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ARTICLE

Effects of extracellular vesicles released by peritoneal B-1 cells on experimental *Leishmania* (*Leishmania*) *amazonensis* infection

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Abstract

B-1 cells are a B-lymphocyte subtype whose roles in immunity are not completely defined. These cells can produce cytokines (mainly IL-10) and natural and specific antibodies. Currently, extracellular vesicles (EVs) released by immune cells have emerged as new important entities in cell-cell communication. Immune cells release EVs that can activate and/or modulate other immune cells. Here, we characterized the EVs released by peritoneal B-1 cells infected or not with Leishmania (*Leishmania*) *amazonensis*. This *Leishmania* species causes cutaneous leishmaniasis and can infect macrophages and B-1 cells. Our results showed that peritoneal B-1 cells spontaneously release EVs, but the parasite stimulated an increase in EVs production by peritoneal B-1 cells. The treatment of BALB/c and C57BL/6 bone marrow-derived macrophages (BMDM) with EVs from infected peritoneal B-1 cells led to differential expression of iNOS, IL-6, IL-10, and TNF-α. Additionally, BALB/c mice previous treated with EVs released by peritoneal B-1 cells showed a significant lower lesion size and parasite burden. Thus, this study demonstrated that peritoneal B-1 cells could release EVs that can alter the functions of macrophages in vitro and in vivo these EVs altered the course of *L. amazonensis* infection. These findings represent the first evidence that EVs from peritoneal B-1 cells can act as a new mechanism of cellular communication between macrophages and B-1 cells, contributing to immunity against experimental leishmaniasis.

KEYWORDS

B-1 cells, extracellular vesicles, *Leishmania* (*L.*) *amazonensis*, macrophages

1 | INTRODUCTION

B-1 cells are a subtype of B lymphocytes with peculiar functions in immunity. Although these cells belong to the B-lymphocyte population, certain phenotypic characteristics distinguish B-1 cells, especially those related to cell surface markers, ontogeny, anatomic distribution, self-renewing ability, and precursors.[1–3] In the steady state, B-1 cells produce natural antibodies[4] and large amounts of IL-10[5]. However, after stimulation, they may differentiate into phagocytic cells,[6] migrate to the inflammatory milieu,[7] produce different types of pro and anti-inflammatory cytokines,[5,8] present antigens,[9] and produce specific antibodies.[10,11] Currently, extracellular vesicles (EVs) released by immune or nonimmune cells have been proposed as a new player in cell-to-cell communication.[12,13] However, data on the production and release of EVs released by B-1 cells, as well as their role in the modulation of other cells, have not yet been studied.

EVs have acquired great importance in the field of immunology due to their ability to mediate the signaling and transfer of biomolecules (communication) between the cells of the immune system.[12,13] EVs are released by prokaryotic and eukaryotic cells and can carry different types of biomolecules (proteins, lipids, nucleic acids, and sugars) that play pivotal role during interaction with the host.[14–16] Additionally, communication by EVs dispenses cell-cell contact and allows to the delivery of messages to remote sites.[13] Furthermore,

Abbreviations: ADCL, anergic diffuse cutaneous leishmaniasis; BMDM, bone marrow-derived macrophages; CL, cutaneous leishmaniasis; DCL, disseminated cutaneous leishmaniasis; EVs, extracellular vesicles; Th, T helper.

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pathogen-infected immune host cells can release EVs containing different compositions, leading to a different response in uninfected cells.[12] Although the study of the communication among cells of the immune system by EVs is relatively recent, several studies have shown that EVs released by activated cells can act as messengers carrying mediators and signals from one cell to another.[12,13,17,18]

This observation has been corroborated by studies with intracellular protozoan pathogens showing that they induce the release of exosomes from the cells that they infect.[19,20] For *Leishmania*, macrophages in vitro treated with EVs released from cells infected with *Leishmania mexicana* up-regulated the phosphorylation of signaling proteins and significantly increased the immune-related response.[21] EVs from *Leishmania* (*Leishmania*) *amazonensis*-infected macrophages stimulated naïve macrophages to initiate antiparasitic Th1 responses through the release of the inflammatory cytokines IL-12, IL-1β, and TNF-α[19]. However, the role of EVs released by infected cells in the pathogenesis of leishmaniasis, as well as their influence on other cells of the immune system, is still poorly understood.

Leishmania amazonensis causes cutaneous leishmaniasis (CL), anergic diffuse cutaneous leishmaniasis (ADCL), and disseminated cutaneous leishmaniasis (DCL). This species is often associated to drug resistance and treatment failure[22,23] and to induce immunologic anergy by subverting the host innate defense machinery.[24,25,26] *L. amazonensis* infects different cell types; however, in macrophages, the parasites are rapidly phagocytosed, survive, and multiply. Our group demonstrated that B-1 cells could phagocytose more efficiently *L. amazonensis* promastigotes than macrophages[27] and that these cells participated in parasite resistance in experimental infection.[28] These findings led us to question the mechanisms by which peritoneal B-1 cells could participate in the immunity against CL. Thus, here, we characterized EVs released by peritoneal B-1 cells, their immunomodulatory effects on different macrophages lineages and their role in the progression of experimental leishmaniasis.

2 | METHODS

2.1 | Animals

Pathogen-free female BALB/c and C57BL/6 mice (6–8 wk of age) were purchased from the Center for the Development of Experimental Models for Medicine and Biology (CEDEME, Universidade Federal de São Paulo—UNIFESP, São Paulo, Brazil). All animals were treated according to the guidelines of the National Council for Control Animal Experimentation (CONCEA) of Brazil. Animal procedures were approved by the Committee on Ethics of Animal Experiments (CEUA) of UNIFESP (protocols 1359120716 and 5076200215). All efforts were made to minimize the animals' suffering.

2.2 | Parasites

Leishmania amazonensis (MHOM/BR/1973/M2269) promastigotes (kindly provided by Dr. Clara Lucia Barbieri—Universidade Federal de São Paulo) were cultured in 199 medium (Gibco, Life Technologies Brand, Grand Island, NY, USA) supplemented with 4.2 mM sodium bicarbonate, 4.2 mM HEPES, 1 mM adenine, 5 μg/mL hemin (bovine type I) (Sigma, St. Louis, MO, USA), and 10% inactivated FBS (Gibco). Parasites were cultured at 26°C until the stationary growth phase. At this stage, parasites were washed 5 times with PBS and used for in vitro and in vivo infections.

