

## Basic Study

**B-1 cells modulate the murine macrophage response to *Leishmania major* infection**

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**Abstract****AIM**

To investigate the modulatory effect of B-1 cells on murine peritoneal macrophages infected with *Leishmania major* (*L. major*) *in vitro*.

**METHODS**

Peritoneal macrophages obtained from BALB/c and

BALB/c XID mice were infected with *L. major* and cultured in the presence or absence of B-1 cells obtained from wild-type BALB/c mice. Intracellular amastigotes were counted, and interleukin-10 (IL-10) production was quantified in the cellular supernatants using an enzyme-linked immunosorbent assay. The levels of the lipid mediator prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were determined using a PGE<sub>2</sub> enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI), and the number of lipid bodies was quantified in the cytoplasm of infected macrophages in the presence and absence of B-1 cells. Culturing the cells with selective PGE<sub>2</sub>-neutralizing drugs inhibited PGE<sub>2</sub> production and confirmed the role of this lipid mediator in IL-10 production. In contrast, we demonstrated that B-1 cells derived from IL-10 KO mice did not favor the intracellular growth of *L. major*.

## RESULTS

We report that B-1 cells promote the growth of *L. major* amastigotes inside peritoneal murine macrophages. We demonstrated that the modulatory effect was independent of physical contact between the cells, suggesting that soluble factor(s) were released into the cultures. We demonstrated in our co-culture system that B-1 cells trigger IL-10 production by *L. major*-infected macrophages. Furthermore, the increased secretion of IL-10 was attributed to the presence of the lipid mediator PGE<sub>2</sub> in supernatants of *L. major*-infected macrophages. The presence of B-1 cells also favors the production of lipid bodies by infected macrophages. In contrast, we failed to obtain the same effect on parasite replication inside *L. major*-infected macrophages when the B-1 cells were isolated from IL-10 knockout mice.

## CONCLUSION

Our results show that elevated levels of PGE<sub>2</sub> and IL-10 produced by B-1 cells increase *L. major* growth, as indicated by the number of parasites in cell cultures.

**Key words:** *Leishmania major*; Macrophages; B-1 cells; Interleukin-10; Prostaglandin E<sub>2</sub>; Infection

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**Core tip:** This original manuscript describes the modulatory effect of B-1 cells on *Leishmania major*-infected macrophages. We demonstrated the participation of soluble mediators in a co-culture system and characterized prostaglandin E<sub>2</sub> and interleukin-10 (IL-10) as key factors involved in increased intracellular parasite replication. We also demonstrated that cell-cell contact is not important. The same effect was not observed when we used B-1 cells from IL-10 knockout mice, as no significant difference in parasite multiplication was observed. Thus, the current manuscript may be of interest for scientists working in the fields of immunoparasitology or immunomodulation.

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## INTRODUCTION

B-1 lymphocytes are murine cellular subpopulations that have their development and other characteristics that are distinct from the conventional B-2 cell populations<sup>[1]</sup>. The B-1 cell population is a minor B-cell compartment that has been identified in mice and is involved primarily in innate immune responses<sup>[2]</sup>. The interest in this cellular population has increased considerably due to its relationship with leukemia, autoimmunity and auto-reactivity<sup>[3-6]</sup>. Recently, several manuscripts demonstrated that B-1 cells are able to transform into a type of phagocyte called a B-1 cell-derived phagocyte (B-1CDP) and play a role as antigen presenting cells (APCs)<sup>[7-10]</sup>. Borrello and Phipps<sup>[11]</sup> demonstrated that B-1 lymphocytes from the peritoneum of mice differentiate into phagocytic cells that are similar to macrophages.

*Leishmania major* (*L. major*) is the causative agent of cutaneous leishmaniasis (CL) in the Old World and is transmitted by the bite of the female phlebotomine sandfly, which injects the infective metacyclic promastigote forms into the dermis of the host<sup>[12]</sup>. Cutaneous leishmaniasis is characterized by an ulcerative lesion that appears on the skin at the site of the sandfly bite and generally heals spontaneously. In experimental models of susceptibility, *L. major* infection induces a Th2-type immune response. In models of susceptibility to *L. major* infection, there is a production of anti-inflammatory mediators, which negatively modulate the response of the vertebrate host, favoring the establishment of infection<sup>[13-15]</sup>. Graf and collaborators demonstrated that B-1 cells express COX-1 and up-regulate COX-2 and prostaglandin production in response to inflammatory signals. Our group recently demonstrated that B-1 CDP cells are easily infected by *L. major* and exhibit high susceptibility to infection and that this mechanism is dependent on prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)/interleukin-10 (IL-10) production<sup>[16]</sup>.

Based on these data, we investigated the interaction between B-1 cells and *L. major*-infected macrophages from BALB/c mice and BALB/c XID mice (a lineage that is genetically depleted of B-1 cells) to elucidate the possible influence of this minor B-cell population on the progression of infection *in vitro*. We performed experiments to investigate whether B-1 cells could mediate any effect on macrophage susceptibility to infection based on cell direct interaction or cytokine-driven modulation.

## MATERIALS AND METHODS

### Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of

Laboratory Animals of the National Institutes of Health (United States). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of the Federal University of Rio de Janeiro (CEUA-CCS, Permit Number: IBCCF 062/14), and all efforts were made to minimize suffering.

### Mice and parasites

BALB/c and BALB XID (a lineage that is genetically depleted of B-1 cells) mice of both sexes, aged 6–8 wk, were obtained from the Oswaldo Cruz Institute Animal Care Facility (Fiocruz, Rio de Janeiro, Brazil). C57BL/6 IL-10 knockout (KO) and C57BL/6 wild-type mice were kindly donated by Professor João Santana Silva from the Department of Pharmacology, School of Medicine, USP, Ribeirão Preto. *L. major* strain LV39 (MRHO/Sv/59/P) was isolated each month from the footpads of infected BALB/c mice and maintained *in vitro* as proliferating promastigotes. The parasites were maintained in Schneider's medium (Gibco, Life Technologies) supplemented with 10% FCS, 1% glutamine and 2% human urine maintained in the animal facility at the Federal University of Rio de Janeiro (UFRJ).

### B-1 cells

B-1 cells were isolated from BALB/c mice using the protocol described by Abrahão *et al.*<sup>[1]</sup>. Briefly, macrophages were harvested *via* peritoneal lavage of BALB/c mice using cold DMEM medium (Gibco, Life Technologies). The total population of cells from the peritoneum was plated into 25 cm<sup>2</sup> tissue culture flasks (Corning) and incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere for 120 min. The non-adherent cells were discarded, and DMEM medium containing 2 mmol/L glutamine, 50 µmol/L 2-ME, 10 µg/mL of gentamicin, 1 mmol/L sodium pyruvate, and 100 µmol/L MEM nonessential amino acids plus 10% fetal calf serum (FCS) was added to the adherent monolayer. The cultures were maintained for 5 d without changing the medium under the conditions described above. The non-adherent cell population was composed primarily of B-1 cells, as indicated by flow cytometry (results not shown), whereas the adherent cells represented an enriched macrophage population.

