Institut Pasteur
Microbes and Infection 17 (2015) 295–303
www.elsevier.com/locate/micinf

Original article

E-NTPDase (ecto-nucleoside triphosphate diphosphohydrolase) of *Leishmania amazonensis* inhibits macrophage activation

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Received 21 October 2014; accepted 16 December 2014
Available online 30 December 2014

Abstract

Leishmania amazonensis, the causal agent of diffuse cutaneous leishmaniasis, is known for its ability to modulate the host immune response. Because a relationship between ectonucleotidase activity and the ability of *Leishmania* to generate injury in C57BL/6 mice has been demonstrated, in this study we evaluated the involvement of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) activity of *L. amazonensis* in the process of infection of J774-macrophages. Our results show that high-activity parasites show increased survival rate in LPS/IFN-γ-activated cells, by inhibiting the host-cell NO production. Conversely, inhibition of E-NTPDase activity reduces the parasite survival rates, an effect associated with increased macrophage NO production. E-NTPDase activity generates substrate for the production of extracellular adenosine, which binds to A_{2B} receptors and reduces IL-12 and TNF-α produced by activated macrophages, thus inhibiting NO production. These results indicate that E-NTPDase activity is important for survival of *L. amazonensis* within macrophages, showing the role of the enzyme in modulating macrophage response and lower NO production, which ultimately favors infection. Our results point to a new mechanism of *L. amazonensis* infection that may pave the way for the development of new treatments for this neglected disease.

Keywords: *Leishmania amazonensis*; E-NTPDase; Adenosine; Nitric oxide; Cytokines

1. Introduction

The success of *Leishmania* infection in macrophages depends on the ability of the parasite to adhere to these cells, to be phagocytosed, and to survive the effects of microbicidal mechanisms [1]. After phagocytosis, macrophages are activated to produce nitric oxide and ROS (reactive intermediates of oxygen), which are highly toxic to the parasite [2–4]. NO production by iNOS is stimulated by numerous inflammatory cytokines such as IFN-γ, TNF-α, and IL-1, and inhibited by anti-inflammatory cytokines such as IL-4, TGF-β, and IL-10 [5].

In both animal models and humans infected with *Leishmania major* or *Leishmania braziliensis*, the host immune response is usually able to control the infection. On the other hand, one of the main features of infection by *Leishmania amazonensis* is its ability to modulate the host immune response and sustain the infection, both in experimental models and in humans [6–8]. Evidence shows that *L. amazonensis* inhibits macrophage NO production, which favors the survival of the parasite inside the cell [9–11]. The reduction in iNOS expression, and the consequent reduction in NO production, in *L. amazonensis* infection is the result of a number of immune alterations, such as increase in IL-10 and TGF-β

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http://dx.doi.org/10.1016/j.micinf.2014.12.009
1286-4579/

production, reduction in IL-12 JACK/STAT inactivation, activation of phosphotyrosine phosphatases, and inhibition of TNF-α production [12].

NTPDases, or nucleoside triphosphate diphosphohydrolases, are enzymes of the apyrase family with the ability to hydrolyze nucleotide triphosphates to their monophosphate form under stimulation of divalent ions such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) [13]. In trypanosomatids, the E-NTPDase is important to the salvation of purine nucleotides [14], since these parasites are unable to perform *de novo* synthesis of these nucleotides [15]. E-NTPDases have been extensively characterized in *L. amazonensis* [16] as well as in other species of protozoa, such as *Toxoplasma gondii* [17] and *Trypanosoma cruzi* [18,19]. Data from our research group have shown the importance of NTPDase activity in establishing *Leishmania* infection [20–22] and driving the clinical form of the disease [23]. Adenosine, the product of the combined action of E-NTPDase and 5′-ectonucleotidase, is an important molecule in the regulation of macrophage cell function, which is mediated by its binding to specific receptors on the surface of these cells [24]. It has been shown that adenosine can prevent respiratory burst in macrophages [25] and suppress LPS-stimulated cellular NO production [26]. Adenosine, therefore, plays a role in reducing the production of TNF-α and IL-12, by acting either on A_{2A} or A_{2B} receptors [27].

The *Leishmania* E-NTPDases can be divided into two different groups: E-NTPDase-1, also named guanosine diphosphatase, which is similar to the E-NTPDase-1 of *T. cruzi*; and E-NTPDase-2, or ATP diphosphohydrolase or nucleoside diphosphatase [28]. Although the role of E-NTPDase of *L. amazonensis* in developing and maintaining *in vivo* infection is well established, the role of this parasite's enzyme in macrophage infection remains unknown. Here, we observed that the activity of E-NTPDase in *L. amazonensis* plays a critical role in the modulation of the macrophage's immune response and increases the parasite's survival.

