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Original article

Extracellular nucleotide metabolism in *Leishmania*: influence of adenosine in the establishment of infection

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Abstract

Leishmaniasis is a parasitic disease with a variety of clinical forms, which are related to the *Leishmania* species involved. In the murine model, *Leishmania amazonensis* causes chronic non-healing lesions in *Leishmania braziliensis*- or *Leishmania major*-resistant mouse strains. In this study, we investigated the involvement of the pathway of extracellular nucleotide hydrolysis, with special focus on the role of extracellular adenosine, in the establishment of *Leishmania* infection. Our results show that the more virulent parasite—*L. amazonensis*—hydrolyzes higher amounts of ATP, ADP and AMP than the two other species, probably due to the higher expression of membrane NTPDase. Corroborating the idea that increased production of adenosine is important to lesion development and establishment of tissue parasitism, we observed that increased 5′-nucleotidase activity in *L. braziliensis* or addition of adenosine at the moment of infection with this parasite resulted in an increase in lesion size and parasitism as well as a delay in lesion healing. Furthermore, inhibition of adenosine receptor A_{2B} led to decreased lesion size and parasitism. Thus, our results suggest that the conversion of ATP, a molecule with pro-inflammatory activity, into adenosine, which possesses immunomodulatory properties, may contribute to the establishment of infection by *Leishmania*.

Keywords: Leishmania; Ecto-nucleotidase; Adenosine; Extracellular ATP

1. Introduction

Amongst the several species causative of cutaneous leishmaniasis, *Leishmania amazonensis* occupies a distinctive position due to its involvement in the diffuse form of the disease. Patients infected with this species usually present a defective cellular immune response and do not respond to conventional treatment. Also, in the murine model, this parasite is able to cause chronic non-healing infections in most mouse strains. In this model, although animals are able to mount a Th1 response, which is capable of controlling the infection by other species such as *Leishmania braziliensis* and *Leishmania major*, lesions do not heal and become chronic [1,2]. This discrepancy clearly points to differences between parasite species that are, currently, not known.

ATP, released in the extracellular milieu by injured or pathogen-stimulated cells, is interpreted by the immune system as a ‘‘danger signal’’ and participates in many aspects of the establishment of an inflammatory response such as cytokine secretion and cellular migration [3], mainly by the activation of $P2X_7$ receptors. The response induced by extracellular ATP is controlled in the organism mainly by the action of

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CD39 (ecto-nucleoside triphosphate diphosphohydrolase; E-NTPDase) and CD73 (5′-nucleotidase), which sequentially hydrolyze ATP to ADP, AMP and, finally, adenosine, which, in turn, counteracts the effects of ATP by inhibiting the release of pro-inflammatory cytokines and inducing the release of interleukin (IL)-10 [4–6] via activation of P1 receptors.

Leishmania are devoid of *de novo* synthesis of purine nucleotides. Thus, they need to make use of salvage of preformed purines from the host environment to satisfy their nucleotide requirement, translocating these molecules across the plasma-membrane [7]. Interestingly, studies have shown that *L. amazonensis* promastigotes exhibit ecto-ATPase as well as 5′-nucleotidase activity [2,8,9]. The action of these enzymes on extracellular ATP has been associated with adenosine production that is, then, internalized by the parasite. Simultaneously, however, this same adenosine may activate P1 receptors in the host cells interfering with the establishment of the immune response.

To investigate the possible involvement of ecto-nucleotide hydrolysis and, specially, adenosine production in the establishment of *Leishmania* infection, we compared the ecto-nucleotidasic activity of metacyclic promastigotes from three *Leishmania* species causative of cutaneous leishmaniasis. Our results showed that *L. amazonensis* presents higher enzymatic activity than *L. braziliensis* and *L. major*. Furthermore, we demonstrate that the presence of increased levels of adenosine early in infection by *L. braziliensis* causes an increase in lesion size and parasitism and a delayed lesion control.

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*.

