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# Differential regulation of E-NTPdases during *Leishmania amazonensis* lifecycle and effect of their overexpression on parasite infectivity and virulence

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ABSTRACT

Infections caused by *Leishmania amazonensis* are characterized by a persistent parasitemia due to the ability of the parasite to modulate the immune response of macrophages. It has been proposed that ecto-nucleoside triphosphate diphosphohydrolase (*E*-NTPDases) could be able to suppress the host immune defense by reducing the ATP and ADP levels. The AMP generated from E-NTPDase activity can be subsequently hydrolyzed by ecto-nucleotidases, increasing the levels of adenosine, which can reduce the inflammatory response. In the present work, we provide new information about the role of *E*-NTPDases on infectivity and virulence of L. *amazonensis*. Our data demonstrate that not only the *E*-NTPDase activity is differentially regulated during the parasite development but also the expression of the genes *ntpd1* and *ntpd2*. E-NTPDase activity increases significantly in axenic amastigotes and metacyclic promastigotes, both infective forms in mammalian host. A similar profile was found for mRNA levels of the *ntpd1* and *ntpd2* genes. Using parasites overexpressing the genes *ntpd1* and *ntpd2*, we could demonstrate that L. *amazonensis* promastigotes overexpressing *ntpd2* gene show a remarkable increase in their ability to interact with macrophages compared to controls. In addition, both *ntpd1* and *ntpd2*-overexpressing parasites were more infective to macrophages than controls. The kinetics of lesion formation by transfected parasites were similar to controls until the second week. However, twenty days post-infection, mice infected with *ntpd1* and *ntpd2*-overexpressing parasites presented significantly reduced lesions compared to controls. Interestingly, parasite load reached similar levels among the different experimental groups. Thus, our data show a non-linear relationship between higher *E*-NTPDase activity and lesion formation. Previous studies have correlated increased ecto-NTPDase activity with virulence and infectivity of *Leishmania* parasites. Based in our results, we are suggesting that the induced overexpression of *E*-NTPDases in L. *amazonensis could* increase extracellular adenosine levels, interfering with the balance of the immune response to promote the pathogen clearance and maintain the host protection.

## 1. Introduction

Leishmaniasis is a neglected tropical disease caused by unicellular eukaryotic protozoa belonging to the genus *Leishmania*. It is estimated that 0.7–1 million new cases of leishmaniasis occur worldwide, and some 26,000 to 65,000 deaths occur annually [68]. *Leishmania* parasites develop as extracellular flagellated promastigote stages in the digestive tract of the sand fly vector. After injection into a mammalian host, the

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promastigotes are phagocytosed by various host cells, including neutrophils, dendritic cells (DC) and macrophages. Subsequently, parasites differentiate to a non-motile and spherical amastigote stage, which proliferates inside of phagolysosome compartments of macrophages found in the hosts, such as humans, dogs, cats and rodents [10].

In humans, different phenotypes of the disease can occur depending on the species of the parasite, including localized cutaneous lesions to disseminated muco-cutaneous and lethal visceral infections. About 8% of cutaneous leishmaniases in Brazil are attributed to *Leishmania amazonensis*. This species can cause simple and diffuse forms of cutaneous leishmaniasis and has been recently implicated in borderline disseminated cutaneous leishmaniasis, an intermediate form of the disease [6,58]. Infections caused by L. *amazonensis* are characterized by a persistent parasitemia due to the ability of the parasite to modulate the immune response of the macrophages [1,29,45].

The enzyme ecto-nucleoside triphosphate diphosphohydrolase (*E*-NTPDase, CD39_GDA1 protein superfamily, EC 3.6.1.5) hydrolyzes a series of tri-and diphosphates nucleosides to the monophosphate forms [69,70]. In pathogenic trypanosomatids, nucleotidases/*E*-NTPDases have been implicated in purine salvage pathways, adhesion in the cell host, infectivity and virulence processes [16,20,42,54,57]. Extracellular ATP is sequentially hydrolyzed to ADP and AMP by Ecto-NTPDase (CD39). The AMP generated can be subsequently hydrolyzed by Ecto-5′-nucleotidase (CD73) of either pathogen or host cells, increasing the levels of adenosine, which can reduce the inflammatory response. It has been proposed that *E*-NTPDases could be able to suppress the host immune defense by reducing the ATP and ADP levels [3,19,53].

Purinergic signaling is mediated *via* two families of purinergic receptors, P1 and P2. P2 receptors are responsive to ligands tri- and diphosphonucleotides, while P1 receptors use adenosine as a ligand. Extracellular nucleotides, including adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) can modulate the function of cells through ligand-gated ion channels (P2X) or G protein-coupled receptors (P2Y), respectively [31]. An increase in the levels of extracellular ATP is interpreted by immune system as a danger signal and triggers an inflammatory response by activating $P2X_7$ receptor [30,63]. Extracellular ATP is a potent inducer of inflammation characterized by macrophage and dendritic cells activation and increased production of IL-12 and TNF-α by these cells, leading to elimination of intracellular pathogens.

On the other hand, extracellular adenosine, acting on P1 receptors, modulates the inflammatory response by increasing the intracellular cAMP concentration [17]. Thus, adenosine inhibits the production of inflammatory cytokines by macrophages and dendritic cells and the production of microbicidal effectors by neutrophils and macrophages. In addition, adenosine stimulates the synthesis and release of IL-10, one of the major regulatory cytokines [2,16,26,67]. Thus, both ATP and adenosine may have dual effects on immune responses, depending on concentration, the duration of the exposure, and the conditions of the *in vivo* environment. The ultimate effect of ATP and adenosine during immune responses depends on the balance between the two molecules [71].

The sequential hydrolysis of extracellular ATP to adenosine indicates that not only *E*-NTPDases but also ecto-5′-nucleotidases are present in several protozoan parasites including those belonging to the genera *Leishmania* and *Trypanosoma* [20,38,47,54]. The activity of ecto-nucleotidase has been demonstrated in L. *amazonensis* [48]. An active and antigenic NTPDase isoform was isolated from the plasma membrane fraction from L. *amazonensis* promastigote [7,8]. Posteriorly, a study identified a specific NTPDase 1 by western blot and immunolocalization. The NTPDase 1 is localized on the membrane surface and subcellular organelles, including mitochondria and nucleus of L. *amazonensis* promastigotes [13].

