The leishmania-macrophage interactions: role of E-NTPDases and purinergic signaling

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Leishmaniasis comprises a group of diseases caused by protozoan parasites of the genus Leishmania. The transmission occurs by the bite of phlebotomine female sand flies of the genera Phlebotomus and Lutzomyia. In the mammalian host, these parasites infect and proliferate mainly into the macrophages, avoiding the harmful effects of the innate inflammatory response. It has been reported in several works that purinergic signaling, including purine receptors P1 and P2, are dynamically modulated during both the development and activation of cells from the immune system to achieve homeostasis or combat infections as well as harmful damage. Many pathogens are able to subvert the host immune response in order to promote the success or maintenance of infection. This subversion can be related to the influence of pathogens on purinergic signaling leading to decreased levels of the pro-inflammatory molecule ATP and increased levels of the immunosuppressive molecule adenosine. This scenario can be produced as the final product of the joint activity of E-NTPDases and 5’-ectonucleotidases. Thus, this review will discuss the cellular and molecular events occurring during the interaction of Leishmania and macrophages focusing on the purinergic signaling pathways and their relationships with E-NTPDases from pathogenic species of Leishmania.

**Keywords:** Leishmaniasis; E-NTPDases, purinergic signaling, nucleotides, host-pathogen interaction


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**Introduction**

*Leishmaniasis, leishmania infection and macrophage relationships*

Leishmaniasis are human diseases caused by protozoan parasites of the genus Leishmania. The three major clinical forms of the diseases have distinct clinical signs and symptoms and are comprised of the following: (i) cutaneous Leishmaniasis (CL), (ii) muco-cutaneous Leishmaniasis (ML; also known as espundia) and (iii) visceral Leishmaniasis (VL; also known as kala-azar). CL is characterized by the presence of long-term ulcerative skin lesions, which in most cases are self-healing. In CL, the patient generally presents with one or several ulcer(s) or nodule(s) on the skin. Different species of
Leishmania can infect the macrophages in the dermis with variable clinical presentations and prognoses [1]. *Leishmania braziliensis* is responsible for most cases of ML.

VL is the most severe form in which the parasites leave the site of inoculation and proliferate in the liver, spleen and bone marrow, resulting in chronic infection, immunosuppression of the host and death if it left untreated [2]. VL is caused by the *Leishmania donovani* complex, which is *L. donovani* in East Africa and the Indian subcontinent, *Leishmania infantum* in Europe and North Africa and Leishmania chagasi in Latin America [3, 4].

Leishmania parasites are well suited to initiate infection, resist the arsenal of defense molecules of innate immunity and achieve a long-term proliferative stage inside host cells. All species exhibit a pronounced tropism for macrophages, although they have the ability to infect a variety of other phagocytic and non-phagocytic mammalian cells [5-8]. Unlike most of intra-macrophage pathogens, the Leishmania proliferative stage dwells in mature phagolysosomes inside the host macrophages. Leishmania’s ability to evade the self-healing mechanisms of macrophages and their anti-parasitic functions is directly correlated with their ability to modulate several signaling pathways that regulate the defense responses in these host cells [9-12]. The outcomes of Leishmaniasis diseases are strongly dependent on the initial stages of infection and involve molecular interactions and cell signaling between macrophages and the parasite [13-15] (and for a complete review see [16]).

The infection of a mammalian host is initiated by the transmission of the parasite by the female sand flies from the genus Lutzomyia or the genus Phlebotomus. The bite of the sandfly induces a rapid neutrophil infiltration and substantial macrophage recruitment to the skin, regardless of the presence of parasites [6, 7, 17, 18]. Then, the metacyclic promastigotes forms are phagocytosed by neutrophils, but they do not differentiate into amastigotes or undergo intracellular proliferation within neutrophils [6, 19]. Although neutrophils are the predominant type of infected cell, at the initial stage of infection their population gradually decreases at the site of inoculation, followed by an increase in the frequency of infected macrophages [16]. In fact, neutrophils are phagocytic polymorphonuclear cells that have a very short life span and then spontaneously undergo apoptosis. The Leishmania major infection of polymorphonuclear neutrophils leads to a significant delay in the apoptotic cell death program, meaning the parasites do not multiply and remain as promastigote forms [20]. Additionally, the neutrophils secrete a high level of MIP-1β chemokine, which is known to attract macrophages to the site of infection [21, 22]. When macrophages are recruited, they

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**Table 1. Overview of the biological roles of E-NTPDases and ecto-nucleotidases from Leishmania**