2. Materials and methods

2.1. Parasites

Promastigotes of *Leishmania (Leishmania) amazonensis*, PH8 strain (IFLA/BR/67/PH8), and 1IIId clone of the same strain were cultured at 25 °C, in Grace's insect medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS – LGC, Cotia, SP, Brazil), 2 mM L-glutamine (Sigma–Aldrich), and 100 U/mL penicillin G potassium (Sigma–Aldrich), pH 6.5. The original strain was also maintained in the presence of adenine 5 mM, for a few passages, to reduce ectonucleotidase activity of the parasite, as described previously [16]. Metacyclic promastigotes were obtained by gradient centrifugation of parasites at the late log phase of culture (day 5) over Ficoll 400 (Sigma–Aldrich), as previously described [21]. Parasites were kept in culture for no more than 20 passages.

2.2. Ectonucleotidase activity measurement

The activity of ecto-ATPase, ecto-ADPase and ecto-AMPase was measured by incubation of intact parasites for 1 h at 30 °C in reaction buffer 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 5 mM of ADP, ATP, or AMP (Sigma–Aldrich) [18]. The reaction was initiated with the addition of live metacyclic promastigotes and terminated by addition of 0.2 M HCl [14]. Nonspecific hydrolysis was determined by the addition of parasites after the reaction was stopped. We used a pelleted parasite suspension, and aliquots of the supernatant were used for measuring the liberated inorganic phosphate (Pi) as previously described [29]. Enzyme activities were expressed as nmol of Pi released by 1×10^8 parasites in 1 h.

2.3. Western blotting

Enriched plasma membrane preparations were obtained by 14,000 × g centrifugation of metacyclic promastigote or axenic amastigote extracts as described elsewhere [30] 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–Aldrich). The determination of protein was performed by the Lowry method [31]. 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 *Leishmania infantum* NTPDase (1:2000) followed by peroxidase-goat anti-rabbit IgG conjugate (Zymed Laboratories, San Francisco, CA, USA) (1:10,000), and revealed by reaction with DAB/4-chloro naphtol/methanol/H_2O_2 solution.

2.4. Infection of J774 cells

J774 cells were plated at 5×10^5 cells per well (0.5 mL) onto round coverslips in Dulbecco's minimal essential medium containing 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin G potassium, 25 mM N-2-hydroxiethylpiperazine-N9-2-ethanosulfonic acid (HEPES; USBiological, Swampscott, MA, USA), and 50 mM β-mercaptoethanol (Pharmacia Biotech AB, Uppsala, Sweden) in 24-well plates. Cells were incubated for 90 min at 37 °C, 5% CO_2. Non-adherent cells were removed by washing with warm PBS. Metacyclic promastigotes were added to the culture at a 3:1 parasite-to-cell ratio. After 3 h co-culture, cells were washed with PBS to remove non-internalized parasites and coverslips were collected to evaluate infectivity. Fresh medium was added to the cultures and the macrophages were stimulated, or not, with 10 U/mL IFN-γ and 100 pg/mL lipopolysaccharide (LPS) (Sigma–Aldrich). After 48 h, coverslips were collected for evaluation of infectivity and supernatants were collected for measurement of nitrite and cytokines. Coverslips were fixed with methanol (Vetec Fine Chemistry) for

