



## The influence of ecto-nucleotidases on *Leishmania amazonensis* infection and immune response in C57B/6 mice

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### ABSTRACT

Previous results from our laboratory and from the literature have implicated the expression of ecto-nucleotidases in the establishment of *Leishmania* infection. In the present study we evaluated the correlation between ecto-nucleotidasic activity and the infectivity of *L. amazonensis* promastigotes that were kept in culture for short or extended numbers of passages, a condition that is known to decrease parasite infectivity. We also analyzed the immune response associated with the infection by these parasites. As expected, we found that long-term cultured parasites induce the development of smaller lesions than the short-term cultured counterparts. Interestingly, long-term cultured parasites presented reduced ecto-nucleotidasic activity. In addition, cells recovered from animals infected with long-term cultured parasites produced higher amounts of IFN- $\gamma$  and have smaller parasite load, after 8 weeks of infection. Furthermore, after 1 week of infection, there is increased expression of the chemokine CCL2 mRNA in animals infected with short-term cultured parasites. Finally, infection of peritoneal macrophages by these parasites also shows marked differences. Thus, while short-term cultured parasites are able to infect a greater proportion of macrophages, cells infected by long-term cultured parasites express higher amounts of CXCL10 mRNA, which may activate these cells to kill the parasites. We suggest that the enzymes involved in metabolism of extracellular nucleotides may have an important role in infection by *L. amazonensis*, by acting directly in its adhesion to target cells and by modulating host cell chemokine production.

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### 1. Introduction

Parasites of the genus *Leishmania* are the causative agents of leishmaniasis, a group of diseases that affect 88 countries worldwide. Due to species differences in tissue tropism, virulence and their interaction with the host's immune system, infection by these parasites can result in a variety of clinical manifestations ranging from single self-healing ulcers to life threatening visceral dissemination (Alexander et al., 1999; Desjeux, 2004).

*Leishmania (Leishmania) amazonensis* is clinically important in the New World, where it can cause localized cutaneous

leishmaniasis or diffuse cutaneous leishmaniasis, especially in immunocompromised hosts (McMahon-Pratt and Alexander, 2004). Diffuse cutaneous leishmaniasis is characterized by the development of disseminated parasite laden nodules that is frequently associated to anergy to parasite's antigens (Convit et al., 1972). In the context of murine models, the majority of mouse strains is susceptible to infection by *L. amazonensis* (Pereira and Alves, 2008).

It is generally accepted that resistance to infection by *Leishmania* is associated with the development of a type 1 immune response, whereas disease progression is promoted by several proposed mechanisms, such as the development of a type 2 immune response, absence of type 1 immune response or co-existence of both responses (McMahon-Pratt and Alexander, 2004; Sacks and Noben-Trauth, 2002). Concerning the infection by *L. amazonensis*, it has been long known that, in C57BL/10 mice, the absence of a type 1 response is sufficient to cause parasite multiplication and lesion development (Afonso and Scott, 1993).

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The study of the role of extracellular nucleotides and nucleosides as immunomodulatory molecules has been the focus of several laboratories over the last decade. It has been demonstrated that increased concentrations of extracellular ATP can be interpreted as “danger signals” that trigger an inflammatory response. On the other hand, adenosine production is generally associated with decreased inflammation and tissue remodeling (Coutinho-Silva et al., 2007; Di Virgilio, 2007).

Several enzymes are important to control the extracellular concentrations of nucleotides and their metabolic products (Bours et al., 2006). These enzymes also exist in prokaryotic and eukaryotic organisms (Komoszynski and Wojtczak, 1996) and have been identified in several protozoan parasites such as *Trypanosoma cruzi* (Bisaggio et al., 2003; Fietto et al., 2004), *Trypanosoma rangeli* (Fonseca et al., 2006), *Trypanosoma brucei* (Leite et al., 2007), *L. tropica* (Meyer-Fernandes et al., 1997), *L. amazonensis* (Berredo-Pinho et al., 2001), *Acanthamoeba* sp. (Sissons et al., 2004), *Trichomonas foetus* (Jesus et al., 2002) and *Balamuthia mandrillaris* (Matin and Khan, 2008). It has been shown that these parasites are able to hydrolyze extracellular ATP in order to produce adenosine (de Sa Pinheiro et al., 2008; Leite et al., 2007). This enzymatic activity is extremely important given the fact that these organisms do not present *de novo* pathways for synthesis of purine nucleotides and depend on a salvage pathway to acquire these molecules (Cohn and Gottlieb, 1997).

The enzymes ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), and 5'-nucleotidase sequentially hydrolyze extracellular ATP to adenosine (Robson et al., 2006). Many evidences support the pro-inflammatory effects of extracellular ATP, whereas adenosine has anti-inflammatory effects. By mimicking this enzymatic cascade, parasites could suppress the host immune response (Sansom et al., 2008). This supports the idea that enzymes involved in the extracellular metabolism of nucleotides can also act as virulence factors for parasites.

Previous studies from our group corroborate this hypothesis. In a comparative study with metacyclic promastigotes of *L. amazonensis*, *L. major* and *L. braziliensis*, a potential correlation between enzymatic activity and virulence *in vivo* was demonstrated (Marques-da-Silva et al., 2008). In the present study we further develop this hypothesis by evaluating the ecto-nucleotidase activity on parasites that were rendered less infective by multiple passages in culture as well as by analyzing the activity of non-infective clones derived from an infective isolate. Our results confirmed the positive correlation between increased ecto-nucleotidase activity and infectivity. In addition, we observed that parasites with high enzymatic activity presented enhanced infectivity to macrophages *in vitro* which was associated with decreased production of CXCL-10.

## 2. Materials and methods

### 2.1. Mice

Female C57BL/6 mice (4–8 weeks old) were obtained from the University's animal facility (Biotério Central - NUPEB/UFOP, Ouro Preto, Brazil). Animals were given water and food *ad libitum*. All procedures involving animals were cleared by the University's Ethical Committee in Animal Experimentation.

### 2.2. Parasites

*Leishmania (Leishmania) amazonensis*, PH8 strain (IFLA/BR/67/PH8) was cultured in Grace's insect medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Cripion, Andradina, SP,

Brazil), 2 mM L-glutamine (GIBCO BRL) and 100 U/ml penicillin G potassium (USB Corporation, Cleveland, OH, USA), pH 6.5, at 26 °C. Metacyclic promastigotes were obtained by gradient centrifugation of parasites at the late log phase of culture (day 5) over Ficoll® 400 (Amersham Biosciences do Brasil, São Paulo, SP, Brazil) (Marques-da-Silva et al., 2008) adapted from Späth and Beverley (2001). Parasites were sub-cultured every 2 or 3 days at  $1 \times 10^5$  parasites/ml.