<table>
<thead>
<tr>
<th>Biological observation</th>
<th>Specie</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecto-ATPase Mg++ dependent involved in adenosine acquisition and virulence</td>
<td><em>L. amazonensis</em></td>
<td>Berredo-Pinho, 2001</td>
</tr>
<tr>
<td>E-NTPDase is expressed at the surface of the plasma membrane, flagellum, and several organelles</td>
<td><em>L. amazonensis</em>; <em>L. braziliensis</em></td>
<td>Coimbra, 2002; Rezende-Soares et al., 2010; Vasconcellos et al., 2014</td>
</tr>
<tr>
<td>Presence of Ecto-ATPase, ADPase and 5’-nucleotidase in three Leishmania species and relation with immunosuppression in lymph node cells.</td>
<td><em>L. amazonensis</em>, <em>L. braziliensis</em>, <em>L. major</em></td>
<td>Maioli 2004</td>
</tr>
<tr>
<td>E-NTPDase participates in adhesion to macrophages and the purine salvage pathway. The ecto-ATPase activity is corresponding to the CD39 family. Higher E-NTPDase 2 and AMPase activity in <em>L. amazonensis</em> compared to <em>L. braziliensis</em> and its relationship with lesions and virulence</td>
<td><em>L. amazonensis</em></td>
<td>Marques-da-Silva et al., 2008</td>
</tr>
<tr>
<td>Ecto-nucleotidase activity is associated with lesion development and parasite multiplication</td>
<td><em>L. amazonensis</em>; <em>L. braziliensis</em></td>
<td>De Souza, 2010; Leite, 2012</td>
</tr>
<tr>
<td>Ecto-nucleotidase activity is associated with infectivity and lesions in mice</td>
<td><em>L. amazonensis</em>; <em>L. infantum</em></td>
<td>De Souza, 2010; Vasconcellos et al., 2014</td>
</tr>
<tr>
<td>Ecto-nucleotidase activity is associated with virulence</td>
<td><em>L. amazonensis</em></td>
<td>Paletta-Silva, 2011</td>
</tr>
<tr>
<td>High ecto-nucleotidase activity inhibits NO production by activated macrophages</td>
<td><em>L. braziliensis</em></td>
<td>Leite, 2012</td>
</tr>
<tr>
<td>E-NTPDase is associated with disease progression in mice</td>
<td><em>L. amazonensis</em></td>
<td>Detoni et al., 2013</td>
</tr>
<tr>
<td>E-NTPDase induces specific immune response in naturally infected dogs</td>
<td><em>L. infantum</em></td>
<td>de Souza et al., 2013; Maia et al., 2013</td>
</tr>
<tr>
<td>E-NTPDase is expressed in amastigotes in naturally infected dogs</td>
<td><em>L. infantum</em></td>
<td>Vasconcellos et al., 2014</td>
</tr>
<tr>
<td>E-NTPDase activity inhibits macrophage activation</td>
<td><em>L. amazonensis</em></td>
<td>Gomes et al., 2014</td>
</tr>
<tr>
<td>E-NTPDase is associated with adhesion and infectivity in macrophages</td>
<td><em>L. infantum</em></td>
<td>Vasconcellos et al., 2014</td>
</tr>
</tbody>
</table>
phagocytose apoptotic infected neutrophils and become definitive hosts for the amastigote replicative form of the parasite. Leishmania uses neutrophils as intermediate host cells and modulates their spontaneous apoptosis and ability to attract macrophages. These data indicated that Leishmania can use neutrophils as "Trojan horses" to silently enter macrophages [22]. Thus, the macrophage is an important host cell for establishing infection and the persistence of the parasite during Leishmania infection.

When macrophages are infected by Leishmania promastigotes, the parasites are recruited to a vacuolar compartment with characteristics of mature phagolysosomes, where they transform into non-motile amastigotes, which proliferate by binary cell division within the acidic and hydrolase-rich fagolysosomal compartment. The ability of these pathogens to target and replicate within the mature phagolysosomal compartment is remarkable. As macrophages respond to infection, tissue damage often occurs, contributing to the clinical manifestations of the various forms of Leishmaniasis [23, 24].

The persistence of the parasite infection correlates with the parasite’s ability to adapt to the hostile environment and counter the defenses of the host macrophage. Like other infectious agents, Leishmania species have evolved effective strategies to dodge the innate immune response during the early stages of infection, quickly modulating the signaling pathways of the host cell. Interestingly, several Leishmania molecules secreted or expressed on the surface of the parasite have been found to be involved in the inactivation of the key functions of the host macrophage, including the production of nitric oxide (NO), interleukin-12 (IL-12), tumoral necrosis factor alpha (TNF-α) and reactive oxygen species (ROS) [25]. In fact, many Leishmania molecules have been studied in-depth and have been described as potential virulence factors. For example, liphosphoglycan (LPG), glycosylinositol phospholipids (GIPLs), proteophosphoglycans (PPGs), secreted acid phosphatases (SAPs) and cysteine protease B have been well-explored in this matter [26-33]. The GP63, a zinc-metalloprotease named glycoprotein 63 (GP63), is another critical virulence factor that has been fully explored [34-38]. However, the E-NTPDases (ecto-nucleoside triphosphatase diphosphohydrolases), while less-extensively studied, have been demonstrated and believed to be important infectivity and virulence factors [39-48].