Two genes encoding putative NTPDases (NTPDase 1 and NTPDase 2) can be found in L. *amazonensis* genome [42,53]. The peptide sequences deduced from the *ntpd1 and ntpd2* genes contain all the five conserved ACR domains called “apyrase conserved regions” (ACRs), which are defining feature of prokaryotic and eukaryotic NTPDases [27,44,54].

In the present work we demonstrated that the *E*-NTPDase activity is differentially regulated throughout the lifecycle of L. *amazonensis,* increasing significantly in amastigotes and metacyclic promastigotes, both infective forms in mammalian host.

In addition, our results showed that induced overexpression of the genes *ntpd1* and *ntpd2* increased the parasite infection in axenic macrophages. However, mice transfected with parasites overexpressing *ntpd1* and *ntpd2* presented reduced lesion when compared to control. Based on this contradictory effect, we hypothesize that the induced overexpression of *E*-NTPDases in L. *amazonensis could* increase extracellular adenosine levels, interfering with the balance of the immune response to promote the pathogen clearance and maintain the host protection.

## 2. Materials and methods

### 2.1. Materials

The reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), Cripion Biotecnologia LTDA (Andradina, SP, Brazil), LGC Biotecnologia (Cotia, SP, Brazil), GE Healthcare Biosciences (Pittsburgh, PA, USA), Thermo Fisher (Waltham, MA, USA), Zymo Research (Irvine, CA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA). Water used in preparation of all solutions was purified with a four-stage Milli-Q system (Millipore Corp., Bedford, MA, USA), [γ-$^{32}$P] ATP was prepared as described by Glynn and Chappell [23].

### 2.2. Parasites and growth conditions

The MHOM/BR/75/Josefa strain of L. *amazonensis* was used in this work. This strain was isolated by Dr. Cuba-Cuba (Universidade de Brasília, Brazil) from a human case of diffuse cutaneous leishmaniasis in Brazil and has been maintained within our laboratory in both axenic culture and through hamster footpad inoculation. Promastigotes were maintained in axenic culture in Schneider’s insect medium supplemented with 10% (*v*/v) heat-inactivated fetal bovine serum (FBS, Cripion) at 22 °C for two days. Afterwards, the parasites were harvested from the culture medium by centrifugation at 1500*g* at 4 °C for 10 min and washed three times in a cold buffer solution containing 100 mM sucrose, 20 mM KCl and 50 mM Tris (pH 7.2). In order to obtain axenic amastigote forms, parasites were maintained in Schneider’s medium culture (pH: 5.4) supplemented with 5% fetal calf serum (FSC) at 32 °C with a generation time of approximately 7–10 days [61]. Metacyclic promastigotes form were purified from stationary phase Schneider’s medium cultures (day 7–8) by differential centrifugation over a Ficoll gradient as previously described [60]. Parasites were maintained in culture for no more than 10 passages. The number of parasites was estimated by counting in Neubauer chamber. Cell viability was then estimated using the MTT assay [39]. Cell viability was not compromised under the conditions employed in this work.

### 2.3. Ecto-ATPase and Ecto-5′-nucleotidase activities measurements

Intact promastigote, amastigote and metacyclic promastigote forms were collected by centrifugation and washed twice with buffer A. After that, $5 \times 10^7$ cells/mL were incubated for 1 h at 22 °C in 0.5 mL of a mixture containing 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM D-Glucose, 50.0 mM Hepes–Tris buffer, pH 7.2, 5.0 mM ATP in the absence or presence of 5.0 mM $MgCl_2$, unless otherwise specified. The Mg-dependent ecto-ATPase activity was calculated from the total activity measured in the presence of 5 mM $MgCl_2$ minus the basal activity measured in the absence of $MgCl_2$. The ATPase activity was determined by measuring the hydrolysis of [γ-$^{32}$P] ATP ($10^4$ Bq/nmol ATP; specific activity of approximately $10^4$ Bq/nmol ATP) [4]. The experiments were started by the addition of the cells and stopped by the addition of 1.0 mL

of ice-cold 25% charcoal in 0. 1 M HCl. To avoid inorganic phosphate (Pi) contamination, the charcoal suspension was washed at least 20 times with 0.1 M HCl before it was used [24]. The tubes were then centrifuged at 1500 *g* for 20 min at 4 °C. Aliquots (0.5 mL) of the clear supernatants containing the released $^{32}$Pi (inorganic phosphate) were dried on filter paper, added to 9 mL of scintillation liquid (2.0 g PPO in 1 L of toluene) and counted in scintillation counter. The ecto-ATPase activity was also assayed by measuring the release of Pi from ATP hydrolysis in a spectrophotometer. The hydrolytic activities were measured under the same conditions as those describe above. After the reaction, the tubes were centrifuged at 1500 *g* for 10 min at 4 °C, and 0.1 mL of the supernatant was added to 0.1 mL of Fiske-Subbarow reactive mixture to measure the concentration the Pi released into the supernatants by spectrophotometry at 650 nm [18]. The concentration of Pi was determined using a standard curve for comparison. The values obtained for the ATPase activities (ATP hydrolysis) were exactly the same for both the spectrophotometric and radioactive methods.

To evaluate the ecto-5′-nucleotidase activity, *L. amazonensis* promastigotes ($5 \times 10^7$ cells/mL) were incubated for 1 h at 37 °C in 0.5 mL of a mixture containing 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM D-Glucose, 50.0 mM Hepes–Tris buffer, pH 7.2 and 5.0 mM 5′-AMP as substrate. The ecto-5′-nucleotidase activity was determined spectrophotometrically by measuring the release of Pi from the nucleotides, under the same conditions described above for ecto-ATPase activity.