### Macrophages and infection

Primary BALB/c or BALB/c XID peritoneal macrophages ( $2 \times 10^5$  cells/well) were cultured in 24-well plates (Corning) containing sterile round glass coverslips (13 mm) and allowed to attach for 2 h at 37 °C in 5% CO<sub>2</sub>. The adherent macrophages were infected for 24 h with stationary phase *L. major* promastigotes at a multiplicity of infection of 10:1 (parasite/macrophage) and were then incubated at 37 °C in 5% CO<sub>2</sub>. After 24 h, the monolayers were washed extensively with warm HBSS (Gibco, Life Technologies) to remove extracellular parasites. All cultures were maintained in medium containing 1% Nutridoma-SP (Roche, Basel, Switzerland) instead of FCS. B-1 cells were added at a 10:1 ratio (B-1 cell/macrophage) in the presence or absence of antibodies, solvents and reagents. After 3 d, infected

macrophages monolayers were extensively washed to remove non-phagocytosed promastigotes, and medium was replaced by Schneider medium (Life Technologies), supplemented with 20% FCS and 2% human urine. Infected monolayers were cultured at 26 °C for additional 3 d. The number of motile promastigotes released into the cellular supernatant was evaluated using a Neubauer chamber.

### Assessment of the intracellular load of *L. major*

The relative intracellular load of *L. major* was assessed by counting the number of motile extracellular promastigotes released in each well. Infected peritoneal macrophages cultured on glass coverslips in the presence or absence of B-1 cells for 3 d at 37 °C. After this time the cultures were washed and stained with May-Grunwald Giemsa (Sigma-Aldrich), and intracellular amastigotes were counted in 100 infected macrophages. The results are shown as the number of intracellular amastigotes per macrophages and as the percentage of infected macrophages. All results are presented as the mean and SE of triplicate cultures.

### Antibodies, cytokines and inhibitors

Peritoneal macrophage monolayers were treated with 10 µg/mL of aspirin (Sigma-Aldrich). Aspirin acts as an inhibitor of PGE<sub>2</sub> production<sup>[17,18]</sup>. Neutralizing anti-transforming growth factor (TGF)-β and normal chicken IgY (R and D System), anti-IL-10 and rat IgG1 isotype control (BioSource Europe, Nivelles) antibodies were used at a concentration of 10 µg/mL.

### Cytokine and PGE<sub>2</sub> measurement

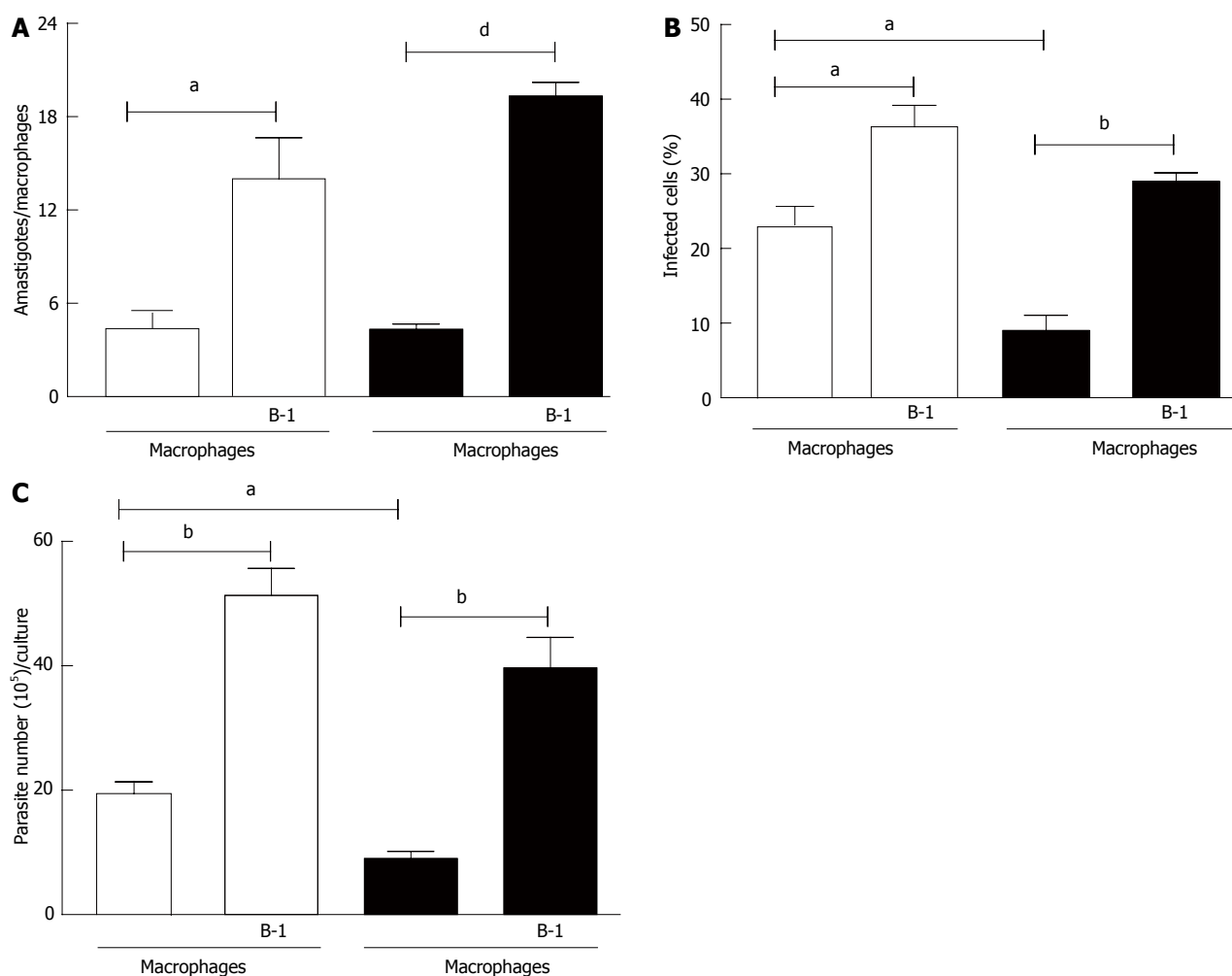
The concentrations of cytokines in the supernatants obtained from infected cell cultures were quantified after 24 h of incubation using the sandwich immunoassay (ELISA) method according to the methodology recommended by the manufacturer (R and D). The optical density was obtained by reading the absorbance in a plate spectrophotometer (Versamax Microplates Reader Molecular Devices, United States), with a filter of 405 nm. The concentrations of cytokines were calculated from a standard curve of recombinant cytokines. PGE<sub>2</sub> was quantified using a PGE<sub>2</sub>-specific EIA kit, according to the methodology recommended by the manufacturer (Cayman Chemical, Ann Arbor, MI).