In the last few years, there has been accumulating evidence that the E-NTPDases can be potential virulence factors due to the relationship between ecto-nucleotidase activity and the ability of Leishmania to generate injury in mice. Moreover, these enzymes have the potential to regulate the concentrations of tri- and diphosphate nucleosides at the interaction of the microenvironment/pathogen, and by-products of nucleotide degradation, such as adenosine, can lead to indirect control of the immune response via purinergic signaling [49-52]. Therefore, this review will focus mainly on the description of some cellular and molecular events occurring during the pivotal multifaceted interaction of Leishmania and the macrophage. In particular, we will discuss the role of E-NTPDases and purinergic signaling pathways in the context of the modulation of regulatory mechanisms that support the inactivation of macrophage signaling pathways and their functions, allowing the Leishmania to dodge the harmful effects of the innate inflammatory response.

Table 2. Overview of the influence of Leishmania infection on the purinergic system in the context of the immune system (Purine receptors in parasite infections)

<table>
<thead>
<tr>
<th>Host cells</th>
<th>Paraphyte</th>
<th>Purine receptor</th>
<th>Mainly biological effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td><em>P. falciparum</em></td>
<td>P2Y1</td>
<td>Can participate in the permeability of the infected cell.</td>
<td>Li, 1998</td>
</tr>
<tr>
<td>Monocytes</td>
<td><em>M. tuberculosis</em></td>
<td>P2X7</td>
<td>mRNA increased</td>
<td>Placido, 2006</td>
</tr>
<tr>
<td>Macrophage</td>
<td><em>M. tuberculosis</em></td>
<td>P2X7</td>
<td>mRNA increased</td>
<td>Placido, 2006</td>
</tr>
<tr>
<td>Macrophage</td>
<td><em>L. amazonensis</em></td>
<td>P2X7</td>
<td>mRNA increased, receptor is functional</td>
<td>Chaves, 2009</td>
</tr>
<tr>
<td>Macrophage</td>
<td><em>T. gondii</em></td>
<td>P2X7</td>
<td>P2X7 activation may help in the elimination of infection</td>
<td>Correa, 2010</td>
</tr>
<tr>
<td>Macrophage</td>
<td><em>L. amazonensis</em></td>
<td>P2X7</td>
<td>Changed the receptor selectivity</td>
<td>Marques-da-Silva, 2011</td>
</tr>
<tr>
<td>Macrophage</td>
<td><em>L. amazonensis</em></td>
<td>P2Y1 and P2Y4</td>
<td>mRNA increased and receptor is functional</td>
<td>Marques-da-Silva, 2011</td>
</tr>
<tr>
<td>Cardiac mast cells</td>
<td><em>T. cruzi</em></td>
<td>P2X7</td>
<td>mRNA increased</td>
<td>Meuser-Batista, 2011</td>
</tr>
<tr>
<td>Peritoneal mast cells</td>
<td><em>T. cruzi</em></td>
<td>P2X7</td>
<td>Didn’t change mRNA level</td>
<td>Meuser-Batista, 2011</td>
</tr>
<tr>
<td>Macrophage</td>
<td><em>S. mansoni</em></td>
<td>P2X7</td>
<td>Decreased population of P2X7 in membrane surface. No change in mRNA level.</td>
<td>Oliveira, 2014</td>
</tr>
</tbody>
</table>
Biochemical and biological roles of ecto-nucleotidases and the E-NTPDases of leishmania