### 2.4. Western blot analysis

Total protein extracts from promastigotes, axenic amastigotes and metacyclic promastigotes were prepared for western blot analysis. The cells were harvested, washed twice with PBS, and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM EGTA, 2 mM EDTA, 20% glycerol (*v*/v), 0.1% Triton X-100 (v/v), 1 mM PMSF and protease inhibitor mixture (Sigma®). The lysates were clarified by centrifugation (16,000 *g* for 15 min at 4 °C), and the protein concentrations of the supernatants were determined by the Bradford method using bovine serum albumin (BSA) as a standard [5]. Samples (50 μg each) were subjected to 10% SDS polyacrylamide gel electrophoresis, and subsequently, the proteins were electrotransferred to nitrocellulose membrane (GE Healthcare). Membranes were blocked with 5% (*w*/*v*) nonfat dry milk in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl containing 0.1% *v*/v Tween-20). The membranes were washed three times for 20 min with TBS-T buffer and incubated with polyclonal goat antisera CD39 of human origin (1:500) (Santa Cruz Biotechnology, USA) and monoclonal mouse anti-α-tubulin (10D8):sc-53,646 (1:5000 – used as control) for 2 h at 25 °C. After washing with TBS-T, the membranes were incubated with the secondary antibody goat anti-mouse IgG horseradish peroxidase (Sigma®) diluted to 1:10000. Protein signals were revealed using the Super-Signal® West Pico Chemiluminescent ECL substrate (Thermo Fisher Scientific) following the manufacturer's instructions. Densitometry analyses was performed using ImageJ software version 1.50i (NHI Image, Bethesda, MD, USA) with background correction.

### 2.5. RNA isolation and quantitative PCR (qPCR)

Total RNA was extracted from the samples ($10^8$ cells) using TRIzol® Reagent (Invitrogen Corporation, Carlsbad, USA) according to the manufacturer's instructions. Total RNA concentrations were determined spectrophotometrically at 260 nm using a Nanodrop ND-1000 system (Thermo Scientific, Wilmington, USA). Only RNA samples that showed an A260/A280 ratio between 1.8 and 2.0 were used for cDNA synthesis. Total RNA integrity was checked by means of agarose gel electrophoresis. The cDNA synthesis was carried out using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) and one microgram of total RNA, previously treated with RNase-free DNAse I (Fermentas International Inc., Burlington, Canada). For the quantitative polymerase chain reaction assays (qPCR), 10 ng/μL cDNA were used per well in a reaction medium of 15 μL, containing 5 μM of primer mix and 7 μL of PowerUp Sybr Green Master Mix (Thermo Fisher) were used according to the following conditions: one cycle for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C. Fluorescent readings were performed by Step One PlusTM Real-Time PCR (Thermo Fisher). Genes were amplified using the primers described in Table 1. All assays were performed in triplicate, and gene expression data were normalized to an endogenous reference (α-Tubulin, GAPDH and 18S ribosomal RNA) (Table 1). All primers were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). In order to check amplification specificity, qPCR amplification was followed by melting curve analysis, and the reaction products were subjected to agarose gel electrophoresis. The determination of the expression ratios was performed using the cycle threshold (ΔΔCT) method described by Livak and Schmittgen [34].

### 2.6. Construction of the pXG-NTP1 and pXG-NTP2 and parasite transfection

The full-lengh ORFs encoding ***ntpd 1*** (GenBank: AFJ75402.2, ATP diphosphohydrolase) and ***ntpd 2*** (TriTrypDB: LAMA_000158100.1, GDA1/CD39 nucleoside phosphatase family) genes were amplified by PCR using genomic DNA of L. *amazonensis* as the template (primers described in Table 1). Amplification products were subcloned into restriction site *BamHI* of pXG-neo expression vector [25]. Purified plasmids pXG-NTPD1 and pXG-NTPD2 were obtained using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen) and sequenced to confirm the insertion. The constructs were used for cell transfections of L. *amazonensis* promastigotes by electroporation using an Amaxa Nucleofector II device with human T-cell buffer (Lonza). To this end, $10^7$ promastigotes were harvested by centrifugation, washed with PBS, and resuspended in Nucleofector™ Solution for Human T Cells (Lonza) and the cell suspension was mixed 10 μg of plasmid DNA in a 0.2 cm gap cuvette. The parasites were placed on ice for 10 min and subjected to 1 electric pulse under program U-033 in the device. After electroporation, the cells were placed on ice for 10 min, transferred to 2 mL Schneider's insect medium supplemented with 20% FBS, 1% penicillin-streptomycin cocktail and incubated at 22 °C over 24 h. After this period, transfected parasites were selected by four weeks of culturing in the presence of G418 antibiotic concentrations (100 μg/mL). Wild-type (WT) parasites, mock overexpressor (transfected with an empty version of pXG-*neo*), pXG-NTPD1 and pXG-NTPD2 were obtained and used in our experiments. The expression levels of *ntpd 1* and *ntpd 2* genes were analyzed by measuring the enzyme activities and qPCR, as detailed above.

### 2.7. Parasite-macrophage interaction assay

Immortalized Bone marrow-derived macrophages (IBMM) were plated at $1 \times 10^6$ cells per well (0.5 mL) onto round coverslips in RPMI

**Table 1**
Primers used for PCR and qPCR reactions.

| Primer forward | Primer reverse |
|---|---|
| (PCR)LaNTPDase1 5′-**GGATCC**ATGCGTCCGTACTCCTCGGT-3′ | 5′-GGATCCTTCCATCTTGAGCAGGAAGG-3′ |
| (PCR)LaNTPDase2 5′-**GGATCC**ATGGGTCGCCTCGTCGTTG-3′ | 5′-GGATCCGCCGCCCCGCCCTCCACTA-3′ |
| (RT)LaNTPDase1 5′-GTACGCGAACGTCTACGACA-3′ | 5′-CCCGGCTCTATACGTTTGAA-3′ |
| (RT)LaNTPDase2 5′-AGAACGCCGTGATGAAAAGT-3′ | 5′-GATGCTCACCAGCTCCTCTC-3′ |
| (RT) GAPDH 5′-TGGAGCTGCGGTTGTCATT-3′ | 5′-AGCGCGCGTCTAAGACTTACA-3′ |
| (RT) a-Tubulin 5′-TCAAGTGCGGCATCAACTAC-3′ | 5′-GAGTTGGCAATCATGCACAC-3′ |

1640 medium in 24-well plates. Cells were incubated overnight at 37 °C under a 5% $CO_2$ atmosphere. Non-adherent cells were removed by washing with phosphate-buffered saline (PBS). *L. amazonensis* promastigotes were added to the culture at 1:1 or 5:1 parasite-to-cell ratio to measure association and survival indexes, respectively. After 3 h or 24 h of co-culture, coverslips were collected for evaluation of parasite adherence and internalization. Coverslips were fixed and stained with Panoptic kit (Laborclin, Pinhais, PR, Brazil), according to manufacturer's instructions. The percentage of infected macrophages was determined by counting 200 cells in triplicate coverslips. The indexes were determined by multiplying the percentage of infected macrophages by the mean of the parasites per cell. Statistical analysis all experiments were performed in triplicate, with similar results obtained from at least three separate cell suspensions.

