Intralesional uridine-5'-triphosphate (UTP) treatment induced resistance to *Leishmania amazonensis* infection by boosting Th₁ immune responses and reactive oxygen species production

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Abstract

Leishmania amazonensis is the etiologic agent of cutaneous leishmaniasis, an immune-driven disease causing a range of clinical symptoms. Infections caused by *L. amazonensis* suppress the activation and function of immune cells, including macrophages, dendritic cells, and CD4⁺ T cells. In this study, we analyzed the course of infection as well as the leishmanicidal effect of intralesional UTP treatment in *L. amazonensis*-infected BALB/c mice. We found that UTP treatment reduced the parasitic load in both footpad and lymph node sites of infection. UTP also boosted Th1 immune responses, increasing CD4⁺ T cell recruitment and production of IFN- γ , IL-1 β , IL-12, and TNF- α . In addition, the role of UTP during innate immune response against *L. amazonensis* was evaluated using the air pouch model. We observed that UTP augmented neutrophil chemoattraction and activated microbicidal mechanisms, including ROS production. In conclusion, our data suggested an important role for this physiological nucleotide in controlling *L. amazonensis* infection, and its possible use as a therapeutic agent for shifting immune responses to Th1 and increasing host resistance against *L. amazonensis* infection.

Keywords UTP · *Leishmania amazonensis* · Leishmaniasis · P2Y receptors · ROS

Abbreviations

ATP	Adenosine 5'- triphosphate	LDA	Parasite-limiting dilution assay
CEUA	Commission for Ethical Use of Research Animals	LTB4	Leukotriene B4
DMEM	Dulbecco's modified Eagle's medium	LN	Lymph node
DPI	Days post-infection	M199	199 medium
FBS	Heat-inactivated fetal bovine serum	NO	Nitric oxide
IL	Interleukin	ROS	Reactive oxygen species
		UTP	Uridine-5'-triphosphate
		MOI	Multiplicity of infection

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Introduction

Leishmaniasis is a severe parasitic disease, considered by the World Health Organization (WHO) to be one of the most important neglected diseases globally. Approximately 12 million people are infected with more than 20 different pathogenic *Leishmania* species worldwide (i.e., tropical and subtropical regions of Asia, the Middle East, sub-Saharan Africa, and South America) [1, 2]. *Leishmania* infection symptoms range from disfiguring cutaneous ulcers to lethal visceral forms, depending on the species [2, 3].

Leishmania amazonensis (*L. amazonensis*) is one of the causative agents of cutaneous leishmaniasis in South America [4]. The disease is transmitted by sandflies that inoculate promastigotes—the *Leishmania* infective form—into the host skin, that in turn are internalized by tissue resident phagocytes. Once inside the phagocytes, promastigotes become amastigotes and multiply until the host cells are lysed, releasing parasites and host danger signals (i.e., nucleotides—adenosine 5'-triphosphate (ATP)/uridine 5'-triphosphate (UTP)). Once released, the parasites infect and activate neighboring cells, thus propagating the infection and inflammation [5, 6].

As the drugs currently available for treating leishmaniasis (i.e., pentavalent antimonials, liposomal amphotericin-B, miltefosine, and paromomycin) have side effects [7], the most effective mechanisms to protect the host against *Leishmania* involve efficient immune responses with the generation of Th1 CD4⁺ T cells. The activation of phagocytes, including neutrophils, macrophages, and dendritic cells, by interferon- γ (IFN- γ) produced by Th1 cells, inducing the release of reactive oxygen species (ROS) and nitric oxide (NO), leading to parasite elimination [2, 8].