Extracellular nucleotides have been described as messengers in many kinds of cells (muscular, neuronal, and immune). In the immune system, they participate in the mediation of pro-inflammatory responses, including lymphocyte proliferation, the production of reactive oxygen species (ROS), and the secretion of cytokines as well as chemokines [53]. In the extracellular environment, nucleotides (e.g., ATP, UTP, ADP, and NAD), nucleosides and their derivatives are released in a controlled manner by different cell types to provide the initial responses of purinergic signaling [54]. The final components of regulatory purinergic signaling comprise the ecto-nucleotidases, and those hydrolyze extracellular nucleotides to generate other nucleotides and nucleosides (e.g., hydrolysis of ATP to ADP and ADP to AMP by E-NTPDases and hydrolysis of AMP to adenosine by 5’-ecto-nucleotidase, see Figure 1A). In this context, ecto-nucleotidases can turn off the signaling through the decrease in the concentration of a substrate that activates the purinergic receptor, or they can turn on the signaling if the product of their activity would be an activator of purinergic signaling [55, 56]. Among the important enzymes of this family are the diphosphohydrolases from ecto-nucleoside triphosphate. The diphosphohydrolase family (E-NTPDases) [57] can be found on the cell surface, on the membranes of some organelles, dissolved in the cytosol or even secreted [58]. This family of proteins shares five conserved domains called "apyrase conserved regions" (ACRs), which are essential for their catalytic activity [55, 59, 60]. Briefly, E-NTPDases hydrolyze ATP to ADP and ADP to AMP, which can be hydrolyzed to adenosine by 5’-ecto-nucleotidase [61]. Eight different E-NTPDase genes encode members of the E-NTPDase protein family [62]. Four of the E-NTPDases are typical cell surface-located enzymes with an extracellularly facing catalytic site (E-NTPDase 1, 2, 3, and 8). E-NTPDases 5 and 6 exhibit intracellular localization and undergo secretion after heterologous expression. E-NTPDases 4 and 7 are entirely intracellularly located and face the lumen of cytoplasmic organelles [62]. The E-NTPDases have several roles in eukaryotic organisms [63-68]. Although rare in bacteria, Legionella pneumophila secretes E-NTPDases that act as virulence factors [69]. Many pathogens are able to subvert the host immune response by the production of adenosine that is the end product of the joint action of E-NTPDase and 5’-ecto-nucleotidase. This nucleoside modulates cell function via membrane receptors coupled to G proteins (A1, A2A, A2B, A3) [70]. It is well appreciated that adenosine receptor expression is dynamically altered during both development and activation on the surfaces of macrophages and dendritic cells (DCs). The differential expression of adenosine receptors at various stages of inflammation is important for fine-tuning the responsiveness of adenosine receptors to maximize their ability to alter cell function in a way that generally leads to the restoration of homeostasis [71]. A summary of the general view of ecto-nucleotidases, ecto-nucleosides and mammalian immune system homeostasis control is shown in Figure 1. Additionally, E-NTPDases had been found in several protozoan parasites including the following: Toxoplasma gondii [72] Trichomonas vaginalis [73] Trypanosoma cruzi [40] Leishmania amazonensis [74], Leishmania braziliensis [75] and other organisms [46]. In Trypanosomatids, these enzymes are important components of the pathway of the salvage of purines [40] because these parasites are unable to perform de novo synthesis of purines [76]. In Leishmania, literature data suggest that E-NTPDases also participate in the establishment of infection [41, 44] and in the development of clinical signs [42]. In these organisms, the following two E-NTPDases were identified: E-NTPDase-1 and E-NTPDase-2. The isoform called as E-NTPDase-1 (approximately 70 kDa) is also known as guanosine diphosphatase and is more similar to E-NTPDase-1 from T. cruzi that was described for the first time by our research group [40]. Unfortunately, the literature on this topic is not uniform and other research groups have named these proteins differently, e.g., E-NTPDase-2 (approximately 40 kDa) is also called as ATP-diphosphohydrolase or nucleoside diphosphatase [48]. In order to stimulate a uniformity in the parasite E-NTPDase nomenclature, we have performed phylogenetic and bioinformatic analyses and proposed the following uniform nomenclature: TpNTPDase-1 and TpNTPDase-2 (T = trypanosomatids NTPDases; isoform 1 is approximately 70 kDa and isoform 2 is 40 kDa) [46].

There are many studies concerning ecto-nucleotidases and E-NTPDases in different species of Leishmania, and a general concept is that the higher the activity/expression of ecto-nucleotidases, in particular E-NTPDases, the higher the infection and virulence of the Leishmania strain or species. Below we will present the state of progress in this field and more deeply discuss the data involving macrophages.

The first group to study ecto-nucleotidases on the surface of Leishmania was the Mayer-Fernandes research group, who showed for the first time the presence of Mg-dependent ecto-ATPases in live promastigotes of Leishmania tropica [77]. Then, the same group demonstrated the presence of an Mg-dependent ecto-ATPase in Leishmania amazonensis and its possible role in adenosine acquisition and virulence [78]. They showed for the first time that virulent L. amazonensis presented higher ecto-ATPase activity than the avirulent promastigotes. In 2006, Pinheiro and co-workers demonstrated that the surface E-NTPDase from L. amazonensis is a protein from the CD39 family and that this enzyme may play a role in the purine salvage pathway [74].
Figure 1. Summary overview of purinergic signaling in the context of the immune system during homeostasis, dangerous stimuli or in the face of Leishmania infection. 1A) Summary representation of the purinergic signaling under homeostasis conditions. During homeostasis self-ectonucleotidases (e.g., ENTPDases and 5´-NT) maintain lower concentrations of purinergic signaling mediators (e.g., ATP, ADP, AMP and ADO) in order to maintain the ecto-nucleotide dependent homeostasis of the innate immune system. 1B) Summary of known changes in the purinergic signaling pathway through nonspecific dangerous stimuli (e.g., infection, inflammation, and necrosis). After a dangerous stimuli, the concentration of purinergic signaling mediators increases leading to the activation of purinergic receptors, which could culminate with increased levels of inflammation, apoptosis, chemotaxis, phagocytosis and ROS response. 1C) Summary of known influences of Leishmania infection on the purinergic signaling. During Leishmanial infection, the action of ectonucleotidases from the parasite (e.g., ENTPDases and 5´NT) can subvert the host immune system through the modulation of purinergic signaling, leading to the inhibition of P2 activation and inducing P1 activation by ADO. Additionally, E-NTPDases from Leishmania can act as pro-adhesion molecules to facilitate the infection of host cells (e.g., macrophages).
Furthermore, they showed higher expression and activity in amastigote than in promastigote forms and that adenosine down-regulated the expression of this enzyme. Additionally, they showed that adenosine and anti-E-NTPDase antibodies decreased the adhesion of promastigotes to mouse peritoneal macrophages.