### 2.8. In vivo infection in a murine model

BALB/c mice (5 animals per experimental group) were maintained under specific pathogen-free conditions. $5 \times 10^6$/10 μL promastigotes of L. *amazonensis* (wild type, pXG-NTPD1, pXG-NTPD2 or empty pXG) were inoculated intradermally in the right ear using a $27^{1/2}$-gauge needle. The lesion sizes were measured twice per week using a dial caliper until the end of experiment (day 36) when the animals were euthanized.

### 2.9. Ethics statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto Oswaldo Cruz (CEUA-IOC, License Number: L-11/2017). All data were analyzed anonymously.

### 2.10. Parasite load quantification

Parasite load was determined using a quantitative limiting dilution assay as described previously [28]. The infected ears were excised, weighed and minced in Schneider's medium with 10% fetal calf serum, and the resulting cell suspension was serially diluted. The number of viable parasites in each ear was estimated from the highest dilution at which promastigotes could be grown at 26 °C.

### 2.11. Statistical analysis

All values presented in this work represent the mean ± S.E.M. of at least three independent experiments. For *in vivo* infection and parasite load quantification experiments, the results were analyzed statistically using two-way analysis of variance (ANOVA), followed by Bonferroni's post-test. In the other experiments, the data were submitted to one-way analysis of variance (ANOVA), followed by Tukey's Multiple Comparison test. All statistical analyses were performed using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was considered at $p < 0.05$.

## 3. Results

### 3.1. Differential regulation of E-NTPDase throughout L. amazonensis lifecycle

The genes encoding the putative NTPDases were obtained after a search in L. *amazonensis* genome database [51]. As previously described, a smaller isoform named NTPDase 1 (~47 kDa) (GenBank: AFJ75402.2) and a larger isoform named NTPDase 2 (~72 kDa) (TriTrypDB: LAMA_000158100.1) contain the five ACR domains typical of prokaryotic and eukaryotic NTPDases [32,42,44,53].

These genes are conserved among all sequenced *Leishmania* species, with homologues present in L. *infantum, L. braziliensis, L. donovani, L. major,* L. *tropica* and *L. mexicana* [42,44,54]. Using the nomenclature previously proposed for parasite NTPDases [54], we will refer to AFJ75402.2 as NTPD1 and LAMA_000158100.1 as NTPD2.

Ecto-ATPase activity was determined in the three life forms of L. *amazonensis* using intact promastigote, amastigote and metacyclic promastigote parasites. As shown in Fig. 1A, enzyme activity was significantly different among the three life stages. Axenic amastigotes and metacyclic promastigotes exhibited the highest ecto-ATPase activity and promastigote form exhibited the lowest activity level. A similar profile was found for mRNA levels obtained through qPCR experiments for both NTPD1 and NTPD2 (Fig. 1B). Western blot analysis using a CD39 antibody was able to recognize NTPD1. The antibody showed reactivity only against NTPD1 probably due to the higher sequence similarity (Supplementary Fig. S1A and B). As shown in Fig. 1C, NTPD1 was poorly expressed in promastigote form, increased significantly in axenic amastigotes, and reached the highest expression level in metacyclic promastigotes. Densitometry analysis of such bands confirm that NTPD1 is differentially expressed among the three life forms of *L. amazonensis* (Fig. 1D). Taken together, these data demonstrate that not only the genes *ntpd1* and *ntpd2* are differentially regulated among the different parasite stages but also the ecto-ATPase activity and the NTPD1 protein expression.

### 3.2. Evaluation of overexpression of E-NTPDase on L. amazonensis infectivity

We studied the effect of the overexpression of NTPD1 and NTPD2 on infectivity and virulence of the parasites. For this purpose, the genes *ntpd1* and *ntpd2* were cloned in pXG expression vector and then transfected into wild type L. *amazonensis* promastigotes. Ecto-ATPase activity was four-fold higher in promastigotes transfected with *ntpd1* and *ntpd2* when compared to controls (*L. amazonensis* promastigotes wild-type and promastigotes transfected with the empty vector, pXG) (Fig. 2A). Similar results were obtained with amastigotes and metacyclic promastigotes overexpressing the genes *ntpd1* and *ntpd2* (Supplementary Fig. S2A and B). It is worth mentioning that parasites overexpressing the genes *ntpd1* and *ntpd2* showed ecto-5′-nucleotidase activities similar to that observed in controls (WT and pXG) (Fig. 2B).

Transfectant parasites were subjected to qPCR, and the results showed that transcription levels were seven-fold higher in overexpressor parasites than in controls (Fig. 2C), confirming the efficiency of transfection. In addition, parasites overexpressing the genes *ntpd1* and *ntpd2* showed similar growth curve in comparison with the controls, WT and pXG (Fig. 2D).

To evaluate the role of NTPD1 and NTPD2 in the macrophage interaction, transfectants promastigotes were incubated with IBMM macrophages for 3 and 24 h. The adhesion and the survival of parasites-macrophages were evaluated by optical microscopy. Our results demonstrate that L. *amazonensis* promastigotes overexpressing the *ntpd2* gene show a remarkable increase in their ability to interact with macrophages compared to controls, when incubated with macrophage for 3 h (Fig. 3A and B). On the other hand, both *ntpd1* and *ntpd2*-overexpressing parasites were more infective to macrophages than controls at 24 h of infection (Fig. 3A and C).