### Lipid body evaluation

To observe lipid bodies (LBs), macrophages were fixed with 3.7% formaldehyde for 10 min and stained by osmium tetroxide. The morphology of fixed cells was observed, and osmium-stained lipid bodies were counted by light microscopy with a 100 × objective lens in 50 consecutively scanned leukocytes.

### Statistical analysis

Statistical analysis was performed using the program GraphPad InStat version 3.01 (San Diego, CA, United States). The data were analyzed using a *t*-test. Differences



**Figure 1** Effect of B-1 cells co-cultured with macrophages infected with *Leishmania major*. B-1 cells and peritoneal macrophages from BALB/c mice (white bars) or XID mice (black bars) were cultured (105/well) and infected with metacyclic promastigotes of *L. major*. After 24 h, the cell culture was washed and phagocytes were cultured for another 3 d with DMEM supplemented with 10% FBS at 37 °C. After this period, cells were stained and amastigotes inside the phagocytes were counted under the light microscope (A) and set the percentage of infected cells (B). To quantify promastigotes forms in the supernatants, the cells were cultures and infected with *L. major*. After 24 h of infection, so were washed and cultured at a temperature of 27 °C in Schneider medium 20% FBS during 5 d. After this period, the promastigotes were quantified in the supernatant of the cultures of infected phagocytes (C). All cultures were performed in triplicate and bars show the mean  $\pm$  SD. Statistical analysis were performed by t-test from representative results of three different experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.005$  and <sup>d</sup> $P < 0.0001$ .

with a  $P$  value of 0.05 or lower were considered to be significant.

## RESULTS

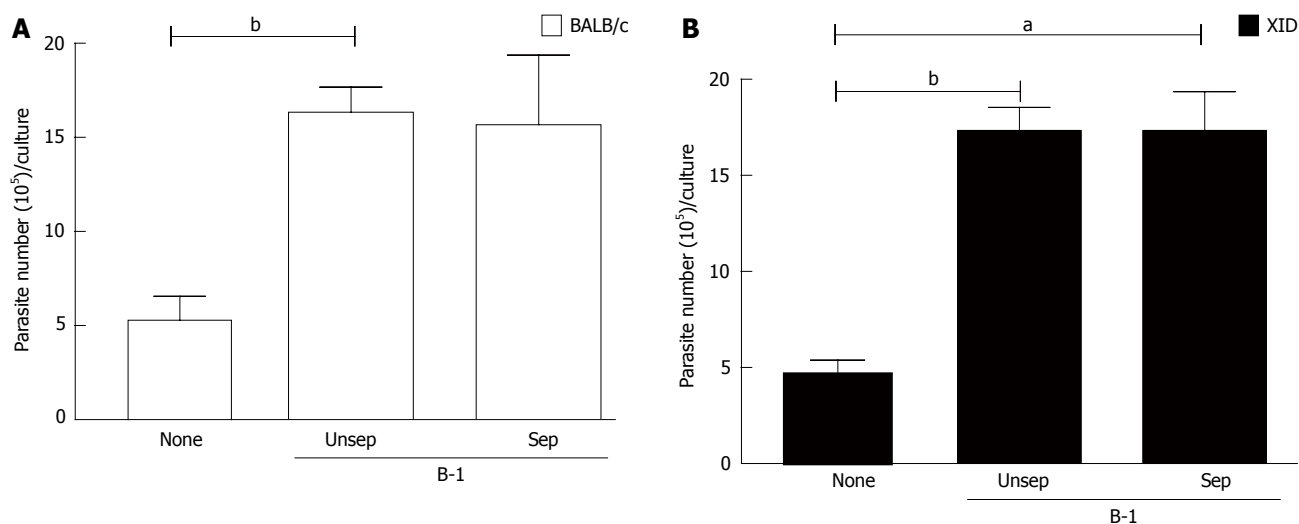
### Macrophage interactions with B-1 cells induce the growth of *L. major*

In this study, we analyzed the role of B-1 lymphocytes in modulating the replication of *L. major* in infected peritoneal macrophages *in vitro*. B-1 cells obtained from wild-type BALB/c mice and co-cultured with peritoneal macrophages from BALB/c and BALB XID mice infected with *L. major* promastigotes induced not only accentuated intracellular amastigote growth (Figure 1A) but also an increase in the percentage of infected cells (Figure 1B). The results obtained by counting the number of amastigotes in infected macrophages were confirmed *via* the quantification of motile promastigote forms in the supernatants after 5 d of interaction between B-1

cells and *L. major*-infected macrophages (Figure 1C). It is important to mention that XID macrophages were more resistant to infection with *L. major* than BALB/c macrophages (Figure 1).

### The immunomodulatory factor of B-1 cells is not dependent on physical contact

We then assessed whether B-1/macrophage physical contact was required for the increased intracellular amastigote replication that was observed in our co-cultures. Increasing *T. cruzi* replication in macrophages requires recognition with apoptotic cells, thus directing the process of adhesion and phagocytosis<sup>[19-21]</sup>. However, when using a Trans-well system in our model of intracellular *L. major* growth, we found that parasite multiplication was independent of cell contact between B-1 cells and infected macrophages from both types of mice (Figure 2). These results indicate that in our system, the observed parasite growth is mediated by a soluble factor(s) that is released



**Figure 2** The immunomodulatory effect of co-cultured infected macrophages and B-1 cells is independent of contact. Peritoneal macrophages from BALB/c (A) and XID (B) mice were plated in 24 wells vessels at 105 cells/well in complete culture medium and infected with *L. major* (10<sup>6</sup>/well). After 24 h of infection, cells were washed and B-1 cells were added at same compartment (Unsep), or separated (Sep) by a cell-impermeable culture insert. The cells were cultured in Schneider medium for 5 d at a temperature of 27 °C. After this period, the promastigotes were quantified in the supernatant of the cultures of infected phagocytes. All cultures were performed in triplicate and bars show the mean  $\pm$  SD. Statistical analysis were performed by *t*-test from representative results of three different experiments. <sup>a</sup>*P* < 0.05 and <sup>b</sup>*P* < 0.005.

into the cultures.