There is clear evidence of the presence of E-NTPDases on the Leishmania surface, and the known main roles of nucleotides in the immune system stimulated other researchers to investigate E-NTPDases in Leishmania too. In 2004, Maioli and co-workers showed that L. amazonensis had a higher AMPase activity than L. braziliensis and that these data had a positive correlation with mouse immunosuppression. They observed lower levels of IFN-γ and TNF-β in lymph node cells caused by L. amazonensis and hypothesized that this event may be through adenosine, a known immunomodulator molecule produced by the conjunct action of E-NTPDase and 5′-ecto-nucleotidase (5′-NT or CD73) observed in live and intact parasites. Then, in 2008 Marques-da-Silva studied the influence of extracellular nucleotides and adenosine on Leishmania infection in a murine model susceptible to infection by L. amazonensis but resistant to infections by L. braziliensis and L. major. This work described that the more virulent parasite (L. amazonensis) hydrolyzed higher amounts of ATP, ADP and AMP and that this result correlated with higher expression of E-NTPDase-2 on the L. amazonensis membrane. Furthermore they have shown that the presence of adenosine or higher ecto-AMPase activity by 5′-NT on the parasite led to an increase in lesion size, parasitism and a delay in lesion healing. On the other hand, inhibition of a specific adenosine receptor (A2B) resulted in a decrease in lesion size. These data suggested that higher ecto-ATPase conjugated with higher ecto-AMPase activity on the Leishmania surface could convert the pro-inflammatory ATP molecule to the immunomodulatory adenosine molecule that may influence the establishment of Leishmania infection.

De Souza and co-workers investigated the relationship between ecto-nucleotidase activities and L. amazonensis infection more deeply. They have shown that higher ecto-nucleotidase activity in parasites from short-term culture positively correlates with higher lesions in mice models and higher infection rates in macrophages. On the other hand, lower ecto-nucleotidase activity in parasites from long-term cultures led to the development of smaller lesions and higher levels of IFN-γ production in lymph node cells from infected mice than the short-term culture parasites. In addition, infection with the long-term parasites expressed higher amounts of CXCL10 mRNA, which might activate macrophage cells to kill the parasites. This paper reinforced the evidence that the enzymes involved in the metabolism of extracellular nucleotides positively influence the adhesion to target cells and modulate the host cell chemokine production. Recently, Gomes et al. demonstrated the role of E-NTPDase2 from L. amazonensis in macrophage infection. This paper shows that in Leishmania amazonensis, the pathogenic agent of diffuse Leishmaniasis, the high activity of E-NTPDase-2 at the surface of the parasites, increased the survival rate in LPS/IFN-γ-activated cells. On the other hand, the inhibition of surface E-NTPDase2 led to a lower survival rate and higher macrophage activation. The inhibition of this enzyme activity resulted in decreased parasite survival in activated J774 macrophages, which was associated with increased production of NO and inflammatory cytokines (TNF-α and IL-12). The authors also demonstrated that the ecto-nucleotidase activity in L. amazonensis is also related to the inhibition of the inflammatory profile of macrophages. Therefore, the expression of E-NTPDase at the parasite surface plays an important role in the modulation and infection of macrophages.

Another group of researchers demonstrated that L. amazonensis resistant to vinblastine (a cell division inhibitor) showed an increase in the Mg2+-dependent ecto-ATPase activity and an increase in the severity of disease in infected mice.

