginase activity were low in lesional $CD11b^+$ and $CD11b^-$ cells compared with infected and uninfected peritoneal macrophages, and the capacity to control infection in response to IFN-γ and LPS was lower than that of peritoneal $CD11b^+$ cells. Despite the high phenotypic heterogeneity of macrophages, the most prevalent model still describes M1/M2 polarization. Briefly, M1 macrophages are mainly characterized by inflammatory cytokine secretion and antimicrobial properties, and M2 macrophages have high phagocytosis capacity, decreased ability to produce proinflammatory cytokines and increased production of arginase and IL-10 (Giorgio 2013; Röszer 2015). Taken together, our results suggest that lesional $CD11b^+$ cells cultivated *ex vivo* exhibit some of the characteristics of M1 macrophages (low arginase and phagocytic activities) and M2 macrophages (IL-10 production). Other studies confirm the presence of functional variants of macrophages during bacterial infections with *Haemophilus ducreyi*, *Heliobacter pylori* and *Neisseria meningitidis* (Quiding-Jarbrink, Raghavan and Sundquist 2010; Li, Katz and Spinola 2012; Wang *et al.* 2016). Further studies are needed to better characterize the complex phenotype of cells subpopulations in leishmaniosis lesions.

In summary, an *ex vivo* model of lesional cells from mice infected with *L. amazonensis* was developed for first time in this study. The *ex vivo* model of lesional cell cultures allowed us to not only identify cells, but also analyze cytokine production, leukocyte surface markers, arginase and phagocytic capacities. The main cell type in *ex vivo* cultures are myeloid $CD11b^+$ $CD14^+$ $F4/80^+$ $CD68^+$ cells. Low levels of IFN-γ and IL-4, IL-10 production and low arginase and phagocytic activities were detected in *ex vivo* lesional cultures. This report also opens perspectives for identifying molecular profiles and surface biomarkers and studying the biology of leishmanial lesions in cellular subpopulations and at the single-cell level.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

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Conflict of Interest. None declared.

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