Clones from the PH8 strain of *L. amazonensis* were obtained by plating in solid medium as described previously (Gomes et al., 1991; Tanuri et al., 1985) except for the modification of the culture medium (1% agar in alpha-MEM). Each cloned population was re-cloned three times.

### 2.3. Infection

C57BL/6 mice were inoculated in the left hind footpad with  $1.0 \times 10^5$  metacyclic promastigotes and lesion development was followed weekly with a dial micrometer (model 1015MA; L.S. Starret Co., Itu, SP, Brazil). The results were expressed as the difference between measures of infected and contra lateral non-infected footpad (Afonso and Scott, 1993).

### 2.4. Antigen preparation

*Leishmania* antigens were obtained from logarithmic phase cultures of promastigotes, which were washed twice in PBS. The pellets obtained were submitted to seven cycles of freezing in liquid nitrogen followed by thawing at 37 °C. The preparations were observed under microscope for the presence of intact parasites (Afonso and Scott, 1993). Protein content of preparations was determined by the Lowry method (Lowry et al., 1951) and adjusted to 1 mg/ml. Antigen preparation was aliquoted and stored frozen at –70 °C and thawed immediately before the use.

### 2.5. Parasite load estimation

The number of parasites in the footpad was estimated by a limiting dilution assay (Afonso and Scott, 1993). Mice were sacrificed and the whole lesion was removed and ground in Grace's insect medium, pH 6.5, in a glass tissue grinder. Tissue debris was removed by centrifugation at  $50 \times g$  at 4 °C/1 min, and supernatant was transferred to another tube and centrifuged at  $1540 \times g$  at 4 °C/15 min. The pellet was resuspended in 0.5 ml Grace's insect medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and 100 U/ml penicillin G potassium, pH 6.5. The parasite suspension was, then, serially diluted in duplicates in a final volume of 200  $\mu$ l in 96-well plates. Pipette tips were replaced for each dilution. Plates were incubated for 15 days at 26 °C and examined under an inverted microscope for the presence of parasites. Results were expressed as –log of the parasite titer corresponding to the last dilution in which parasites were detected.

### 2.6. Analysis of cytokine production

Single-cell suspensions were prepared from the lymph nodes of mice infected for 3 weeks. Cells were adjusted to a concentration of  $5 \times 10^6$  cells/ml in Dulbecco's minimal essential medium (GIBCO BRL) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin G potassium, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; SIGMA) and 50 mM 2-mercaptoethanol (Pharmacia Biotech AB, Uppsala, Sweden), and were plated at 0.5 ml/well in 48-well tissue culture plates. Cells were stimulated with parasite antigen at 50  $\mu$ g/ml. TNF was evaluated by bioassay using WEHI cells (Lattime et al., 1988)

in supernatants collected after 24 h incubation. IFN- $\gamma$ , IL-4 (Souza-Neto et al., 2004) and IL-10 (PeproTech Inc., Rock Hill, NJ, USA) levels were measured by ELISA in 72 h supernatants.

### 2.7. Enzymatic activity measurements

ATPase, ADPase and 5'-nucleotidase activities were measured by incubation of intact parasites for 1 h at 30 °C in a mixture containing 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 5 mM MgCl<sub>2</sub>, and 50 mM Hepes-Tris buffer, in the presence of ATP, ADP or AMP (SIGMA) 5 mM (Bisaggio et al., 2003). The reaction was started by the addition of living promastigotes and terminated by the addition of ice cold HCl 0.2 M (Fietto et al., 2004). Nonspecific hydrolysis was determined by adding the parasites after the reaction was stopped. The suspensions were pelleted and aliquots of supernatant were used for the measurement of released inorganic phosphate (Pi) as previously described (Taussky and Shorr, 1953). Enzymatic activities were expressed as nmol of Pi release induced by  $1.0 \times 10^8$  parasites in 1 h.

### 2.8. Infection of peritoneal macrophages

Peritoneal macrophages were obtained as Zhang et al. (2008), with few modifications. C57BL/6 mice were inoculated, in the peritoneum, with a solution of thioglycolate 3% (Biobrás, Montes Claros, MG, Brazil). After 5 days, peritoneal cells were harvested and plated at  $1 \times 10^6$  cells/ml onto round coverslips in supplemented DMEM in 24-well plates. Cells were incubated for 90 min at 37 °C, 5.0% CO<sub>2</sub>. Non-adherent cells were removed by washing with warm PBS. Parasites were added to the culture at a 5:1 parasite to cell ratio. After 3 h co-culture, cells were washed with PBS to remove non-internalized parasites and fresh medium was added to the cultures. After 72 h of infection, samples were harvested for RNA extraction and for the assessment of cellular parasitism. Coverslips were fixed in methanol for 10 min (Vetec Fine Chemistry), dried and stained using the kit Panótico Rápido (Laborclin, Pinhais, PR, Brazil), according to manufacturer's instructions. The analysis was performed using an Olympus BX50 optical microscope (Olympus, Center Valley, PA, USA). The number of infected and uninfected cells and the number of parasites present in infected cells were determined. A minimum of 200 macrophages per coverslip was evaluated.

TNF (Lattime et al., 1988), IL-12 (Vieira et al., 1994) and IL-10 (PeproTech, Inc.) were measured in 24 h supernatants.