#### **IL-10 released into the culture medium by B-1 cells controls *L. major* growth**

B-1 cells are known to produce high levels of IL-10<sup>[13,22,23]</sup>. However, the importance of B-1-derived IL-10 in infected macrophages must be clarified. Recently, our group demonstrated that the IL-10 produced by B-1 CDP cells is important to facilitate intracellular infection and increase the number of motile *L. major* promastigotes in the supernatants<sup>[13]</sup>. Based on this information, we investigated whether B-1 cell-derived IL-10 should be related in the susceptibility of macrophages to intracellular infection.

Our data show that the amount of IL-10 in the supernatants of macrophages infected with *L. major* is increased in the presence of B-1 cells (Figure 3A). The cytokine TGF- $\beta$ , which is another important mediator involved in the modulation of macrophages infected with intracellular parasites<sup>[19-20]</sup>, was not detected in the present study model (data not shown).

To determine the real importance of B-1-derived IL-10 in *L. major*-infected macrophages, we added a neutralizing anti-IL-10 antibody to the cell cultures. Our results demonstrated that the neutralization of IL-10 induced an important reduction in the number of promastigotes released into the supernatant (Figure 3B and C). The data previously described demonstrate that the production of this cytokine by B-1 lymphocytes is relevant for increasing parasite growth in peritoneal macrophages.

#### **The presence of B-1 cells induced lipid body formation and PGE<sub>2</sub> production in infected macrophages**

Lipid bodies (LBs) are organelles that are related in arachidonic acid metabolism and produce lipid mediators

in response to inflammatory stimulus<sup>[24,25]</sup>. Infection with intracellular pathogens alters and increases the number of the lipid bodies<sup>[26,27]</sup> in infected cells. In recent years, we have described a progress of the number of lipid bodies in phagocytic cells infected with *Trypanosoma cruzi* and *L. major* and evidenced the role of these lipid bodies in disease development<sup>[13,26]</sup>.

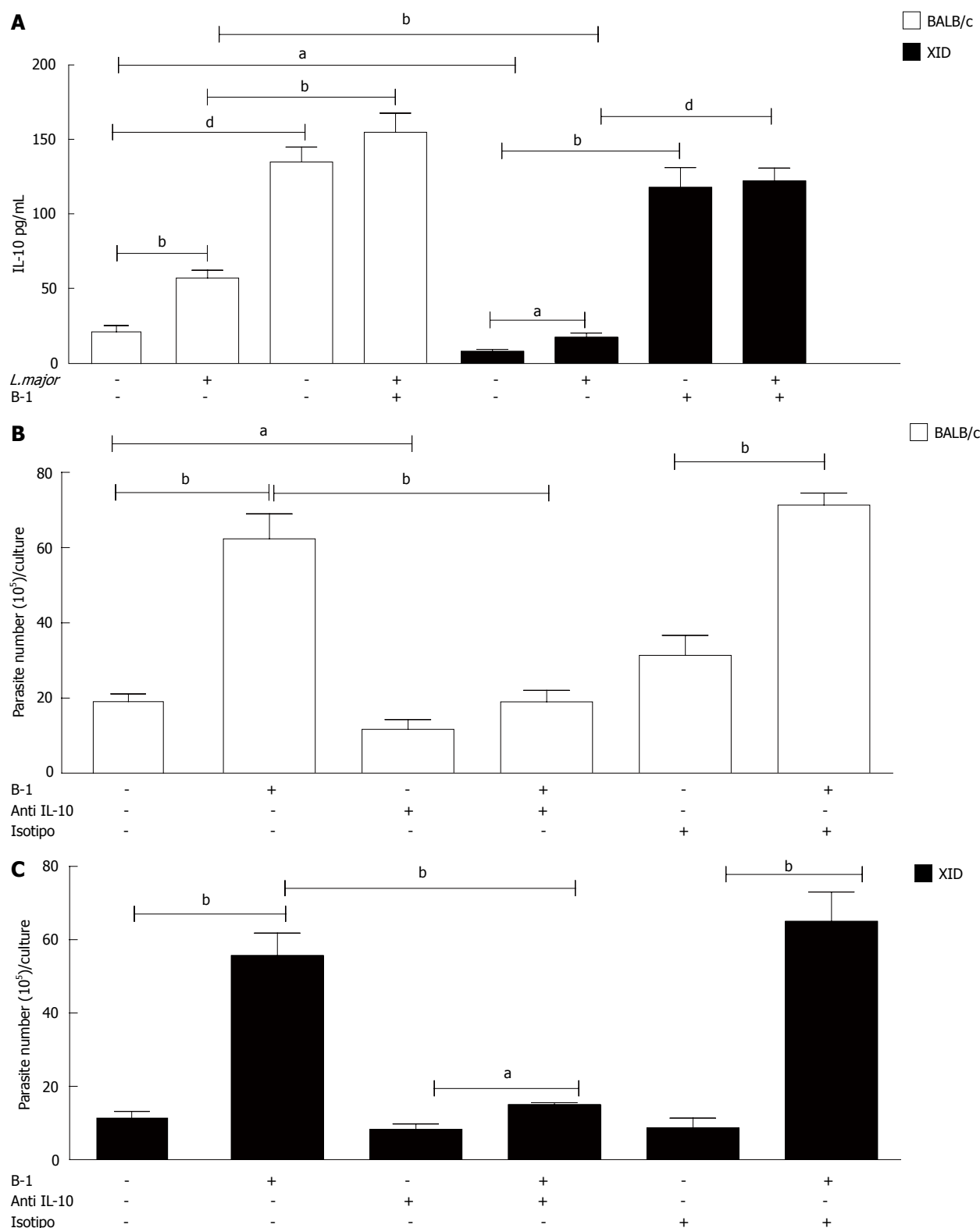
The results shown in Figure 4 demonstrate that infected macrophages co-cultured with B-1 cells contain a large number of lipid bodies in comparison to infected macrophages cultivated without B-1 cells (Figure 4). Our data demonstrate that macrophages from BALB/c mice contain more lipid bodies than XID macrophages, even in absence of a stimulus (Figure 4).