Extracellular nucleotides play an important role in the immunopathogenesis of intracellular/extracellular parasite infections by boosting microbicidal mechanisms and inflammatory immune responses [9–14]. Classically, ATP and UTP are cytosolic nucleotide constituents of nucleic acids serving as energy sources; however, they also act as signaling molecules in the extracellular space [15, 16] when released from cells by lytic and non-lytic mechanisms. Once in the extracellular compartment, nucleotides activate P2 receptors [17, 18]. P2 purinergic/pyrimidinergic receptors can be subdivided into metabotropic G-protein-coupled P2Y receptors (P2Y_{1,2,4,6,11–14}), and ionotropic P2X receptors (P2X_{1–7}) [19]. P2X and P2Y₁₁ receptors are chiefly activated by extracellular ATP; P2Y_{2,4} by ATP and UTP; P2Y₁, P2Y₁₂, and P2Y₁₃ by ADP; P2Y₆ by UDP; and P2Y₁₄ by UDP-glucose [20]. P2 receptor expression can be constitutive or regulated under pathological conditions by the immune system [20]. Therefore, these receptors are involved in a range of immunological reactions that are being widely considered in studies of inflammation [21, 22].

In the context of *Leishmania* infections, it was shown that P2X₇ purinergic and P2Y_{2/4} pyrimidinergic receptors are up-regulated in *L. amazonensis*-infected murine macrophages, and both receptors have anti-parasitic action once activated by their agonists ATP and UTP, respectively [6, 23, 24]. Recently, we reported the involvement of ATP-P2X₇ receptor signaling in mouse susceptibility to *L. amazonensis* infection [25]. In the present article, we aimed to investigate the anti-parasitic effect of intralesional UTP treatment during *L. amazonensis* infection. We found that UTP treatment induced resistance to *L. amazonensis* infection, boosting Th1

immune responses and ROS production, suggesting a crucial role for UTP-gated pyrimidinergic receptors in the immune response to *Leishmania* infections.

Materials and methods

Parasites

L. amazonensis amastigotes (strain MHOM/BR/75/Josefa) and green fluorescent protein (GFP)-transfected *L. amazonensis* (strain MHOM/BR/75/Josefa) were isolated from a mouse lesion and were allowed to transform into axenic promastigotes. They were maintained at 27 °C in 199 medium (M199) (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Cultilab), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25% hemin (Sigma). Promastigotes in stationary phase were used as described previously [23] to perform the in vitro and in vivo infections.

Experimental infection

We subcutaneously injected 8–12-week-old female BALB/c mice in the right footpad with 2×10^6 *L. amazonensis* promastigotes in 20 µl PBS. Lesion size was determined with traditional calipers (Mitutoyo®) by measuring the thickness of the infected footpad subtracted by the thickness of the uninfected footpad (initial measurement on day 0). In order to evaluate the UTP treatment as a therapeutic approach, we started the intralesional treatment with 10 mM UTP (20 µl in PBS, pH = 7.2) or vehicle (20 µl PBS) 21 days post-infection (d.p.i.) when the lesion was large enough to characterize the disease (lesion size about 30×10^{-2} mm). In BALB/c mice, the exacerbation of the disease (characterized by parasite migration to secondary sites and formation of metastatic lesions) occurs markedly at 21 d.p.i. [26]. Alternatively, for evaluating the lesion appearance that is correlated with the second wave of neutrophils observed within the first days post-infection (5–10 d.p.i.) [27], we initiated UTP treatment at 7 d.p.i. The animals in both experimental protocols were treated twice a week with UTP for 3 weeks, for a total of six administrations. Forty-eight hours after the last injection, the animals were subjected to euthanasia and analyses were performed.