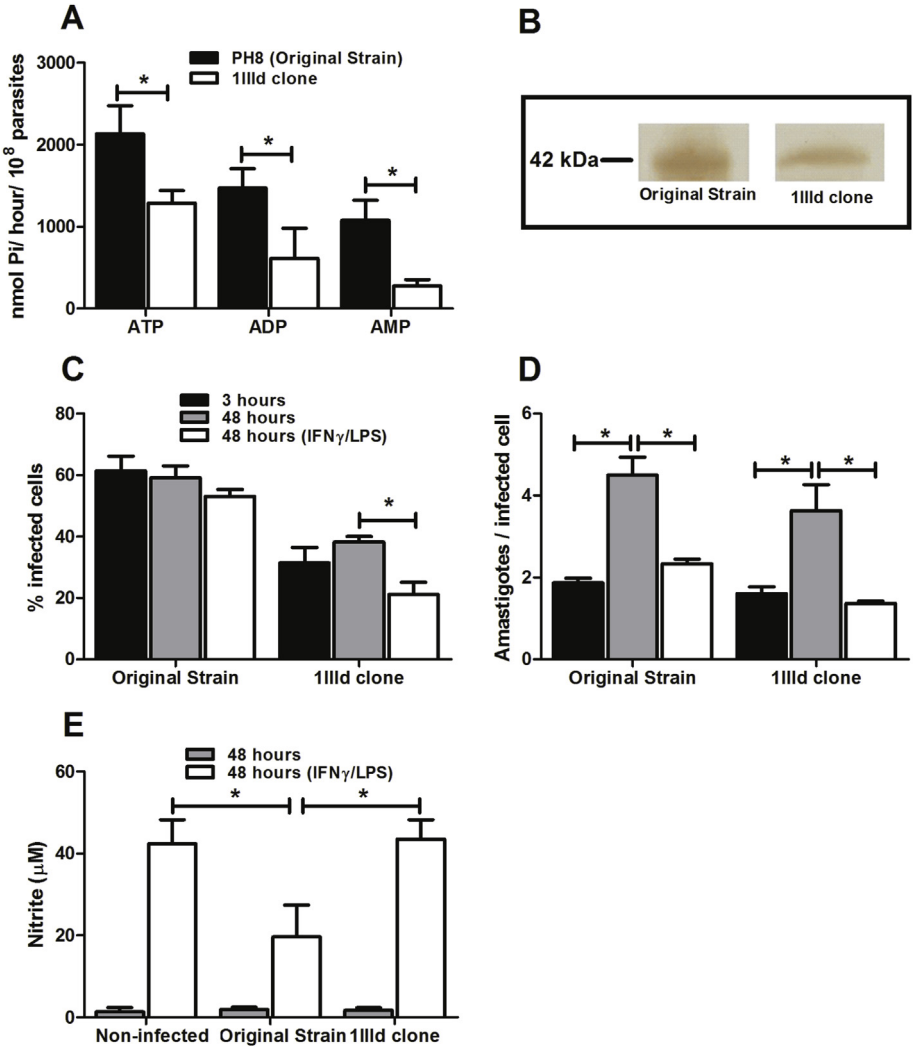

Fig. 1. *L. amazonensis* E-NTPDase activity correlates with survival of parasites within stimulated cells. Parasites were incubated with ATP, ADP, or AMP for 1 h at 30 °C. (A) Enzymatic activity was evaluated by the measurement of inorganic phosphate released. (B) Analysis of the expression of E-NTPDase by Western blotting on membrane preparations of metacyclic promastigotes. J774-macrophages were infected with metacyclic promastigotes for 3 h, washed and incubated for 48 h, in presence or not of IFNγ/LPS. (C) Percentage of infected cells. (D) Amastigotes per infected cell. (E) NO production in 48-h supernatants. Bars represent the mean ± SD of three independent experiments performed in duplicate. *$p < 0.05$.

10 min and then stained with Panótico Rápido kit (Laborclin, Pinhais, PR, Brazil), according to manufacturer's instructions. The analysis was performed by counting the cells containing adhered or internalized parasites using an optical microscope Olympus BX50 (Olympus, Center Valley, PA, USA). We evaluated the percentage of macrophages containing internalized parasites and the number of parasites per 100 cells. At least 200 cells were evaluated.

2.5. Nitric oxide and cytokine quantification

Quantification of NO produced by the cells was performed by the indirect Griess method to detect nitrite [32], and the production of IL-12, IL-10, and TNF-α was evaluated by indirect ELISA, according to the manufacturer (BD Biosciences).

2.6. Statistical analysis

Statistical analysis was performed by 1way-ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ectonucleotidase activity in L. amazonensis is related to the parasite's survival within macrophages

Given that a relationship has been demonstrated between the level of ectonucleotidase activity and the ability of *Leishmania* to generate injury in C57BL/6 mice [21,22], here we investigated whether the presence and activity of *L. amazonensis's* E-NTPDases also related to the survival of parasites in infected macrophages. To this end, metacyclic