2.3 | Bone marrow-derived macrophages (BMDMs)

BMDMs were obtained according to procedures previously described by Zamboni and Rabinovitch (2003)[29] with some modifications. The femurs of BALB/c and/or C57BL/6 mice were removed, and bone marrow cells were collected by washing with RPMI 1640 medium. The cells were cultured for 3 d at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 20% inactivated FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin (all from Sigma), and 50% conditioned medium derived from L929 cultures (LCCM) (source of granulocyte/macrophage-colony stimulating factor). After 3 d, the medium was replaced with fresh RPMI 1640 medium supplemented with 30% LCCM, 20% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 3 d, the medium was replaced with fresh medium with the same composition. On the ninth day, the cells were cultivated using RPMI 1640 supplemented with 10% inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin for 24 h prior to stimulus.

2.4 | Purification of peritoneal B-1 cells

Total peritoneal cells obtained from lavage of peritoneal cavity of BALB/c and C57BL/6 mice were subjected to magnetic cell sorting using negative selection with anti-CD23 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and positive selection with anti-CD19 microbeads (Miltenyi Biotec) to obtain enrichment of B-1 cells.[30] B-1 cell purity was evaluated by flow cytometry (FACSCalibur; BD, San Diego, CA, USA) after labeling with anti-CD11b monoclonal antibodies conjugated with phycoerythrin (PE) and anti-IgM conjugated to FITC (all from BD).

2.5 | Isolation and characterization of EVs from peritoneal B-1 cells

Approximately 1×10^6 purified peritoneal B-1 cells were seeded per well in 24-well culture tissue plates (Costar; Corning, Inc., NY, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% inactivated FBS (previously ultracentrifuged at 100,000 $\times g$ for 12 h at 4°C to eliminate EVs from serum; FBS-EVs depleted), 100 U/mL penicillin, and 100 μg/mL streptomycin for 4 h prior to infection. B-1 cells were infected with *L. amazonensis* promastigotes in the stationary phase at a multiplicity of infection (MOI) of 5 (5 parasites/cell). After 24 h, the supernatants were collected to obtain EVs from the infected cells. Uninfected B-1 cells were cultured under the same conditions to collect EVs used as a control in all experiments. The supernatants were maintained at −20°C until the isolation of EVs. To obtain EVs from B-1 cells, the supernatants from infected and uninfected B-1 cells

were filtered through 0.22 μm sterile cartridges and were subjected to serial centrifugation: 500 ×*g* for 10 min at 4°C; 1500 ×*g* for 10 min at 4°C; 10,000 ×*g* for 10 min at 4°C; and 100,000 ×*g* for 1.5 h at 4°C. The pellets were resuspended in PBS (filtered through 0.22 μm) and ultracentrifuged at 100,000 ×*g* for 1.5 h at 4°C. The ultracentrifugation apparatus was the Sorvall WX Ultra Thermo Scientific—rotor T890 (Thermo Fisher Scientific, Waltham, MA, United States). The pellets were diluted in sterile, endotoxin free, and filtered PBS. EV samples were stored at −20°C until use.

Scanning electron microcopy (SEM) was performed using peritoneal B-1 cells seeded into 24-well culture tissue plates containing glass cover slips previously treated with poly-L-Lysine (Sigma), for complete cellular adhesion. All cultures and infection procedures were performed as described earlier. Fixation and SEM were performed according to previously described protocols.[31,32] The samples were processed and analyzed at the Center for Electronic Microscopy (CEME) at UNIFESP. The concentration and size distribution of EVs from infected or uninfected B-1 cells were evaluated by nanoparticle tracking analysis (NTA) using a Nanosight NS300 instrument (Malvern Instruments Ltd., Malvern, United Kingdom) equipped with a CCD camera and a 405 nm laser. The samples were diluted to 10- to 100-fold in filtered PBS, and each sample was captured in triplicate for 1 min (20 frames per second) at 20°C. The camera level was set to 14, and the threshold used was always the same. The results obtained were analyzed using NTA software (version 2.3 build 0017).

EVs from infected or uninfected peritoneal B-1 cells were evaluated by flow cytometry using the CytoFLEX flow cytometer (Beckman Coulter). Previously, annexin V conjugated to FITC (Molecular Probes, Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States) and antibody anti-MHC II-PE (BD) were centrifuged at 20,000 ×*g* for 30 min (no breaking) to remove the precipitates. Next, 5×10^6 EVs from infected or uninfected B-1 cells were labeled for 2 h at room temperature under protection from light. The instrument setup was performed according to the manufacturer's instructions. Before the analysis of the EVs, Gigamis beads (BioCytex a Stago group company, Marseille, France) were used to set the population with a size of approximately 90 nm to 100 nm. In total, 10,000 events were acquired from the gate of 100 to 500 nm for each EV sample analyzed. The data were analyzed using FlowJo software (FlowJo, LLC, Tree Star, Inc., Ashland, OR, USA).

2.6 | Treatment of macrophages with EVs isolate from B-1 cells

BMDM from BALB/c or C57BL/6 mice were treated with EVs from infected or uninfected B-1 cells from mice with the same background. Briefly, 2×10^5 macrophages were seeded into each well of 24-well tissue culture plates (Costar) and were maintained at 37°C in 5% CO_2 for 24 h with RPMI 1640 supplemented with 10% FBS-EVs depleted. Thereafter, the macrophages were treated with 50 EVs/cell because uninfected B-1 cells release approximately 50 particles/B-1 cell. So, this quantity is closer of physiologic environment. As 2×10^5 macrophages were placed by well, 1×10^7 EVs from B-1 cells were added by each well. This quantity (1×10^7 EVs) is equivalent to 4 μg of EVs. Macrophages were treated by 48 h to evaluate for sustained long-lasting activity. Cells were cultured under the same conditions until mRNA extraction.