Based on the importance of E-NTPDases in L. amazonensis infection and on the evidence of the presence of the same two orthologous E-NTPDase1 and 2 in L. infantum, a study published by our group in 2013 investigated the use of recombinant E-NTPDase2 from L. infantum (Lic-E-NTPDase2) as an antigen in the diagnosis of canine visceral Leishmaniasis (CVL) by an ELISA assay. In this work, anti-E-NTPDase2 antibodies were detected in 100% of the dogs with CVL from an endemic region of Brazil, regardless of the stage of the disease (asymptomatic, oligosymptomatic or polysymptomatic). This work has shown the expression of E-NTPDase2 on this parasite and highlighted its antigenicity. Next, our group studied the ecto-nucleotidase activity of recombinant Lic-E-NTPDase2, its expression on promastigotes and its possible roles in macrophage infection. We demonstrated that this enzyme is a genuine nucleotidase from the CD39 family, and its hydrolytic capability to use triand diphosphate nucleosides (ATP, ADP, GTP, GDP, UTP and UDP but not AMP) depends on divalent cations (Mg2+ or Ca2+) and on the use of known partial inhibitors from the CD39 family. The nucleotidase activity of this recombinant protein was similar to that of live promastigotes. In addition, we showed the expression of E-NTPDases on the surfaces of these cells. The role of Lic-E-NTPDase2 during Leishmania...
infantum chagasi adhesion and infection of macrophages was also investigated, and the results suggest that this protein participates as a facilitator of adhesion and infection. These roles were indicated because when we used the recombinant protein as a competitor or the anti-E-NTPDase2 antibody in adhesion and infection assays, we observed significant decreases in the adhesion and macrophage infection indices. However, the proliferation of the parasite inside macrophages was not affected. These results suggested the existence of a possible binding site (like an unknown receptor) for the Lic-E-NTPDase2 in macrophages that could participate in facilitating adhesion and infection [46]. These data are in accordance with previous results also observed in T. cruzi [45].

In 2011, Maia et al. demonstrated the occurrence of another conserved domain, which differs from the characteristic conserved domains (ACRs) of the ATP diphosphohydrolase family, as a functional region in ATP diphosphohydrolase isofoms of Schistosoma mansoni and L. braziliensis. In addition, there was associated antigenicity of this domain in Schistosomiasis and Leishmaniasis. The r-Domain B shares an identity with the conserved domain r78–117 within potato apyrase and has demonstrated immunostimulatory properties by activating humoral immune responses in healthy BALB/c mice as well as increasing the production of IgG2a and IgG1 subtypes. Along with the other results demonstrated, the authors suggested that for the human immune system, the conserved domain B (with high similarity to the domain B of potato apyrase) within either S. mansoni SmATPDase2 (r156–195) or L. braziliensis NDPase (r83–122) is rich in B-cell epitopes [80]. In another work, this same group reported an antigenic and catalytically active E-NTPDase1 isoform of approximately 50 kDa from L. infantum promastigotes. In the tests performed, the E-NTPDase activity was not affected by inhibitors of adenylate kinase and ATPases, and an antigenic conserved domain (r82–121) rich in B cell epitopes was identified [81]. As can be seen, L. amazonensis was the main investigated species in this field. All of the published papers agree that when the ecto-nucleotidase (ATPase, ADPase and AMPase) is higher, the infection and virulence are higher too. In addition, the roles of E-NTPDase as a facilitator of adhesion and infection of macrophages were indicated based on when the nucleotidase activity of E-NTPDase seems to be inactive. In this manner, we believe that E-NTPDases could bind to specific receptors on target host cells (like macrophages) and directly influence their adhesion or modulate the host purinergic signaling inhibiting the ATP and ADP signalizations and promoting the adenosine signaling. In this last role, the result of purinergic signaling may be dependent on the global ecto-nucleotidase capability of E-NTPDases and 5‘-NT from the parasite and may be influenced by the host purinergic system as well (see Figure 1).

In this context, in the next section we will discuss the purinergic signaling in the context of Leishmania infection.

**Purinergic signaling in the immune system in the context of leishmania infection and macrophages**

Purinergic signaling is present in many organisms as an important communication mechanism between the cells and the extracellular medium or cell to cell. Although we do not know the purine receptors involved in this signaling in all organisms, including in bacteria and protozoa, there are many studies concerning the purinergic signaling in mammals. The known purine receptors are cell membrane-anchored proteins that recognize purines and pyrimidines, which bind to the receptor triggering a series of reactions that can change cell behavior. These purine receptors are divided into two types, the P1 and the P2 receptors [82]. The P1-type receptors are divided into A1, A2a A2b and A3, all of which are activated by adenosine. These receptors are serpine type receptors coupled to G protein (GPCR) in the cytosolic portion acting in the activation or inhibition of adenyl cyclase [82, 83]. The P2-type receptors are subdivided into the following two major groups: P2X and P2Y. The P2X channel-type receptors (P2X1, 2) are activated by ATP and allow the flux of cations across the membrane as a non-selective ion channel. They are activated by different ATP concentrations depending on the subtype. The P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11,14) are GPCR [82-85]. The P2Y receptors may be activated by ATP, ADP, UTP, UDP, ITP and nucleotides bound to sugars [83].

The P1 and P2 receptors are expressed in several types of immune cells, such as neutrophils, monocytes, macrophages, lymphocytes, natural "killer" cells and dendritic cells. However, it is important to note that the variability of the population and expression of receptors in each cell type is crucial to determine the physiological response for each stimulus [82, 83, 86-94].