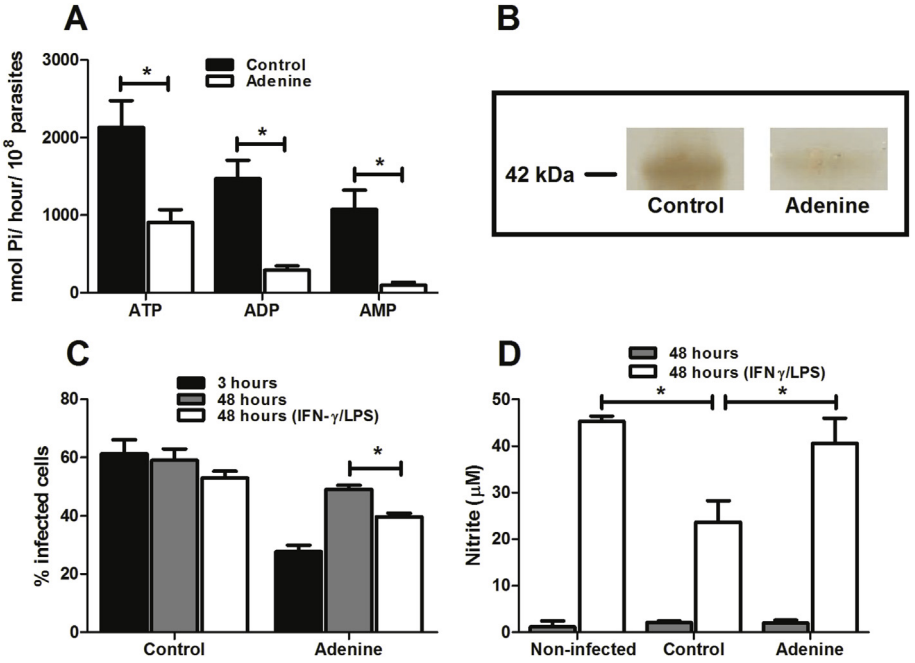

Fig. 2. The modulation of E-NTPDase activity alters the ability of *L. amazonensis* to inhibit NO production by activated J774 cells. J774-macrophages were incubated for 3 h with metacyclic promastigotes of *L. amazonensis* (3 parasites/cell) grown in adenine supplemented medium, washed and incubated for additional 48 h, in presence or not of IFN-γ/LPS. (A) Enzymatic activity was evaluated by the measurement of inorganic phosphate released. (B) Analysis of the expression of E-NTPDase by Western blotting on membrane preparations of metacyclic promastigotes. (C) Percentage of infected cells. (D) NO production in 48-h supernatants. Bars represent the mean ± SD of three independent experiments performed in duplicate. *$p < 0.05$.

promastigotes of both the original strain of *L. amazonensis* and a clone of the same strain (1IIId clone) were incubated with ATP, ADP, or AMP for 1 h and the amount of inorganic phosphate was evaluated. As shown in Fig. 1A, the 1IIId clone presents lower ectonucleotidase activity for the three nucleotides when compared to metacyclic promastigotes of original strain. In addition, the expression of the protein on the membrane of the parasites is lower in the 1IIId clone than in the original strain (Fig. 1B).

J774 cells were infected with metacyclic promastigotes of the original strain or 1IIId clone of the same strain, for 3 h, washed to remove non-internalized parasites, and stimulated with LPS and IFN-γ for 48 h. Our results show that at three hours of incubation the percentage of macrophages infected by the 1IIId clone was smaller than that of the original strain. More importantly, whereas activation of macrophages infected by the 1IIId clone reduced the percentage of infected cells after 48 h, no effect was observed when infections were initiated by the original strain (Fig. 1C). The number of amastigotes per infected cell was similar in both groups in each incubation condition (Fig. 1D). Given that the percentage of infected macrophages was decreased when 1IIId infected cells were activated, the combined results from Fig. 1C and D indicate that some macrophages were able to complete eliminate the parasite while in cells infected by the original strain, activation allowed only for a containment of the parasite growth. Interestingly, unlike the 1IIId clone, the original strain reduced NO production by activated cells, which may explain the parasite's survival capacity, even under inflammatory stimuli (Fig. 1E). This suggests that the level of E-NTPDase activity may influence parasite survival by inhibiting NO production by activated macrophages.

3.2. The modulation of E-NTPDase alters the ability of L. amazonensis *to inhibit NO production by activated J774 cells*

Given the role of the E-NTPDase in increasing extracellular AMP, which is hydrolyzed to adenosine by 5′-ectonucleotidase to supply the purine salvage pathway [33], we expected that an increased purine concentration in the culture medium would affect the expression of the E-NTPDase on the parasite surface. To modulate the enzyme activity, promastigotes of the original strain were grown in medium supplemented with adenine prior to evaluating enzyme activity. Our data show that maintenance of parasites in the presence of adenine led to a reduction in the activity and expression of E-NTPDase in the original strain (Fig. 2A and B).

To verify if the modulation in the activity of E-NTPDase would also interfere with the ability of the parasite to survive within activated macrophages, the original strain grown in adenine-supplemented medium was used to infect J774 cells. After 3 h, cells were washed to remove non-internalized parasites and cells were incubated for 48 h in the presence of IFN-γ and LPS. Our results demonstrate that the original strain grown in the presence of adenine is less able to resist activation by IFN-γ and LPS. As shown in Fig. 2C, the percentage of infected cells was reduced after 48 h of