2.2. Parasites

Leishmania (Viannia) braziliensis, M2903 strain (MHOM/BR/75/M2903), *L. (Leishmania) amazonensis*, PH8 strain (IFLA/BR/67/PH8), and *L. (L.) major*, FRIEDLIN strain (MHOM/IL/80/Friedlin) were 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. In the experiments using ammonium molybdate, this salt was added to the culture medium, at the final concentration of 5 μM, daily for 5 days. 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). Briefly, parasites were washed twice in 0.9% NaCl, the pellet was re-suspended in Dulbecco's minimal essential medium (Gibco BRL) or RPMI medium (Sigma, St. Louis, MO, USA), pH 7.2, and overlaid on 10% Ficoll. This preparation was centrifuged at 1070 × *g*/25 °C/15 min, the fraction that laid on top and inside 10% Ficoll was collected, and parasites were washed twice with 0.9% NaCl for use in enzymatic activity measurements or PBS, pH 7.2 for use in infection (adapted from [10]). In the experiment testing the effects of MRS 1754 (Sigma) on parasite growth, *L. braziliensis* promastigotes were cultured in Grace's insect medium (2×10^6 parasites/ml) in the presence of this drug (50 μM), which was pre-diluted in DMSO (Research Organics, Cleveland, OH, USA; 1% final concentration). Parasite growth was measured by daily counting of the parasites up to 72 h from the culture preparation.

2.3. Infection

C57BL/6 mice were inoculated in the left hind footpad with 1×10^5 metacyclic promastigotes/50 μl PBS alone or in association with 2.5 nmol adenosine or MRS 1754 pre-diluted in DMSO (1% final concentration), 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 contralateral non-infected footpad [1].

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 [1]. Protein content of preparations was determined by the Lowry method [11] and adjusted to 1 mg/ml protein. Antigen preparation was aliquoted and stored frozen at −70 °C and thawed immediately before use.

2.5. Parasite load estimation

The number of parasites in the footpad was estimated by a limiting dilution assay [1]. Mice were euthanized 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 × *g*/4 °C/1 min, and supernatant was transferred to another tube and centrifuged at 1540 × *g*/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 10-fold dilutions in duplicates to a final volume of 200 μl 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 last dilution in which they were detected.

2.6. Analysis of cytokine production

Single-cell suspensions were prepared from the lymph nodes of mice infected for 3 or 4 weeks. Cells were adjusted to

a concentration of 5×10^6 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-hydroxiethylpiperazine-*N*′-2-ethanosulfonic 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 μg/ml. Supernatants were harvested after 72 h for interferon (IFN)-γ [2] and IL-10 (PeproTech Inc., Rock Hill, NJ, USA).

2.7. Histology

Histological analysis of footpad lesions was done in paraffin-embedded sections stained by hematoxylin–eosin. Sections were inspected for the presence of the parasite and evaluation of the inflammatory infiltrate was performed under an optical microscope.

2.8. 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 $MgCl_2$, and 50 mM HEPES–Tris buffer, in the presence of ATP, ADP or AMP (Sigma) 5 mM. The reaction was started by the addition of living metacyclic promastigotes and terminated by the addition of ice-cold 0.2 M HCl [12]. 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 [13]. Enzymatic activities were expressed as nmol of Pi release induced by 1×10^8 parasites in 1 h.

2.9. Western blotting analysis

Plasma membrane enriched preparations were obtained by 14,000 × *g* centrifugation of metacyclic promastigote extracts as described elsewhere [14] and stored until use at −20 °C in the presence of the protease inhibitors: 200 μM EGTA, 4 mM PMSF, 40 μM TPCK, 40 μM TLCK, 4 mM DTT and 40 mM NEM (Sigma). Protein determination was performed by the Lowry method [11]. Membrane preparations were treated with 0.4 IU of glycopeptidase F (Sigma) to remove glycosylation residues [15]. For Western blotting analysis, membrane preparations were run on 10% SDS–PAGE followed by semi-dry transfer to nitrocellulose membranes. Blotted nitrocellulose membranes were incubated with serum from a rabbit immunized with recombinant *Trypanosoma cruzi* NTPDase-I (1:2000) followed by peroxidase-goat anti-rabbit IgG conjugate (Zymed Laboratories, San Francisco, CA, USA) (1:10000), and revealed by reaction with DAB/4-chloro naphtol/methanol/H_2O_2 solution.

2.10. Quantification of the inflammatory infiltrate on the skin and morphometric studies

Morphometric studies were performed by image analysis (Leica QWin), counting all cellular nuclei in skin fragments in five randomly-selected fields (total area 0.4 mm^2) on a single slide per animal. All analyses were performed using the 40× objective lens.

2.11. Statistical analysis

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

3. Results

3.1. Leishmania species with different virulence display different patterns of nucleotide hydrolysis and NTPDase expression

In order to establish a possible correlation between the degree of virulence in the mouse model and the ecto-nucleotidase activity of the parasites, we initially inoculated C57BL/6 mice with metacyclic promastigotes from three different *Leishmania* species. As shown in Fig. 1A, mice infected with *L. amazonensis* developed chronic lesions that did not heal spontaneously. On the other hand, mice inoculated with *L. braziliensis* completely healed their lesions by 7 weeks of infection. Mice infected with *L. major* had controlled lesion progression by 7 weeks and were able to completely heal by 10–11 weeks (data not shown).