Previous studies demonstrated the effect of activation of these receptors in immune cells and their importance in the modulation of the response through the control of cytokine release. For example, it was demonstrated that the P2Y2 and P2Y12 receptors as well as the P1A2a, A2b and A3 receptors are important in the mechanism of macrophage chemotaxis induced by a C5a gradient that is dependent on ATP signaling [85]. In this work, the addition of potato apyrase (an E-NTPDase with high ATPase and ADPase activities) led to a decrease in chemotaxis because the potato apyrase removed extracellular nucleotides released after the stimulation [95]. In addition, the authors observed a decrease in cytokine release [95]. A similar result was observed when the authors used a cocktail of purinergic receptor inhibitors including MRS-2179
(P2Y1), AR-C69931MX (P2Y12), NF449 (P2X1 and P2X4) and the non-selective inhibitor for the P1 receptor 8- (p-sulfophenyl) theophylline in macrophages with deletion for P2Y2. Interesting, P2Y12 and P1 inhibitors are enough to disorient a macrophage P2Y2-deleted cell in a chemotactic gradient [95]. In another study using human neutrophils, it was shown that the polarized release of ATP led to the activation of P1A3 and P2Y2 receptors. These effects are related to the amplification of the signal that controls the cell orientation. In this study, authors showed that neutrophil chemotaxis changed when extracellular nucleotides were hydrolyzed by apyrase. Another effect observed was the reduction in the formation of superoxide [96]. Furthermore, activation or inhibition of P2 receptors on monocytes can cause effects in other cells, such as changes in neutrophil migration. In this context, it was shown that macrophages stimulated by lipopolysaccharides (LPS) were capable of releasing IL-8 that stimulates the migration of neutrophils. In addition, the secretion of IL-8 is dependent on cell stimulation by LPS, which in its turn leads to nucleotide release activating P2Y2 and P2Y4 receptors. Moreover, a decrease in IL-8 release was shown when P2Y2 and P2Y4 receptors were inhibited or knocked down. Similar results were obtained when potato apyrase was added during stimulation with LPS [93, 97].

In another study using mouse macrophages, it has been shown that the activation of the P2X7 receptor is regulated by E-NTPDase-1 on the cell surface. In experiments for the induction of apoptosis by extracellular ATP, it was found that normal cells have a much lower mortality rate than the E-NTPDase-1 gene knocked out cells. This suggests that E-NTPDase-1 acts as a regulator of P2X7 activation and blocks sudden apoptosis [98]. Taken together, those data indicate that the purine receptors play important roles in immune system cells and that ecto-nucleotidases and ecto-nucleotidases are closely related with purinergic signaling (a summary of the data is presented in Figure 1).

Thus, it is important to emphasize that E-NTPDases (synonym apyrase) can act in the regulation of purinergic signaling and that this activity is related with self-purinergic signaling control. This control could be by an ecto-nucleotidase from the same organism (e.g., E-NTPDase1 from the CD39 protein family) or by non-self-purinergic signaling control if the ecto-nucleotidase is from a distinct organism. Thus, we can imagine that the expression/activity of E-NTPDase on the surface of parasites or secreted by them can influence the host purinergic signaling, simulating the regulatory effect of the host cell enzymes but in this case favoring infection. In fact, several studies support this mechanism. For example there are many papers describing that the activation of P2X7 is required for the activation of cells from the host immune system and elimination of infection.

P2X7 activation is one important regulatory mechanism to start specific immune response against intracellular pathogens [91, 92, 99-101]. This receptor also acts in the control of apoptosis in monocytes and macrophages infected with *Mycobacterium tuberculosis*, and this is an important pathway through which cells can eliminate intracellular infection as well. In this manner, it was demonstrated that the P2X7 expression is increased when monocytes are infected by *M. tuberculosis* and that apoptosis induced by ATP is also increased. In addition, the apoptosis induced by ATP decreased when the P2X7 receptor was inhibited in infected cells [91]. Similar results were observed in cardiac mast cells infected by *T. cruzi*. In this study, infected cells showed increased expression of P2X7, and the parasite regulated the activation of this receptor. However, the specific mechanism of this regulation was not clear [102]. Studies with *T. gondii* showed that the P2X1 receptor was also important for the elimination of infection [103]. This paper has shown that infected macrophages were capable of eliminating the parasite when they were stimulated with ATP. In Schistosomiasis, a chronic inflammatory disease caused by the extracellular parasite *S. mansonii*, peritoneal macrophages from infected mice had less sensitivity to ATP than cells from uninfected animals. Despite that *S. mansonii* infection did not increase the global expression of P2X7, the authors observed a significant decrease in the P2X7 population on the membrane surfaces of cells from infected mice [104]. In human erythrocytes infected by *Plasmodium falciparum*, another work showed evidence that P2Y1 participates in cellular permeabilization. In this manner, infected cells were more sensitive to permeabilization induced by ATP than uninfected cells, and P2Y1-deficient cells showed lower levels of permeabilization than wild-type cells [105].