In addition, we also investigated the *in vivo* infectivity of L. *amazonensis* overexpressing the genes *ntpd1* and *ntpd2*. For this purpose, transfected promastigotes were inoculated intradermally in the ear of BALB/c mice ($2 \times 10^5$ cells in 10 μL of PBS). The kinetics of lesion formation by transfected parasites were similar to controls until the second week. However, twenty days post-infection, the mice infected with *ntpd1* and *ntpd2* presented significantly reduced lesions compared to the lesions of mice infected with controls, WT and pXG ($p < 0.05$ and $n = 5$). This difference persisted until the end of the experiment (day thirty-six), when the mice were sacrificed (Fig. 4A). After euthanasia,

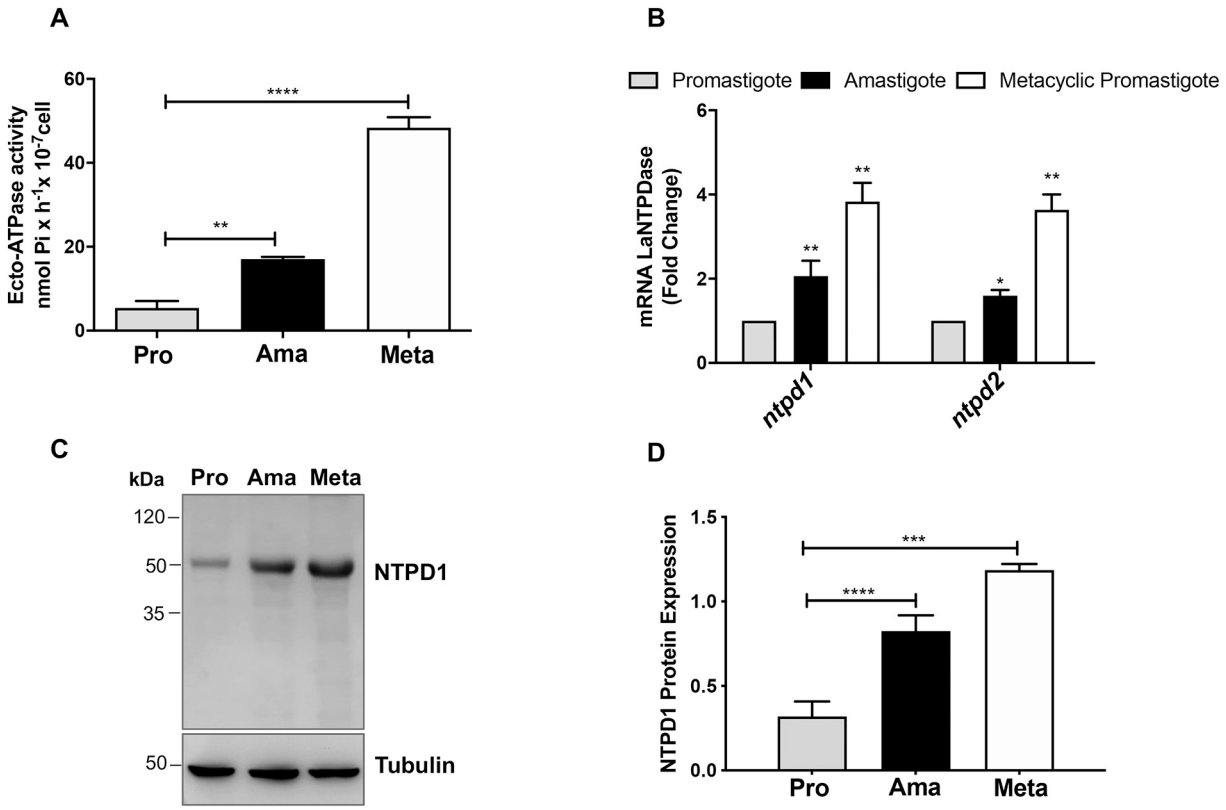


**Fig. 1.** Ecto-ATPase activity and *E*-NTPDases expression are differentially regulated during the life stages of L. *amazonensis*. (A) Activities $Mg^{2+}$-dependent ecto-ATPase of L. *amazonensis* promastigotes, amastigotes and metacyclic promastigotes. The cells were collected after three days at 22 °C in Schneider's medium, washed and then assayed. Enzymatic activity was evaluated by the measurement of inorganic phosphate released. Results represent the mean ± S.E.M of five independent experiments performed in triplicate.
(B) The mRNA levels of the *ntpd1* and *ntpd2* genes were determined by qPCR using the relative expression of Tubulin, GAPDH and RNA18S, as housekeeping genes. The expression ratio was calculated by the $2^{-\Delta\Delta Ct}$ method, in which promastigote stage expression was defined as 1. Bars represent mean ± S.E.M. of five independent experiments with technical triplicates.
(C) Protein levels were analyzed by western blot using antibodies CD39 and α-tubulin as a loading control and for normalization of the densitometric analysis. Image shown is representative of three independent experiments (D) Relative protein amounts were determined by densitometry using ImageJ software. Data represent means ± S.E.M of three independent biological samples. In all cases the significant differences were assessed by one-way ANOVA followed by Tukey's Multiple Comparison Test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ indicate significant differences relative to promastigote stage.

the parasite load in the infected ears was determined. Fig. 4B shows that the parasite load reached similar levels among the different experimental groups, although the lesions were smaller in the mice infected with *ntpd1* and *ntpd2*-overexpressing parasites, compared to controls.

## 4. Discussion

Nucleoside triphosphate diphosphohydrolases have been implicated in a variety of cellular processes in *Leishmania* parasites, including cell growth, cellular adhesion, infectivity and virulence [11,42]. The modulation of *ntpd1* and *ntpd2* gene expression among the different parasite stages as well as the higher Ecto-ATPase activity levels in infective forms (promastigotes metacyclic and amastigotes) corroborates the hypothesis that these enzymes could carry out an important role in *Leishmania* infectivity. In *T. cruzi,* the *ntpd1* gene is also overexpressed in infective forms, trypomastigote and amastigote [56].

Nucleotidase activity is higher in infective metacyclic promastigotes of L. *amazonensis* than in other *Leishmania* species known to be less virulent, such as L. *braziliensis* and L. *major*. Such increased virulence has been attributed to the higher expression of NTPDase on L. *amazonensis* surface [35].

Furthermore, intracellular amastigote stage of L. *amazonensis* showed increased Ecto-ATPase activity when compared to promastigote form [48]. While *E*-NTPDase activity in amastigote forms has been associated to nutritional requirements of the parasite inside mammalian macrophages, E-NTPDase activity in metacyclic promastigote forms is involved in virulence, influencing the host inflammatory responses [48,59].

As previously demonstrated, in L. *amazonensis* and L. *braziliensis* promastigotes, the enzyme NTPDase 1 was identified on plasma membrane surface, flagellar pocket and flagellum, and in subcellular organelles, including mitochondria and nucleus [7,13,48–50].