Parasite load estimation

Parasite load was measured in both the infected footpads and in popliteal lymph nodes (LN). The number of live *L. amazonensis* parasites was determined using the parasite-limiting dilution assay (LDA) [28, 29]. Whole footpads or LNs were collected, weighed, and homogenized in M199

using a cell strainer (BD®). Large debris was removed by centrifugation at $150\times g$ for 5 min with subsequent collection of the supernatant. Cells were concentrated by centrifugation at $2000\times g$ for 10 min. Fifty microliters (50 μ l) of the cell suspension were plated onto 96-well flat bottom microtiter plates (Becton Dickinson, Franklin Lakes, NJ) and serially diluted in complete M199 with successive 4:1 ratio dilutions, ranging from 1 to 0.25×10^6 . Each sample was plated in triplicate and cultured at 26–28 °C for at least 7 days, when the assay was read microscopically. A positive titration was scored when at least one parasite was observed in a particular dilution, and parasite load was estimated according to the highest dilution at which parasites could still be detected, as described previously [29].

Promastigote growth curves

Promastigotes of *L. amazonensis* were incubated with or without 1 mM UTP in supplemented culture medium M199 for 5 days at 27 °C. The growth curves were initiated with 1.0×10^6 /mL promastigotes on day 0. The growth and viability of *Leishmania* were measured by quantification of parasites in a hemocytometer after cell dilution and fixation in phosphate-buffered saline (PBS) containing 0.5% paraformaldehyde.

Air pouch model

Female BALB/c mice (8–12-week old) were subjected to the air pouch model. This model allows for differential quantification of leukocytes that accumulate in the air pouch wall (tissue) as well as those that transmigrate into the air pouch cavity (lavage). Air pouches were raised by injection of 5 ml of sterile air into the skin on the dorsum of each mouse on day 0 and were maintained by re-injecting 3 ml of sterile air on day 3. Before the injection of air, mice were briefly anesthetized with isoflurane. On day 6, infection was induced by injecting 2×10^6 *L. amazonensis* GFP-transfected promastigotes into the air pouch. Then, 1 mM UTP was injected into the air pouch and washed 6 h later with PBS to isolate recruited cells, after which analyses were performed.

THP-1-derived human macrophages

THP-1 cells (ATCC® TIB-202™) were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 IU penicillin/mL and 100 μ g streptomycin/mL. THP-1 cells were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 h to differentiate into human macrophages and were then infected with promastigotes of *L. amazonensis* in a multiplicity of infection (MOI) of 10:1. Forty-eight hours post-infection, cells were treated with 100 μ M UTP for 30 min, washed with PBS, and

maintained in culture for an additional 24 h, when the cells were fixed and stained with Meyer-Grunnwarld-Giemsa (Laborclin, Pinhais, PR, Brazil), according to the manufacturer's instructions. Infection index was calculated as previously described [6].

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells of draining popliteal LN using the TRIzol® reagent (Life Technologies Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA was quantified using an ND-1000 spectrophotometer (NanoDrop, ThermoFisher, Waltham, MA, USA), and cDNA was synthesized from 500 ng total RNA using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Invitrogen Carlsbad, CA, USA). The SYBR® Select Master Mix (Life Technologies Carlsbad, CA, USA) was used for qRT-PCR, to detect double-stranded DNA synthesis. Reactions were carried out in a final volume of 10 μ l, using 2 μ l of diluted cDNA (1:10) and 300 nM each of reverse and forward primers. The following primers were used for qRT-PCR: for *P2ry2*, 5'-TGACGACTCAAGACGGACAG-3' (forward) and 5'-GTCCCCTACAGCTCCCCTAC-3' (reverse); for *P2ry4* 5'-ACTGGCTTCTGCAAGTTCGT-3' (forward) and 5'-AGGCAGCCAGCTACTACCAA-3' (reverse); for *P2ry6* 5'-TGCTGCTTGGGTAGTGTGTGG-3' (forward) and 5'-GTAAGGCTATGAAGGGCAGC-3' (reverse); for *Actb*, 5'-TATGCCAACACAGTGCTGCTGCTGG-3' (forward) and 5'-TACTCCTGCTTGCTGATCCACAT-3' (reverse); and for *Gapdh*, 5'-GGTCATCCAGAGCTGAACG-3' (forward) and 5'-TTGCTGTTGAAGTCGCAGGA-3' (reverse). Reactions were performed in a 7500 Fast Real-Time System (Life Technologies Carlsbad, CA, USA). Relative expression levels were determined using the Sequence Detection Software v.2.0.5 (Life Technologies Carlsbad, CA, USA), and the efficiency per sample was calculated using LinRegPCR 11.0 software. β -Actin and *Gapdh* were used as internal controls to calculate the relative amount of the genes of interest. We used the comparative threshold cycle ($\Delta\Delta CT$) method with efficiency correction, using the mean qRT-PCR efficiency for each amplicon, as previously described [30, 31].