2.7 | Cytokine and iNOS relative expression

Quantitative RT-PCR (qRT-PCR) was used to evaluate the cytokine and iNOS expression in macrophages treated with EVs from infected or uninfected B-1 cells. Briefly, total cellular RNA was extracted from macrophages using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified by UV absorption using a spectrophotometer (Nanodrop 2000c, Thermo Fisher), and only RNA samples with a reading range of 1.8–2.0 at 260/280 nm and 260/230 nm were analyzed by electrophoresis in 1.5% agarose gels to assess RNA integrity. High-quality RNAs (1 μg) were treated with identical amounts of DNAse (RQ1 RNase-free DNase; Promega, Madison, WI, USA) prior to cDNA synthesis using the Proto Script First Strand cDNA Synthesis Kit (New England Biolabs, Inc., Ipswich, MA, USA). Equal amounts of each cDNA were submitted to real-time PCR (qRT-PCR) using the SYBR Green Real-Time PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States) in the thermocycler Step OnePlus system (Applied Biosystems). The primer sequences of each target gene were as follows: IL-6 sense 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and anti-sense 5′-TTGGTCCTTAGCCACTCCTTC-3′; IL-10 sense 5′-GCTGGACAACATACTGCTAACC-3′ and antisense 5′-ATTTCCGATAAGGCTTGGCAA-3′; TNF-α sense 5′-CCCTCACACTCAGATCATCTTCT-3′ and antisense 5′-GCTACGACGTGGGCTACAG-3′; iNOS sense 5′- ACATCGACCCGTCCACAGTAT-3′ and antisense 5′- CAGAGGGGTAGGCTTGTCTC-3′ (all sequences from PrimerBank)[33]; glyceraldehyde-3-phosphate dehydrogenase (gapdh) sense 5′-AAATGGT-GAAGGTCGGTGTG-3′ and antisense 5′-TGAAGGGGTCGTTGATGG-3′; and ribosomal protein, large, P0 (rplp0) sense 5′-AGCTGAAGCAAAGGAAGAGTCGGA-3′ and antisense 5′-ACTTGGTTGCTTTGGCGGGATTAG-3′. The qRT-PCR samples comprised 1 μL of cDNA, 5.0 μL of master mix Sybr Green (Thermo Fisher), and 2.0 μL of each oligonucleotide (1.0 μM). The cycling parameters were as follows: 10 min at 50°C for enzyme activation, denaturation for 5 min at 95°C, and 40 cycles of 95°C per 30 s and 60°C for 1 min. A nontemplate control (NTC) was included in each reaction as a negative control to enable the detection of contamination. Step OnePlus software (Applied Biosystems) was used to analyze the dissociation curves and baseline and threshold cycle values (C_T). The reaction quality was evaluated by dissociation curves, the baseline was adjusted to three or two cycles prior to the detection of the fluorescent signal, and the threshold cycle (C_T) was defined in the region of exponential amplification across all plots. All primers were previously evaluated for their efficiency by constructing standard curves with serial dilution of the cDNA ($E = 10^{[-1/\text{slope}]} - 1$), E corresponding to the efficiency and slope is the slope of standard curve). The relative quantification of reactions with efficiencies ranging from 90% to 110% were calculated according to the $2^{-\Delta\Delta CT}$ method.[34] All reactions were

performed in triplicate using at least two biologic samples following the MIQE guidelines.[35] Differences in the relative expression levels of target genes were determined by comparing RNA from macrophages stimulated with EVs from uninfected B-1 cells as reference samples with the respective cell type stimulated with EVs from infected B-1 cells. The gene expression from the reference sample was always adjusted to be equal to 1.

2.8 | *Leishmania amazonensis* experimental infection

Groups of 6 BALB/c mice were subcutaneously treated in the right footpad with EVs derived from infected or uninfected B-1 cells (10^7 EVs/animal). After 48 h, the mice were inoculated with 10^6 *L. amazonensis* promastigotes in the right footpad. Animals treated with PBS were used as controls. Parasites and EVs were resuspended in 50 μL of sterile, endotoxin-free PBS. The presence of edema was checked weekly by monitoring the induration diameter. The parasite burden was evaluated in the paws after 7 wk of infection. The paws were aseptically removed from euthanatized mice and individually homogenized in M199 medium. The number of parasites was determined using a previously described limiting dilution method.[36] For histologic analysis, the paws were removed, fixed in 10% neutral buffered formalin, cut into 5 μm sections, stained with H&E, and examined under a Zeiss AxioScope II microscope (Carl Zeiss, Thornwood, NY, USA). The number of inflammatory cells was quantified in the sections stained with H&E in 10 fields (×400).[37] In addition, the inflammatory infiltrate was graded using the following classification[38]: (–), no inflammatory infiltrate; (+), isolated foci of inflammatory cells; (++), isolated to coalescing areas of inflammatory infiltrate; and (+++), diffuse areas of inflammatory infiltrate in areas with inflammatory infiltrate.

2.9 | Statistical analysis

The data were shown as means ± SD. Student's *t*-test was used to compare two groups, and ANOVA followed by Bonferroni's or Kruskal-Wallis's posttest was carried out to compare multiple groups. *P*-values <0.05 were considered significant. Graph Pad Prism version 7 for Mac was used to perform statistical tests (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). All experiments were performed with three or more replicates and repeated at least twice.

3 | RESULTS

3.1 | Peritoneal B-1 cells release EVs

The production of EVs by peritoneal B-1 cells was evaluated in the absence or presence of *L. amazonensis* promastigotes. SEM showed that EVs are released from uninfected (Fig. 1A and B) and infected B-1 cells (Fig. 1C and D). These vesicles were observed both on the cell surface and in the supernatant (adhered to the coverslip) (Fig. 1C and D). The images also showed the increase in EV production by infected B-1 cells compared with that in uninfected cells. This observation was confirmed by NTA. The parasite led to an increase in the release of EVs by B-1 cells (Fig. 1E), especially those with an average size of 100 nm (Fig. 1F).

Cell surface markers were evaluated in EVs released by B-1 cells. The percentage of EVs (particles with 100 to 500 nm) from infected or uninfected B-1 cells showed low labeling to annexin V (Fig. 2A and B), an important exosome marker that excludes apoptotic bodies (Fig. 2C and D). On the other hand, EVs from uninfected B-1 cells express MHC II (22.7%), but a significant reduction in the expression of this marker was observed in EVs from infected B-1 cells (1.85% of the population present between 100 and 500 nm) (Fig. 2C and D). No labeling was seen for CD19, CD80, CD86, CD40, CD5, CD11b, and F4/80 (data not shown).

3.2 | EVs from infected B-1 cells alter cytokine and iNOS expression by naïve macrophages

Immune cell-derived EVs can stimulate or modulate the activity of other immune cells.[12] Next, we investigated the ability of EVs from uninfected and infected B-1 cells to stimulate cytokine and iNOS RNA expression by naïve macrophages. All experiments were performed with BMDM because this population is more homogeneous as compared to total peritoneal macrophages population and to avoid the contamination with peritoneal B-1 cells.[39,40] EVs released by peritoneal B-1 cells from BALB/c mice were used to treat BMDM from BALB/c and BMDM from C57Bl/6 mice were treated with EVs derived of B-1 cells from C57Bl/6 mice. The cytokine expression in BMDM from BALB/c mice showed a different pattern. BMDMs from BALB/c mice treated with EVs from infected B-1 cells not altered TNF-α expression but had a significant increase in expression of IL-6 and IL-10 ($^{***}P < 0.001$) (Fig. 3A–C) compared with the same cells stimulated with EVs from uninfected B-1 cells. However, BMDMs from C57BL/6 mice stimulated with EVs from infected B-1 cells showed a significant increase in TNF-α expression ($^{***}P < 0.001$) (Fig. 3D) and no differences in IL-6 expression (Fig. 3E); however, these cells showed a significant decrease in IL-10 transcription ($^{***}P < 0.001$) compared with BMDM-treated with EVs from uninfected cells (Fig. 3F).