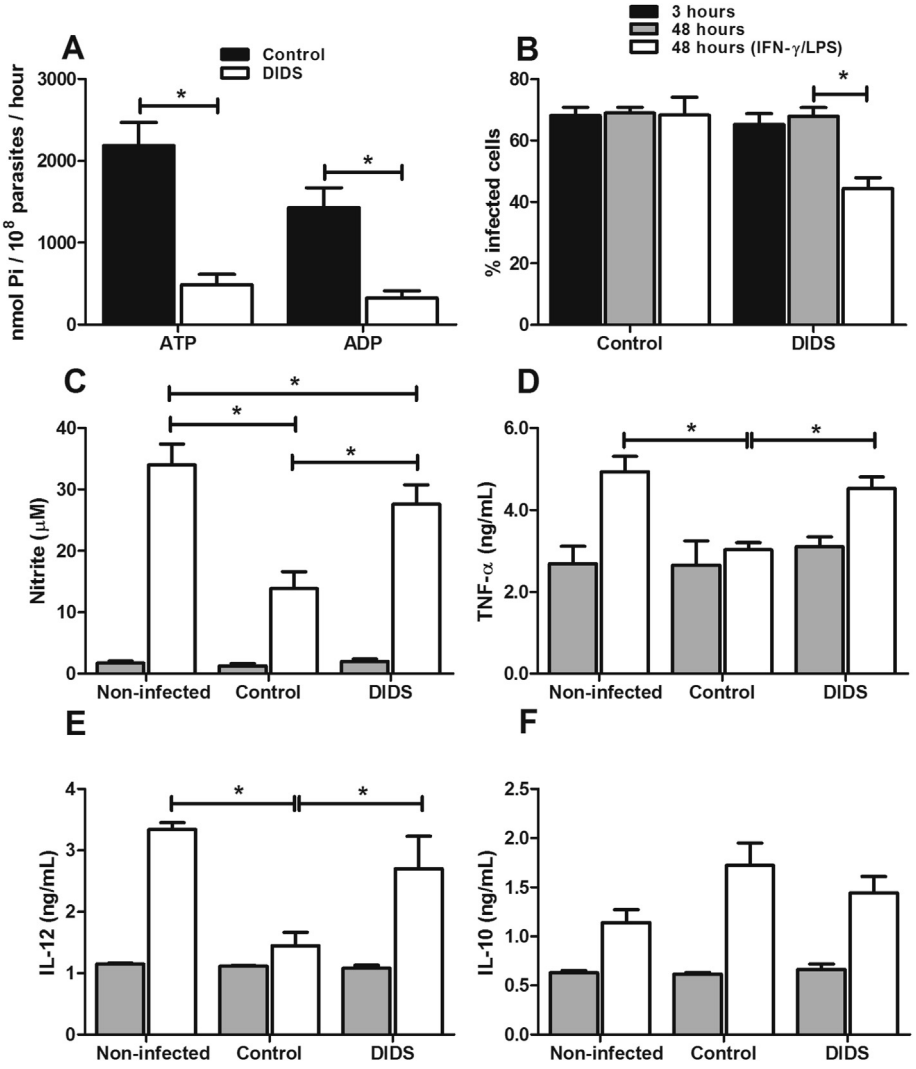

Fig. 3. Inhibition of ectonucleotidase activity of *L. amazonensis* reduces the survival and modulation capacity of the parasite. J774-macrophages were incubated, for 3 h, with metacyclic promastigotes of *L. amazonensis* (3 parasites/cell) that have been pre-incubated with 100 μM DIDS, washed and incubated for additional 48 h, in presence or not of IFN-γ/LPS. (A) Enzymatic activity was evaluated by the measurement of inorganic phosphate released. (B) Percentage of infected cells. (C) NO, (D) TNF-α, (E) IL-12, and (F) IL-10 production in 48-h supernatants. Bars represent the mean ± SD of three independent experiments performed in duplicate. *$p < 0.05$.

activation, when the original strain was grown in the presence of adenine. The decrease in the number of infected cells was associated with an infection rate (amastigotes per infected cell) similar to that observed in infection with the 1IIId strain (Fig. 1D and data not shown) indicating a similar mechanism of parasite control. In fact, the reduced resistance to activation by IFN-γ and LPS was accompanied by an increased NO production by cells infected with adenine-treated parasites (Fig. 2D). Our results strongly suggest that the activity of E-NTPDase on the surface of the parasite is involved in the parasite's survival within the macrophage by downmodulating the NO production by activated cells. To our surprise, although the adenine-treated parasites were less phagocytized than the control strain (similar to the 1IIId clone), these parasites seem more capable to disseminate to other cells than the original strain (Fig. 1C). The reason for this behavior remains unclear.

3.3. The ectonucleotidase activity of L. amazonensis *is important for down-modulation of TNF-α and IL-12*

The strong correlation between the activity of E-NTPDase and the ability of *L. amazonensis* in reducing NO production and favoring the survival of the parasite, as shown above, prompted us to evaluate the effect of the inhibition of this enzyme on the infective capacity of the parasite. Thus, we infected J774 cells with the original strain, which was pre-incubated for 30 min with 100 μM DIDS (4,4′-diisothiocyanatostilbene 2,2′-disulfonic acid). As previously shown [16], we observed that DIDS reduces the ectonucleotidase activity of E-NTPDase in *L. amazonensis* (Fig. 3A). As expected, inhibition of activity by DIDS decreased parasite survival (Fig. 3B) and prevented the reduction of NO production by stimulated J774 cells (Fig. 3C). These results confirm the role of E-NTPDase activity in modulating cells and increasing