These parasites were, then, tested with regard to their competence to hydrolyze ATP, ADP or AMP nucleotides. We observed a higher ecto-nucleotidasic activity for the three nucleotides tested in *L. amazonensis* metacyclic promastigotes when compared with the other two species (Fig. 1B).

The differences in nucleotide hydrolysis observed in the metacyclic promastigotes (infective forms) described above were partially confirmed by the analysis of NTPDase expression by Western blotting (Fig 1C). Given the homology in Apyrase Conserved Regions between *T. cruzi* and *L. major* NTPDase genes [12], we used an anti-*T. cruzi* NTPDase-I serum to evaluate the expression of this enzyme in the membrane of *Leishmania* metacyclic promastigotes. The anti-*T. cruzi* NTPDase-I serum was produced using recombinant purified NTPDase-I that is homologue to GDPase from *Leishmania*. This anti-serum reacted strongly with membrane preparations of *L. amazonensis* promastigotes revealing a band of approximately 50.3 kDa (NT), slightly larger than the 47.2 kDa molecular weight of the *L. major* NTPDase predicted by gene analysis [12]. Treatment of membrane preparations with glycopeptidase F altered the migration pattern of the band and revealed a band of 48.2 kDa similar to the *L. major*-NTPDase predicted value (T).

Curiously, we were not able to detect the expression of NTPDase in the enriched membrane preparations of *L. braziliensis* or *L. major* even using 10 times more protein than used for *L. amazonensis*.

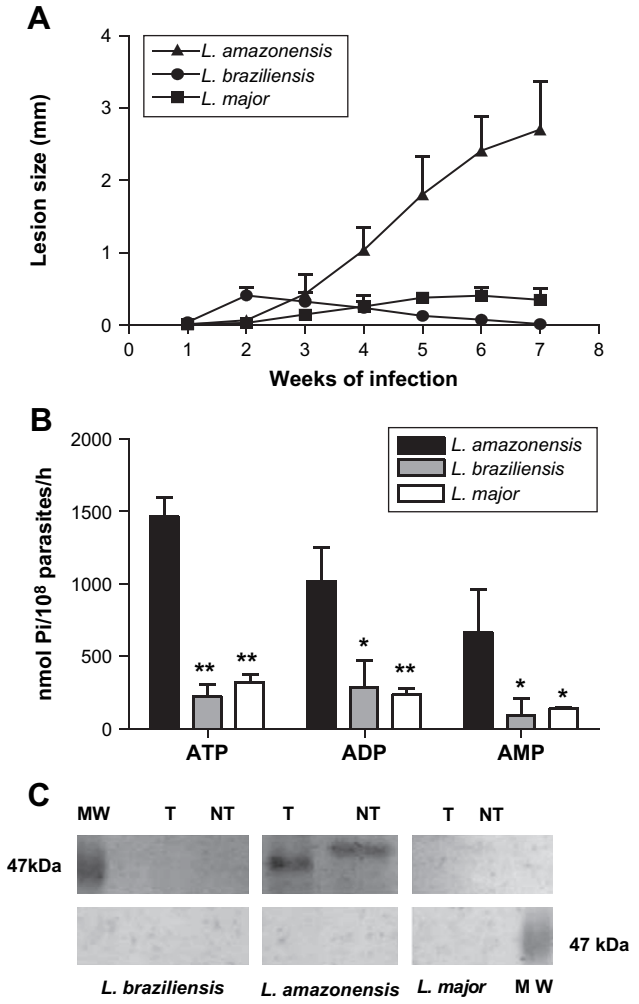

Fig. 1. Lesion development in C57BL/6 mice inoculated with *Leishmania*, ecto-nucleotidasic activity and NTPDase expression in metacyclic promastigotes. (A) Mice were inoculated with 1×10^5 metacyclic promastigotes on the left hind footpad and lesion sizes were measured weekly. Data represent mean + 1 SD from two or three separate experiments with three animals per group. (B) 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. Data represent mean + 1 SD from three separate experiments. Asterisks represent statistical difference against *L. amazonensis* group. $*p < 0.05$, $**p < 0.01$. (C) Glycopeptidase F treated (T) or non-treated (NT) plasma membrane enriched fractions from metacyclic promastigotes were submitted to SDS–PAGE followed by Western blotting as described in materials and methods. Blottings were incubated with anti *T. cruzi*-NTPDase (upper panel) or pre-immune rabbit sera (lower panel). MW - molecular weight marker. 20 μg of membrane protein preparations from *L. braziliensis* and *L. major* were loaded per lane. Lanes from *L. amazonensis* were loaded with 2 μg.