Differences in iNOS expression were not observed in BMDMs from BALB/c mice stimulated with EVs from infected or uninfected B-1 cells (Fig. 4A). On the other hand, BMDMs from C57BL/6 mice showed a significant reduction in iNOS expression after treatment with EVs from infected B-1 cells compared with treatment with EVs from uninfected B-1 cells ($P < 0.05$) (Fig. 4B).

3.3 | EVs from peritoneal B-1 cells partially protect mice in experimental *L. amazonensis* infection

Considering that EVs from infected B-1 cells altered the cytokine and iNOS production in naïve macrophages, we investigated the possibility that these effects influence the course of experimental *L. amazonensis* infection. Figure 5A shows the experimental design and experimental groups. The lesion size was evaluated weekly; after 8 wk postinfection, a significant reduction in the lesion size was detected in the groups of BALB/c mice treated with EVs from uninfected and infected peritoneal

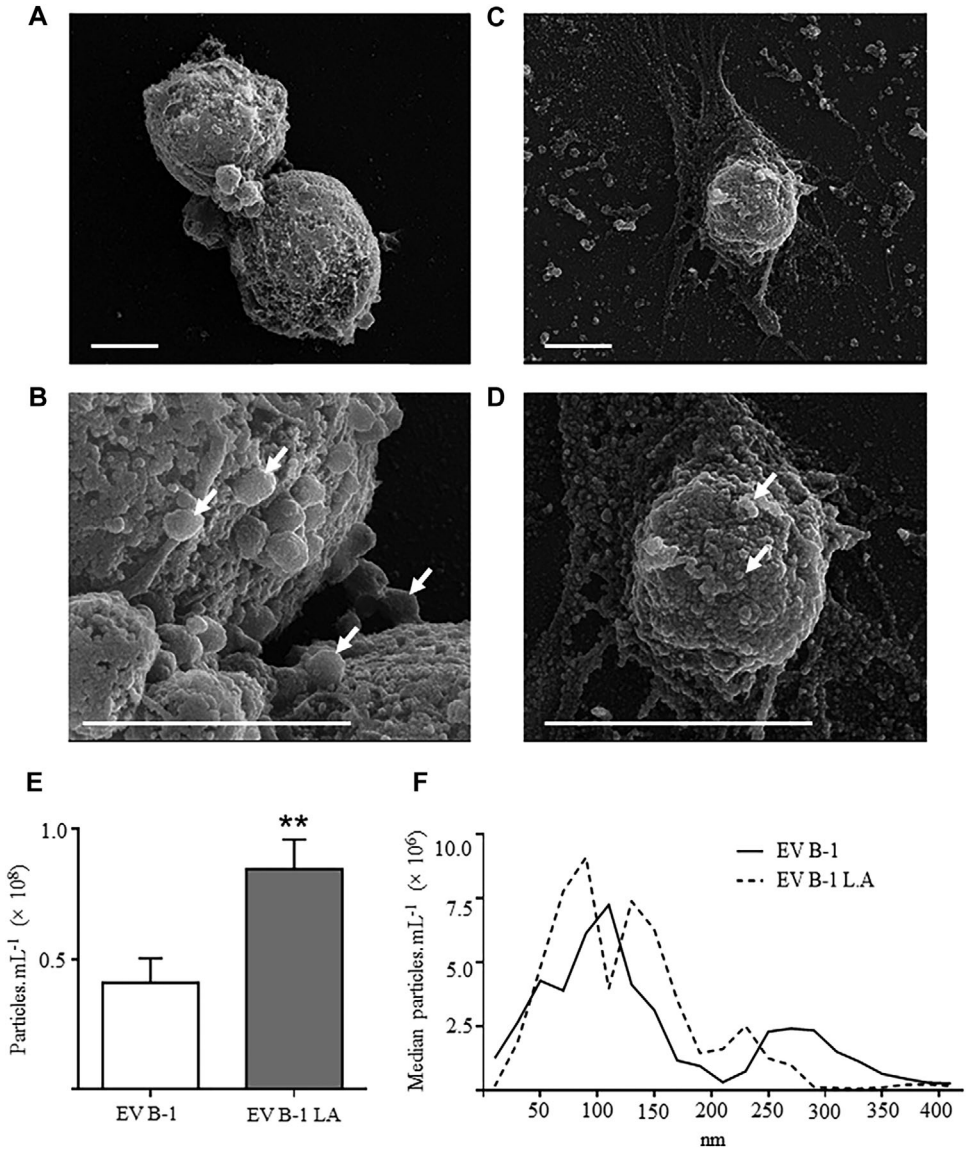

FIGURE 1 **Peritoneal B-1 cells release extracellular vesicles (EVs).** B-1 cells were obtained from BALB/c mouse peritoneal cavities using negative selection with anti-CD23 and positive selection with anti-CD19, both antibodies coupled with magnetic microbeads. Next, the cells were cultivated for 24 h in the presence or absence of *L. amazonensis* promastigotes (multiplicity of infection [MOI] = 5). Cells were submitted to scanning electronic microscopy (SEM), and the supernatants were ultracentrifuged and analyzed by nanoparticle tracking analysis (NTA). (**A, B**) SEM of uninfected B-1 cells. (**C, D**) SEM of infected B-1 cells. The arrows show the release of EVs from B-1 cells. Scale bars represent 2 μm. (**E**) Concentration of EVs released by uninfected (EV B-1) and infected B-1 cells (EV B-1 LA). (F) Size distribution of EV populations released from uninfected (EV B-1) and infected B-1 cells (EV B-1 LA). The data are representative of three independent experiments, and error bars denote SD. Student's *t*-test: $^{**}P < 0.01$

B-1 cells compared with that in the control group (mice treated with PBS and subsequently infected with the parasites—PBS + LA) (Fig. 5A). After 9 wk, the animals were euthanized, and the parasite load was evaluated in the paws by limiting dilution. The group treated with EVs from uninfected B-1 cells (EV B-1) exhibited a significant reduction in the number of parasites/g of tissue compared with the control group (PBS + LA) ($^{**}P < 0.001$) and the group of animals treated with EVs from infected B-1 cells (EV B-1 LA) ($^{*}P < 0.05$) (Fig. 5B).