Cytokine dosage

To measure the secretion of cytokines from infected tissues, the supernatants obtained from macerated paws were used to quantify IL-10, IL-1 β , IL-12, TNF- α , and IFN- γ production by ELISA according to the manufacturer's instructions (Peprotech, Rocky Hill NJ, USA). The amount of cytokine in the footpad was expressed in nanograms of cytokine per gram of tissue.

ROS production measurement

ROS production was measured from suspended cells obtained from the air pouches using the CM-H₂DCFDA probe (20 µg/mL) (Invitrogen, Carlsbad, CA, USA) for 40 min at 37 °C, 5% CO₂, in a humidified incubator to allow loading of the dye. Spontaneous ROS production was analyzed by flow cytometry, comparing mean fluorescence intensity at 530 nm when the sample was excited at 485 nm between samples. At least 10,000 events were acquired using FACScan (Becton Dickinson, USA). Production of ROS was quantified after debris exclusion in forward versus side scatter (FSC × SSC) using WinMDI software (Scion Corporation, USA).

Fluorescence microscopy

In order to visualize ROS production in pouch cells, slides were mounted and images were analyzed by fluorescence microscopy at room temperature. Previously, leukocytes from pouch washes were loaded with dihydroethidium (DHE) according to the manufacturer's instructions (superoxide detection probe, Invitrogen, Carlsbad, CA), adjusted to 2×10^5 cells, and seeded onto slides using a cytospin (750 g for 5 min). The cells were incubated with 1 µg/mL diamidino-2-phenylindole (DAPI) for 10 min for nuclear counterstaining. Slides were viewed with a Carl Zeiss (Jena, Germany) microscope using a ×100 oil immersion objective lens (filter set 46 HE YFP; excitation wavelength, 500/20 nm; emission wavelength, 535/30 nm). Fluorescent images were captured with an AxioCam camera (Carl Zeiss), processed using AxioVision software, version 3.1, and saved as TIFF files. Further processing was performed using Adobe Photoshop, version 7.0 (Adobe Systems Incorporated, CA).

Phenotypic analysis

Cells obtained from LNs and air pouch washes were adjusted to 1×10^6 (LN) and 2×10^5 (pouch) cells per sample plated on 96-well plates, blocked (1 µg/mL Fc block) for 1 h and then labeled with one of the following antibodies: anti-CD3 FITC, anti-CD4-Alexa488, anti-CD8-PE, anti-CD11b-PE, anti-B220-Alexa 488, anti-F4/80-eFluor 450, anti-CD25-PE, anti-FoxP3-APC, or anti-Ly6G-FITC (all from e-Bioscience, San Diego, CA, USA). Samples were used at a final concentration of 1 µg/ml (FACS Buffer) for 30 min according to the manufacturer's instructions. Cells were washed twice in PBS, and at least 10,000 events were acquired for each sample using FACScan (Becton Dickinson, USA). Cells were maintained at 4 °C throughout the experiment. The percentage of positive cells for each antigen was applied to the total number of cells in each mouse to produce estimates of absolute numbers of cells of each cell

type. Cells were initially gated at FSC × SSC to exclude cellular debris and then by type-specific antibodies. T lymphocytes were individually identified on CD3⁺ cells within the lymphocyte gate (FSC × SSC). Data were analyzed using WinMDI software (Scion Corporation, USA).