The distribution pattern of these NTPDases among *Leishmania* species could indicate different roles for these enzymes, such as involvement in distinct metabolic pathways, modulation of growth and morphological changes during parasite lifecycle [13,55]. In L. *major,* NTPDase 1 was found in the Golgi apparatus and is required for normal lesion development triggered by promastigote forms, whereas its counterpart NTPDase 2 is preferentially secreted and is dispensable for virulence [55].

In L. *infantum chagasi,* NTPDase showed a ubiquitous distribution in the cell, having been found on the cell surface, mitochondria, nuclei, kinetoplasts, flagella, flagellar pocket regions and internal vesicles [64]. Recently, the NTPDase 1 was also identified in amastigotes of L. *infantum* using an immunocytochemical approach [36].

The overexpression of *ntpd2* gene increased the adherence of parasite on macrophage, suggesting the involvement of this ecto-nucleotidase in the early stage of infection by L. *amazonensis,* when the attachment of parasites to the host cell occur (Fig. 3A). In fact, different hypotheses have been proposed to explain the involvement of NTPDase 2 in the

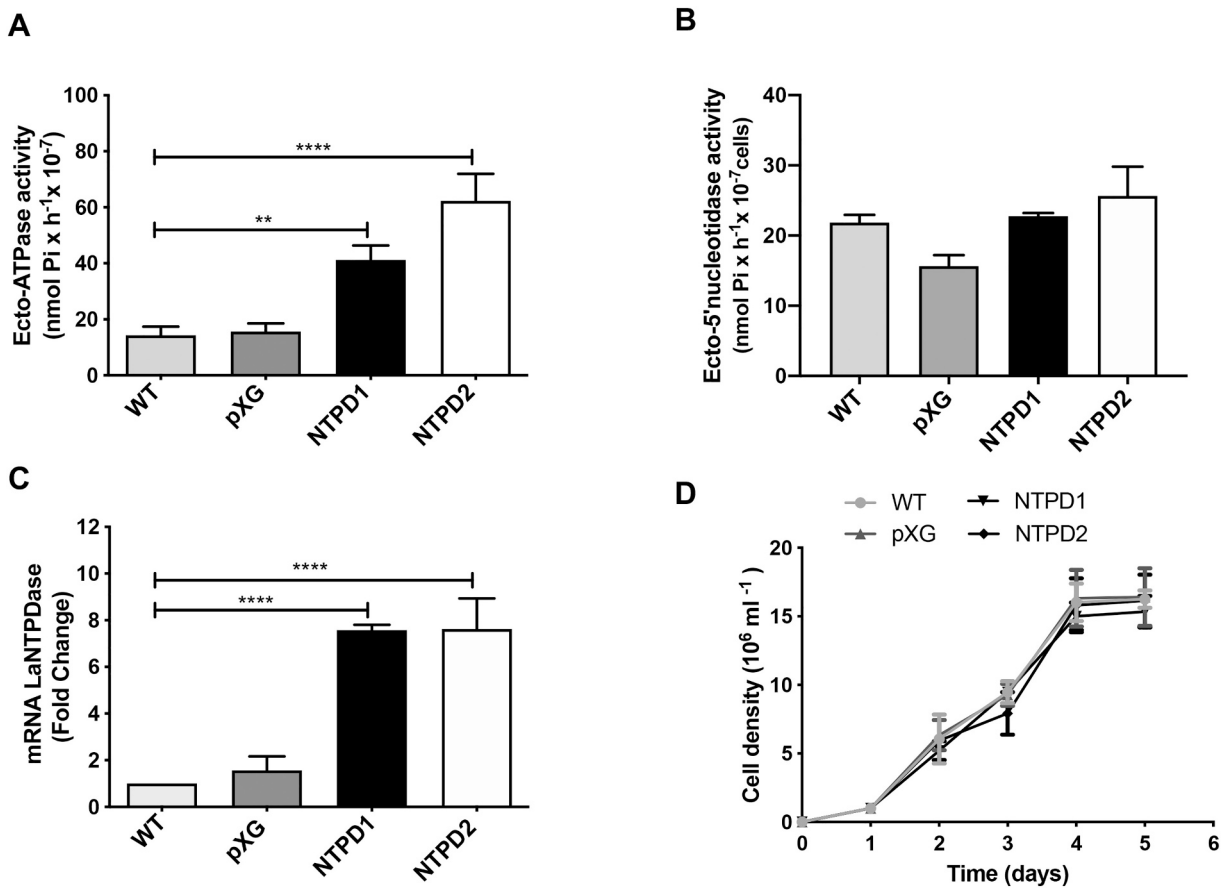


**Fig. 2.** Validation of parasite cell lines generated in this study. (A) $Mg^{+2}$-dependent ecto-ATPase and (B) ecto-5'nucleotidase activities were determined in control cell lines, *L. amazonensis* promastigotes wild type, WT and pXG (mock-complemented) and overexpressing cell lines for NTPD1 (pXG-NTPD1) and NTPD2 (pXG-NTPD2). (C) Quantification of transcripts were determined by qPCR for both *ntpd1* and *ntpd2* genes in cells lines (WT, pXG, NTPD1 and NTPD2). (D) Growth curves in Schneider's Insect medium of L. *amazonensis* promastigotes (WT) and pXG, NTPD1 and NTPD2. Curves represent the means ± S.E.M. of cell densities determined by cell counting from four independent experiments. $^{**}p < 0.01$ and $^{****}p < 0.0001$ indicate significant differences relative to the control group (WT and pXG).

adherence of parasite on macrophage. For instance, Peres et al. [46] showed that the incubation of macrophages with recombinant NTPDase 2 prior to addition of L. *infantum* parasites reduced both adhesion and infection. The authors suggest that not only the NTPDase 2 activity is important in establishing the infection, but also the enzyme localization on the surface of the parasite might favor adhesion to phagocytes.

In L. *amazonensis,* pretreatment of promastigotes with anti-NTPDase antibodies also reduced the interaction with macrophage [48]. In addition, the incubation of promastigotes with CrATP, an analogue of ATP and inhibitor of ecto-ATPase, reduced significantly the adhesion and endocytic indices of L. *amazonensis*, indicating the involvement of ecto-ATPases in the macrophage invasion process [14,40].

