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ORIGINAL ARTICLE

A CELL-CULTURING SYSTEM FOR THE STUDY OF INTERACTION BETWEEN MACROPHAGES INFECTED WITH *Leishmania amazonensis*

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ABSTRACT

The cell culture insert system is a culturing system for the study of contact-independent cellular communication. Leishmaniasis is a neglect tropical disease with no vaccines and the available drugs present toxic side effects. Studies on *Leishmania* interaction with host macrophages aim to develop strategies for parasite control and drug development. The purpose of this study was to evaluate the effects of interaction between non-infected and *L. amazonensis*-infected human macrophages, by using the cell culture system. The results showed that the infection index was reduced by 56.2% as compared to controls only when infected macrophages were inserted on both sides of the Transwell membranes. An improvement in macrophage viability was also observed in this cell culture. The levels of interleukin-1β, an inflammatory cytokine, and nitric oxide, a microbicidal molecule, did not increase in *L. amazonensis*-infected macrophage cultures in the Transwell system; thus other soluble factors were responsible for parasite control.

KEY WORDS: Leishmaniasis; *Leishmania amazonensis*; macrophages; co-cultures.

INTRODUCION

The development of cell and tissue techniques is absolutely necessary to basic and applied biomedical research. The cells communicate with each other through chemical signals. Two cell populations are used in co-culture systems to study the conditions and mechanisms of this two-way communication (Goers et al., 2014; Revenfeld et al., 2016). Initially, Grobstein studied the effects of embryonic mouse epithelial and mesenchymal tissue interaction. Distinguishing between effects due to direct cellular contact and those due to diffusion of molecules that applied the so-called membrane/molecular filter system was important (Grobstein, 1953). This cell system consisted of fragments of tissues cultured on either side of 20 μm thickness molecular filter sheets in glass dishes (Grobstein, 1953). Adapted over the years, the cell culture insert system is now recognized as an excellent cell culturing system for

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studies on contact-independent cellular communication. As shown in Figure 1, the permeable support is a porous membrane insert placed within a well in a culture plate creating upper and lower compartments. It is available in a range of pore sizes (0.4, 3.0 and 8.0 μm) and different materials (polycarbonate, polyester, and collagen-coated polytetrafluoroethylene). The recommended pore sizes for cell-cell interaction studies are 0.4 and 3.0 μm to prevent cell migration/chemotactic responses and to allow the passage of soluble factors and small vesicles (Herron et al., 2013).

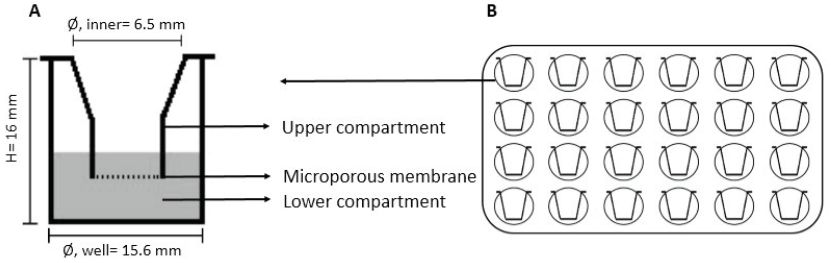

Figure 1. Schematic illustration of the co-culture system. The 24-well culture plate with membrane inserts (Transwell). The insert placed within a well creates upper and lower compartments.

Our laboratory research focuses on the many aspects of leishmaniasis (Araujo et al., 2012; Cyrino et al., 2012; Negrão et al., 2017; Moraes et al., 2018). This neglected tropical disease is characterized by diffuse or localized cutaneous and mucocutaneous lesions or visceral disease and transmitted by *Leishmania* infected sandflies (Burza & Croft, 2018). Annually, 0.7-1 million new cases are reported from 98 countries including Brazil (Muller et al., 2018). There are no vaccines and the available drugs present toxic side effects (Burza & Croft, 2018). Cutaneous lesions induced by *L. amazonensis* consist predominantly of macrophage accumulation, namely mononuclear phagocytes (host cells of *Leishmania*) and a small number of infiltrating neutrophils, mast cells, and lymphocytes (Araujo et al., 2012). Studies on parasite interaction with macrophages and lesional environments aim to develop strategies for parasite control and drug development.

