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# Comparative effect of ion calcium and magnesium in the activation and infection of the murine macrophage by *Leishmania major*

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**ABSTRACT**

Amastigotes of *Leishmania major* have a great ability to evade destruction in host cells. This study investigated the activation in resident, inflammatory macrophages and J774 cells *in vitro* treated with lipopolysaccharide (LPS), soluble *Leishmania* antigen (SLA), calcium ionophore (CaI) and magnesium ($Mg^{2+}$) alone or combined. An increase in nitric oxide (NO) production was observed in J774 or inflammatory macrophages treated with LPS alone or in combination with SLA and CaI. The same treatments did not affect the NO release by resident macrophages. There was no interference in uptake of *L. major* but CaI decreased intracellular proliferation of the parasite. This study demonstrated the importance of CaI in decreasing *L. major* proliferation inside murine macrophages while $Mg^{2+}$ seemed to increase parasite proliferation. These finding may help to understand the events involved in host cells' clearance of this pathogen.

**Key terms:** calcium, *Leishmania major*, macrophage, magnesium, nitric oxide.

## INTRODUCTION

*Leishmania* are dimorphic tripanosomatidae (amastigotes and promastigotes). When in contact with phagocytic cells of their vertebrate hosts, they replicate as non-flagelate amastigotes inside endocytic organelles called parasitophorous vacuoles (Alexander and Russell, 1992; Probst et al., 2001; Chang et al., 2003). Metacyclic promastigotes are introduced into the organism by sand fly bites, after the parasites are phagocytosed by macrophages through interactions with CR3 receptors and also parasite glycoproteins and glycolipids, such as gp63 and lipophosphoglycan (LPG), respectively (Turco and Descoteaux, 1992).

Among the various products of macrophage activation, nitric oxide (NO) is an important mediator of a number of biological functions including tumoricidal effects, vascular relaxation, neurotransmission, immunosuppression and cytotoxic activity against several pathogens, including *Leishmania major, Plasmodium falciparum, Schistosoma mansoni* and *Toxoplasma gondii* (Vouldoukis et al., 1995; Huang et al., 1998; Carreras et al., 2000; Rubbo et al., 2000).

Macrophages play a pivotal role in the course of leishmaniasis, acting not only as host cells but also as antigen-presenting cells capable of modulating the specific immune response. Macrophages also act as effector cells against the parasite, after proper activation by cytokines such as interferon (IFN)-gamma and tumor necrosis

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factor (TNF)-alpha (Drapier et al., 1988; Auger and Ross, 1991; Rogers et al., 2002). Calcium ionophore has been shown to mimic the effect of IFN-gamma in priming macrophages for leishmanicidal activity (Buchmüller-Rouiller and Mauël, 1991). However, questions regarding the mechanisms of parasite persistence for several years in the host and the strategies devised by *Leishmania* to escape and/or modulate specific immune responses remain unclear.

In the present work we analyzed the microbicidal activity of macrophages during infection by *Leishmania major,* evaluating macrophage activation as well as the ability of these cells to impair parasite replication.

## MATERIALS AND METHODS

### *Animals and parasites*

Inbred strains female of BALB/c mice at 6-8 weeks of age were used for obtaining resident and inflammatory macrophages. *Leishmania major*, strain MRHO/SU/59/P, were obtained from footpads of infected BALB/c mice and cultivated in complete RPMI medium, consisting of RPMI 1640 medium supplemented with 2mM of L-glutamine (Life Technologies, Grand Island, N.Y.), 10 % fetal bovine serum (Life Technologies), 100 U/ml of penicillin (Sigma Chemical Co., St Louis, MO, USA), 100 μg/ml of streptomycin (Sigma), 1 mM of sodium pyruvate and 100 mM of MEM non-essential amino acids (Life Technologies) at 25ºC. All animal experiments were performed according to the standards of the Colégio Brasileiro de Experimentação Animal (CoBEA).