It has been proposed that the hydrolysis of ATP and ADP nucleotides by *E*-NTPDases could be able to down-modulate the host immune response [19,53]. The mechanism by which such modulation takes place requires the involvement of two ecto-enzymes, an E-NTPDase and an ecto-5′-nucleotidase. The first enzyme hydrolyzes ATP to ADP and subsequently to AMP, which is hydrolyzed by the second enzyme, increasing adenosine levels. Aside from these enzymes, *L. amazonensis* also express another remarkable nucleotidase, the ecto-3′-nucleotidase/nuclease (ecto-3′-NT/NU) as an important source of adenosine *via* 3′-AMP hydrolysis [21,43].

ATP exerts its effects by binding to P2 receptors which are divided in two subtypes: P2X receptors, that are associated to ionic channels and protein G coupled-P2Y. In immune cells, P2X7 is the main ATP receptor and its activation accounts for most of the inflammatory effects of extracellular ATP. ADP activates $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, which are members of the G protein coupled P2Y receptor [31], and the activation of P2 receptors by nucleotide secretion is likely to represent a physiological mechanism for parasitism control [9]. Recent studies show that the $P2Y_2$ and $P2X_7$ receptors (activated by UTP/ATP and ATP, respectively) cooperate to control L. *amazonensis* infection [62].

In L. *amazonensis,* extracellular adenosine binds to specific receptors on macrophage membrane, the P1 receptors [15]. This interaction leads to the reduction of IL-12 and TNF-α cytokines by activated macrophages, which in turn inhibit nitric oxide ($NO^{\bullet}$) production resulting in the persistence of parasite in the infected cells [22,41,65].

The presence of adenosine at the time of *Leishmania* infection augments the host-parasite interaction, increases the tissue parasitism and delays the lesion resolution [22]. On the other hand, interference in the adenosine signaling, such as blockade or ablation of adenosine receptor or enzymatic removal of adenosine at the time of infection, results in reduced host-pathogen interaction [17,66].

Thus, the expression of ecto-nucleotidases in *Leishmania* and the presence of adenosine are linked to lesion size upon infection, with high expression of these enzymes associated to decreased immune response and bigger lesions [11]. However, in this work, *ntpd1* and *ntpd2*-overexpressing parasites exhibited high ecto-nucleotidase activity and high parasite load, but reduced lesion size (Fig. 4A and B). Thus, our results showed a non-linear relationship between adenosine concentration and lesion size.

In addition, all the parasites showed the same ability to hydrolyze 5′-AMP to adenosine. Taking into account the fact that overexpressor parasites have greater *E*-NTPDase activity, we can hypothesize that

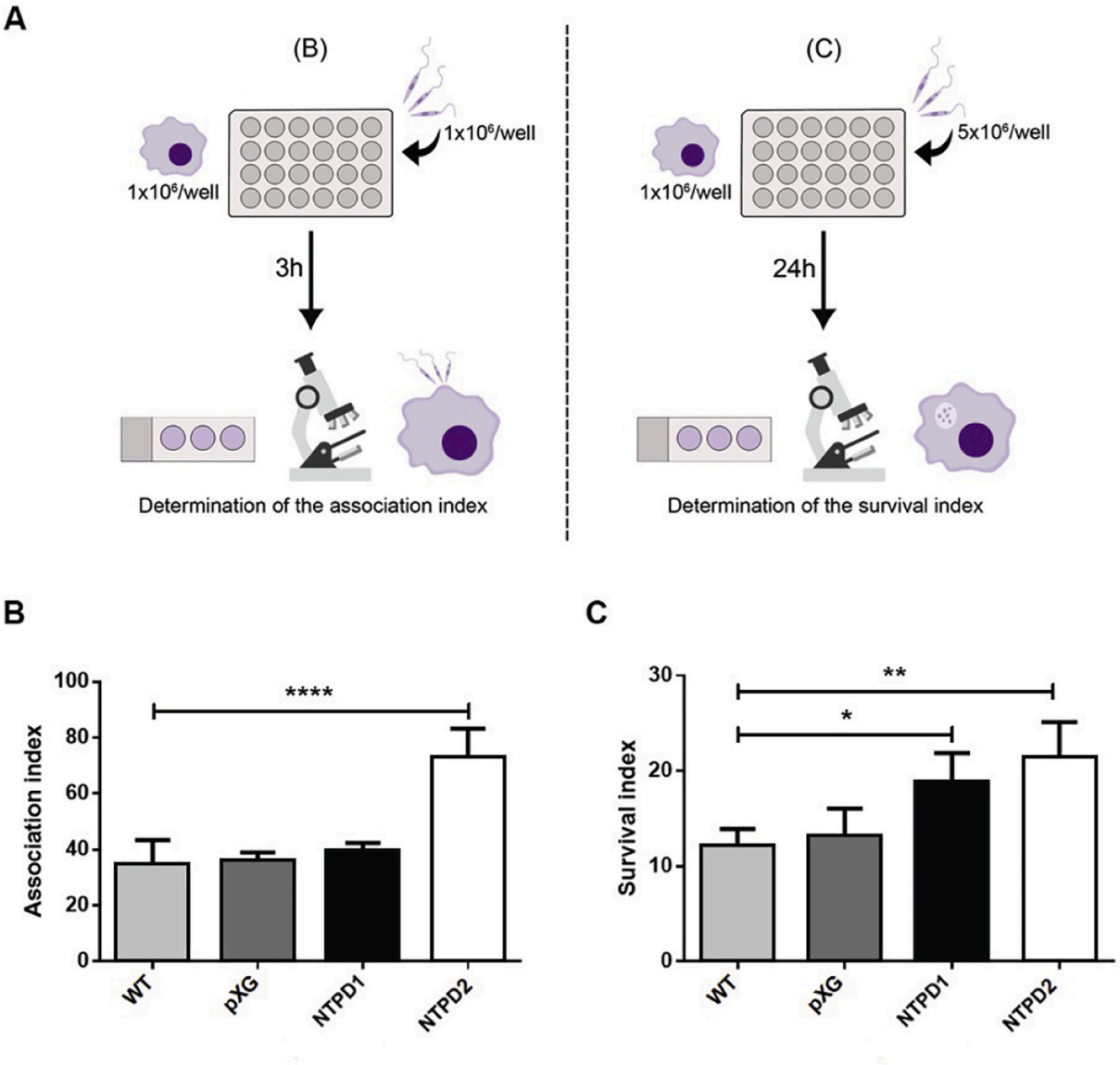


**Fig. 3.** Adherence and survival of promastigotes in parasite-macrophage interaction. Immortalized Bone marrow-derived macrophages (IBMM) were infected with promastigotes of L. *amazonensis* overexpressing cell lines for NTPD1 and NTPD2. To determine the association and survival indexes, the parasites were added at 1:1 or 5:1 parasite-to-cell ratio, respectively. After 3 h or 24 h of co-culture, coverslips were collected, fixed, and stained for observation in the light microscope (A). The association (B) and survival (C) indexes (mean number of parasites per infected macrophage multiplied by the percentage of infected macrophages) were determined by counting 200 cells on triplicate coverslips. Bars represent the means ± S.E.M. of at least three independent experiments performed in triplicate. In all cases the significant differences were assessed by one-way ANOVA followed by Tukey's Multiple Comparison Test. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$ indicate significant differences relative to the control group (WT and pXG). No statistical differences were observed between WT and pXG group.