3.2. Increase in the activity of 5′-nucleotidase induces larger lesion development and parasitism in L. braziliensis infected mice

In the face of the results described above, we decided to investigate if increasing the ecto-nucleotidasic activity would interfere with the course of infection in *L. braziliensis* infected mice. To this aim, we initially treated *L. braziliensis* cultures for 5 days with ammonium molybdate, an inhibitor of 5′-nucleotidase. As shown in Fig. 2A, this treatment led to an increase in AMP hydrolysis by metacyclic promastigotes, probably due to increased expression caused by the persistent presence of the inhibitor. C57BL/6 mice inoculated with these

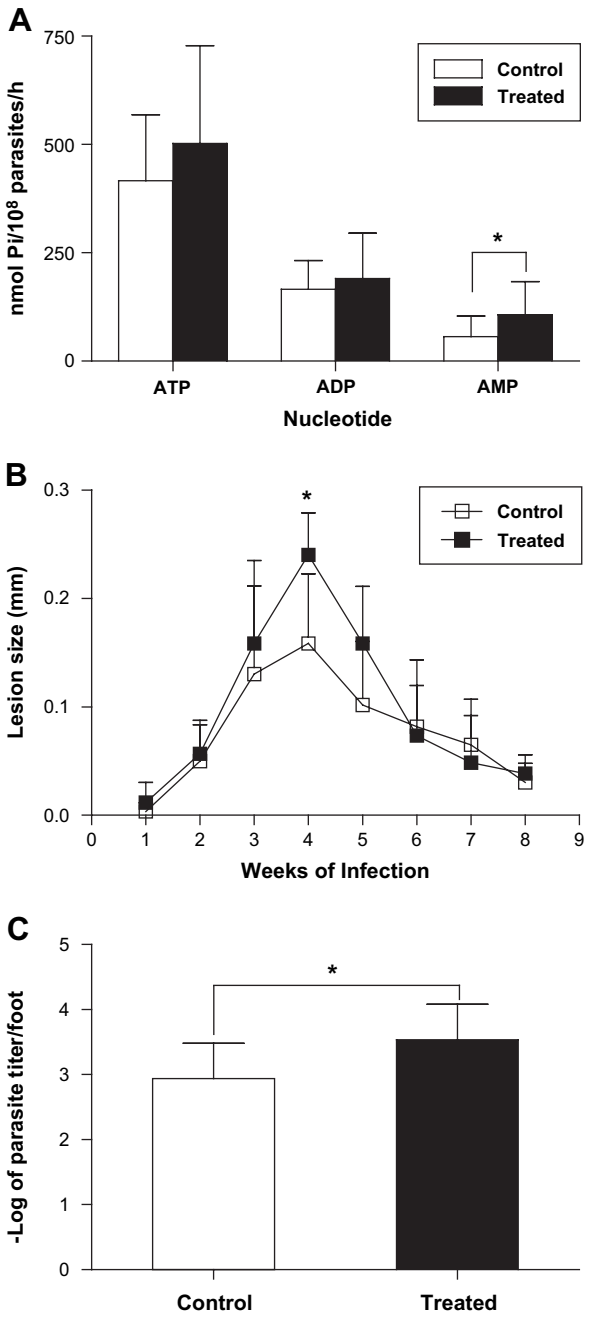

Fig. 2. Effects of ammonium molybdate addition to *L. braziliensis* cultures in ecto-nucleotidase activity and infectivity of metacyclic promastigotes. Cultures of *L. braziliensis* were treated daily with ammonium molybdate for 5 days. (A) Metacyclic promastigotes were isolated and ecto-nucleotidasic activity measured as described in materials and methods. Bars represent mean + 1 SD from six separate experiments. (B) Lesion development in C57BL/6 mice inoculated with 1×10^5 metacyclic promastigotes in the hind footpad. (C) Tissue parasitism at the 4th week of infection. Lesions from infected mice were excised and parasitism evaluated by limiting dilution. Symbols and bars represent mean + 1 SD from two separate experiments and four mice were used per group. Asterisk indicates statistical difference between treated and control groups ($p < 0.05$).

parasites were able to heal the infection. However, an increased lesion development and tissue parasitism were observed (Fig. 2B and C), suggesting a correlation between extracellular AMP hydrolysis and infectivity.