As noted for the pathogens described above, there are many papers that studied purinergic signaling in Leishmania infection. Most of these works studied infection in *L. amazonensis*, the causative agent of diffuse Leishmaniasis that is known to lead to non-ulcerative metastatic lesions related to an immunomodulatory action of the parasite in relation to the host [100, 106-109]. The literature has shown increased expression of P2X7, P2Y2 and P2Y4 receptors in infected macrophages in comparison to uninfected cells. In addition, the activation of these receptors was important for the elimination of the parasite, and infected cells were more sensitive to apoptosis induced by UTP or ATP [100, 108]. Interesting data concerning the P2X7 receptor showed that macrophages infected with *L. amazonensis* and uninfected macrophages have altered selectivity for ion flux [108]. One recent work more deeply elucidated the mechanism of action of the P2X7 receptor in the elimination of *L. amazonensis*. They observed that P2X7 activation is necessary for the formation and release of leukotriene B4 that in turn binds to the leukotriene B4 receptor and assists in parasite elimination pathways. When the P2X7...
receptor was blocked, it was observed that ATP did not elicit the expected response in infected macrophages, and when the leukotriene B4 receptor was blocked, a reduction in the effect of ATP on the infected cell was observed because the leukotriene B4 released could not bind to its receptor \[^{106}\]. We can conclude that \(\text{P2X}_7\) is very important for the activation of immune cells, mainly in macrophages, and the elimination of parasites, including Leishmania, but this receptor is not the only one involved in infection.

It has already been demonstrated that \(\text{P2Y}_2\) and \(\text{P2Y}_4\) can also participate in \(L.\ \text{amazonensis}\) elimination \[^{100, 108}\]. In fact, parasites can modulate the activation of purinergic receptors, including macrophage receptors, but it is still necessary to understand more details about how the parasite modulates these receptors, and it is necessary to expand the studies to other purinergic receptors.

The \(\text{P2X}_7\) receptor is the most studied receptor concerning its relationship with Leishmania infection, but we still need more details to better elucidate the relationship between purinergic signaling and Leishmania infection including the following details: the specific receptors involved in infections by different Leishmania species; and the commonly activated receptors. It is also important to understand the signaling cascade involved and the biological outcomes. We believe that many receptors and pathways could be activated at the same time, and this can make research in this field difficult. The expansion of knowledge in these fields will certainly contribute molecular details that can be applied in the future development of better and new drugs to treat Leishmaniasis or other types of intervention to control these diseases.

The figure below proposes a hypothetical signaling pathway that occurs during the interaction of Leishmania and macrophages.

**Conclusion and future perspectives**

Currently, there are 12 million people infected with Leishmania and every year there are 12 million new cases reported. However, there are not effective vaccines, and the parasites are increasing their resistance to the few drugs available. The Leishmaniasis are prevalent in 98 countries in Asia, Africa, Central and South America and some European countries, with at least 20 pathogenic species of Leishmania. Additionally, most drugs have several side effects increasing the difficulty of treatment. In general, the variation in drug sensitivity and the specific way that each species interacts with the host often translates into limitations in the drug choice.

The advancement in understanding the immune responses directed by the host towards the infection and how the parasites can interfere with these mechanisms will assist in efforts to minimize the symptoms of this disease. Similarly, the expansion of the knowledge of the functions of macrophages and Leishmania survival strategies will aid in making accurate decisions and increased efforts to develop drugs and vaccines.

Several studies cited here demonstrate the importance of the activation of purine receptors in host cells in order to have an effective response against the parasite. \(\text{P2X}_7\) is the receptor that we have the most details about until now, but more research is needed to better illustrate what receptors are involved and the specific role of each receptor \[^{91, 98-101, 103, 104, 106, 110-114}\]. As for the parasites, there have also been several studies demonstrating the importance of ecto-nucleotidases for the virulence mechanism. There is a direct relationship between the ability to hydrolyze nucleotides and the ability to sustain infection \[^{39, 42-44, 47, 74, 78, 115-119}\]. It has also been shown recently that the \(E\)-NTPDase of parasites can act as an adhesion protein during the early stages of infection \[^{45, 46, 120}\]. Inhibiting Leishmania molecules that actively modulate the macrophage signaling pathways is essential to prevent the intracellular survival of Leishmania. Thus, the demonstrated waterways are a good field to search for the development of new drugs.

**Conflicting interests**

The authors have declared that no competing interests exist.

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**Author contributions**

F.G.P., M.S.B., R.F.S., and J.L.R.F. conceived the paper and written the reviewed, C.M.M. done the table 1, M.S.B. done the table 2, F.G.P. and J.L.R.F. done the Figure 1, G.C.B., A.S.J., M.R.A., and J. L.R.F. analysed and reviewed the text and figures.

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