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Review Article

Purinergic signaling: A new front-line determinant of resistance and susceptibility in leishmaniasis

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ABSTRACT

Leishmaniasis is a neglected tropical disease that causes several clinical manifestations. Parasites of the genus *Leishmania* cause this disease. Spread across five continents, leishmaniasis is a particular public health problem in developing countries. *Leishmania* infects phagocytic cells such as macrophages, where it induces adenosine triphosphate (ATP) release at the time of infection. ATP activates purinergic receptors in the cell membranes of infected cells and promotes parasite control by inducing leukotriene B₄ release and NLRP3 inflammasome activation. Moreover, uridine triphosphate induces ATP release, exacerbating the immune response. However, ATP may also undergo catalysis by ectonucleotidases present in the parasite membrane, generating adenosine, which activates P₁ receptors and induces the production of anti-inflammatory molecules such as prostaglandin E₂ and IL-10. These mechanisms culminate in *Leishmania*'s survival. Thus, how *Leishmania* handles extracellular nucleotides and the activation of purinergic receptors determines the control or the dissemination of the disease.

Leishmaniasis is a neglected tropical disease found in almost 100 countries worldwide. It affects 12 million people globally, and about 1.5–2 million new cases are reported annually [1,2]. Leishmaniasis is the second-leading cause of death related to parasite infections, causing 20,000 to 30,000 deaths per year [3]. This parasitic disease can be generated by at least 20 different protozoan species belonging to the

family Trypanosomatidae, order Kinetoplastida, and genus *Leishmania*. Three clinical manifestations depend on the *Leishmania* species, ranging from self-healing cutaneous lesions and mucosal tissue damage to life-threatening visceral disease. Cutaneous leishmaniasis (CL) manifests primarily in the infectious site with self-resolving lesions; however, mucosal damage often occurs. In some cases,

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more severe variants of CL may occur, including diffuse or disseminated CL [4]. Some *Leishmania* species may localize in nasal and oropharyngeal mucosa sites, where they cause mucocutaneous leishmaniasis. The parasites also can migrate to the spleen, liver, and bone marrow, leading to fatal visceral leishmaniasis (VL) [5].

Leishmania spp. are transmitted by the bite of female vectors called sand flies, genus *Phlebotomus* and *Lutzomyia*, transmitters of Old World and New World disease, respectively [2]. The parasites exist in two forms during their life cycle: flagellar promastigotes, found in the vector's midgut, and amastigotes, mandatory intracellular forms found within phagolysosomes of phagocytic cells in the vertebrate host [6]. Infected sand flies bite the mammalian host during blood-feeding and regurgitate the infectious forms, called metacyclic promastigotes, into the dermis [7]. *Leishmania* parasites infect phagocytic cells present in the skin, mainly neutrophils and dermis-resident macrophages, in the first hour post-transmission [8]. The phagocytosed promastigote differentiates in amastigote form approximately 12–24 h later [9]. The *Leishmania* life cycle continues when the vector aspirates amastigote-infected cells or free parasites. Aflagellate amastigotes become virulent and non-proliferative metacyclic promastigotes in the vector's digestive tract [10].

Currently, leishmaniasis treatment is a challenge for researchers and physicians. Researchers struggle to develop vaccines for humans and domestic reservoirs such as dogs. Medications for treating leishmaniasis are toxic and generate drug resistance [11]. Thus, new therapeutic strategies are vital for leishmaniasis management. In this effort, the elucidation

of immune response is critical to the identification of new therapeutic targets.

Innate immune system cells present in the dermis, including dendritic cells (DC) and dermis-resident macrophages (DRMs), are the first line of defense against *Leishmania* [12,13]. After the infected vector injects the parasite into the dermis, neutrophils are quickly recruited to the infection site, promoting phagocytosis and becoming the first circulating cells to reach the infected tissue [9,14]. Recently, a population of DRMs was identified whose main characteristic is the expression of high levels of mannose receptor. In addition, these cells do not have progenitors from bone marrow, and IL-4 maintains them derived mainly from eosinophils in the skin. This inflammatory environment is required for DRM proliferation and maintenance of its M2-like phenotype [15,16]. DRMs are the first cells to become infected after transmission; they are the main target for infection and a niche for *Leishmania major* proliferation [9,15,17].