3.3. *Adenosine treatment at the time of* L. braziliensis *inoculum delays lesion resolution and induces greater parasitism*

To investigate the possibility that the increased lesion development and parasitism induced by ammonium molybdate-treated parasites was caused by an increased production of adenosine by 5′-nucleotidase, we treated mice with this nucleoside at the moment of *L. braziliensis* inoculation. Interestingly, addition of adenosine to the inoculum also induced an increase in lesion development very similar to that observed in the previous experiment, corroborating our hypothesis (Fig. 3). Adenosine-treated animals developed larger lesions than control animals from the 3rd week of infection onwards (Fig. 3A), resulting in a small delay in the control of the infection, that correlated with an increase in tissue parasitism and evident macrophage infiltrate at 3 weeks of infection (Fig. 3B and D). Since cytokine production has been correlated in several instances to the outcome of infection with *Leishmania* parasites, we evaluated the production of IFN-γ and IL-10 by lymph node cells at 3 weeks of infection. Stimulated cultures from treated and control animals produced similar levels of the cytokines, indicating that adenosine treatment, at least at the concentration used in our experiments, was not sufficient to induce significant changes in the adaptive immune response (data not shown). Interestingly, however, at 3 weeks of infection, lesions from control mice presented larger inflammatory infiltrates than those from adenosine treated group (Fig. 3C–E), corroborating the hypothesis of a delayed control of the lesion development caused by adenosine production at the early stages of infection.

3.4. *A_{2B} receptor blockage induces decrease in lesion size and parasitism from* L. braziliensis *infected C57BL/6 mice*

In an attempt to confirm that the effects observed above were due, predominantly, to the action of adenosine on the host rather than the parasite, C57BL/6 mice were inoculated with *L. braziliensis* metacyclic promastigotes in the presence of MRS 1754, an antagonist of the A_{2B} adenosine receptor. Blockage of this receptor at the moment of parasite inoculation led to lower lesion size and tissue parasitism after 4 weeks of infection (Fig. 4A and B, respectively). MRS 1754 treatment was not able to induce changes in IFN-γ or IL-10 production by lymph node cells compared to non-treated group (data not shown). In addition, parasites cultured up to 72 h in the presence of MRS 1754 did not show changes in growth (Fig. 4C), indicating that the effects observed after *in vivo* administration of this drug did not result from a possible action directly on parasite viability.

4. Discussion

Several virulence factors have been associated with the establishment of *Leishmania* infection, including LPG and gp63 expression. These molecules interfere with the immune response either by altering the cytokine expression or by protecting the parasite from the deleterious action of the Complement system [16]. In this study, we present the hypothesis that enzymes involved in the degradation of extracellular ATP and production of adenosine by parasites of the genus *Leishmania* may be associated with their virulence.

Amongst the enzymes involved in ecto-nucleotide metabolism, E-NTPDases are probably the most studied. The presence of these enzymes has been described in different parasites, including *Toxoplasma gondii* [17], *Trypanosoma cruzi* [12,18], *Tritrichomonas foetus* [19], *Entamoeba histolytica* [20], and *Leishmania tropica* [21]. Our results showed that the more infective metacyclic promastigotes from *L. amazonensis* presented higher levels of extracellular adenine nucleotide hydrolysis than those from *L. braziliensis* or *L. major* (Fig. 1). *In silico* molecular analysis showed that there are two putative NTPDases in the *L. major* genome named NTPDase and GDPase with predicted molecular weights of 47.2 and 73.4 kDa, respectively[12]. Data also show the presence of a predicted amino-terminal transmembrane domain for both enzymes, but only the GDPase possess a putative signal peptide that could generate a 70.4 kDa soluble excreted protein. This same organization is observed in the *L. braziliensis* genome, showing putative NTPDase and GDPase with 47.7 and 74.8 kDa deduced proteins. Furthermore, the putative *L. braziliensis* peptide contains an amino-terminal putative signal peptide that could generate a 71.3 kDa soluble excreted protein. Although genomic analysis indicates the presence of NTPDase genes in both *L. major* and *L. braziliensis*, we did not detect the expression of these enzymes in the membrane preparations of either *L. braziliensis* or *L. major* metacyclic promastigotes even when we loaded the gels with 10 times more protein (Fig. 1C). We cannot exclude the possibility that, due to differences in sequence between parasite's NTPDases, the serum used was not able to detect this enzyme in the membrane of *L. major* and *L. braziliensis*. However, a more plausible hypothesis would be that, in the case of *L. major* and *L. braziliensis*, the putative secreted GDPases were responsible for the detected nucleotidasic activity or that the NTPDases are being held inside the cells due to problems in the glycosylation-dependent secretory pathway. However, in several instances the activity of E-NTPDases has been correlated with parasite virulence [17,19,22,23], acting, probably, as a protecting mechanism against the cytolytic effects of extracellular ATP [24] or by increasing parasite adhesion to the host cell [25]. The reasons for the differences observed as well as the actual involvement of these specific enzymes in the establishment of infection are under investigation in our laboratory.