Differences in the inflammatory process were observed in the tissue histologic analyzes. Figure 6A shows the histologic pattern of paws from uninfected mice, and Figure 6B presents the histologic findings in mice infected for 9 wk with *L. amazonensis* promastigotes. Animals previously treated with EVs from uninfected B-1 cells showed better tissue preservation, in addition to having a lower necrotic areas and cell infiltrate (Fig. 6C and F), compared with the untreated group (Fig. 6B and E). Animals treated with EVs from infected B-1 cells showed an intermediate pattern of inflammatory infiltration (Fig. 6D and G) but also showed reduction in necrotic areas. The intensities of cellular inflammatory infiltration in the footpad of animals previously treated with EVs from uninfected B-1 cells were significantly lower as compared to control group (PBS + LA) ($^{*}P < 0.05$) (Fig. 6H). In addition, histopathologic examination revealed changes in the pattern of dermal

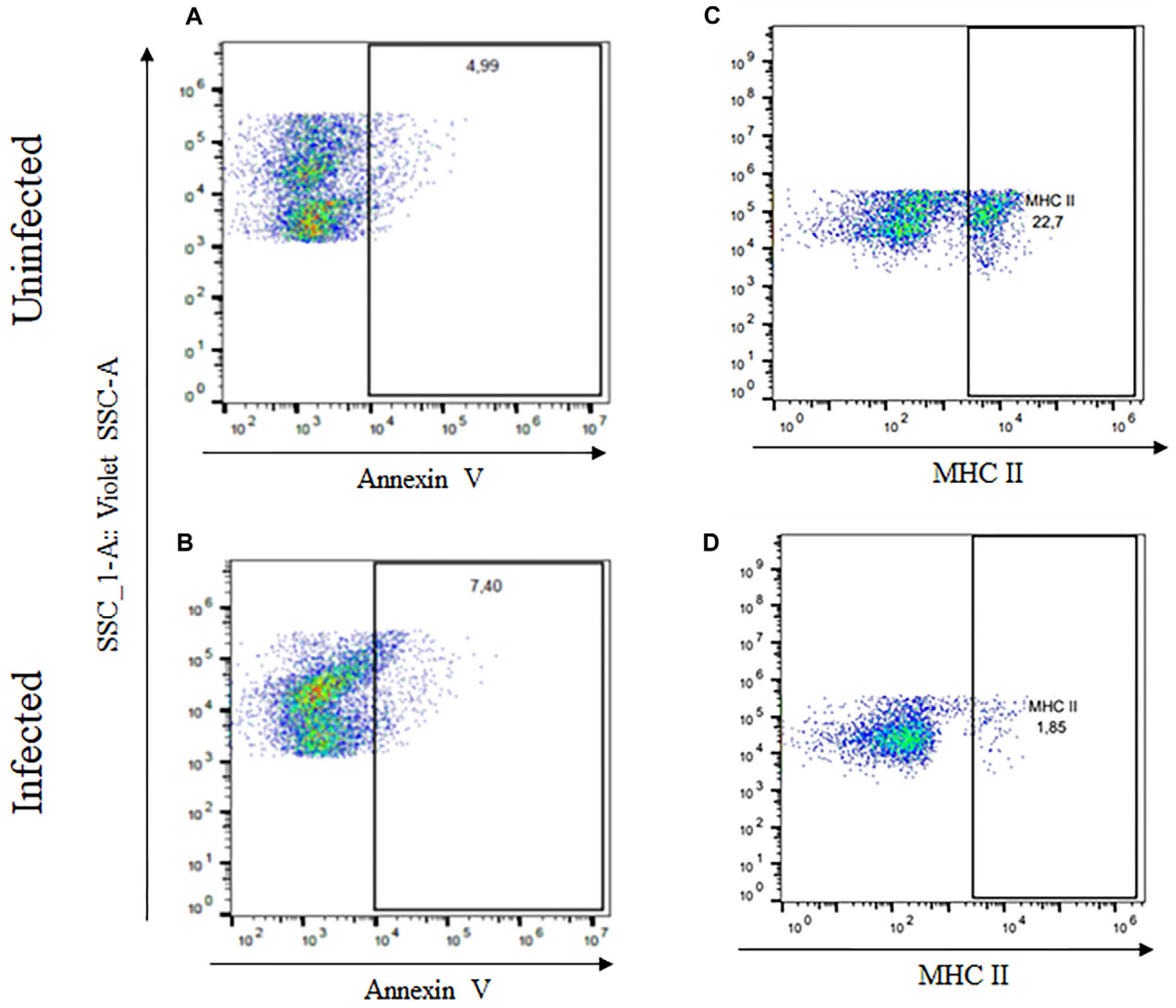

FIGURE 2 ***Leishmania*-infection reduces MHC class II into extracellular vesicles (EVs) from peritoneal B-1 cells.** B-1 cells were obtained from BALB/c mouse peritoneal cavities and by using anti-CD23 (negative selection) and anti-CD19 (positive selection) coupled with magnetic microbeads. The cells were cultivated in the presence or absence of *L. amazonensis* promastigotes (multiplicity of infection [MOI] = 5). Next, the supernatants were collected, and EVs obtained by ultracentrifugation were analyzed by flow cytometry using annexin V or anti-MHC II PE. (**A**) EVs from uninfected B-1 cells labeled with FITC annexin V. (**B**) EVs from B-1-infected cells stained with FITC annexin V. (**C**) EVs from uninfected B-1 cells stained with anti-MHC class II PE conjugated. (**D**) EVs from infected B-1 cells stained with anti-MHC class II PE conjugated

inflammation in foot skin of animals treated with EVs from uninfected or infected B-1 cells (Fig. 6I).

4 | DISCUSSION

EVs are released by virtually all eukaryotic and prokaryotic cells.[41] Spontaneous release of EVs by immune cells have been reported and also their role in physiologic and immunologic processes.[42,43] Depending on the cell origin, EVs released by immune cells can participate in innate and acquire immune response, antigen presentation, NK and T cells activation, Treg polarization, immunomodulation, and have anti-inflammatory and pro- and anti-inflammatory effects.[44,45] These wide effects have been studied and evaluated as potential applications on treatment and vaccination protocols.[12,44]

B-1 cells are unique cells in immunity because, in the steady state, they produce cytokines (mainly IL-10) and are a source of natural antibodies; however, after stimulation, these cells can respond by differentiating into phagocyte-like cells, producing more specific antibodies and secreting different cytokines.[1,5,46,47] Herein, we describe, for the first time, the ability of peritoneal B-1 cells to release EVs spontaneously or after stimulation.