these parasites would be able to generate a higher level of adenosine from the same concentration of ATP when compared to the control cells. This is related to the sequential dephosphorylation of ATP to adenosine performed by E-NTPDases (ATP → ADP → 5′-AMP) and ecto-5′-nucleotidase (5′-AMP → Adenosine). A higher level of NTPDase activity results in an increase of 5′-AMP, which is a substrate for ecto-5′-nucleotidase. By confirming that these parasites present ecto-5′-nucleotidase activity we can propose that the major availability of the substrate 5′-AMP results in greater generation of adenosine.

This apparently paradoxical result could be explained by the findings of Ribeiro et al. [52]. Using a cellular automate/lattice-gas model, the authors demonstrated the importance of Adenosine in the inhibition of inflammatory Th cells to study host lesion size for several species of *Leishmania*. In hypothetical simulations, the authors found that using sufficient levels of Adenosine to inhibit Th cells in the range of 0%–20% did not have a strong effect on the maximum lesion size. Inhibition from 20 to 40% led to an increase in the lesion size. Higher inhibition (40 to 100%) of Th cells by adenosine resulted in decreased host lesion size. In this extreme case (>40% of inhibition) the number of parasites continuously increased but immune response was almost completely inhibited, which explains smaller lesions [52]. In addition, delay in lesion development as a result of the increased ecto-ATPase activity of the parasite was demonstrated in a study using *L. (L.) amazonensis* isolates obtained from patients with different clinical forms [59]. Moreover, promastigotes from mucosal/mucocutaneous lesions showed delayed lesion development and presented higher ecto-nucleotidase activity than those from cutaneous lesions [33]. Such as suggested by Ribeiro et al. [52], a moderate inhibition of Th-cells activation may lead bigger lesions, in agreement with Marques-da-Silva et al. [35], Maioli et al. [37] and Ji et al. [29].

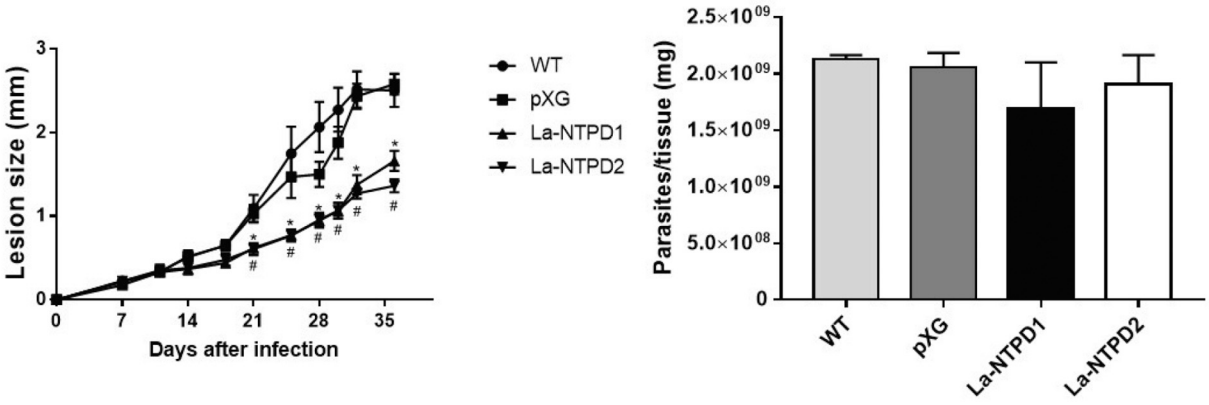


**Fig. 4.** *In vivo* infection of mice with transfected L. *amazonensis* promastigotes. Mice were subcutaneously infected with L. *amazonensis* promastigotes WT, pXG and overexpressing parasites NTPD1 and NTPD2 (A) The course of lesion development was monitored for 36 days. (B) At the end of the experiment, the mice were euthanized, and ear parasite loads were determined *via* a limiting dilution assay. Data are expressed as the means ± S.E.M., $n = 2,5$ animals per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences relative to the control group (WT and pXG). No statistical differences were observed between WT and pXG group.

## 5. Conclusions

Previous studies have shown that increased ecto-nucleotidase activity is correlated with virulence and infectivity of *Leishmania* parasites [11,12,16]. This correlation was observed in parasites from different species and in isolates from the same species or in clones from a single isolate [22,33,35]. In the current work we provided information about the role of *E*-NTPDases on infectivity and virulence of L. *amazonensis*. We show that not only the genes *ntpd1* and *ntpd2* are significantly up-regulated in the infective form metacyclic promastigote but also that the E-NTPDase activity is significantly higher in the same infective stages of L. *amazonensis*. Using an E-NTPDase1-specific commercial antibody we show that at least the isoform 1 presents the same expression profile found in qPCR experiments. Unfortunately, the same antibody was not able to recognize the isoform 2, probably due to the low identity shared between them.

While the overexpression of the genes *ntpd1* and *ntpd2* increased the infection of axenic macrophages, transfected parasites were not able to increase the lesion size in mice, as could be expected. We hypothesize that such apparently paradoxical result could be due to the high levels of adenosine, which could be produced by transfected parasites due to the activity of at least two ecto-enzymes, E-NTPDase and ecto-5′-nucleotidase.

Thus, the results obtained here suggest that not only the reduction of extracellular ATP, caused by E-NTPDase activity, but also the increase in extracellular adenosine levels triggered by the action of ecto-5′-nucleotidase might play an important role in the balance of the immune response to promote the pathogen clearance and maintain the host protection.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2021.102423.

## Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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