Besides its role in parasite metabolism, the adenosine produced by the action of the ecto-nucleotidases may also influence the establishment of infection due to its immunomodulatory effects [26]. Given the fact that inhibiting adenosine production

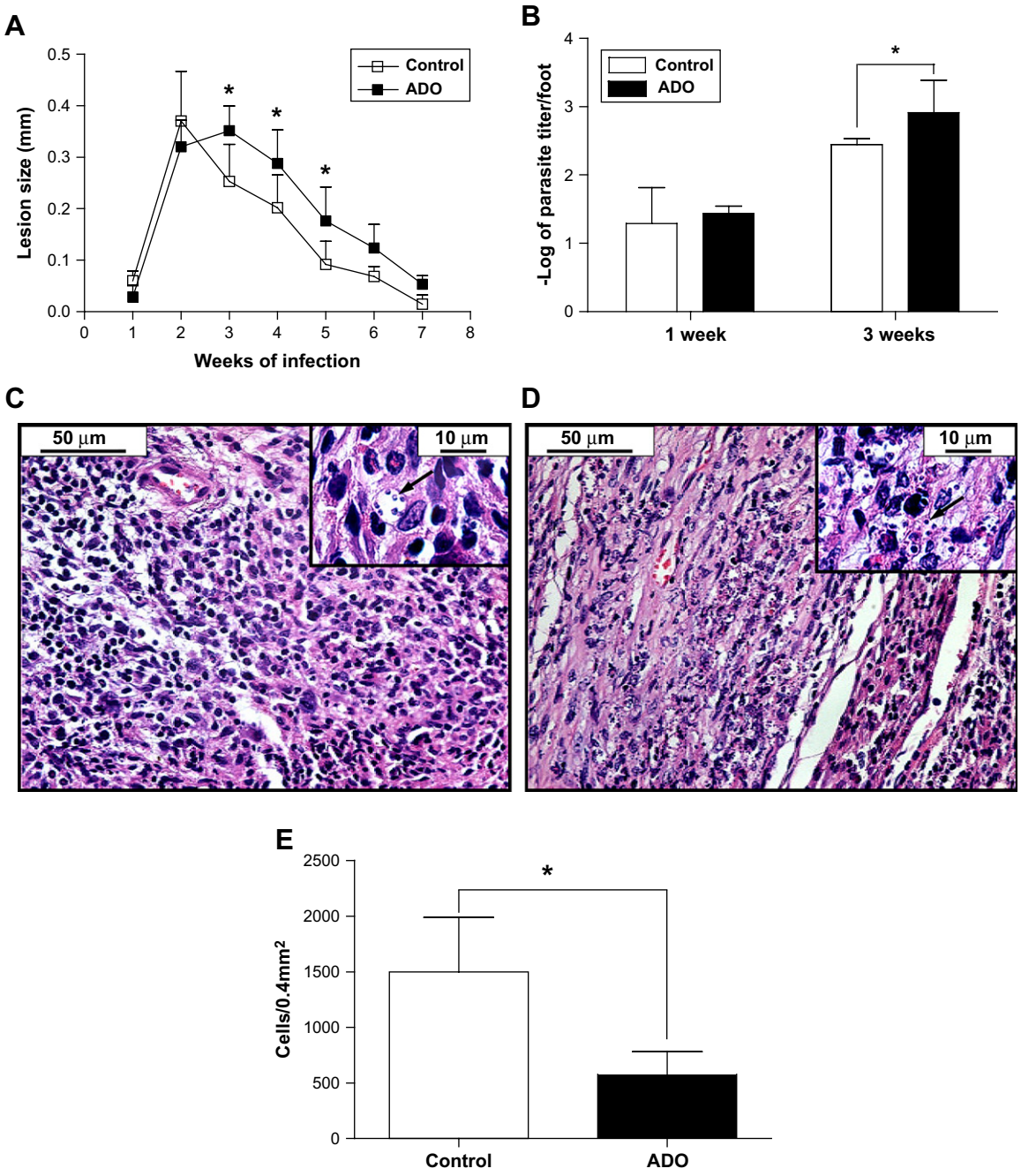

Fig. 3. Effect of adenosine in *L. braziliensis* infectivity. C57BL/6 mice were inoculated in the hind footpad with 1×10^5 *L. braziliensis* metacyclic promastigotes alone (Control) or associated with 2.5 nmol adenosine (ADO). (A) Lesion sizes were measured weekly, as described in Section 2. (B) Tissue parasitism from two grouped independent experiments using three mice per group. Values represent mean + 1 SD. *$p < 0.05$. (C, D) Histological analysis of footpad lesions. Lesions from non-treated (C) and adenosine-treated mice (D) were harvested and stained by hematoxylin–eosin. Insets show magnified section from the correspondent picture and depict differences in tissue parasitism. Arrows point to amastigote forms. (E) Inflammatory infiltrate on the lesion was quantified as described in Section 2. The results represent the average number of cells per 0.4 mm^2. Values represent mean + 1 SD from two separate experiments and four mice were used per group. Asterisk indicates statistical difference between adenosine-treated and control groups ($p < 0.05$).

by *L. amazonensis* promastigotes would probably have direct effects on parasite survival and based on previous results from the literature [18], which described an increase in *T. cruzi*'s apyrase activity when the cultures were treated with suramin (an inhibitor of this enzyme), we decided to evaluate the role of adenosine production in the early phase of *Leishmania* infection by increasing the 5′-nucleotidase activity of *L. braziliensis*. Treatment of *L. braziliensis* promastigotes cultures with an inhibitor of 5′-nucleotidase led to a small but significant increase in extracellular AMP hydrolysis with no alterations in ATP or ADP degradation. Similar results were observed when we treated *L. amazonensis* or *Leishmania chagasi* promastigote cultures (our unpublished observations) demonstrating that this effect was not restricted to *L. braziliensis* promastigotes.