#### **Production of PGE<sub>2</sub>**

The mechanism of LB formation and PGE<sub>2</sub> synthesis during *L. major* infection in co-cultures of macrophages and B-1 cells was investigated. Phagocytes derived from B-1 cells (B-1CDP cells) constitutively express COX enzymes and produce large amounts of PGE<sub>2</sub> in response to inflammatory signals or infection with *Leishmania* spp.<sup>[13]</sup>. However, the production of the lipid mediator PGE<sub>2</sub> by B-1 cells has not yet been described. Based on this information, we investigated the production of PGE<sub>2</sub> by *L. major*-infected peritoneal macrophages obtained from BALB/c and XID mice. As described in Figure 5, the presence of B-1 cells induced considerable amounts of PGE<sub>2</sub> in both types of infected macrophages. Our data also demonstrate that infected macrophages from XID mice produce less PGE<sub>2</sub> than macrophages from BALB/c mice, even in the absence of B-1 cells (Figures 5 and 6).

To characterize the importance of PGE<sub>2</sub> for parasite load, we added two different non-steroidal anti-inflammatory drugs (NSAIDs) to the B-1 cell/macrophage cultures:

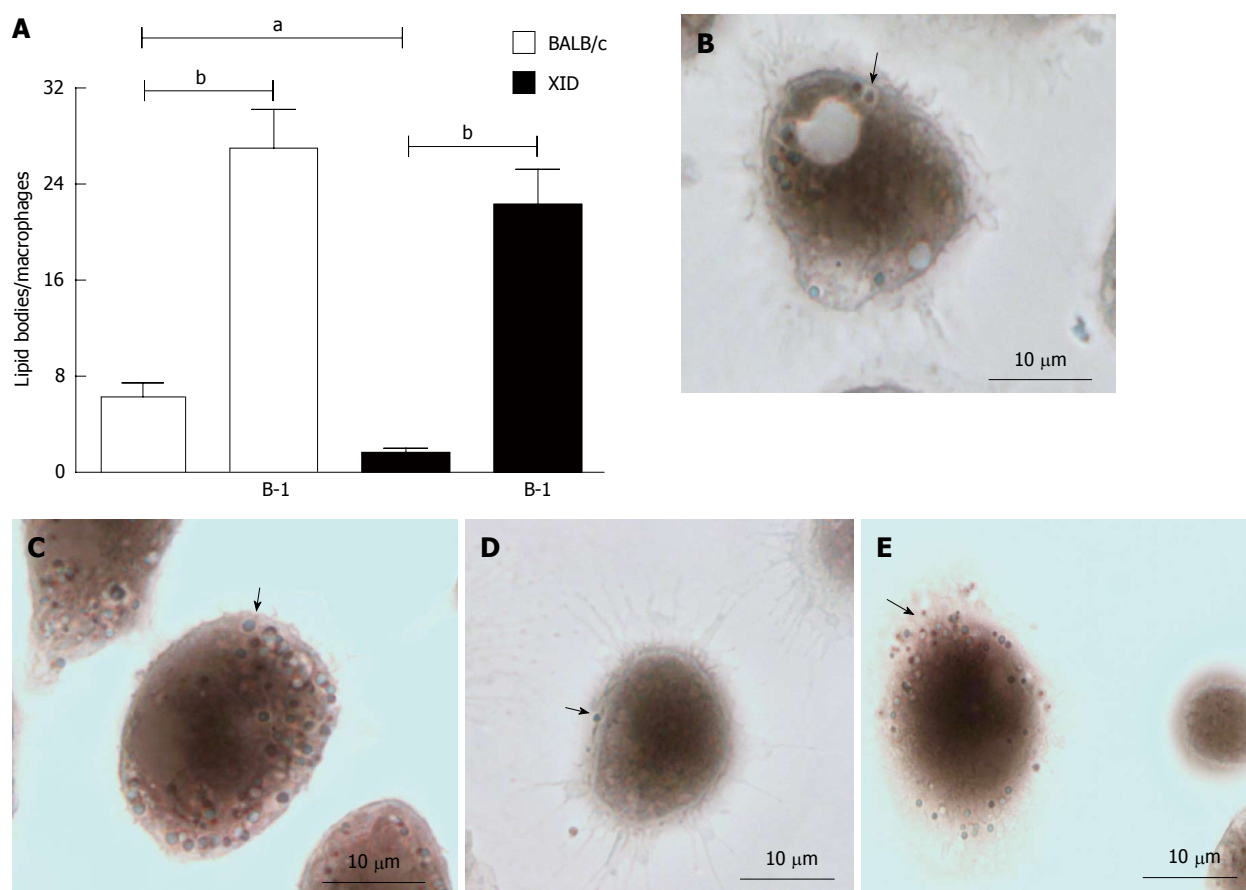




**Figure 3 Interleukin-10 is a determinant factor for the increased parasite load in macrophages.** Peritoneal macrophages from BALB/c or XID mice co-cultured with B-1 cells were cultured in the presence or absence of *L. major* (MOI 10:1). After 24 h, the supernatant was collected and IL-10 was measured by ELISA (A). All cultures were performed in triplicate and bars show the mean  $\pm$  SD. Representative result of three different experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.005$  and <sup>c</sup> $P < 0.0001$ . Co-cultures of B-1 cells and infected macrophages from BALB/c or XID mice were treated or not with doses of monoclonal neutralizing anti-IL-10 or control isotype. Once were infected with *L. major*, after 24 h, the cell cultures were washed with DMEM and incubated 3 d and then passed to Schneider medium. After 5 d in medium Schneider, promastigotes were counted in the supernatant (B). Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  and <sup>b</sup> $P < 0.005$ .

aspirin and indomethacin. These two drugs are recognized as potent inhibitors of PGE<sub>2</sub> production<sup>[19,20]</sup>. Our data

demonstrate that the blockade of PGE<sub>2</sub> production affected the parasite burden inside the macrophages (Figure 6).



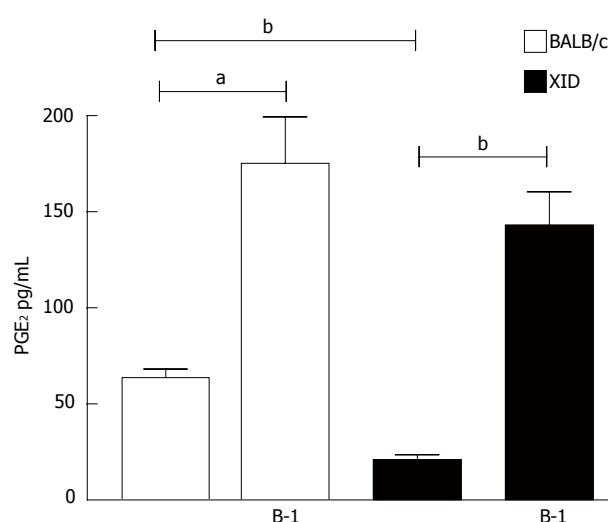
**Figure 4 Presence of B-1 cells increased numbers of lipid bodies in infected macrophages.** Peritoneal macrophages from BALB/c or XID mice were incubated with glass coverslips; some cultures were infected with *L. major*. Stained with Osmium tetroxide, the slides were washed and stained with DAPI (Sigma). The morphology of fixed cells was observed, and Nile red LBs were counted by light microscopy with a 100 × objective lens in 50 consecutively scanned leukocytes (A). Representative images of lipid body formation in infected macrophages from BALB/c mice (B), infected macrophages from BALB/c mice cultured in the presence of B-1 cells (C), infected macrophages from XID mice (D) and infected macrophages from XID mice co-cultured with B-1 cells (E). Black arrows pointing to the LB. Statistical analysis were performed by *t*-test from representative results of three different experiments. <sup>a</sup>*P* < 0.05 and <sup>b</sup>*P* < 0.005.