Our results showed that peritoneal B-1 cells spontaneously release EVs, and the stimulation with *L. amazonensis* promastigotes leads to increased EV production. Although the composition of these EVs has not yet been fully characterized, flow cytometric analyses have shown that EVs spontaneously released by B-1 cells carry MHC II molecules. Interestingly, after contact with the parasite, EVs released by B-1 cells showed decreased expression of these molecules, suggesting that the

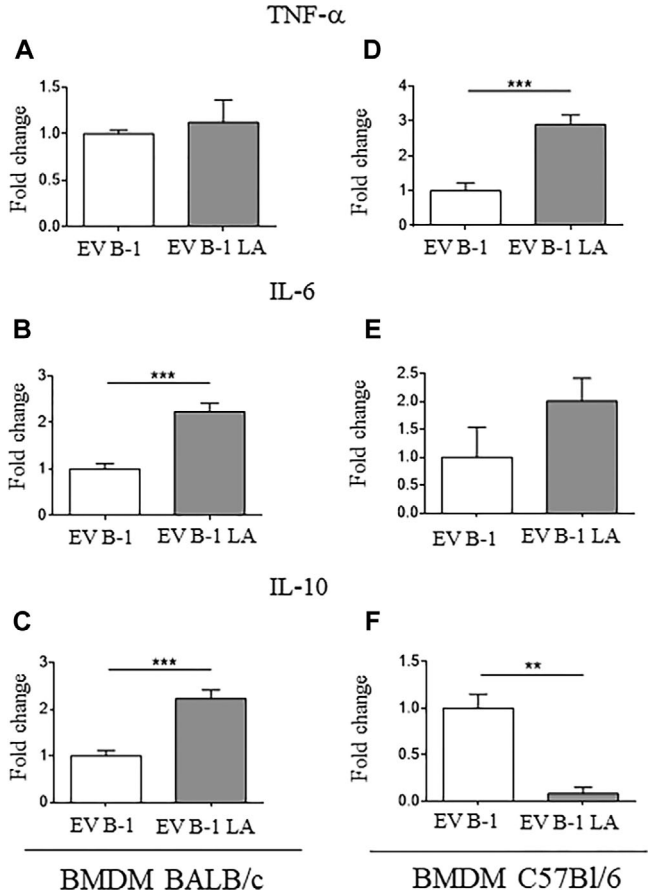

FIGURE 3 **Extracellular vesicles (EVs) released by infected peritoneal B-1 cells stimulate the production of different types of cytokines depending on the origin of macrophages.** Bone marrow-derived macrophages (BMDM) from BALB/c or C57BL/6 mice were treated with EVs from uninfected B-1 cells (EV B-1) or from infected B-1 cells (EV B-1 L.A). After 48 h, RNA was extracted and the relative cytokine mRNA expression of TNF-α (**A, D**), IL-6 (**B, E**), and IL-10 (**C, F**) was determined by qRT-PCR. The bars show the average of three measurements, and error bars denote the SD. The data are representative of three independent experiments. Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the respective control

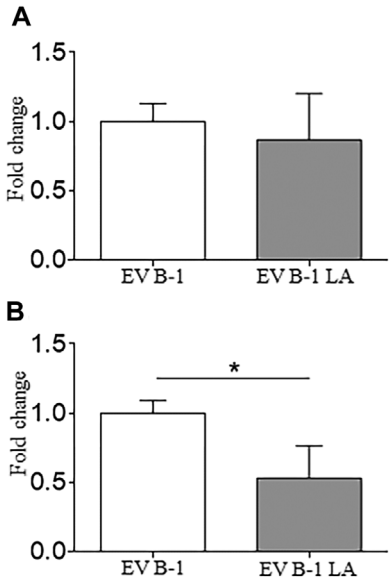

FIGURE 4 **The iNOS gene expression in bone marrow-derived macrophages (BMDM).** BMDMs from BALB/c and C57BL/6 mice were treated with extracellular vesicles (EVs) from uninfected B-1 cells (EV B-1) or from infected B-1 cells (EV B-1 L.A). After 48 h, RNA was extracted and the relative iNOS mRNA expression was determined by qRT-PCR. iNOS gene expression in (**A**) BMDMs from BALB/c and (**B**) BMDMs from C57BL/6 mice. The bars show the average of three measurements, and error bars denote the SD. The data are representative of three independent experiments. Student's *t*-test: $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the respective control

parasite could modulate the sorting of MHC class II to the vesicles. Previous studies have shown that *L. amazonensis* modulates the expression of MHC II molecules by reducing their expression in murine and human macrophages infected with promastigotes.[48,49] The MHC II molecules were found to be associated with the parasites very early after they are phagocytosed, but these molecules were not detected after 48 h of infection.[49] This time coincides with the increase in the protease activity of the parasite, suggesting that the parasite could internalize and very likely degrade host MHC II molecules.[49] B-1 cells can phagocytose *L. amazonensis* promastigotes; thus, the parasite could also induce the reduction of MHC II molecules in infected B-1 cells, impairing the export of these molecules to EVs.

EVs are a new player in cell-to-cell communication, including immune cells.[12,50,51] The interaction between B-1 cells and macrophages has been demonstrated,[52–55] but the possible communication of these cells by EVs has not yet been reported. Our results showed that macrophages treated with EVs from infected B-1 cells differently express iNOS, and pro- and anti-inflammatory cytokines compared with cells stimulated with EVs from uninfected B-1 cells. Hassani and Olivier showed that exosomes released by macrophages infected with *L. mexicana* exhibit changes in their composition compared with exosomes from uninfected macrophages.[21] In this study, the metalloprotease gp63 was the only protozoan protein identified in the exosomes.[21] This change in the composition of the exosomes probably reflected the profile activation of MAPKs in naïve macrophages stimulated with exosomes from macrophages infected by the parasite in relation to the exosomes from uninfected cells.[21] These data suggest that *Leishmania*-infected cells release EVs with different compositions that can influence the response of other immune cells. Using specific monoclonal antibodies we did not detect the presence of gp63 and lipophosphoglycan (two important virulence factors) in EVs released by infected B-1 cells (data not shown), but we observed a dramatic reduction in MHC II expression, suggesting that the parasite can also induce alterations in EVs released by infected B-1 cells. Future studies will be carried out using more sensitive methodologies to identify differences in the composition of the vesicles released by B-1 cells cultured in the presence or absence of the parasite.