### 2.9. Chemokine analysis

RNA extraction was performed using RNeasy Total RNA Isolation System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Samples were treated with RQ1 DNase (Promega Corporation) and the reverse transcription was performed using reverse transcriptase ImProm-II Reverse Transcriptase (Promega Corporation) according to manufacturer's instructions. The complementary DNA (cDNA) obtained was subjected to amplification by PCR using Taq DNA polymerase from Phoeutria (Belo Horizonte, MG, Brazil). The primers (listed as 5' to 3') and conditions used for amplification were as follows: HPRT: forward (FOR): GTTGATACAGGCCAGACTTTGTT, reverse (REV): GATTCAACTTGGCTCATCTTAGGC, 58 °C; CCL2: FOR: CACTCACCTGCTGCTACTCATTCA, REV: GGATTCACAGAGAGGGAAAAATGG, 62 °C; CCL5: FOR: CCACGTCAAGGAGTATTTCTACACC, REV: CTGTTTCTTGGGTTGCTGTGG, 54 °C; CXCL10: FOR: TGAGCAGA-GATGTCTGAATC, REV: TCGCACCCTCACATAGCTTACAG, 62 °C. The products were submitted to electrophoresis on acrylamide gels and stained with silver nitrate (Righetti, 2005). The densitometric quan-

tification of bands was performed with the software Quantity One 4.5.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### 2.10. Regulatory T cell analysis

The commercial kit Mouse Regulatory T Cell Staining Kit (eBioscience, San Diego, CA, USA) was used for the detection of regulatory T cells. C57BL/6 mice were inoculated in both footpads, with  $1 \times 10^5$  metacyclic promastigotes of *L. amazonensis*, as described earlier. After 1 or 4 weeks, the cells from their popliteal lymph nodes were harvested and stained according to manufacturer's instructions. Cells were analyzed on FACScalibur (BD Biosciences, San Jose, CA, USA) and the software CellQuest Pro (BD Biosciences).

### 2.11. Statistical analysis

Statistical analysis was performed by Student's *t* test.  $p < 0.05$  was considered statistically significant.

## 3. Results

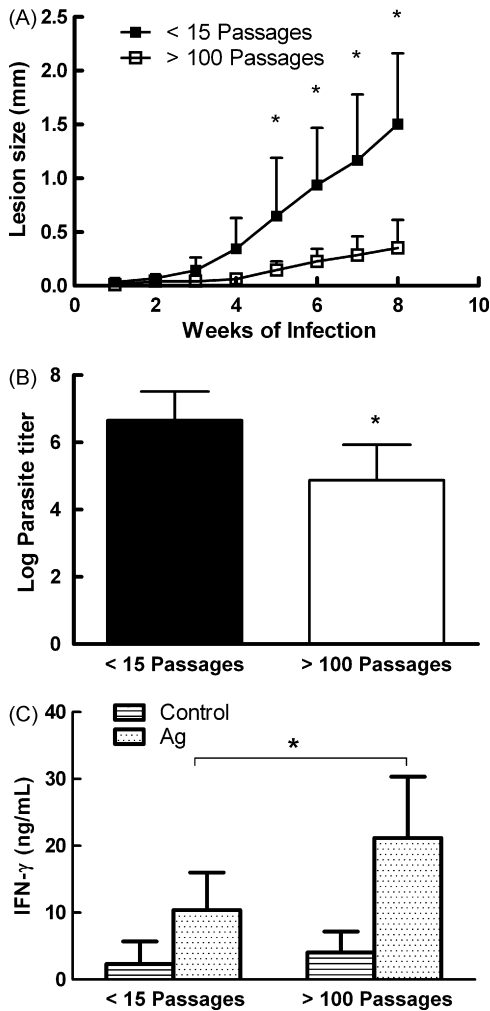
### 3.1. Maintenance of parasites in culture decreases infectivity

It has been generally accepted that maintenance of *Leishmania* parasites in culture decreases the infectivity of the culture. To avoid loss of infectivity parasite strains are regularly re-isolated from infected hamsters or mice. In order to evaluate the effect of long-term culture on the infectivity of *L. amazonensis* promastigotes, parasites were kept for over 100 passages in culture. Long-term maintenance of parasites in culture decreased by 50% the yield of metacyclic promastigotes after 5 days of culture (data not shown). To compensate for this discrepancy, mice were inoculated with metacyclic promastigotes and lesion development followed over the course of 8 weeks. As shown in Fig. 1, mice inoculated with parasites maintained in culture for less than 15 passages develop larger lesions than those infected with long-term culture parasites. This difference in lesion development is also associated with a 100-fold decrease in parasitism in the footpad and increased IFN- $\gamma$  production by stimulated lymph node cells. IL-4 production was not detected in significant levels as previously shown (Afonso and Scott, 1993; Souza-Neto et al., 2004).

### 3.2. Infectivity of *L. amazonensis* is associated to the level of promastigotes ecto-nucleotidase activity

Previous studies raised the idea that ecto-nucleotidase activity could be related with parasite virulence in vivo. To address this, we determined the enzymatic activity of short- or long-term cultured parasites. As shown in Fig. 2A, long-term maintenance of parasites in culture led to a significant decrease in the activity of metacyclic promastigotes over extracellular ATP and ADP. This result reinforces the hypothesis that ecto-nucleotidase, in particular, ecto-NTPDase expression in *Leishmania* promastigotes is a virulence factor for these parasites.

To further corroborate the correlation between ecto-nucleotidase activity and infectivity in *L. amazonensis* infection, we added adenosine to the culture medium of short-term cultured parasites. This treatment led to a decreased activity of metacyclic promastigotes over extracellular nucleotides, especially over ATP (Fig. 2B). This observation expands on the results from Berredo-Pinho et al. (2001) where a decreased ATPase activity was demonstrated in parasites grown in the presence of added adenosine. Given the proposed role of ecto-nucleotidases in obtaining purine bases, the increased availability of adenosine in the culture media may have



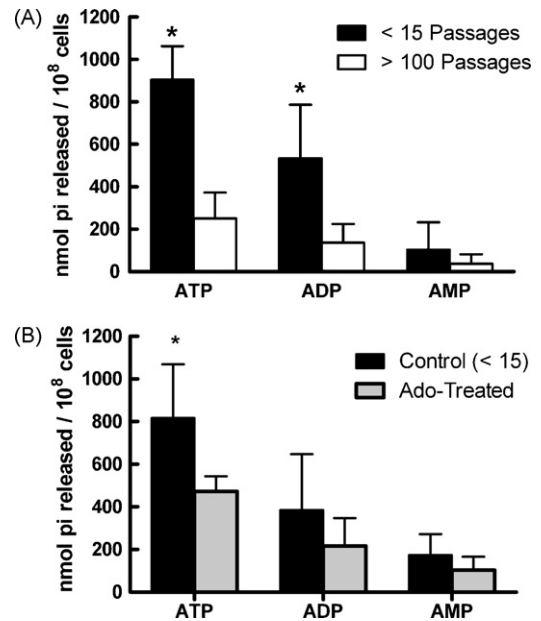
**Fig. 1.** Long-term culture decreases the infectivity of *L. amazonensis* promastigotes. C57BL/6J mice were inoculated in the left hind footpad with  $1 \times 10^5$  *L. amazonensis* metacyclic promastigotes obtained from parasites that were kept in culture for few (<15) or many (>100) passages. Lesion development was followed weekly (A). Tissue parasitism was evaluated by limiting dilution at the 8th week of infection (B). IFN- $\gamma$  production by draining lymph node cells from mice infected for 8 weeks. Cells were stimulated for 72 h with particulate Ag (C). Results are expressed as mean + 1 SD of three independent experiments with four animals per group. \* $p < 0.05$ .

contributed to a down modulation of the enzymatic activity.