Statistical analysis

Normal distribution and homogeneous variance were tested for all data. The Shapiro–Wilk test was used to assess normality for all variables. For data with a non-Gaussian distribution, the non-parametric Mann–Whitney test was used. Parametric data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple range tests. The observed differences were considered significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism software (GraphPad Software 7.0, La Jolla, CA, USA).

Results

P2Y₂ receptor gene expression was upregulated during *L. amazonensis* infection

Previous data showed that macrophages infected with *L. amazonensis* upregulated P2Y₂ and P2Y₄ receptor expression. In addition, exogenous UTP, P2Y₂ and P2Y₄ agonist, induced apoptosis and activated anti-leishmanicidal mechanisms [24]. In order to study the implications of P2Y receptors in an in vivo approach, we infected the footpad of BALB/c mice with *L. amazonensis* and the *P2ry2*, *P2ry4*, and *P2ry6* gene expression in popliteal LN from infected mice was analyzed. We found a significant increase in P2Y2 receptor gene expression 21 d.p.i. in LN cells from infected mice when compared to cells from uninfected LN cells. These data suggest that this receptor might play a role in leishmaniasis (Fig. 1). However, we could not rule out the possibility that this result explained by changes in LN cells subpopulations. Further studies are required to identify the composition of LN cell subpopulations expressing P2Y2 receptor during leishmaniasis.

Intralesional injection of UTP improved resistance against *L. amazonensis* infection

In order to evaluate UTP treatment as a potential therapeutic molecule, BALB/c mice were infected intradermally with *L. amazonensis* and were treated with 10 mM UTP or PBS (vehicle) intralesionally as described in the “Materials and methods” section. We observed that the UTP treatment significantly reduced the lesion size in both experimental protocols, whether starting the

Fig. 1 P2Y₂ receptor gene expression was upregulated in popliteal lymph node cells from *L. amazonensis*-infected mice. Mice were infected via footpad dermis with 2×10^6 stationary-phase *L. amazonensis* promastigotes. Twenty-one days post-infection, mice were subjected to euthanasia and popliteal lymph nodes were excised and processed for evaluation of P2Y₂, P2Y₄, and P2Y₆ gene expression. Data are representative of two independent experiments. Each experiment was performed with three mice per group. Means $\pm$ S.E.M. ($n = 6$; * $p < 0.05$)

treatment at 21 or 7 d.p.i. (Fig. 2a, b). Forty-eight hours after the last UTP administration, mice were subjected to euthanasia and the parasitic load and number of cells

Fig. 2 Intralesional UTP treatment reduced lesion size and parasite load in *L. amazonensis*-infected mice. Mice were injected in the footpad with 2×10^6 stationary-phase *L. amazonensis* promastigotes. Intralesional UTP treatment was initiated on **a** 21 days post-infection (21 d.p.i.) or **b** 7 days post-infection (7 d.p.i.), and the lesion size was measured on the indicated days. **c**, **e** Parasite loads in footpad and **d**, **f** LN from mice starting UTP treatment on 21 d.p.i. (**c**, **d**) or 7 d.p.i. (**e**, **f**). **g**, **i** Total number of cells in footpad and **h**, **j** in LN from infected mice that started to receive intralesional UTP treatment 21 d.p.i. (**g**, **h**) or 7 d.p.i. (**i**, **j**). One representative experiment is shown from three experiments. Each experiment was performed with at least four mice per group. Means $\pm$ S.E.M ($n \geq 4$) * $p < 0.05$; ** $p < 0.01$ in relation to PBS

were evaluated in the footpad and LN. UTP treatment significantly diminished the parasitic load (Fig. 2c–f) and the total number of cells (Fig. 2g–j) in the footpad and LN from infected mice in both experimental protocols. Of note, UTP had no direct effect on *L. amazonensis* survival and proliferation (Fig. 3).