### Blockade of PGE<sub>2</sub> by NSAIDs reduced IL-10 production by infected macrophages

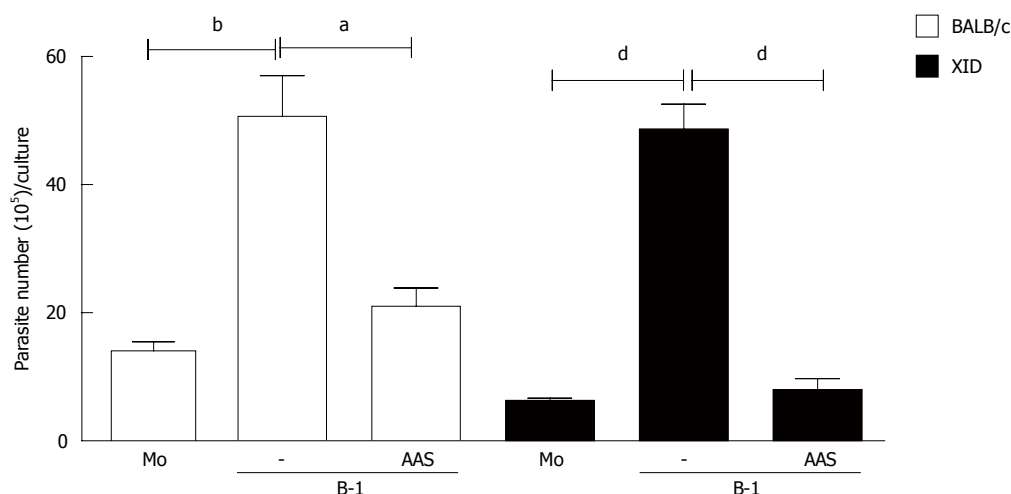
Following the rationale that the inhibition of PGE<sub>2</sub> decreased the release of promastigotes into the supernatant of *L. major*-infected macrophages co-cultured with B-1 cells, we investigated whether that lipid mediator has an effect on IL-10 production. The results shown in Figure 7 reveal that an important reduction of IL-10 production occurred when the cultures were treated with NSAIDs (Figure 7).

### B-1 cells isolated from IL-10 KO mice do not stimulate *L. major* growth

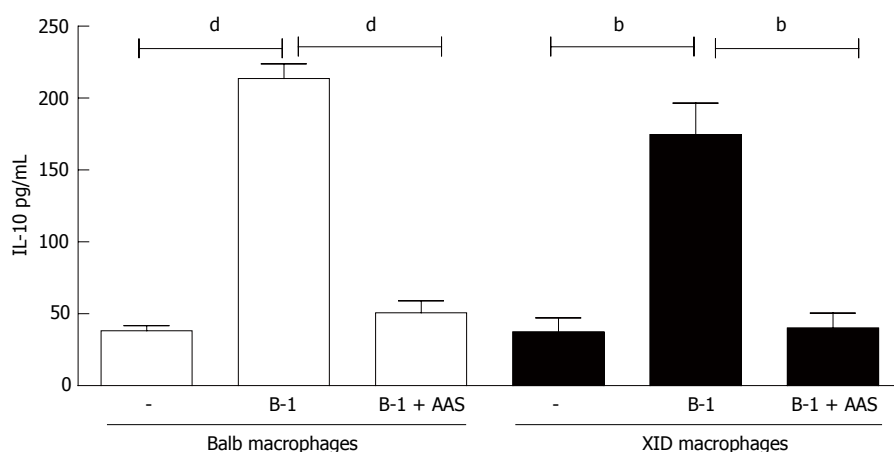
To demonstrate that the presence of the soluble cytokine IL-10 was essential for favoring intracellular infection, we used murine B-1 lymphocytes from IL-10 KO animals. When *L. major*-infected macrophages were co-cultured with B-1 cells from wild-type mice, the number of intracellular amastigotes (Figure 8A), the percentage of infected macrophages (Figure 8B) and the number of promastigotes released (Figure 8C) all increased. On the other hand, when infected macrophages were co-cultivated with B-1 cells from IL-10 KO mice, the modulatory effect that favors the parasite was not



**Figure 5 Infected macrophages co-cultured with B-1 cells secrete high levels of prostaglandin E<sub>2</sub>.** Infected macrophages from BALB/c or XID mice were incubated in the presence or absence of *L. major* (MOI 10:1). After 24 h of infection the supernatant was collected, and B-1 cells were added to the culture. After 24 h the level of PGE<sub>2</sub> secreted was measured by EIA. All cultures were performed in triplicate and bars show the mean ± SD. Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean ± SD. <sup>a</sup>*P* < 0.05 and <sup>b</sup>*P* < 0.005.



**Figure 6 Blockage of the cyclooxygenase pathway induces protective phenotype in the *Leishmania major* infection.** Infected macrophages from BALB/c or XID mice macrophages were incubated in the presence or absence of *L. major*, 24 h after the cellular cultures were washed and B-1 cells were added. The co-culture was treated or not with aspirin (AAS) (10 mg/mL). After 24 h of incubation, the cells were washed and incubated again for 3 d. After this time, the promastigotes were counted in the culture supernatant. All cultures were performed in triplicate and bars show the mean  $\pm$  SD. Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean  $\pm$  SD. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.005 and <sup>d</sup>*P* < 0.0001.



**Figure 7 Effect of prostaglandin E2 inhibition on interleukin-10 production.** Infected peritoneal macrophages from BALB/c or XID mice were co-cultured with B-1 cells and incubated with or without aspirin (AAS) (10  $\mu$ g/mL). After 24 h of incubation, the supernatant was collected and interleukin-10 measured by enzyme-linked immunosorbent assay. All cultures were performed in triplicate and bars show the mean  $\pm$  SD. Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean  $\pm$  SD. <sup>b</sup>*P* < 0.005 and <sup>d</sup>*P* < 0.0001.

observed. These results confirm the importance of IL-10 released by B-1 cells in the modulation of macrophage infection by *L. major*.