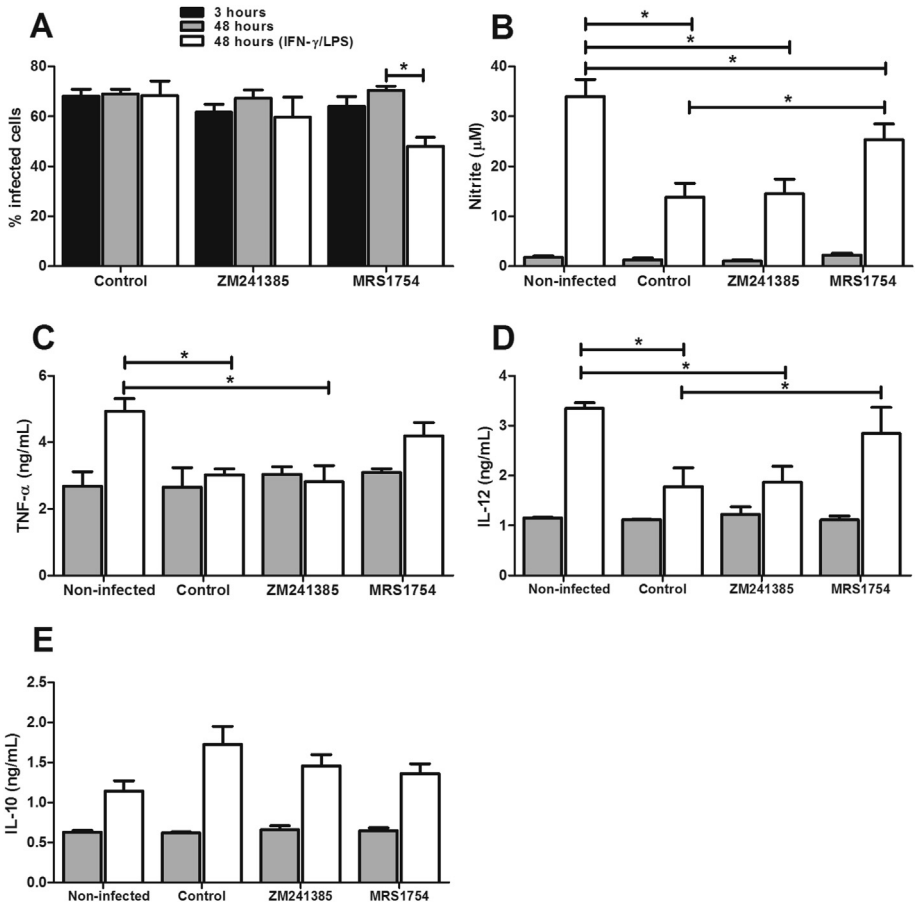

Fig. 4. Modulation of infected macrophages is mediated by the activation of the adenosine A_{2B} receptor. J774-macrophages were pre-incubated with 5 μM of ZM241385 (A_{2A}R antagonist) or MRS1754 (A_{2B}R antagonist) and infected with metacyclic promastigotes of *L. amazonensis* (3 parasites/cell) for 3 h, washed and for additional 48 h, in presence or not of IFN-γ/LPS. (A) Percentage of infected cells. (B) NO, (C) TNF-α, (D) IL-12, and (E) IL-10 production in 48-h supernatants. Bars represent the mean ± SD of three independent experiments performed in duplicate. *$p < 0.05$.

infection. To analyze whether this activity is also important for modulating cytokine production, we used indirect ELISA to investigate the profile of cytokines produced by infected cells. Infection with the original strain reduced the production of IL-12 and TNF-α by stimulated cells. The inhibition of E-NTPDase activity with DIDS reduced *L. amazonensis*'s capacity to down-modulate the release of inflammatory cytokines (Fig. 3D–E). No alteration was observed in IL-10 production, and DIDS treatment for 3 h did not alter the production of any of the cytokines or the production of NO by stimulated non-infected cells (data not shown). These data indicate that *L. amazonensis* ectonocleotidase activity is important for the down-modulation of inflammatory cytokines, which are essential for NO production.