In addition, animals inoculated with adenosine-treated parasites developed smaller lesions and presented a 10-fold decrease in tissue parasitism (Fig. 3A and B). Interestingly, this decreased infectivity of adenosine-treated parasites was also correlated with an increased IFN- $\gamma$  production by antigen-stimulated lymph node cells from infected mice (Fig. 3C).

Parasite isolates are considered a mixture of several subpopulations of the microorganism. To evaluate the possibility that subpopulations of parasites might display different levels of ecto-nucleotidasic activity we took advantage of the existence in our laboratory of clones of the PH8 *L. amazonensis* strain. These clones were obtained several years prior to the present study and have always shown decreased infectiveness in the mouse model (data not shown).

Mice inoculated with metacyclic promastigotes from each of the two clones developed only small lesions that contained fewer parasites than those from animals inoculated with the parental strain (Fig. 4A and B). Interestingly, the ability of these two clones to hydrolyze extracellular ATP was significantly reduced as compared



**Fig. 2.** Ecto-nucleotidasic activity of *L. amazonensis* metacyclic promastigotes. (A) Metacyclic promastigotes were isolated on the 5th day of culture and incubated with each nucleotide for 1 h at 30 °C. Enzymatic activity was evaluated by the measurement of inorganic phosphate released. (B) Adenosine treatment decreases ecto-nucleotidasic activity of metacyclic promastigotes. Adenosine (5 mM) was added to culture medium of short-term (<15 passages) cultured *L. amazonensis* promastigotes. Metacyclic promastigotes were isolated on the 5th day of culture and incubated with each nucleotide for 1 h at 30 °C. Enzymatic activity was evaluated by the measurement of inorganic phosphate released. Results are expressed as mean + 1 SD of four (A) or five (B) independent experiments. \* $p < 0.05$  between short- and long-term cultured parasites (A) or between control and treated groups (B).

to the original strain while AMP hydrolysis was increased (Fig. 4C). These results further corroborate our hypothesis that lesion development and parasite multiplication within the host are associated with the level of ecto-NTPDase activity by the parasite.

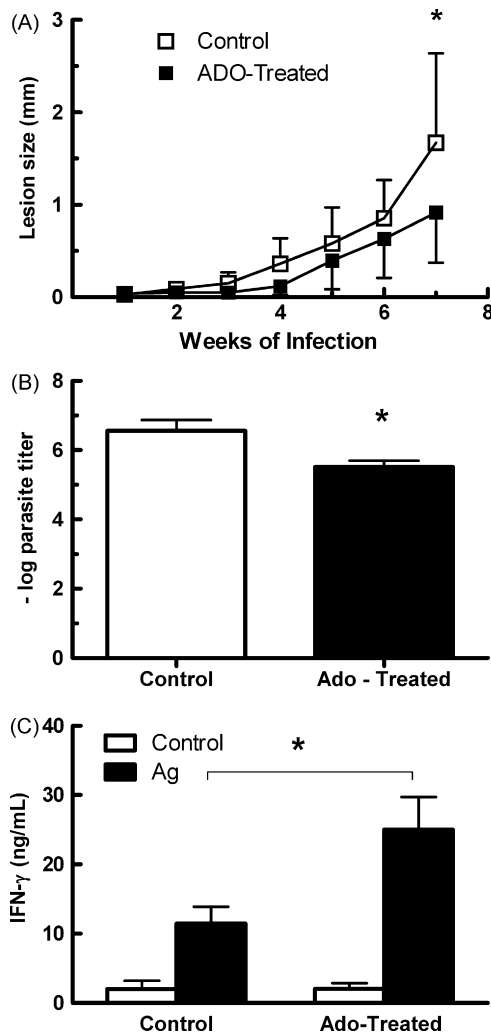
### 3.3. Alterations in immune response

#### 3.3.1. In vivo experiments

To investigate the mechanisms by which the decreased expression of ecto-NTPDase activity contributes to lesion development in the host, mice were inoculated with short- or long-term cultured parasites and the presence of regulatory T cells as well as cytokine and chemokine production was evaluated. In this regard, evaluation of IL-10, TNF and IL-12 production by stimulated cells from the draining lymph nodes also did not reveal differences between the two groups (data not shown).

Regulatory T cells ( $CD4^+CD25^+Foxp3^+$ ) modulate the response of the host during *Leishmania* infection (Belkaid et al., 2002). In the case of *L. amazonensis* infection it has been demonstrated that these cells rapidly disappear from the lesion site thus allowing for lesion development (Ji et al., 2005). Analysis of the presence of  $CD4^+CD25^+Foxp3^+$  at the draining lymph node from infected animals (Fig. 5A) demonstrated that even though a decrease in the percentage of these cells from 1 to 4 weeks of infection was observed, no differences were detected between the two groups, suggesting that differential regulation of the response by Treg cells is not the cause for the distinct lesion development in these animals.