Primary cultures of BMDMs from BALB/c and C57BL/6 mice were treated with EVs from infected B-1 cells and the expression of iNOS, TNF-α, IL-6, and IL-10 were compared with the same cells treated with EVs from uninfected B-1 cells. These mouse strains are widely

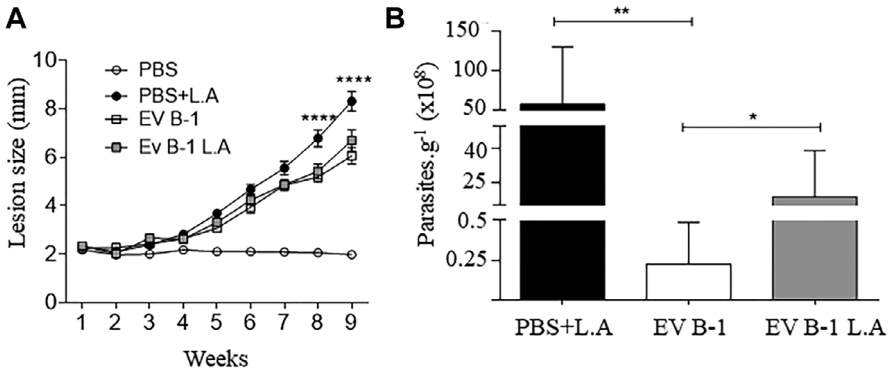

FIGURE 5 **Extracellular vesicles (EVs) from peritoneal B-1 cells alter the course of *L. amazonensis* experimental infection in BALB/c mice.** Groups of 6 animals were treated with EVs from uninfected B-1 cells (EV B-1) or infected B-1 cells (EV B-1 L.A). After 2 d, mice were infected with 10^6 *L. amazonensis* promastigotes in the footpad. Control groups were uninfected mice (PBS) and animals treated with PBS and subsequently infected with parasites (L.A). The infection course was followed by paw measurements. After 9 wk, the animals were euthanized and the parasite load was evaluated by limiting dilution. (**A**) Lesion size (paws measurements) and (**B**) parasite load. The results are representative of two independent experiments. The bars represent the mean of six samples, and the error bars denote the SD. Statistical analysis was performed using ANOVA followed by Kruskal-Wallis: $^*P < 0.05$, $^{**}P < 0.01$. The data are representative of two independent experiments

used in studies with *Leishmania* parasites because of their contrasting responses to infection. BALB/c mice are vulnerable to infection and show a Th2 response pattern, whereas C57BL/6 do not develop chronic infection, because after the initial lesion, the animals resolve the infection in a few weeks, probably due to the polarization to the Th1 profile.[56,57] Therefore, the genetic differences in these two strains could lead to differences in the composition and functionality of the EVs produced by B-1 cells. BMDMs from BALB/c mice showed increased expression of IL-6 and IL- 10 but no changes in the expression of TNF-α and iNOS and were detected after stimulating with EVs from infected B-1 cells comparing with BMDMs treated with EVs from uninfected B-1 cells. On the other hand, BMDMs from C57BL/6 showed a different gene expression pattern. Treatment with EVs from infected B-1 cells led to a significant increase in the expression of TNF-α, but the expression of IL-10 and iNOS was significant reduced compared with stimulation by EVs from uninfected B-1 cells. EVs released by peritoneal macrophages (BALB/c background) infected with *L. amazonensis* promastigotes stimulated naïve macrophages to produce proinflammatory cytokines compared with macrophages treated with EVs released by uninfected macrophages.[19] Similarly, macrophages infected with *Mycobacterium bovis* (BCG) also produced EVs that led to proinflammatory responses both in vitro and in vivo.[58] Other pathogens such as *Mycobacterium tuberculosis*, *Salmonella typhimurium*, and *Toxoplasma gondii* were also used to infect macrophages, and the EVs produced by the infected cells stimulated in vitro proinflammatory responses.[59–61] In the case of *Mycobacterium tuberculosis*, the vesicles released from infected macrophages stimulated the production of TNF-α and IL-12 by naïve macrophages.[58] Altogether these results suggest that EVs released by infected peritoneal B-1 cells stimulated the production of anti and proinflammatory cytokines by BMDMs, probably because these EVs carry molecules with immunoregulatory properties, contributing to stimulate different responses. In addition, differences in the response of macrophages derived from BALB/c or C57Bl/6 mice can be also attributed to the mice genetic background, which show different pattern of immune response.

IL-10, TNF-α, and IL-6 cytokines are important in the inflammatory environment caused by infection with *Leishmania* spp.[62] TNF-α, together with INF-γ, are proinflammatory cytokines responsible for infection control,[62,63] and IL-6 may promote increased recruitment of phagocytes.[49] However, the exacerbated production of these cytokines is also responsible for tissue damage in the inflammatory process.[62] In contrast, IL-10 and TGF-β are anti-inflammatory cytokines responsible for suppressing the immune response, thus reducing tissue damage and the levels of cytotoxicity mediated primarily by $CD8^+$ T cells.[64,65] Although there is a balance between pro- and anti-inflammatory cytokines, many pathogenic microorganisms can alter this balance to promote their survival and avoid immune system responses. Among these pathogens, *Leishmania* may contribute to the exacerbation of the disease by both the activation and suppression of pro- and/or anti-inflammatory cytokines[65,66] Therefore, an equilibrium in the response in the presence of both inflammatory and anti-inflammatory cytokines seems to be one of the factors that contributes to the containment of a harmful response in the host.

The role of EVs from B-1 cells in modulating the course of *L. amazonensis* experimental infection was also evaluated. BALB/c mice show a chronic infection with dominance of Th2 response after infection with *L. amazonensis*.[65] So, we decided to test the treatment of EVs released by B-1 cells in BALB/c mice to evaluate whether these EVs could alter the course of infection even in an environment with Th2 dominance. BALB/c mice previously treated with EVs from uninfected B-1 cells showed a partially control of the lesion size and the parasite burden in infected mice. No differences were observed in BALB/c mice previously treated with EVs from infected B-1 cells, compared to the control group. However, a significant reduction in the parasite load was observed in mice treated with EVs from uninfected B-1 cells. Our group showed that EVs from *L. amazonensis* increased the inflammatory effects and parasite burden when co-injected with the parasite