### *Soluble antigen of Leishmania major*

Soluble *Leishmania* antigen (SLA) was prepared as described elsewhere (Scott et al., 1987), with modifications. To summarize, stationary growth phase *L. major* promastigotes obtained from culture in complete RPMI medium were washed in Hanks balanced salt solution (HBSS, Life Technologies) and centrifuged for 15 minutes at 3000x*g* and 4ºC. Parasite concentration was adjusted to 1 x $10^9$ parasites in HBSS containing 50 μg/ml of leupeptin (Sigma) and 1.6 mM phenylmethylsulphonyl fluoride (PMSF, Sigma). After 10 minutes in ice bath, parasites were sonicated with 4 cycles of 1 minute each. The lysate was centrifuged for 15 minutes at 3000x*g* and 4ºC. The supernatant was harvested and centrifuged at 20000x*g* at 4ºC for 30 minutes. The supernatant then filtered through 0.22 μm membranes and protein concentration was determined by the Lowry method (Lowry et al., 1951). SLA was stored at -70ºC until use.

### *Cell culture*

Inflammatory macrophages were obtained from BALB/c mice previously inoculated with 3% Thioglycollate medium (Sigma), while the resident macrophages were obtained from the peritoneal fluid of untreated BALB/c mice. The J774A.1 cells were purchased from ATCC (Rockville, MD, USA). Cells ($5x10^5$) were cultured in complete RPMI medium in a humidified chamber at 37ºC, containing 5 % $CO_2$, in the presence of either 10 μg/ml lipopolysaccharide (LPS, Sigma), 4 μg/ml of SLA, 1 μM calcium ionophore A23187 (CaI, Sigma), 1 μM magnesium chloride ($Mg^{2+}$, Vetec, Brazil) alone or in combination, in a total volume of 200 μl/well. The stimuli concentration was performed in accordance with Reis et al. (2001). After 48 hours, culture supernatants were collected for nitrite/nitrate [$NO_2^-$ + $NO_3^-$] measurements based on the Griess method (Martinez et al., 2000). Alternatively, cells were infected with stationary growth phase *L. major* promastigotes ($5x10^6$parasites/200μl/well) for a period of three hours, then washed and fixed in cold methanol for 10 minutes. Cells were stained with Giemsa's solution or by immunofluorescence. The percentage of infected cells was determined by counting 500 cells in each experimental condition.

### *Nitrite assay for estimation of NO production*

The concentration of stable nitrite, the end product from NO generation by effector

macrophages, was determined according to the Griess method. Then, 50 µl of Griess reagent [1 % sulfanilamide in 2.5 % $H_3PO_4$ and 0.1% naphtylethylenediamine (NEED) in 2.5 % $H_3PO_4$ (v/v)] were added to 50 µl of each sample, blank and standard curve in a 96-well microplate. The absorbance at 570 nm was measured using a microplate reader (Titertek Multiskan Plus) and software (Microplate Manager 4.0, Hercules, CA, USA). Nitrite content (µM/ $5x10^5$ cells) was quantified by extrapolation from the sodium nitrite standard curve in each experiment.

*Immunofluorescence assay*

For the immunofluorescence assay, fixed macrophages were rinsed with PBS pH 7.3 (3 times for 5 minutes each time) and permeated with 0.1 % Triton X-100 for 10 minutes. After rinsing, cells were incubated for 45 minutes at 37ºC with rabbit anti- *L. major* antibody diluted 1:100 in 2 % normal goat serum in PBS. After rinsing, cells were incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (1:20 diluted). Slides were mounted in glycerol-N-propyl-galate, examined and photographed using the Olympus BX50F-3 microscope (Olympus Optical Co, LTD, Japan).

*Proliferation assay*

Resident and inflammatory macrophages (5 x $10^5$ cells/200µl/well) were cultured. After an overnight incubation, non-adherent cells were removed by vigorous wash and LPS (10 µg/ml), SLA (4 µg/ml), CaI (1 µM) and $Mg^{2+}$ (1 µM) were added alone or combined. Macrophages were cultured for 48 hours, washed and infected with stationary phase *L. major* promastigotes ($5x10^6$parasites/200µl/well) for three hours. Free parasites were removed, and cells were cultured for another 48 hours. Cells were then pulsed with 0.5 µCi/well of [$^3$H]-Thymidine (New England Nuclear, Boston, MA, USA), incubated for 18 hours and then harvested (Cell Harvester, Cambridge Technology, Inc., USA). Radioactive incorporation was measured in a scintillation counter (Beckman LS 6500, Multi-Purpose Scintillation Counter, USA). Parasite proliferation was determined as the average of the proliferation index under each type of stimulus divided by the average of the proliferation index in the absence of stimulation (medium alone). Values above 1 indicated parasite proliferation within macrophages whereas values below 1 meant inhibition of parasite replication.