Given the fact that lesion development in *Leishmania* infection is predominantly a result of cellular infiltration, rather than parasite proliferation (Belkaid et al., 2000), we evaluated chemokine production at the lesion site 1 week after infection. Our results show that while no differences were detected for the production of CCL5 and CXCL-10, lesions from animals infected with long-term cul-

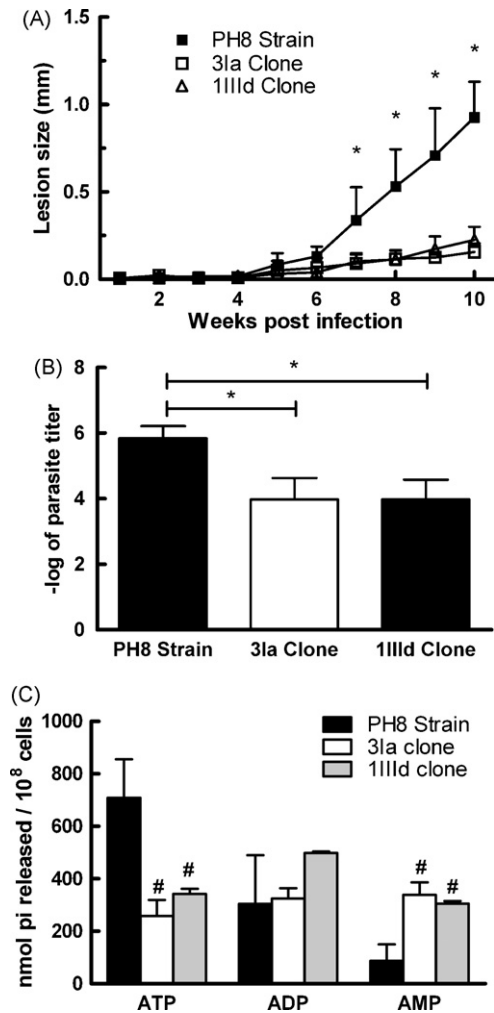


**Fig. 3.** Adenosine treatment decreases the infectivity of *L. amazonensis* promastigotes. C57BL/6J mice were inoculated in the left hind footpad with  $1 \times 10^5$  *L. amazonensis* metacyclic promastigotes obtained from short-term cultured parasites treated or not with 5 mM of adenosine in the culture medium. Lesion development was followed weekly (A). Tissue parasitism was evaluated by limiting dilution at the 8th week of infection (B). IFN- $\gamma$  production by draining lymph node cells from mice infected for 8 weeks. Cells were stimulated for 72 h with particulate Ag (C). Results are expressed as mean + 1 SD of two independent experiments with four or five animals per group. \* $p < 0.05$  between treated and control groups.

tured parasites presented reduced levels of CCL2 as compared to lesions from animals infected with short-term cultured parasites (Fig. 5B).

### 3.3.2. In vitro experiments

To further characterize the interactions between the parasites and the host, we evaluated the infectivity and cytokine production by infected thioglycolate-elicited peritoneal macrophages. A different pattern of infection was observed between short- and long-term cultured parasites. A decreased number of macrophages was found associated with parasites in cultures incubated with long-term cultured promastigotes early after incubation (Fig. 6A–3 h). Furthermore, after 72 h of incubation, the percentage of infected cells was reduced in these cultures, a fact not observed in cells incubated with short-term cultured parasites (Fig. 6A–72 h). Interestingly, the number of parasites per infected cell was similar in both groups at all times (Fig. 6B). These results indicate that short-term cultured promastigotes are capable of not only interacting and infecting a larger number of cells but also, somehow, these



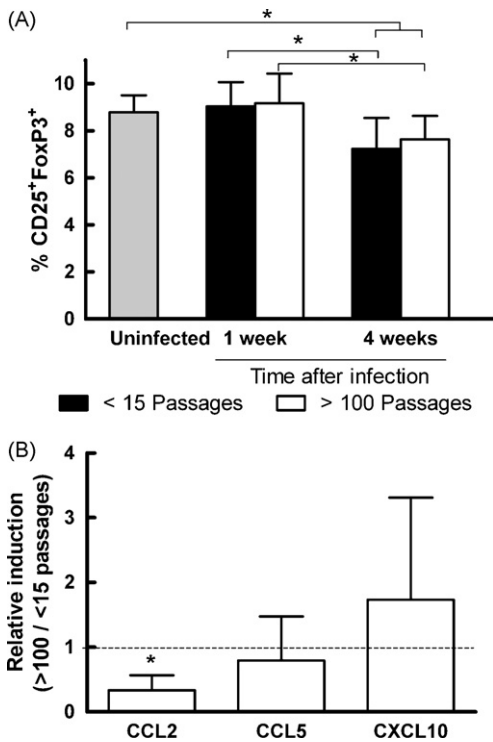
**Fig. 4.** Avirulent clones of *L. amazonensis* PH8 strain show decreased parasitism and ecto-NTPDase activity. (A) C57BL/6J mice were inoculated in the left hind footpad with  $1 \times 10^5$  *L. amazonensis* metacyclic promastigotes obtained from short-term cultured PH8 strain or its clones. Lesion development was followed weekly. (B) Tissue parasitism was evaluated by limiting dilution at the 10th week of infection. Results are expressed as mean + 1 SD of two independent experiments with five animals per group. \* $p < 0.05$  between treated and control groups. (C) Ecto-nucleotidase activity of metacyclic promastigotes was evaluated as described in Fig. 2A. Results represent mean + 1 SD for 3 (PH8) or 2 (clones) independent measurements in triplicate. # $p < 0.05$  as compared to the parental strain (PH8).

parasites interfere with the host cells and inhibit their microbicidal mechanisms.

While we did not observe differences in the production of IL-10 or TNF by infected macrophages (data not shown), analysis of chemokine production by these cells demonstrated a significant increase in CXCL-10 message levels by peritoneal cells infected with long-term cultured promastigotes (Fig. 6C). These in vitro experiments clearly show that not only short-term cultured promastigotes are able to more efficiently infect macrophages but also are less prone to activate this cell, favoring parasite establishment within the host cell.

## 4. Discussion

In this study we observed that long-term maintenance of *L. amazonensis* promastigotes in axenic culture results in virulence loss. These results corroborate previous findings that report the effect of long-term culture on the virulence of *Leishmania* promastigotes (Berredo-Pinho et al., 2001; Cysne-Finkelstein et al., 1998; da Silva