Intralesional UTP treatment boosted Th₁ immune response against *L. amazonensis*

We detected increased levels of IL-1 β , TNF- α , IFN- γ , IL-12, and IL-10 (Fig. 4) in footpads of *L. amazonensis*-infected mice treated with UTP in comparison with that of infected vehicle-treated mice ($p < 0.05$). No statistical difference in these cytokines was observed in infected compared to uninfected draining LN (data not shown). Together, these results showed that UTP-treated mice produced higher levels of inflammatory cytokines that induced resistance against *L. amazonensis* at site of infection.

Performing phenotypic analysis, we observed that UTP treatment reduced the number of CD11b⁺ (Fig. 5a), B220⁺ cells (Fig. 5b), CD8⁺ (Fig. 5c), and CD4⁺CD25⁺FoxP3⁺ (Fig. 5e) and increased the number of CD4⁺ cells (Fig. 5d). The increased levels of IFN- γ and CD4⁺ T cells suggested that

Fig. 3 UTP did not affect the *L. amazonensis* growth in vitro. 10^6 stationary-phase *L. amazonensis* promastigotes (MHOM/BR/75/Josefa) were cultured in medium M199 including 1 mM UTP (pH = 7.0) or PBS at 27 °C for 10 days. An aliquot of parasites was fixed in 0.5% paraformaldehyde, and cell number was estimated with a Neubauer hemocytometer. Each curve point is means of two independent experiments

UTP treatment boosted Th1 immune response against *L. amazonensis*, contributing to control of parasite replication.

Innate immune response against *L. amazonensis* can be modulated by UTP in the air pouch model

In order to explore the role of UTP in activating microbicidal mechanisms in innate immune cells in response to *L. amazonensis* infection, we used the experimental air pouch model in mice, as previously described [32]. We observed that *L. amazonensis* was able to augment the total number of cells into the pouch (Fig. 6a). UTP induced a significant increase in the recruitment of $\text{Ly6G}^+\text{F4/80}^-$ cells (neutrophils) in *L. amazonensis*-infected pouches (Fig. 6b). ROS production by the total group of cells from the air pouch was quantified by flow cytometry after incubation of the cell suspension with

Fig. 4 Intranasal UTP treatment boosted local cytokine production during *L. amazonensis* infection. Mice were infected intradermally in the footpad with 2×10^6 stationary-phase *L. amazonensis* promastigotes. Twenty-one days post-infection, intranasal UTP treatment was initiated. Forty-eight hours after the last UTP injection,

the indicated cytokines were measured in the footpad supernatants by ELISA. One representative experiment is shown from three experiments. Each experiment was performed with at least four mice per group. Means $\pm$ S.E.M. ($n \geq 4$) * $p < 0.05$ compared to PBS

Fig. 5 Intralesional UTP treatment modulated cell recruitment to draining lymph nodes (LN). Mice were infected intradermally in the footpad with 2×10^6 stationary-phase *L. amazonensis* promastigotes. Twenty-one days post-infection, intralesional UTP treatment was initiated. Forty-eight hours after the last UTP injection, the LNs were excised, and single-cell

suspensions were stained with specific antibodies. Absolute number of **a** CD11b⁺, **b** B220⁺, **c** CD4⁺, **d** CD8⁺, and **e** CD4⁺CD25⁺FoxP3⁺ cells of individual LN. One representative experiment is shown from three experiments. Each experiment was performed with at least four mice per group. Means $\pm$ S.E.M. ($n \geq 4$) * $p < 0.05$ in relation to PBS

H₂DCFDA. UTP increased ROS production in cells from *L. amazonensis*-infected pouches (Fig. 6c). ROS induction in cells treated with UTP was visualized using fluorescence microscopy (colocalization in white) (Fig. 6d–h). These results indicated that UTP induced the recruitment of phagocytes, including neutrophils, and activated ROS production that might limit parasitic replication.