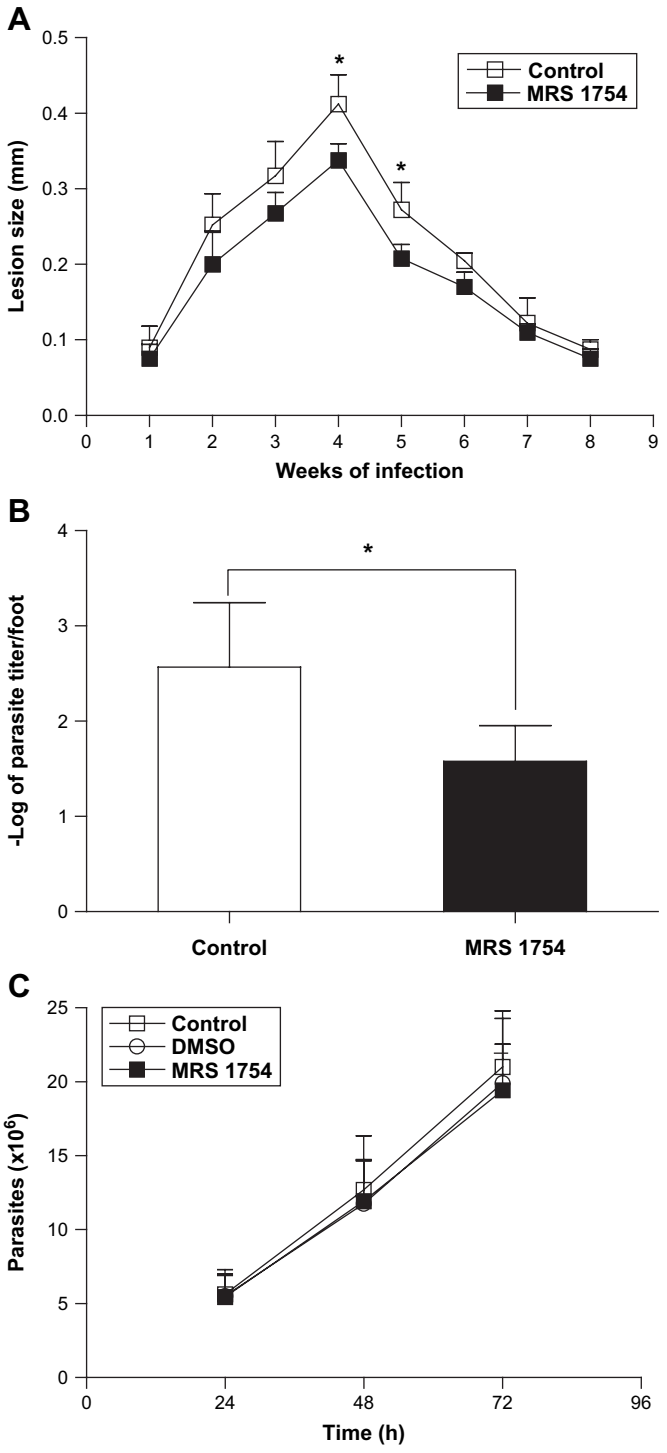

Fig. 4. Effect of adenosine receptor antagonist administration in *L. braziliensis* infection. C57BL/6 mice were inoculated in the hind footpad with 1×10^5 *L. braziliensis* metacyclic promastigotes alone (Control) or plus 2.5 nmol of MRS 1754. Lesion size was measured weekly (A), and lesion parasitism was evaluated at the 4th week of infection (B). Four mice were used per group, and values (mean + 1 SD) are representative of two different experiments (A) or represent grouped data from two independent experiments (B). The effect of the presence of MRS 1754 on parasite growth was evaluated, as described in Section 2, and values (mean + 1 SD) represent grouped data from three independent experiments (C). $*p < 0.05$.

Our results suggest that different mechanisms may control the expression of the enzymes responsible for the hydrolysis of each nucleotide. Further studies are necessary to clarify this issue.

Infection of C57BL/6 mice with ammonium molybdate-treated parasites led to a transient, but significant, increase in lesion size and parasitism, implicating adenosine production in the establishment of infection (Fig. 2B and C). In support to this hypothesis, addition of adenosine to the parasite inoculum had similar effects (Fig. 3).

The effects of adenosine on the host's cell are mediated by four receptors (A_1, A_{2A}, A_{2B} and A_3) which bind to extracellular adenosine with different affinities. Of these, A_{2A} and A_{2B} have immunomodulatory activities (for a review see [26]). If the production of adenosine at the site of infection were to have an effect on the host rather than the parasite, blockage of these receptors would cause an effect opposite to the observed by adenosine administration or by the increased 5′-ecto-nucleotidasic activity. This was exactly what we found when we infected mice with *L. braziliensis* in the presence of MRS-1754, an antagonist of the immunomodulatory A_{2B} adenosine receptor (Fig. 4). The fact that we were able to alter the course of infection by blocking a receptor with low affinity for adenosine [26] suggests that high levels of this nucleotide are produced at the site of infection. These results strongly suggest that adenosine production at the early stages of infection influence the growth of the parasite and lesion development.