3.4. Adenosine generated by E-NTPDase activity acts on the A_{2B} receptor

Adenosine plays an immunomodulatory role by binding to specific cell receptors, i.e., A_{2A} and A_{2B} [26]. To confirm whether E-NTPDase in *L. amazonensis* would exert its effects on the inhibition of pro-inflammatory cytokine production by supplying substrate for adenosine production, we used the metacyclic promastigotes of the original strain to infect J774 cells that were pre-incubated with specific antagonists of adenosine receptors A_{2A} (ZM241385) or A_{2B} (MRS1754). As shown in Fig. 4A, blocking A_{2B} receptors reduced parasite survival within stimulated cells. This result correlates with an increase in NO production (Fig. 4B). No alteration was seen with the A_{2A} antagonist. Interestingly, when we blocked the A_{2B} receptors, the inhibition of IL-12 and TNF-α production observed in stimulated cells infected with the *L. amazonensis* was reverted (Fig. 4C–D). No alteration was observed in IL-10 production (Fig. 4E). Also, treatment of stimulated non-infected cells with either adenosine receptor antagonist for 3 h did not alter the production of any of the cytokines or the production of NO (data not shown). In addition, macrophages were infected with the avirulent clone 1IIId in the presence of 50 μM of NECA, an analog of adenosine. As noted earlier, the avirulent clone with low ectonucleotidase activity has a decreased ability to survive in stimulated cells. However, in infections performed in the presence of NECA, the clone is able to survive within stimulated cells (Fig. 5A). This is due to the fact that the production of NO, IL-12 and TNF were reduced by cells stimulated in the presence of NECA (Fig. 5B–D). No change was observed for IL-10 production

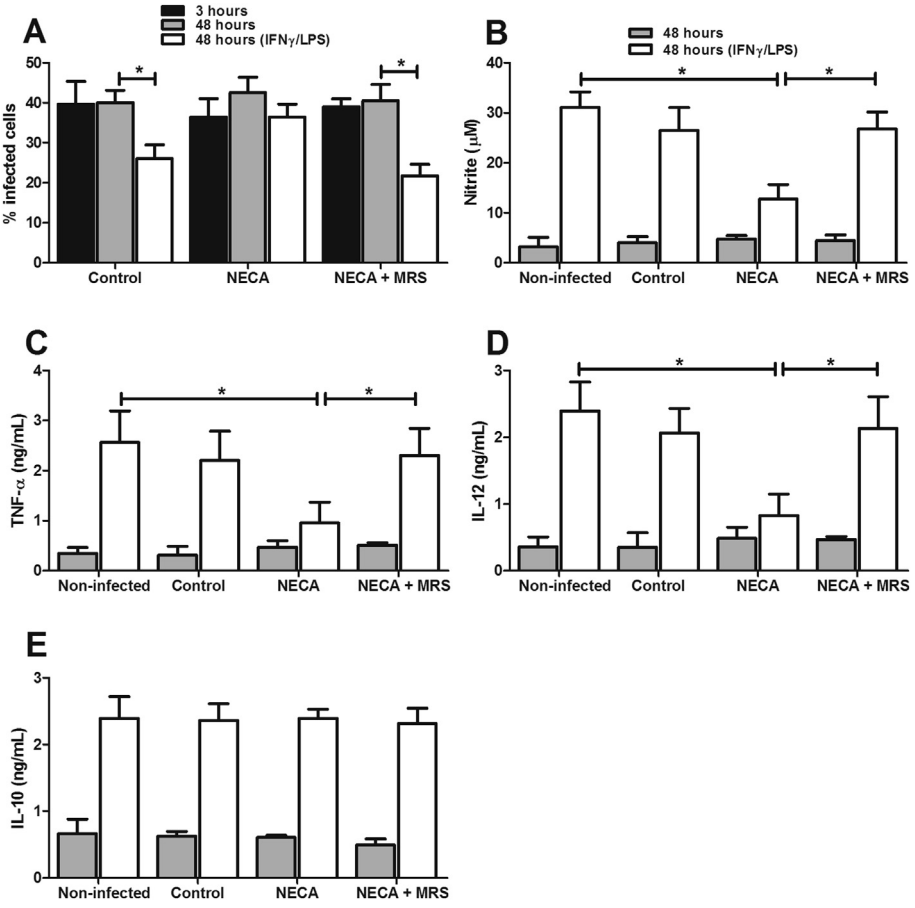

Fig. 5. Infection in the presence of NECA increases survival rates of low-activity parasite in stimulated macrophages. J774-macrophages were incubated, for 3 h, with metacyclic promastigotes of 1IIId clone of *L. amazonensis* (3 parasites/cell) in the presence of 50 μM NECA, washed and incubated for additional 48 h, in presence or not of IFN-γ/LPS. (A) Percentage of infected cells. (B) NO, (C) TNF-α, (D) IL-12, and (E) IL-10 production in 48-h supernatants. Bars represent the mean ± SD of three independent experiments performed in duplicate. *$p < 0.05$.

(Fig. 5E). On the other hand, preincubation of these cells with MRS1754 (antagonist of adenosine receptor A_{2B}) reverted the capacity of avirulent 1IIId clone infected with NECA in reduced inflammatory cytokines and NO production and enhanced survival of this parasite within stimulated cells (Fig. 5). Taken together, these results show that E-NTPDase activity in *L. amazonensis* is important for generating substrate for adenosine production; furthermore, we show here that adenosine acts on the A_{2B} receptor, thus reducing production of IL-12 and TNF-α, which are essential for the release of NO by stimulated cells.