*Statistical analysis*

Data were analyzed by the ANOVA test (multiple comparisons) and results were expressed as the mean ± standard deviation. Significance was considered when $p<0.05$.

## RESULTS

*NO production as a marker of macrophage activation*

High nitrite levels were detected in inflammatory macrophages cultivated in the presence of either SLA or LPS (205 ± 18 and 193 ± 3.5 µM, respectively) compared to the control (medium) (58 ± 4 µM, Fig. 1A, $p < 0.05$). Incubation with CaI+LPS (Fig. 1A, $p<0.05$) resulted in increase of NO release (210 ± 7µM) compared to macrophages stimulated with CaI+SLA. In inflammatory macrophages treated with CaI, $Mg^{2+}$ or CaI+$Mg^{2+}$, no significant difference was observed in NO production (Fig. 1A). Resident macrophages showed the same levels of NO as the medium (Fig. 1B). In J774 cells stimulated with either SLA, LPS+SLA, CaI+LPS or CaI+$Mg^{2+}$, nitrite levels of 84 ± 2.4, 97 ± 1.9, 97 ± 0.3 and 77 ± 0.5 µM, respectively, were detected which were also significantly higher than the medium (Fig. 1C, $p<0.05$). On the other hand, in J774 cells stimulated with CaI alone (59 ± 1.3 µM, Fig. 1C) a decrease in NO release was observed. Supernatants of J774 cells stimulated with LPS (116 ± 2.17 µM) or LPS+$Mg^{2+}$(101 ± 0.8 µM) presented NO levels highly significant (Fig. 1C, $p<0.05$) compared to the control (medium).