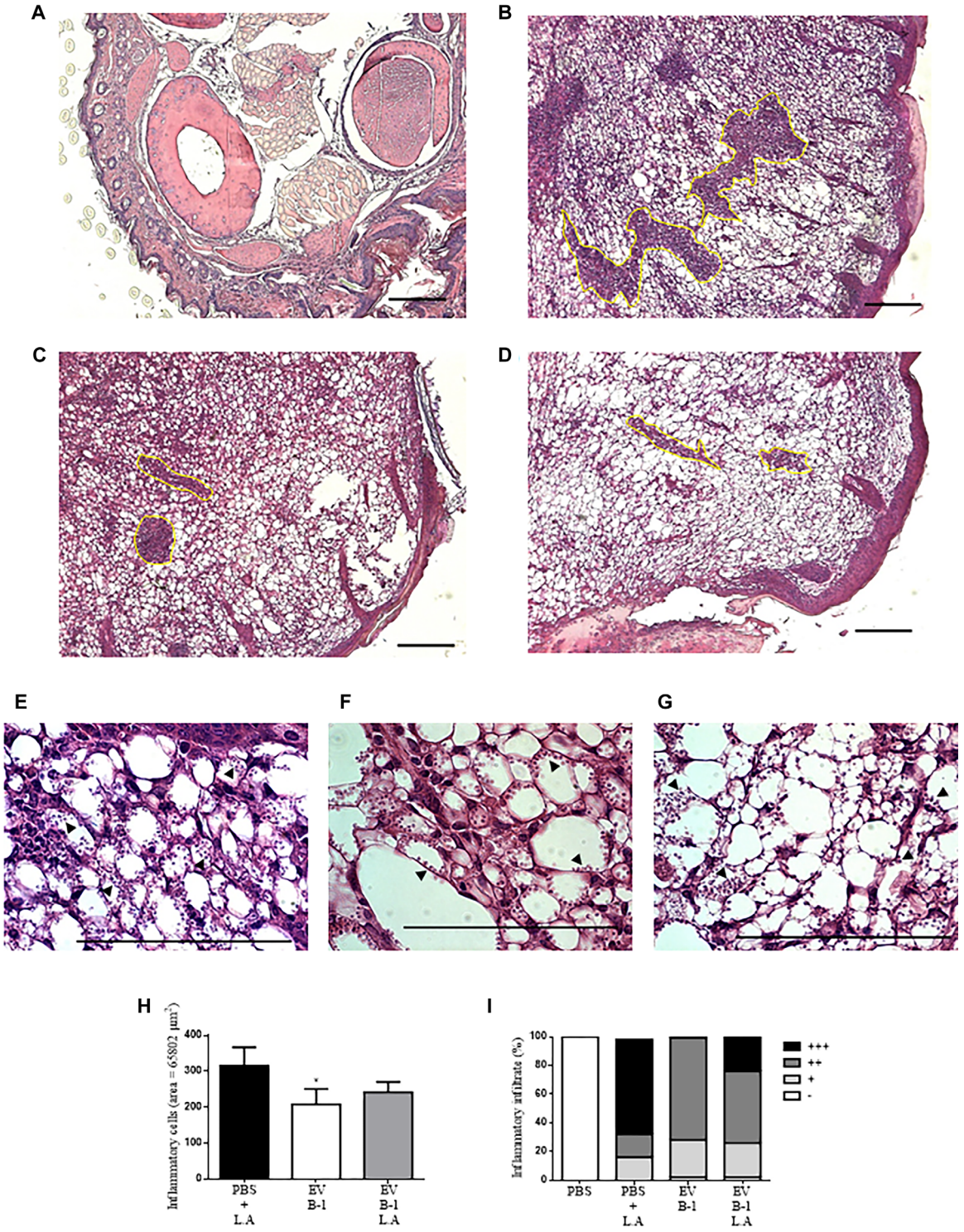

FIGURE 6 **Histopathologic findings in BALB/c mice treated with extracellular vesicles (EVs) from peritoneal B-1 cells.** Animals were treated with EVs from uninfected B-1 cells (EV B-1) or infected B-1 cells (EV B-1 L.A). After 2 d, the mice were infected with 10^6 *L. amazonensis* promastigotes in the footpad. After 9 wk of infection, the animals were euthanized, the paws were removed, fixed, and 2 tissue samples from each animal were cut and stained with H&E. (**A**) Mice treated with PBS and not infected. (**B, E**) Mice treated with PBS and infected with *L. amazonensis* promastigotes. (**C, F**) Mice treated with EVs from uninfected B-1 cells and infected with *L. amazonensis* promastigotes. (D, G) Mice treated with EVs from infected B-1 infected cells and infected with *L. amazonensis* promastigotes. **A–D** scale bars represent 200 µm, and E–G scale bars equal 100 µm. The yellow outlines show necrotic areas, and the black arrows show the presence of *L. amazonensis* amastigotes. Histologic analysis show in (**H**) the number of inflammatory cells counted in 65802 um2 area (400x); and (I) the percentage of inflammatory infiltrated in foot skin graded as follow: white bar [–], absent; gray bar [+], mild; dark bar [++], moderate; and black bar [+++], intense. The results are representative of two independent experiments. The bars represent the measure of 10 fields, and the error bars denote the SD. Statistical analysis was performed using ANOVA followed by Kruskal-Wallis: $^*P < 0.05$. The data are representative of two independent experiments

in BALB/c mice.[67] EVs from infected B-1 cells can cargo some parasites components, as observed by infected macrophages,[68] and then contribute to alter the effects observed by EVs from uninfected B-1 cells. Other study showed that B-1 cells participate in the resistance to experimental infection with *L. amazonensis* because XID mice (animals deficient in B-1 cells) were more susceptible to infection with *L. amazonensis* than infected BALB/c mice (background control).[28] Adoptive transfer of B-1 cells to XID mice resulted in a decrease in the parasite burden compared with the control (XID mice).[28] The mechanisms involved with this process may also be partially related to EVs released by B-1 cells. In our model, the previous treatment with EVs released by peritoneal B-1 cells may have modulated the tissue environment that become more efficient in resolving the parasite infection, promoting a better immune response. Although highly speculative, these hypotheses cannot be ruled out.

In conclusion, peritoneal B-1 cells release EVs that interact with macrophages that alter the expression of cytokines and iNOS. EVs from peritoneal B-1 cells also influence the course of the experimental infection with *L. amazonensis*, leading to a lower lesion size and a lower parasite load in previously treated mice. Thus, this work showed, for the first time, that B-1 cells release EVs that can act as a new mechanism of cellular communication, participating in immunity against experimental leishmaniasis. The data uncover new perspectives in the studies of B-1 cells and their role in CL, especially in the development of different strategies applied for vaccination against *Leishmania* parasites. Therefore, studying EVs released by immune cell and the impact of these EVs on other cells and their function in immunity, especially for cells that are still poorly studied, similar to B-1 cells, can uncover important and relevant information about immunity and its application in disease control.

AUTHORSHIP

M.S.T. performed the experiments, analyzed the data, generated the figures, and wrote the original draft. F.M.C.B assisted in the purification of B-1 cells and with macrophages' cultures. A.C.A. supported the flow cytometry experiments and analysis. N.F.C.R. T.V.D. helped with in vivo experiments. R.P.S. and A.C.T. helped with the data interpretation, discussed the hypotheses, and assisted with the manuscript revisions. P.X. conceived and designed the study, helped with data interpretation, participated in the data analysis, and wrote the final version of the manuscript. All authors read and approved the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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