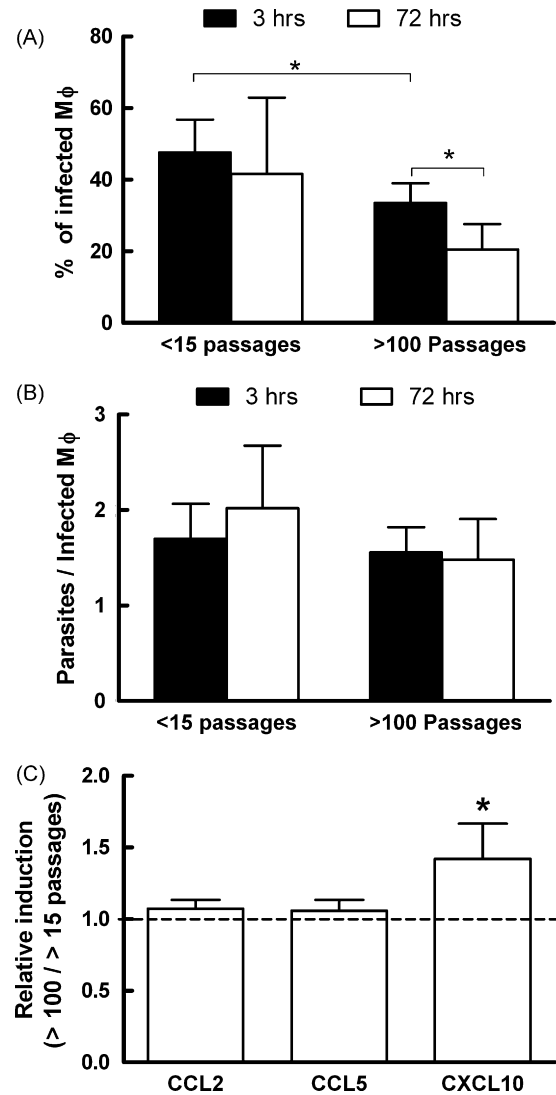
**Fig. 5.** Regulatory T cells and chemokine production in vivo. (A) The presence of regulatory T cells was evaluated at the draining lymph node 1 and 4 weeks after infection with short- and long-term cultured parasites. (B) Relative levels of message for different chemokines at 1 week after infection. Message levels of each chemokine were evaluated relatively to the housekeeping gene – HPRT and the ratio between animals infected with long- and short-term cultured parasites calculated. \* $p < 0.05$  as compared to an equal level of message between the two groups.

and Sacks, 1987; Peres-Sampaio et al., 2008). One of the possible reasons for the decreased infectivity of parasites maintained for long periods in culture would be the decreased number of metacyclic parasites found in these cultures. Although we have also observed a decrease of almost 50% in the number of metacyclic promastigotes in our long-term cultures, this result cannot explain the differences in infectivity observed in our study given the fact that animals were inoculated with purified metacyclic promastigotes. Thus, we decided to evaluate possible causes for the altered infectivity of long-term cultured *L. amazonensis*.

We, initially, investigated nucleotide hydrolysis by these parasites, in order to verify the role of these hydrolytic enzymes in parasite virulence. We found that long-term cultured parasites were less virulent in vivo and showed a reduced hydrolytic activity over ATP and ADP (Fig. 2A). Importantly, when adenosine was added to the culture media, parasites showed a reduction in their ability to hydrolyze nucleotides and became less virulent in vivo (Fig. 2B and Fig. 3). The same relationship was found with distinct clones of parasites (Fig. 4), showing a straight correlation between parasite virulence in the mouse model and hydrolytic activity over nucleotides.

These data corroborate earlier data from our laboratory (Maioli et al., 2004; Marques-da-Silva et al., 2008) and reinforce the hypothesis that the enzymes involved in the extracellular metabolism of nucleotides are important virulence factors for *Leishmania* (Berredo-Pinho et al., 2001; Pinheiro et al., 2006) and *T. cruzi* (Santos et al., 2009).

Next, we evaluated the immune response developed by mice infected with short- or long-term cultured parasites. A reduction in the population of regulatory T cells in the lymph nodes of infected mice from 1 to 4 weeks of infection was observed (Fig. 5A), which is consistent with the increase in lesion development and



**Fig. 6.** Long-term culture reduces infectivity of *Leishmania* parasites to peritoneal macrophages. Thyoglycolate-elicited peritoneal macrophages were infected with metacyclic *L. amazonensis* promastigotes (5 parasites/cell) for 3 or 72 h. (A) Percentage of infected cells \* $p < 0.05$  Student's *t* test as indicated. (B) Number of associated parasites per infected macrophage. (C) Relative levels of message for different chemokines. Message levels of each chemokine were evaluated relatively to the housekeeping gene – HPRT and the ratio between cultures infected with long- and short-term cultured parasites calculated. \* $p < 0.05$  as compared to an equal level of message between the two cultures.

corroborates previous findings by Ji et al. (2005). No differences, however, were observed between animals infected with long- or short-term cultured parasites. On the other hand, we observed that the chemokine CCL2 was preferentially induced by more virulent parasites (Fig. 5B) indicating that this chemokine could be involved in animal susceptibility, in accordance with previous studies that show the involvement of this chemokine with lesion development in *Leishmania* infection (Gu et al., 2000; Santiago et al., 2004; Teixeira et al., 2005).

Finally, we evaluated the infection of peritoneal macrophages by short- or long-term cultured parasites, in order to identify differences in the immune response developed early after *L. amazonensis* internalization. Our results show that peritoneal macrophages were more permissive to infection by short-term cultured parasites, as early as 3 h of co-culture (Fig. 6A). This behavior was also noted 72 h post-infection. This observation is in agreement with the hypothesis that ecto-NTPDases act as adhesion molecules that

facilitate parasite infection (Berredo-Pinho et al., 2001; Pinheiro et al., 2006; Santos et al., 2009).

When the production of chemokines by infected peritoneal macrophages was evaluated, a higher production of CXCL10 by cells infected with long-term cultured parasites was observed (Fig. 6C). It has already been demonstrated that CXCL10 is important in the generation and migration of effector T cells (Dufour et al., 2002). In regard to leishmaniasis, a previous study indicates that the susceptibility of BALB/c mice to *L. major* infection could be related to low expression of the receptor for CXCL10 by activated cells from these animals in comparison to C57BL/6 mice (Barbi et al., 2008). Furthermore, treatment of bone marrow derived macrophages with CXCL10 before infection by *L. amazonensis* resulted in a smaller cellular parasitism (Vasquez and Soong, 2006). In agreement with these data, treatment with CXCL10 enhances the production of IL-12p40 by dendritic cells infected with *L. amazonensis* (Vasquez et al., 2008).