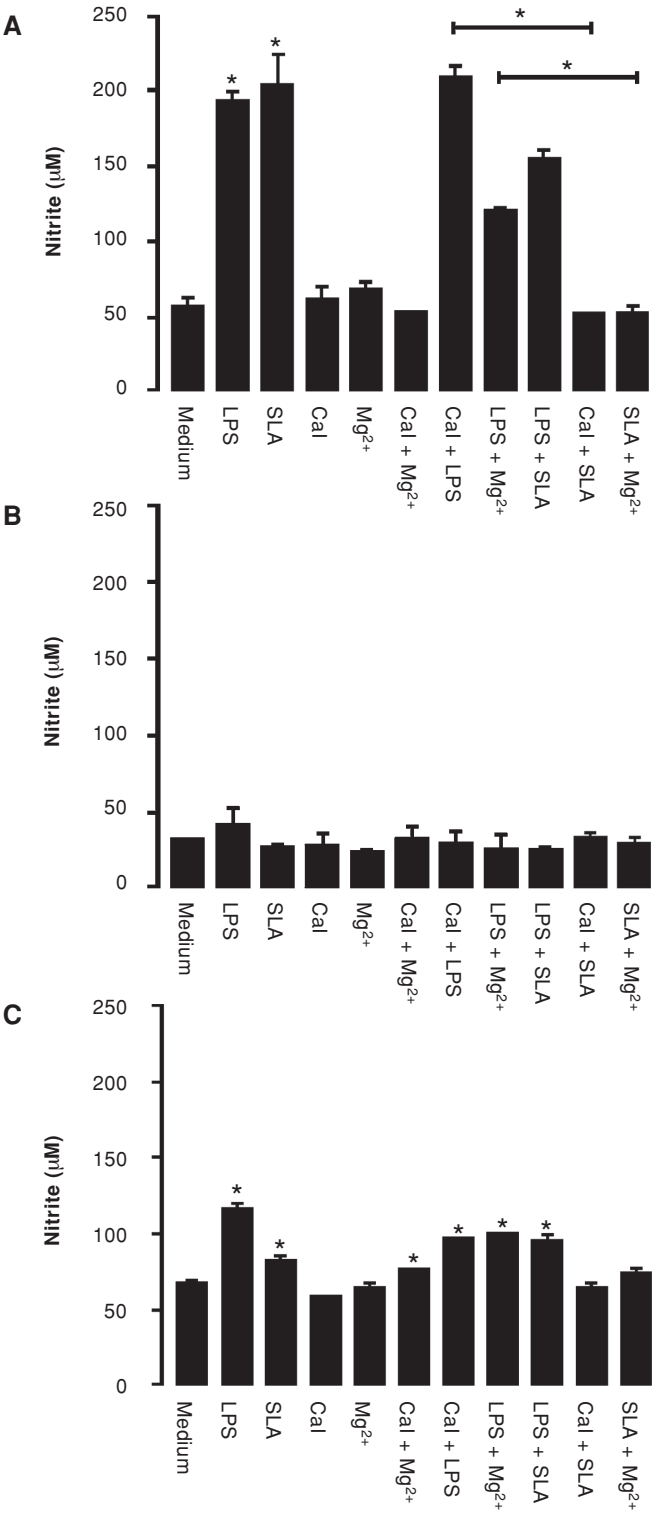


**Figure 1. NO production**. Inflammatory (A), resident macrophages (B) and J774 cells (C) were cultured in the presence or absence of the following treatments: LPS, SLA, CaI, $Mg^{2+}$, CaI+$Mg^{2+}$, CaI+LPS, LPS+$Mg^{2+}$, LPS+SLA, CaI+SLA and SLA+$Mg^{2+}$. After 48 hours of culture, nitrite content was quantified by the Griess method. Columns show nitrite concentration (means ± standard deviations from three independent experiments) expressed in μM. (*) $p<0.05$.

*Uptake of Leishmania major*

Inflammatory macrophages stimulated with either SLA or SLA+$Mg^{2+}$ had uptake rates 51.4 and 51.2 %, respectively when compared to the medium (Fig. 2A, $p<0.05$). After pre-treatment performed with the other stimuli, there were no significant differences compared to the medium. In J774 cells stimulated with SLA, SLA+$Mg^{2+}$ or CaI+$Mg^{2+}$, uptake rates were 38 %, 31 % and 32 %, respectively (Fig. 2B). In J774 cells stimulated with CaI+LPS, CaI, CaI+SLA or LPS, the infection rates were 28 %, 26.8 %, 21 %, 20.2 %, respectively (Fig. 2B). These results were significant different compared from the medium (12.8 %). On the other hand, J774 cells stimulated with $Mg^{2+}$, LPS+SLA or LPS+$Mg^{2+}$ demonstrated uptake rates similar to those observed for medium (Fig. 2B). The Leishmania uptake in resident macrophages was similar to that in inflammatory macrophages (data not shown).