Finally, we show in Fig. 7 that UTP treatment also significantly reduced the parasite load in THP-1 human-derived macrophages, as we have already shown in murine macrophages.

Discussion

In this study, we evaluated the effects of UTP—a P2Y_{2/4} receptor agonist—in an in vivo model of *L. amazonensis* infection. We found that this nucleotide contributed to *L. amazonensis* elimination. In addition, UTP treatment reduced lesion size and cellular infiltrate while increasing

inflammatory cytokine levels and ROS production in infected mice. This suggested that UTP boosted the immune response against *L. amazonensis*.

Several studies have demonstrated the importance of purinergic signaling in parasitic infections [9, 10]. Specifically in the context of leishmaniasis, it was shown that *L. amazonensis* infection upregulated P2X7 and P2Y_{2/4} receptors that, once activated by its agonists ATP and UTP, triggered Ca²⁺-mediated signaling and the consequent elimination of parasites through various microbicidal mechanisms [23, 24]. Recently, it was proposed that the mechanisms of *L. amazonensis* elimination via the ATP-P2X7 receptor were due to an autocrine/paracrine pathway involving leukotriene B4 [6].

Here, we showed that the in vivo treatment with UTP reduced the parasitic load, the immune cell infiltration in the lesion site (paws) and LN, as well as lesion size. In fact, in vitro data showed that UTP reduced the parasitic load in infected macrophages [24]. Fewer parasites infecting tissue might require fewer immune cells at the site of infection,

Fig. 6 UTP treatment induced neutrophil recruitment and increased ROS production in *L. amazonensis*-inoculated subcutaneous mice air pouches. Mouse subcutaneous air pouches were inoculated with 2×10^6 stationary-phase *L. amazonensis* promastigotes, and 30 min later, they received 1 mM UTP in the same site. After 6 h, the pouch exudates were aspirated and centrifuged. The pelleted cells were stained and analyzed by FACS. **a** Absolute number of cells are given from individually analyzed pouches, normalized to 100% as *L. amazonensis*-infected group (average 9.25 and 19×10^5 cells/mL). **b** Ly6G⁺F4/80⁻ were normalized to 100% as *L. amazonensis*-infected group (average 18.9

and 41.7×10^4 cells/mL). **c** ROS production in total pouch cells analyzed by FACS. Representative fluorescence images from pouch cells of the uninfected animals (**d**), *L. amazonensis*-infected (**e**), uninfected-UTP treated (**f**), *L. amazonensis*-UTP treated (**g**), and uninfected treated with 10 mM H₂O₂ (**h**) as positive control. Nuclei stained with DAPI (blue), *L. amazonensis*-GFP⁺ (green), and ROS stained with DHE (reddish). Data are means $\pm$ S.E.M ($n = 6$) from two individual experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Bar = 10 μ m

and fewer parasites are viable to travel to LNs. This idea was corroborated by our phenotypic analysis of LNs from infected mice, in which we observed a significant reduction of phagocytes, T cytolytic lymphocytes (TCD8⁺), regulatory T cells (CD4⁺CD25⁺FoxP3⁺), and B cells (B220⁺). This reduction

was probably due to the diminished parasite load after UTP treatment.

Interestingly, the number of T helper lymphocytes (CD4⁺ T) was increased in LNs from UTP-treated infected mice. The defense against *Leishmania* usually follows the rule of CD4⁺

Fig. 7 UTP induced the control of *L. amazonensis* infection in human macrophages. Parasite load in *L. amazonensis*-infected human macrophages treated or not with 100 μ M UTP for 30 min. The effect of UTP on infection was quantified 24 h after treatment with the nucleotide by determining the infection index (number of infected cells $\times$ mean number of parasites per cell; normalized to the untreated), by direct counting under the light microscope. Data are means of three independent experiments performed in triplicate. Means $\pm$ S.E.M. ($n = 3$). * $p < 0.01$

T cell and IFN- γ -mediated activation of macrophages [33, 34]. Our results showed that, at the site of infection/treatment, IFN- γ was upregulated by UTP, and CD4 $^{+}$ T cells were upregulated in the LNs. These results suggest that UTP may reinforce the organism's arsenal against infections. In the mouse model, the most efficient mechanism of parasite killing is the production of IFN- γ and TNF- α by CD4 $^{+}$ Th1 cells [35], suggesting that UTP may participate in this pathway to modulate the infection.