Another pathogen that infects macrophages is the intracellular bacteria *Mycobacterium tuberculosis*. Hartman & Kornfeld (2011) studied the interaction between naive and infected macrophages that model pulmonary tuberculosis in a cell-culturing system for contact-independent cellular communication, and observed a reduction in bacteria viability. The authors proved that cell contact was not required for anti-mycobacterial activity, and suggested that soluble factors, such as interleukin 1β (IL-1β), an inflammatory cytokine, and nitric oxide (NO) a microbicidal molecule were implicated in the bacterial reduction.

The aim of this study was to evaluate whether, similarly to what was reported with *M. tuberculosis* (Hartman & Kornfeld, 2011), the contact-independent crosstalk between non-infected and *Leishmania*-infected macrophages, which simulates events in cutaneous lesions induced by *L. amazonensis*, can restrict parasite infection and induce IL-1β and NO production. The cell culture insert system is a useful method for these inter-macrophage population communication studies.

MATERIAL AND METHODS

Parasite culture

Leishmania amazonensis (MHOM/BR/73/M2269) promastigotes were grown in RPMI-1640 medium, containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin at pH 7.4 and kept at 26°C.

Macrophage culture and L. amazonensis *infection*

The macrophage cell line THP-1 was obtained from the Banco de Células do Rio de Janeiro, Rio de Janeiro, RJ. It is a human acute monocytic leukemia cell line which was grown in RPMI-1640 medium containing 10% FBS, 50 μg/ml gentamicin and 1 mM sodium piruvate at 37°C in a humidified atmosphere of 5% CO_2 (Tsuchiya et al., 1980). The cells were differentiated into macrophages by exposure to 1 mg/ml phorbol-12- meristate-13-acetate for four days before the experiments (Aldo et al., 2013). For infection, THP-1 macrophages (2×10^5/well) cultured in 24-well plates containing 13 mm diameter glass cover slips were infected with promastigotes in RPMI 1640 medium at a 10:1 parasite to macrophage ratio at 37°C, in a humidified atmosphere with 5% CO_2. Heavily infected macrophage cultures were obtained by the infection of cells with promastigotes at a 20:1 parasite to cell ratio. After 24 hours, all the cell cultures were washed to remove extracellular parasites and used in co-culture experiments. The coverslips were washed with PBS, stained with Giemsa, and mounted on glass slides to evaluate the percentage of infected cells and the average of intracellular amastigotes, (Moraes et al., 2018). The slides were then examined microscopically and at least 200 cells were counted. The results were also expressed as the infection index, which is the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage. Macrophage adherence, as a direct measurement of the cell's viability and integrity, was assessed by counting the cells in 20 random fields per coverslip (Moraes et al., 2018).

Co-culture experiments

L. amazonensis-infected macrophages on the coverslips were incubated in 24-well plates. An equal number of macrophages was added to 0.4 µm pore sized Transwell inserts (Corning). Co-cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. After 24 hours the coverslips were stained with Giemsa and supernatants were collected for IL-1β and nitrite determination according to the experimental conditions shown in Figure 2. Three completely independent experiments were performed for co-culture experiments.