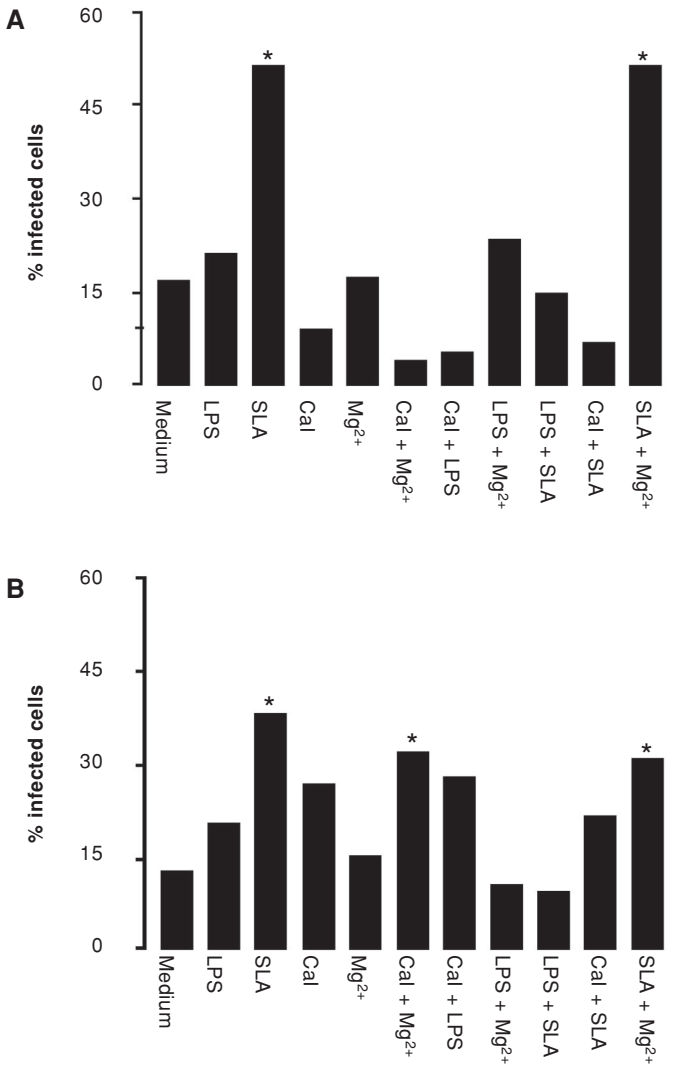


**Figure 2. Cell infection percentage.** Inflammatory (A) and J774 cells (B) were cultured for 48 hours in the presence or absence of treatments: LPS, SLA, CaI, $Mg^{2+}$, CaI+$Mg^{2+}$, CaI+LPS, LPS/$Mg^{2+}$, LPS+SLA, CaI+SLA and SLA+$Mg^{2+}$. Then the cells were infected by *L. major* for 3 hours, stained with Giemsa and analyzed under a microscope. The percentage of infected cells was determined by counting 500 cells from each treatment. (*) $p<0.05$.

*Analysis of L. major proliferation index*

In inflammatory macrophages treated with LPS, SLA or CaI (Fig. 3A), there was a significant decrease ($p<0.05$) in the parasite proliferation index (0.096, 0.30 and 0.20, respectively). Conversely, in those macrophages treated with $Mg^{2+}$ a significant increase in the parasite proliferation index was observed (3.05, Fig. 3A, $p<0.05$). No significant differences were observed in inflammatory macrophages treated with the other stimuli. In resident macrophages pre-treated with CaI+LPS or CaI alone (Fig. 3B, $p< 0.05$), proliferation indexes were significantly inhibited. Parasite proliferation was not evaluated in the J774A.1 immortalized cell line.

*Presence of L. major within activated macrophages*

In inflammatory macrophages as well as in J774 cells, parasites were observed in the cell periphery (data not shown). Figure 4 shows the presence of the parasite inside resident macrophages as long extensions of cytoplasm holding parasites at its ends, which was more evident in macrophages pre-treated with CaI+$Mg^{2+}$ (Fig. 4F), CaI (Fig. 4D), CaI+LPS (Fig. 4G) or $Mg^{2+}$ (Fig. 4E).

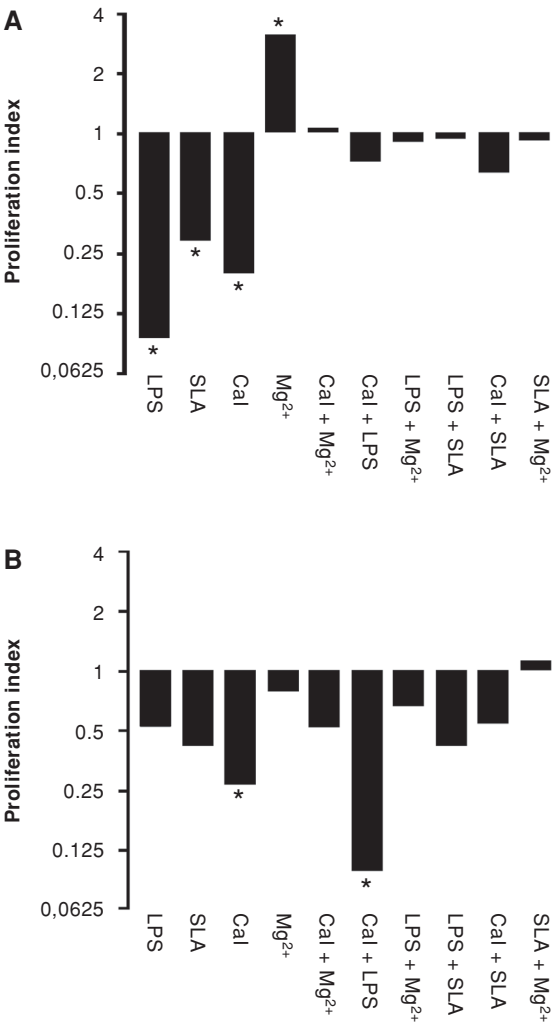


**Figure 3. Proliferation index of *L. major* in activated macrophages**. Inflammatory (A) and resident (B) macrophages were cultured in the presence or absence of treatments: LPS, SLA, CaI, $Mg^{2+}$, CaI+$Mg^{2+}$, CaI+LPS, LPS+$Mg^{2+}$, LPS+SLA, CaI+SLA and SLA+$Mg^{2+}$. After incubation for 48 hours followed infection by *L. major* during 3 hours, $^{3}$[H] thymidine was added. Cells were harvested after 18 hours and counted. The results were expressed as the average proliferation rate with each treatment/ the average proliferation without treatment (proliferation index). Values over 1 indicate increasing intracellular growth of parasites. These data are representative from three other independent experiments. (*) $p<0.05$.