These alterations are probably associated with a delay in the establishment of the immune response as demonstrated by the decreased lymphocytic infiltrate in adenosine-treated animals at three weeks of infection (Fig. 3C and D) as well as by the fact that these animals were eventually capable of controlling the infection and showed no alterations in cytokine production by lymph node cells. This delayed response may be attributed to the inhibitory effects of adenosine on dendritic cell migration [27].

Furthermore, it is quite possible that the effects of the treatments used in our protocols are transient and may be reversed as soon as the parasite differentiates into the amastigote form within the host's macrophages. Our experiments focused on the possible role of ecto-nucleotidasic activity of promastigotes and do not exclude the possibility that the same enzymes and/or adenosine production may be involved in the establishment of chronic lesions if the same differences observed in promastigotes were to be found in the intracellular amastigote. In fact, results from the literature indicate that, at least for *L. amazonensis*, amastigotes present higher levels of ATP hydrolysis than promastigotes [28]. The mechanisms underlying the role of ecto-nucleotidasic activity on amastigotes on the host's immune response and parasite survival are under investigation in our laboratory.

Although, as mentioned before, the correlation between the activity of ecto-nucleotidases and parasite virulence has already been proposed, our results expand on this concept by demonstrating for the first time to our knowledge that this pathway can interfere with the infection, at least in its early steps. We cannot, however, eliminate the possibility that not only the increase

in extracellular adenosine concentration but also a decrease in ATP levels may be involved in this process. Further studies, designed to more deeply evaluate this hypothesis, are warranted and may lead to new target pathways for therapeutic intervention.

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