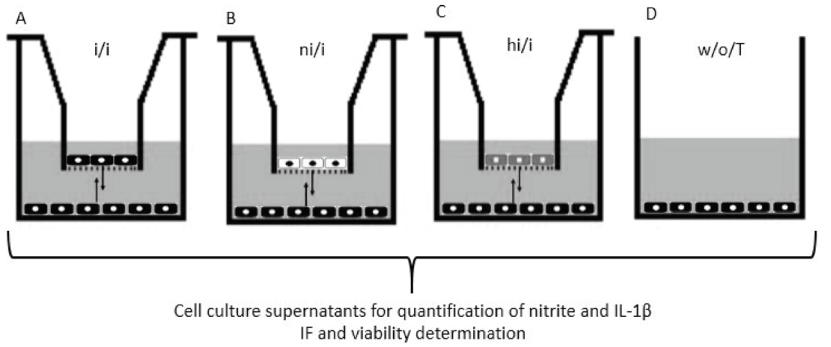

Figure 2. Experimental design. Macrophages were co-cultured under four different conditions using Transwell inserts. After 24 hours cells on coverslips were stained with Giemsa and analysed for infection index (IF) and viability; and supernatants assayed for IL-1β and nitrite. Black circles: *L. amazonensis*-infected macrophages; red circles: heavily *L. amazonensis*-infected macrophages; and white circles: non-infected macrophages. Conditions: i/i (infected macrophages in upper and lower compartments), ni/i (non-infected (naïve) macrophages in upper compartment and infected macrophages in lower compartment), hi/i (heavily infected macrophages in upper compartment and infected macrophages in lower compartment), and w/o/T (infected macrophages cultured without Transwell system).

IL-1β and nitrite determination

Supernatants from co-cultures were collected and frozen at -80°C prior to analysis. The supernatants were assayed for secreted IL-1β by a sandwich Elisa Kit according to the manufacturer's instructions (RayBiotech®, USA). The production of nitrite was evaluated in supernatants after incubation with Griess reagent (Bryan et al., 2007) and the absorbance at 540 nm was

determined using the microplate reader (Synergy HT®, Biotek). The limit detection for IL-1β and nitrite was 0.3 pg/ml and 1 μM, respectively. Three completely independent experiments were performed for the detection of IL-1β and nitrite determination in the macrophage culture supernatants.

Statistical analyses

All experiments were repeated at least three times. Means and standard deviations were calculated by GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA. One-way ANOVA was performed, followed by Dunnett's multiple comparisons test as well as Student t test.

RESULTS

The experiment design is shown in Figure 2; macrophages infected with *L. amazonensis* were cultivated, separately, with a 0.4 μm polycarbonate Transwell filter from non-infected or infected macrophages. After 24 hours, the parasite burden was calculated in macrophages cultured on coverslips in the lower compartment. As shown in Figure 3, the control wells in which infected macrophage cultures were incubated without Transwell inserts (condition w/o/T) showed 47% of infected cells and 3.6 intracellular amastigotes per cell. When Transwell inserts, containing naive macrophages were inserted in the upper compartment (condition ni/i), 38% of the infected cells and 3.6 intracellular amastigotes/cell were counted in the lower side. The infection indices were 169.2 and 136.8 for w/o/T and n/i conditions, respectively. Interestingly when infected macrophages were cultivated on both sides of the Transwell inserts, the percentage of infected cells, intracellular parasites and infection index diminished (37%, 2 and 74, respectively).There was a reduction of 56.2% in the infection index as compared with control (w/o/T). When heavily infected macrophages (20:1) were cultivated in the upper side of the Transwell inserts, the reduction of the infection index in infected macrophages in the lower side was 24% as compared with control, indicating that the Transwell compartmentalization of the macrophage population changed the outcome of the *Leishmania* infection. Cell viability results are equally interesting (Figure 4). *L. amazonensis*-infected macrophage cultures incubated in the presence of infected macrophages separated by a Transwell membrane are more viable than control and other cell culture conditions. In Figure 5, images of macrophage cultures in different conditions can be seen.