DISCUSSION

Macrophage activation under different treatments was investigated to analyze the possible increase of the microbicidal capacity. In this experiment, inflammatory macrophages treated with CaI showed no significant NO production but parasite proliferation was decreased. While nitric oxide has been identified as a key molecule for leishmanicidal function of macrophages (Murray and Nathan, 1999), the data from the present study indicate that other pathways besides NO release are important for parasite clearance.

The microbicidal activity could be restored by excess $Ca^{2+}$ rather than $Mg^{2+}$, which suggests that changes in the concentration of cytosolic $Ca^{2+}$ are sufficient to mediate the molecular events that lead to acquisition of

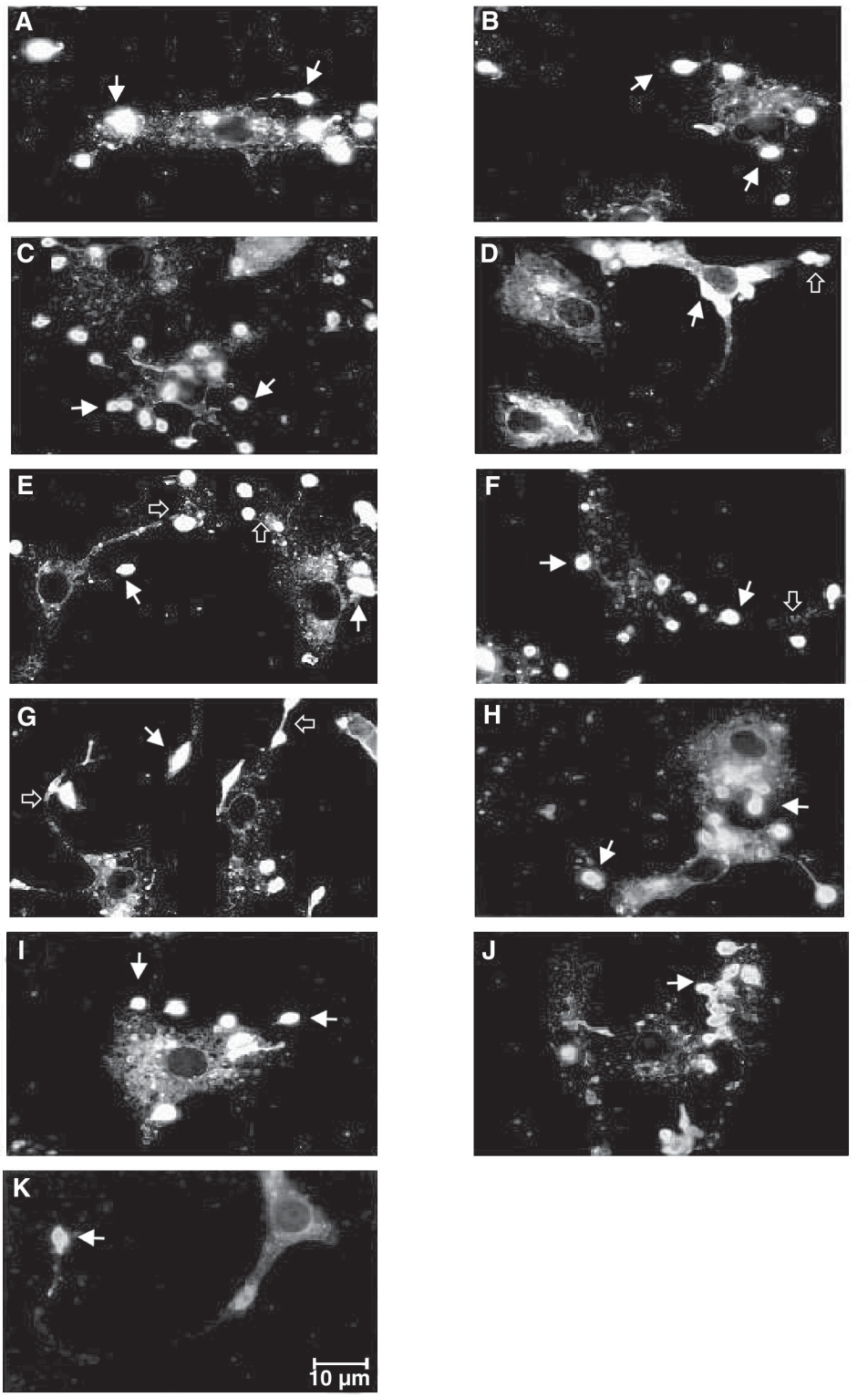


**Figure 4. Immunofluorescence of intracellular amastigotes in resident macrophages.** The resident macrophages were cultured in the presence of the medium (A), LPS (B), SLA (C), CaI (D), $Mg^{2+}$ (E), CaI+$Mg^{2+}$ (F), CaI+LPS (G), LPS+$Mg^{2+}$ (H), LPS+SLA (I), CaI+SLA (J), SLA+$Mg^{2+}$ (K) during 48 hours. After infection by *L. major* for 3 hours an immunofluorescence assay was performed. (—) amastigotes. (⇒) cytoplasm extensions. Bar, 10 µm.

microbicidal potential (Buchmüller-Rouiller and Mauël, 1991). It has been demonstrated that an increase in intracellular $Ca^{2+}$ caused a decrease in the viability of *Mycobacterium tuberculosis* (Malik et al., 2001). Calcium also has been shown to be an effective macrophage activator, favoring the fusion between the parasitophorous vacuoles with lysosomes (Sibley et al., 1991). On the other hand, in the present study scant release of NO was observed in cells treated with $Mg^{2+}$ in accordance with another study which suggested that $Mg^{2+}$ deficiency enhances NO production via iNos by alveolar macrophages isolated from rats (Yokoyama et al., 2003). In addition, it has been demonstrated that $Mg^{2+}$-dependent ecto-ATPase activity in *Leishmania tropica* may play a crucial role in enabling the parasite to avoid the microbicidal activity of macrophages (Meyer-Fernandes et al., 1997).

Resident macrophages did not up-regulate their NO production in response to any stimuli applied, though these macrophages were able to inhibit the proliferation of *L. major* when pre-treated with either LPS, SLA, CaI or CaI+SLA, suggesting that in resident macrophages microbicidal activity through nitrogen reactive species may not play a pivotal role in murine leishmaniasis control. A study comparing resident and inflammatory peritoneal macrophages demonstrated that regulation of phagocytosis by genetically-determined mechanisms is different between those two types of cells (Reichner et al., 2001).