## DISCUSSION

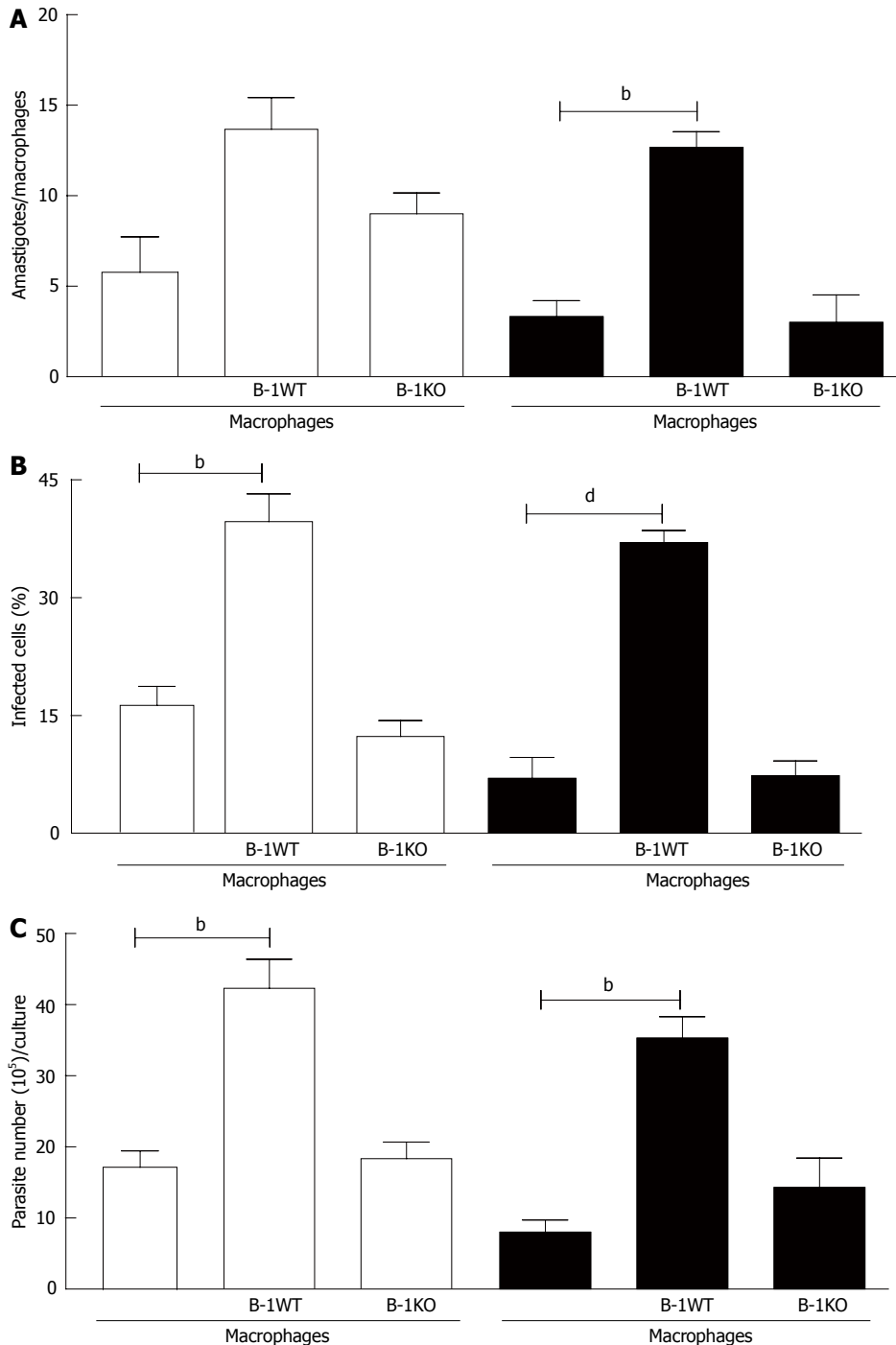
The findings reported in this manuscript are strengthened by experimental data. We co-cultivated B-1 cells with *L. major*-infected peritoneal macrophages from BALB/c mice and BALB XID mice, which are devoid of B-1 cells.

B-1 cells are of interest due to the immunomodulatory effects that these cells have exerted in different models of infection and inflammation<sup>[2,13,23,28-31]</sup>. B-1 cells from the peritoneal cavity migrate to inflammatory and/or infectious sites and release a series of immunomodulatory factors into the environment and also have the ability to differentiate into phagocytes<sup>[2,32]</sup>. Our group recently

demonstrated a role for B-1 cell-derived phagocytes (B-1CDP) in *L. major* infection *in vitro*<sup>[13]</sup>.

In the present study, we demonstrate that B-1 lymphocytes are efficient at inducing parasite replication when co-cultured with *L. major*-infected macrophages. The increased number of amastigotes observed in infected macrophages co-cultured with B-1 cells in our system is related to the number of motile promastigotes in the supernatants. Peritoneal macrophages isolated from XID mice exhibit resistance to infection with *L. major* when compared to BALB/c mice. However, when B-1 lymphocytes from BALB/c were added to the cultures of infected XID peritoneal macrophages *in vitro*, the cells became susceptible to infection, in comparison to co-cultures without B-1 cells. B-1 cells are known to produce large amounts of cytokines that could modulate macrophages and promote the intracellular infection of





**Figure 8 B-1 cells from interleukin-10 deficient mice are more competent to control *Leishmania major* infection in macrophages.** To confirm the production of IL-10 is involved in the susceptibility to infection by *L. major*, we used B-1 cells from BALB/c (white bars) and from IL-10 KO mice (black bars). Our data demonstrate decreased in the number of intracellular amastigotes (A) and percentage of infected cells (B). We also observed the significant decrease in the liberated promastigotes forms by infected macrophages co-cultured with B-1 cells from IL-10 KO mice (C). Statistical analysis was performed by *t*-test from representative results of three different experiments and bars show the mean  $\pm$  SD. <sup>b</sup>*P* < 0.005 and <sup>c</sup>*P* < 0.0001.

macrophages<sup>[13,23]</sup>. To determine if cell-cell physical contact was responsible for macrophage infection and parasite replication, we used a cell-impermeable membrane and observed that macrophage infection occurred in the absence of cell contact. This result demonstrates that soluble factors were being secreted into the medium by B-1 cells.