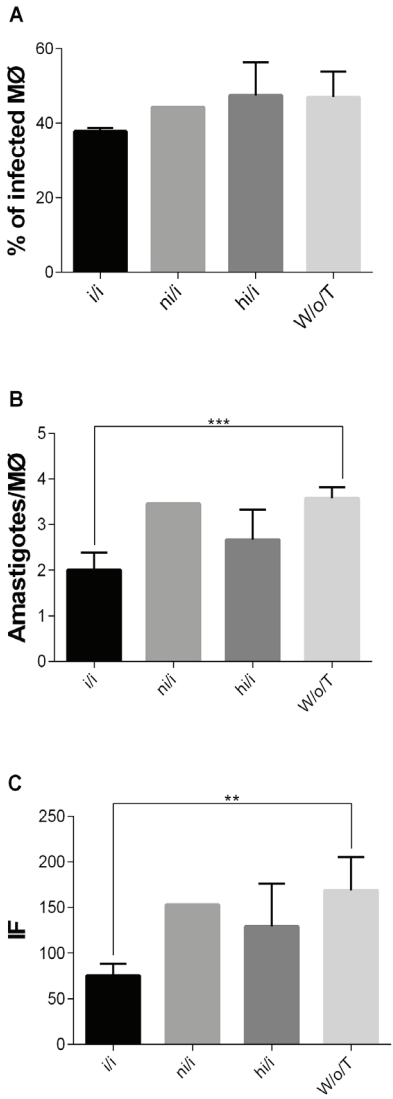

Figure 3. Effect of co-culturing on leishmanicidal activity of THP-1 macrophages. Under different experimental conditions infected macrophages were cultured for 24 hours and then evaluated for % of infected cells (A), intracellular amastigotes (B), and infection index (C; IF) as described in the Material and Methods Section. Values are expressed in mean ± standard error. Experimental conditions are the same as described in the legend to Figure 2. **p ≤ 0.01 and *** p ≤ 0.001.

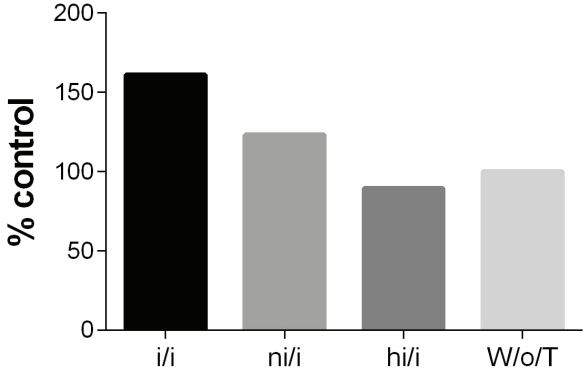

Figure 4. Effect of co-culturing on viability of *L. amazonensis* infected THP-1 macrophages. Under different conditions infected macrophages were cultured for 24 hours and then evaluated for viability, as described in the Material and Methods Section. Values are represented per percentage of cell viability, compared with control (condition w/o/T) and expressed in mean ± standard error. Experimental conditions are the same as described in the legend to Figure 2.

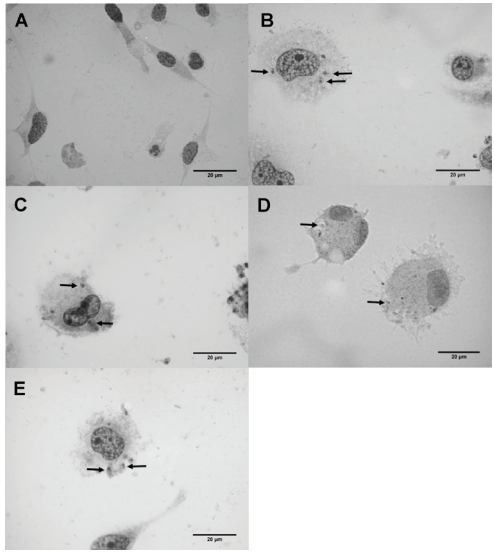

Figure 5. Morphological characteristics of THP-1 macrophages. Images of uninfected macrophages (A); *L. amazonensis*-infected macrophages under i/i (B), n/i (C), hi/i (D) and w/o/T (E) conditions. Experimental conditions are the same as described in the legend to Figure 2.