4. Discussion

In this study, we investigated the role of *L. amazonensis* E-NTPDase in macrophage infection. We provide evidence that, by decreasing extracellular ATP concentration and allowing for increased adenosine production, this enzyme interferes with the activation of the infected macrophage by IFN γ/LPS reducing the production of inflammatory cytokines and NO production. To this aim, we took advantage of a clone of the PH8 strain of *L. amazonensis* that we had previously shown to have lower infectivity *in vivo* [22]. We also downmodulated the ectonucleotidase activity of the PH8 strain by culturing the parasites in excess of adenine. Our data show that the presence of E-NTPDase on the surface of the parasite leads to increased in vitro infectivity to J774 cells, which is associated with decreased NO production upon activation by IFN γ/LPS (Figs. 1 and 2). In addition to its activity in the hydrolysis of extracellular ATP, E-NTPDase has been shown to facilitate parasite adhesion to the host cell [34,35]. Our data confirm these observations by showing an increased parasite uptake (3 h) by macrophages incubated with the PH8 strain when compared to the 1IIId clone (Fig. 1C) or when the parasite was cultured in the presence of increased adenine concentrations (Fig. 2C).

L. amazonensis E-NTPDase is involved in the hydrolysis of extracellular ATP (which is secreted by macrophages under several circumstances) to AMP, which in turn generates adenosine through the activity of a 5′-nucleotidase [16,21]. While extracellular ATP has been shown to increase activated macrophage NO production [36,37], adenosine has been shown to down-modulate macrophage NO production by acting on two surface receptors (A_{2A} and A_{2B}) [38–41].

Inhibition of the parasite's ectonucleotidase activity resulted in reduced parasite survival in activated J774 cells that was associated with increased NO and inflammatory cytokine production (TNF-α and IL-12) (Fig. 3).

It has been shown that TNF-α is essential for the activation of iNOS in macrophages and with subsequent production of NO [3,42]. Furthermore, low IL-12 production should reduce NK and T cell generation of IFN-y required for macrophage activation [43]. Thus, we demonstrate that ectonucleotidase activity in *L. amazonensis* is also linked to a reduction in the macrophage inflammatory profile, when stimulated with IFN-γ and LPS. This shows that E-NTPDase expression on the surface of the parasite plays a key role in macrophage modulation and infection.

The fact that E-NTPDase provides AMP for adenosine production [16] suggests that modulation of the macrophage depends on adenosine production. In fact, blocking A_{2B} receptors increased NO and inflammatory cytokine production resulting in reduced survival of the parasite in activated cells (Fig. 4). Moreover, addition of NECA to low activity parasites resulted in recovery of the ability of these cells to sustain macrophage activation (Fig. 5). This demonstrates that binding of adenosine to the A_{2B} receptors is critical for the modulation of the infected macrophage. Interestingly, this effect was not observed for A_{2A} receptors. Therefore, adenosine acts on A_{2B} receptors and, by increasing cAMP inside cells, reduces the production of inflammatory cytokines and increases the production of regulatory cytokines [44,45]. This study shows an important association between adenosine production and E-NTPDase activity in *L. amazonensis* and that this association modulates infected macrophages.

It has been extensively shown that *L. amazonensis* down-modulates several macrophage functions [9,46,47], including NO production, which is one of the main leishmanicidal mechanisms of the infected cell [4,48]. In addition, several studies demonstrate that the initial steps of the interaction between *Leishmania* and the host cells are important in determining the outcome of the infection [7]. Our research group has demonstrated that E-NTPDase of *L. amazonensis* interferes with the outcome of infection in mice [21,22]. The present results extend these findings by showing that E-NTPDase activity is another mechanism by which this parasite interferes with the host immune response by down-modulating macrophage production of important cytokines such as IL-12 and TNF-α, as well as NO production. Furthermore, our data did not demonstrate an involvement of IL-10 in the control of the activation of the macrophage, a finding that is in agreement with previous findings in the literature that indicate that, in the case of the infection by *L. amazonensis*, IL-10 does not seem to control the response to the parasite [49].

The present work support the evidences for a correlation between activity/expression of E-NTPDase and the capacity of the parasite to induce injury *in vivo*, since the production of adenosine in the early stages of the infection may alter the ability of macrophages to be activated later, preventing the action of inflammatory stimulus on the host cell. This new mechanism represents an important target for therapeutic measures and paves the way for the development of new treatments for this neglected disease.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors wish to thank Leandro H. dos Santos, Marcorelio Divino de Souza, Amanda Braga de Figueiredo and Hellem Damazo for technical and scientific support in the laboratory. Luís C. C. Afonso is a research fellow from CNPq. This work received financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (scholarship to RSG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (304556/2013-0), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (CBB-APQ-01144-11), Rede de Pesquisas em Doenças Infecciosas Humanas e Animais no Estado de Minas Gerais (Rede 20/12), and Rede Mineira de Bioterismo (Rede 31/11).

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