In view of the results described here, we propose that the reduction in extracellular ATP levels and consequent increase in adenosine production by virulent parasites would decrease the ability of the host to mount a protective IFN- $\gamma$  response. Furthermore, these parasites may also bind to phagocytes more easily, due to their higher expression of ecto-NTPDase. In addition, they would stimulate CCL2 synthesis by the host cell, resulting in an increased recruitment of cells, especially monocytes, to the infection site. In an environment with decreased IFN- $\gamma$  production, these recruited monocytes would be more suitable to parasite proliferation. On the other hand, in the presence of less virulent parasites, the increased availability of extracellular ATP would favor the differentiation of IFN- $\gamma$ -producing T cells by activated DC thus increasing the host ability to control the infection. Also, due to the decreased expression of ecto-NTPDase, their adhesion and further uptake by macrophages would be decreased. Furthermore, cells infected by long-term cultured parasites synthesize CXCL10, resulting in phagocyte activation and parasite control.

Taken together, our results support the hypothesis that ectoenzymes involved in nucleotide metabolism may be considered virulence factors to *Leishmania* parasites, at least in the mouse model. The correlation of the expression of these enzymes with the clinical manifestation of the disease in leishmaniasis patients is currently under investigation in our laboratory.

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## References

- Afonso, L.C.C., Scott, P., 1993. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infect. Immun.* 61, 2952–2959.
- Alexander, J., Satoskar, A.R., Russell, D.G., 1999. *Leishmania* species: models of intracellular parasitism. *J. Cell Sci.* 112, 2993–3002.
- Barbi, J., Brombacher, F., Satoskar, A.R., 2008. T cells from *Leishmania* major-susceptible BALB/c mice have a defect in efficiently up-regulating CXCR3 upon activation. *J. Immunol.* 181, 4613–4620.
- Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., Sacks, D., 2000. A natural model of *Leishmania* major infection reveals a prolonged “silent” phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J. Immunol.* 165, 969–977.
- Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M., Sacks, D.L., 2002. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells control *Leishmania* major persistence and immunity. *Nature* 420, 502–507.
- Berredo-Pinho, M., Peres-Sampaio, C.E., Chrispim, P.P., Belmont-Firpo, R., Lemos, A.P., Martiny, A., Vannier-Santos, M.A., Meyer-Fernandes, J.R., 2001. A Mg-dependent ecto-ATPase in *Leishmania amazonensis* and its possible role in adenosine acquisition and virulence. *Arch. Biochem. Biophys.* 391, 16–24.
- Bisaggio, D.F., Peres-Sampaio, C.E., Meyer-Fernandes, J.R., Souto-Padron, T., 2003. Ecto-ATPase activity on the surface of *Trypanosoma cruzi* and its possible role in the parasite–host cell interaction. *Parasitol. Res.* 91, 273–282.
- Bours, M.J., Swennen, E.L., Di Virgilio, F., Cronstein, B.N., Dagnelie, P.C., 2006. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol. Ther.* 112, 358–404.
- Cohn, C.S., Gottlieb, M., 1997. The acquisition of purines by trypanosomatids. *Parasitol. Today* 13, 231–235.
- Convit, J., Pinardi, M.E., Rondón, A.J., 1972. Diffuse cutaneous leishmaniasis: a disease due to an immunological defect in the host. *Trans. R. Soc. Trop. Med. Hyg.* 66, 603–610.
- Coutinho-Silva, R., da Cruz, C.M., Persechini, P.M., Ojcius, D.M., 2007. The role of P2 receptors in controlling infections by intracellular pathogens. *Purinergic Signal* 3, 83–90.
- Cysne-Finkelstein, L., Temporal, R.M., Alves, F.A., Leon, L.L., 1998. *Leishmania amazonensis*: long-term cultivation of axenic amastigotes is associated to metacyclogenesis of promastigotes. *Exp. Parasitol.* 89, 58–62.
- da Silva, R., Sacks, D.L., 1987. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infect. Immun.* 55, 2802–2806.
- de Sa Pinheiro, A.A., Cosentino-Gomes, D., Lanfredi-Rangel, A., Ferraro, R.B., De, S.W., Meyer-Fernandes, J.R., 2008. Giardia lamblia: biochemical characterization of an ecto-ATPase activity. *Exp. Parasitol.* 119, 279–284.
- Desjeux, P., 2004. Leishmaniasis. *Nat. Rev. Microbiol.* 2, 692.
- Di Virgilio, F., 2007. Purinergic signalling in the immune system. A brief update. *Purinergic Signal* 3, 1–3.
- Dufour, J.H., Dziejman, M., Liu, M.T., Leung, J.H., Lane, T.E., Luster, A.D., 2002. IFN- $\gamma$ -inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J. Immunol.* 168, 3195–3204.
- Fietto, J.L., DeMarco, R., Nascimento, I.P., Castro, I.M., Carvalho, T.M., De Souza, W., Bahia, M.T., Alves, M.J., Verjovski-Almeida, S., 2004. Characterization and immunolocalization of an NTP diphosphohydrolase of *Trypanosoma cruzi*. *Biochem. Biophys. Res. Commun.* 316, 454–460.
- Fonseca, F.V., Fonseca De Souza, A.L., Mariano, A.C., Entringer, P.F., Gondim, K.C., Meyer-Fernandes, J.R., 2006. *Trypanosoma rangeli*: characterization of a Mg-dependent ecto-ATP-diphosphohydrolase activity. *Exp. Parasitol.* 112, 76–84.
- Gomes, M.L., Araujo, S.M., Chiari, E., 1991. *Trypanosoma cruzi*: growth of clones on solid medium using culture and blood forms. *Mem. Inst. Oswaldo Cruz* 86, 131–132.
- Gu, L., Tseng, S., Horner, R.M., Tam, C., Loda, M., Rollins, B.J., 2000. Control of Th2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 404, 407–411.
- Jesus, J.B., Lopes, A.H., Meyer-Fernandes, J.R., 2002. Characterization of an ecto-ATPase of *Trichomonas foetus*. *Vet. Parasitol.* 103, 29–42.
- Ji, J., Masterson, J., Sun, J., Soong, L., 2005. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells restrain pathogenic responses during *Leishmania amazonensis* infection. *J. Immunol.* 174, 7147–7153.
- Komoszynski, M., Wojtczak, A., 1996. Apyrases (ATP diphosphohydrolases, EC 3.6.1.5): function and relationship to ATPases. *Biochim. Biophys. Acta* 1310, 233–241.
- Lattime, S.C., Stopacciaro, A., Stutman, O., 1988. Limiting dilution analysis of TNF-producing cells in C3H/HeJ mice. *J. Immunol.* 141, 3422–3428.
- Leite, M.S., Thomaz, R., Fonseca, F.V., Panizzutti, R., Vercesi, A.E., Meyer-Fernandes, J.R., 2007. *Trypanosoma brucei brucei*: biochemical characterization of ecto-nucleoside triphosphate diphosphohydrolase activities. *Exp. Parasitol.* 115, 315–323.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Maioli, T.U., Takane, E., Arantes, R.M., Fietto, J.L., Afonso, L.C., 2004. Immune response induced by New World *Leishmania* species in C57BL/6 mice. *Parasitol. Res.* 94, 207–212.
- Marques-da-Silva, E.A., Oliveira, J.C., Figueiredo, A.B., Lima Jr., D.S., Carneiro, C.M., Fietto, J.L., Afonso, L.C., 2008. Extracellular nucleotide metabolism in *Leishmania*: Influence of adenosine in the establishment of infection. *Microbes Infect.* 10, 850–857.
- Matin, A., Khan, N.A., 2008. Demonstration and partial characterization of ecto-ATPase in *Balamuthia mandrillaris* and its possible role in the host–cell interactions. *Lett. Appl. Microbiol.* 47, 348–354.
- McMahon-Pratt, D., Alexander, J., 2004. Does the *Leishmania* major paradigm of pathogenesis and protection hold for New World cutaneous leishmaniasis or the visceral disease? *Immunol. Rev.* 201, 206–224.
- Meyer-Fernandes, J.R., Dutra, P.M., Rodrigues, C.O., Saad-Nehme, J., Lopes, A.H., 1997. Mg-dependent ecto-ATPase activity in *Leishmania tropica*. *Arch. Biochem. Biophys.* 341, 40–46.
- Pereira, B.A., Alves, C.R., 2008. Immunological characteristics of experimental murine infection with *Leishmania (Leishmania) amazonensis*. *Vet. Parasitol.* 158, 239–255.
- Peres-Sampaio, C.E., de Almeida-Amaral, E.E., Giarola, N.L., Meyer-Fernandes, J.R., 2008. *Leishmania amazonensis*: effects of heat shock on ecto-ATPase activity. *Exp. Parasitol.* 119, 135–143.