As reported in several studies, B-1 cells can produce large amounts of IL-10, which plays an important immunomodulatory action in different cell types<sup>[13,23,33]</sup>. IL-10 is an important mediator, which when released acts directly on different cell populations and is known to be an inhibitor of different types of cytokines, which are

important factors for the activation of phagocytes<sup>[34-37]</sup> and consequently suppress the secretion of nitric oxide (NO). NO is an important factor that has leishmanicidal activity and favors the arginase pathway, which is involved in the activity of ornithine decarboxylase<sup>[19,38-40]</sup>. Arginase would facilitate the intracellular growth of pathogens<sup>[19,20]</sup>.

In addition to IL-10, TGF- $\beta$  is another cytokine that is important in modulating the intracellular growth of parasites<sup>[19,20,26]</sup>. TGF- $\beta$  has been implicated in increased parasite replication in different *in vivo* and *in vitro* models of experimental *Leishmania* infection<sup>[41-44]</sup>. The results reported in this manuscript strongly suggest that

macrophage susceptibility to infection with *L. major* was related to the production of IL-10 by B-1 cells. Our data also indicated that the TGF- $\beta$  was not related in the increased intracellular infection (data not shown). The importance of IL-10 produced by B-1 cells was confirmed in our study when we added a neutralizing antibody anti-IL-10 and observed a marked decrease in the number of parasites released into the culture supernatants.

We also found a large number of lipid bodies in the cytoplasm of *L. major*-infected macrophages co-cultured with B-1 cells. Lipid bodies are lipid-rich cytoplasmic organelles that control the accumulation and hydrolysis of neutral lipids and are found primarily in adipocytes<sup>[45,46]</sup>. Macrophages were previously reported to have few lipid bodies in their cytoplasm, but the number of lipid bodies in these phagocytic cells can increase after stimulation<sup>[13,26]</sup>. *T. cruzi* and *Leishmania* are able to induce lipid bodies in the cytoplasm of infected macrophages<sup>[13,26]</sup>. It is already known that lipid bodies can be used as lipid sources that favor parasite replication or contribute to the production of soluble mediators involved in inflammation<sup>[47]</sup>. Lipid bodies store arachidonic acid, which is important for the production of lipid mediators<sup>[47]</sup>.

Our data demonstrate that the number of lipid bodies in the cytoplasm of infected macrophages increased when these cells were co-cultured with B-1 cells. In addition to the increased number of lipid bodies, we also observed strong production of PGE<sub>2</sub> in the co-cultures. Our data also show that the inhibition of PGE<sub>2</sub> modulated the infection, resulting in a decrease in the number of promastigote forms released in co-cultures of infected macrophages and B-1 cells. PGE<sub>2</sub> is a lipid mediator that plays an important role in the production of factors, such as TGF- $\beta$ <sup>[19]</sup> and IL-10<sup>[13]</sup>.

Our group recently described the production of anti-inflammatory mediators in experimental *T. cruzi* and *Leishmania* models, resulting in the inhibition of leishmanicidal products<sup>[13,19]</sup>.

PGE<sub>2</sub> is an important modulator of the T lymphocytes activation and the production of NO by phagocytes and favors the infection of intracellular parasites<sup>[19,21]</sup>. In addition, PGE<sub>2</sub> is essential for increasing parasite growth in macrophages that have ingested apoptotic cells<sup>[19,21]</sup>. The presence of the lipid mediator PGE<sub>2</sub> acts as a potent inhibitor of not only the immune response mediated by T lymphocytes but also the production of microbicidal factors by macrophages, favoring the growth of intracellular parasites<sup>[13,19,21]</sup>. In addition, PGE<sub>2</sub> is a key lipid mediator that has been implicated in *T. cruzi* amastigote proliferation inside macrophages that have phagocytosed apoptotic bodies<sup>[19-21,26]</sup>.

Our results demonstrate that the production of IL-10 by B-1 cells is involved in the immunomodulatory mechanism in infected macrophages and consequently favors the replication of intracellular parasites. This result clarifies the involvement of IL-10 produced by B-1 cells as the key factor for the modulation of macrophage

activation. A similar result was obtained by Arcanjo *et al.*<sup>[13]</sup>, who used B-1CDP phagocytes from IL-10 KO mice and observed that intracellular infection with *L. major* was impaired. Another elegant work reported similar data in the context of *in vitro* infection by *Coxiella burnetii*. The authors observed that the macrophages of XID mice, which present a defect in the production of B-1 lymphocytes, have a better resistance to intracellular infection by *C. burnetii* when compared to the macrophages of wild-type mice<sup>[48]</sup>.

In summary, our work demonstrates that one role of the B-1 cell population is to produce an anti-inflammatory cytokine and a lipid mediator that exert their effects on macrophages of the innate immune system, contributing to *L. major* growth and replication *in vitro*, probably by inhibition of NO and ROS production. Further studies should be addressed to investigate the importance of B-1 cells in the lesion sites highlighting a possible clinical significance of these cells in the infection.

## COMMENTS

### Background

During infections the pathogens subvert the immune responses to their purpose. This is well demonstrated in chronic persistent infection mediated by *Leishmania* species. This parasite is adapted to grow inside macrophage where they are maintained during the parasitism of the host. Herein, the authors show that infection by *Leishmania major* (*L. major*) triggers the suppressive role of B-1 cells responsible to secrete interleukin-10 (IL-10) cytokine upon contact with infected macrophages. This immunomodulatory cytokine is shown to deactivate macrophage innate responses thus favoring the parasite burst in the host.

### Research frontiers

The findings shown in the present study are of relevance to the immuno-parasitology field.

### Innovations and breakthroughs

The knowledge of the host-parasite interplay is crucial to design therapies aimed at controlling pathogen infections. Here the authors show that *L. major* parasites induce immunosuppression of macrophage cells which is important to their maintenance in the host.

### Applications

The authors' main findings point to a role of B-1 cells mediating the parasite subversion of the host's immune defenses. Further studies should be focused to investigate the molecular mechanisms involved in this immunomodulatory response.

### Terminology

Parasite subversion: Inhibition of protective immunity of the host to favor the parasitism; Chronic persistent infection: A sort of pathogens are able to subvert the host immune responses in order to establish latent infections; Immunomodulatory response: The immune system is counter balanced by homeostatic responses in order to avoid its overactivity; B-1 cells: B lymphocyte subset displaying functional properties different from the conventional B cell population (B-2 cells). B-1 cells are thought to mediate immunomodulatory responses by IL-10 and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) secretion.

### Peer-review

The authors investigate the immunomodulatory effect of B-1 cells in *L. major* infected macrophages. Results suggest that PGE<sub>2</sub> and IL-10 released from B-1 cells increase intracellular parasite replication. The manuscript is generally well-written.

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