Usually, pathogens that cause chronic infections manipulate the host response with a specific polarization of the immune response, to the benefit of the pathogen. *L. amazonensis* infection is known to inhibit the activation and effector functions of macrophages, dendritic cells, and CD4 $^{+}$ T cells. In fact, the determining factor for non-healing disease caused by *L. amazonensis* is the low magnitude of CD4 $^{+}$ T cell activation [36]. In this context, UTP treatment would be an interesting approach to boost Th1 immune responses, thus controlling *L. amazonensis* infection.

Interestingly, the infection of C57Bl/6 mice with *L. amazonensis* did not generate a classical Th1 phenotype as obtained with *Leishmania major* infections. Th1 and Th2 responses are already known to manifest in C57Bl/6 and Balb/c mice when infected by *L. major*, respectively.

L. major infection correlates with Th2 responses driven by IL-4, causing worse pathology. However, in C57Bl/6 mice, the Th1 response is driven by IFN- γ , promoting parasite death and healing [26]. Moreover, during *L. amazonensis* infection and that of other *Leishmania* species, this polarization is not observed. While C57Bl/6 mice are resistant for *L. major* infection, during infection by *L. amazonensis*, these mice are considered partially resistant [37]. The immune response associated with *L. amazonensis* infection is mixed between Th1 and Th2, and the susceptibility is probably a failure of the Th1 response assembly [38]. In this same work, it was shown that IL-4 absence did not alter the *L. amazonensis* infection, suggesting that Th2 cytokines could not promote susceptibility during *L. amazonensis* infection. Therefore, it is reasonable that IL-10 levels observed in this work did not interfere with the infection pathology and that UTP treatment can assist the Th1 response assembly.

Using the air pouch model of infection, we observed that during really early steps of infection (hours), UTP was an important signal to attract neutrophils to the site of infection as well as to stimulate the production of microbicidal molecules, including ROS. These results are consistent with previous reports, where macrophages treated with a UTP subproduct, UDP, secreted more chemoattractant proteins, promoting efficient bacterial clearance [11, 39, 40]. The amastigote forms of *L. amazonensis* parasites are known to be more resistant to neutrophil leishmanicidal molecules than are other species [41, 42]. Our results show increased ROS production in infected pouches treated with UTP, suggesting that this nucleotide overcame parasite subversion mechanisms.

Taken together, this study provides a new vision of pyrimidinergic signaling during a chronic infectious parasitic disease. The data presented here substantiate previous work showing an in vitro leishmanicidal mechanism induced by UTP [24]. Expanding this concept, we showed that UTP had immunomodulatory effects, especially in the early steps of infection. This immunomodulatory effect tended to polarize the Th1 response that more efficiently reduces the severity of the disease. Despite the lack of available human samples, the immune responses seen in *L. amazonensis*-infected mice are very similar to those observed in human infections, showing that this model is of substantial relevance for the study of human cutaneous leishmaniasis [4]. Therefore, these results suggest possible therapeutic approaches based on the administration of intralosomal UTP to boost the immune response and microbicidal mechanisms against *L. amazonensis* infection.

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Compliance with ethical standards

Conflicts of interest The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Ethical approval All experimental protocols were approved by the Commission for Ethical Use of Research Animals (CEUA) of the Federal University of Rio de Janeiro (IBCCF:154 and 077/15).

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