In the present study a significant increase in NO release and a greater ability to inhibit *L. major* proliferation by inflammatory macrophages treated with LPS or SLA alone or in combination were observed. Therefore, treatment of both resident and inflammatory macrophages leads to inhibition of parasite proliferation, suggesting meditated microbicidal activities by LPS. Some authors have attributed microbicidal activities to LPS alone (Cunha et al., 1993) or interferon gama (IFN-γ) and LPS together not only for *L. major* but also for other intracellular parasites, as well (Sibley et al., 1991, Tsang et al., 2000). It has been demonstrated that $Ca^{2+}$ is involved in cell signaling induced by LPS, which regulates NO production by murine RAW 264.7 macrophages, as was observed in the present study when inflammatory macrophages were activated by CaI+LPS (Vivancos and Moreno, 2002).

In macrophages treated with LPS alone or in combination, cell spreading over the dish was observed. This is in keeping with other data demonstrating that cells treated with LPS presented more actin filaments in the periphery, which characterizes the macrophages activation (Chakravortty and Nanda Kumar, 2000). Inflammatory macrophages treated with CaI+LPS, CaI+$Mg^{2+}$ and LPS+SLA (data not shown), as well as resident macrophages treated with CaI+$Mg^{2+}$ and CaI+LPS and J774 cells treated with CaI, SLA and LPS+SLA (data not shown) presented greater number of cytoplasmic elongations, suggesting that calcium ionophore and LPS significantly influence cytoskeleton reorganization. Recently, it was demonstrated that J774 cells activated *in vitro* with CaI increased myosin expression and NO production and also presented greater number of cytoplasmic elongations suggesting the importance of motor molecules on effect functions of these cells (Reis et al., 2001).

The results of this study demonstrated the importance of calcium in decreasing *L. major* proliferation inside murine macrophages. However, $Mg^{2+}$ seemed to increase parasite proliferation as observed in inflammatory macrophages, suggesting that *L. major* may use a $Mg^{2+}$dependent pathway to proliferate and evade microbicidal activity.

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