- Pinheiro, C.M., Martins-Duarte, E.S., Ferraro, R.B., Fonseca De Souza, A.L., Gomes, M.T., Lopes, A.H., Vannier-Santos, M.A., Santos, A.L., Meyer-Fernandes, J.R., 2006. *Leishmania amazonensis*: biological and biochemical characterization of ecto-nucleoside triphosphate diphosphohydrolase activities. *Exp. Parasitol.* 114, 16–25.
- Righetti, P.G., 2005. Electrophoresis: the march of pennies, the march of dimes. *J. Chromatogr. A* 1079, 24–40.
- Robson, S.C., Sevigny, J., Zimmermann, H., 2006. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Signal* 2, 409–430.
- Sacks, D., Noben-Trauth, N., 2002. The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat. Rev. Immunol.* 2, 845–858.
- Sansom, F.M., Robson, S.C., Hartland, E.L., 2008. Possible effects of microbial ecto-nucleoside triphosphate diphosphohydrolases on host-pathogen interactions. *Microbiol. Mol. Biol. Rev.* 72, 765–781.
- Santiago, H.C., Oliveira, C.F., Santiago, L., Ferraz, F.O., de Souza, D.G., de Freitas, L.A., Afonso, L.C.C., Teixeira, M.M., Gazzinelli, R.T., Vieira, L.Q., 2004. Involvement of the chemokine RANTES (CCL5) in resistance to experimental infection with *Leishmania major*. *Infect. Immun.* 72, 4918–4923.
- Santos, R.F., Possa, M.A., Bastos, M.S., Guedes, P.M., Almeida, M.R., DeMarco, R., Verjovski-Almeida, S., Bahia, M.T., Fietto, J.L., 2009. Influence of ecto-nucleoside triphosphate diphosphohydrolase activity on *Trypanosoma cruzi* infectivity and virulence. *PLoS Negl. Trop. Dis.* 3, e387.
- Sissons, J., Alsam, S., Jayasekera, S., Khan, N.A., 2004. Ecto-ATPases of clinical and non-clinical isolates of *Acanthamoeba*. *Microb. Pathog.* 37, 231–239.
- Souza-Neto, S.M., Carneiro, C.M., Vieira, L.Q., Afonso, L.C.C., 2004. *Leishmania braziliensis*: partial control of experimental infection by IL-12p40 deficient mice. *Mem. Inst. Oswaldo Cruz* 99, 289–294.
- Späth, G.F., Beverley, S.M., 2001. A lipophosphoglycan-independent method for isolation of infective *leishmania* metacyclic promastigotes by density gradient centrifugation. *Exp. Parasitol.* 99, 97–103.
- Tanuri, A., de Andrade, P.P., de Almeida, D.F., 1985. *Trypanosoma cruzi*: isolation of cloned strains and characterization of their infectivity. *J. Parasitol.* 71, 397–402.
- Tausky, H.H., Shorr, E., 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* 202, 675–685.
- Teixeira, M.J., Fernandes, J.D., Teixeira, C.R., Andrade, B.B., Pompeu, M.L., Santana, d.S., Brodskyn, C.I., Barral-Netto, M., Barral, A., 2005. Distinct *Leishmania braziliensis* isolates induce different paces of chemokine expression patterns. *Infect. Immun.* 73, 1191–1195.
- Vasquez, R.E., Soong, L., 2006. CXCL10/gamma interferon-inducible protein 10-mediated protection against *Leishmania amazonensis* infection in mice. *Infect. Immun.* 74, 6769–6777.
- Vasquez, R.E., Xin, L., Soong, L., 2008. Effects of CXCL10 on dendritic cell and CD4<sup>+</sup> T-cell functions during *Leishmania amazonensis* infection. *Infect. Immun.* 76, 161–169.
- Vieira, L.Q., Hondowicz, B.D., Afonso, L.C.C., Wysocka, M., Trinchieri, G., Scott, P., 1994. Infection with *Leishmania major* induces interleukin-12 production in vivo. *Immunol. Lett.* 40, 157–161.
- Zhang, X., Goncalves, R., Mosser, D.M., 2008. The isolation and characterization of murine macrophages. *Curr. Protoc. Immunol.* 83, 14.1.1–14.1.14.