Since the Transwell experiments suggested the involvement of soluble factors released from infected macrophage cultures, assays were performed to evaluate IL-1β and nitrite production (Table). Our data showed that nitrite levels are detectable in supernatants, but there were no significant differences among the experimental conditions. Interestingly, infected macrophages cultured on their own (control w/o/T) produced twice the IL-1β level as compared to infected macrophages cultured in Transwells.

Table. Effect of co-culturing on IL-1β and nitrite production by *L. amazonensis*-infected THP-1 macrophages[a]

Experimental situation	IL-1β (pg/mL)	Nitrite (μM)
i/i	44.05 (46.07 – 42.07)[b]	3.50 (3.67 – 3.32)
ni/i	35.65 (37.43 – 33.87)	3.67 (3.85 – 3.49)
hi/i	40.40 (42.42 – 38.38)	4.15 (4.42 – 3.88)
w/o/T	88.32[c] (92.4 – 84.24)	4.97 (5.22 – 4.72)

[a] *L. amazonensis*-infected macrophages were co-cultured under different conditions for 24 hours, and IL-1β and nitrite concentrations were determined in supernatants as described in the Material and Methods Section. Experimental conditions are the same as described in the legend to Figure 2.
[b] 95% confidence intervals in parenthesis.
[c] A significant difference (p=0.01) was observed in regard to other experimental conditions (i/i, ni/i, hi/i).

DISCUSSSION

In this report we evaluated the contact-independent crosstalk between *L. amazonensis*-infected macrophages, using the cell culture insert system. Surprisingly, the separate co-cultivation of two identical populations of infected macrophages by the Transwell system, induced infection reduction as well as improving macrophage viability. There are no studies on macrophage-macrophage communication during *Leishmania* infection using the Transwell system although previous studies using this cell-culturing system showed that cell-cell contact is not required for the leishmanicidal effects of neutrophils (Carmo et al., 2010), mesenchymal stem cells (Dameshgi et al., 2016), $CD4^+$ T cells and B cells (Mukbel et al., 2006). However, studies on mycobacteria have shown that signals released from naive macrophages, from the upper side of Transwell systems, restricted the bacterial viability in *M. tuberculosis*-infected

macrophages (Hartman & Kornfeld, 2011). The authors suggested that IL-1β, an inflammatory cytokine, and NO, a microbicidal molecule, were implicated in bacterial control. Our results did not indicate the involvement of IL-1β and NO in the reduction of *Leishmania* infection; neither IL-1β nor nitrite production are enhanced in the supernatants. Typically human macrophages such as THP-1 differentiated cell line produce low nitrite levels (Thomas & Mattila 2014), which did not increase under the conditions of *Leishmania* infection and the Transwell system. For IL-1β, the levels were lower in macrophages cultured by Transwell as compared with macrophages cultured without Transwell.

The reasons for the compartmentalization of infected macrophage cultures inducing the reduction of *Leishmania* infection are not known. We can hypothesize that a reduction in the direct physical contact between infected macrophages also diminishes the contact with parasitic and cellular fragments; dying cells; autophagic and apoptotic bodies, as well as reducing the upper inflammatory microenvironment and IL-1β levels present in the cell cultures and lesions (Andrade et al., 1984; Giorgio et al., 1998; Wanderley et al., 2006; Cyrino et al., 2012). High levels of IL-1β in the lesional microenvironment are known to cause an exacerbated form of disease whereas sub-physiological doses of IL-1β have induced a less progressive disease (Voronov et al., 2010). Thus, moderate inflammation and low doses of IL1-β can contribute to parasite control. Also, macrophages even though infected, but less busy removing cell corpses from their surrounding, may produce and detect soluble factors that can increase their viability and leishmanicidal activity.

In conclusion the cell-culturing system tested is reliable and useful for contact independent crosstalk studies in *Leishmania in vitro* models. Soluble factors, released from *L. amazonensis*-infected macrophages in compartmentalized co-cultures, reduce parasitic infection. It is important to investigate which are these factors.

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