On the other hand, neutrophils can promote resistance to infection by some *Leishmania* species. During infection by *Leishmania donovani*, neutrophil depletion increased the parasitic burden in mice [18]. When obtained from *Leishmania amazonensis*-infected mice, neutrophils could release NETs, which helped prevent the parasite from spreading [19]. A previous study showed the importance of leukotriene B₄ (LTB₄) in the control of *L. amazonensis* infection [20]. Macrophages release LTB₄ when infected with the parasites, and this mediator induces neutrophil recruitment [21,22]. The interaction between neutrophils and macrophages is relevant in the context of leishmaniasis. The essential role of neutrophils

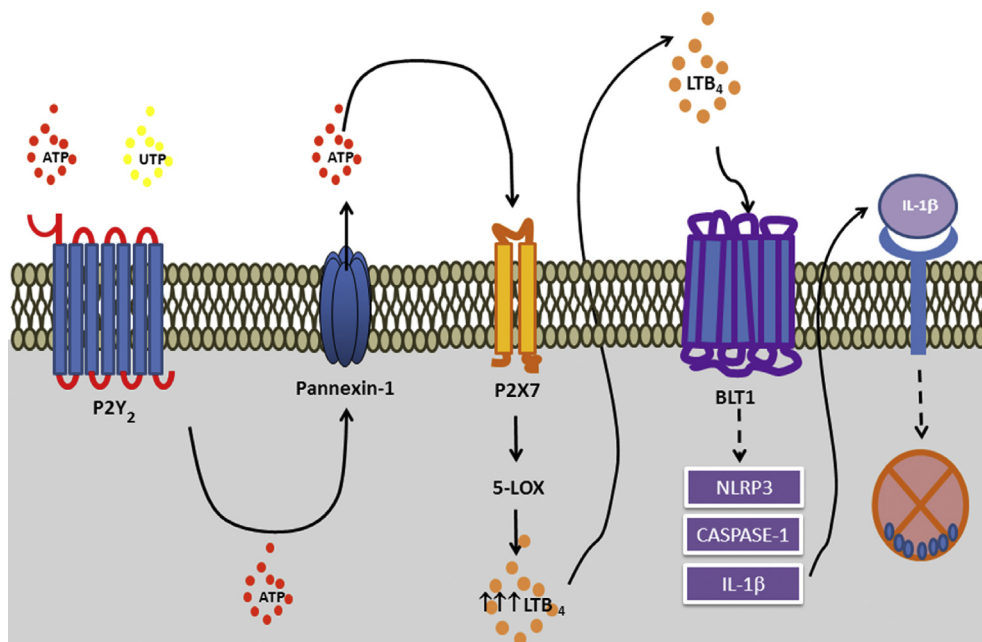


Fig. 1 P2Y₂ and P2X7 receptor cooperation favoring control of *Leishmania* infection. *Leishmania* infection induces P2Y₂ and P2X7 overexpression. Extracellular ATP and UTP activate the P2Y₂ receptor. The activation of the P2Y₂ receptor potentiates ATP release via Panx-1 channels. High levels of ATP in the extracellular media culminate in activating the enzyme 5-LO and consequent LTB₄ production and release. Then, LTB₄ acts in the extracellular environment to activate the leukotriene B₄ receptor 1 receptor that further induces NLRP3 activation and IL-1β secretion, promoting *Leishmania* infection control.

in the persistence of *L. major* infection is because these cells act as a “Trojan Horse.” In this model, neutrophils infected by *L. major* undergo apoptosis and silently enter macrophages, deactivating these cells [8,23] and favoring infection.

Macrophages and neutrophils have pattern recognition receptors (PRRs) activated by molecular patterns associated with pathogens (PAMPs) expressed on the *Leishmania* cell surface. The most well-known PAMPs present on *Leishmania* are lipophosphoglycan and glycoprotein 63, both in humans and mice [24–26]. One of the most well-known PRRs is Toll-like (TLR) receptors. The activation of TLRs was associated with the production and release of inflammatory mediators by macrophages and neutrophils, such as cytokines, lipid mediators, and adenosine 5'-triphosphate (ATP) [27,28].

ATP and other nucleotides are released through both specific and non-specific mechanisms [29]. Nucleotides may be released from live cells in a non-regulated pathway in response to various cell stress conditions, including exposure to cytotoxic agents, hypoxia, or plasma membrane damage [30]. During the inflammatory response, cell membrane disruption can lead to a non-specific release of large amounts of nucleotides. In the extracellular environment, ATP is practically non-existent; under physiological conditions, the extracellular ATP concentration is approximately 10 nM. By contrast, ATP is present in high concentrations in the intracellular space, ranging from 3 to 10 mM [31]. Ion channels in

the cell surface, such as pannexin (Panx), may regulate ATP release in healthy cells and consequently participate in several physiologic and pathologic processes in various cell types and tissues [32]. Three forms of Panx have been described in vertebrates, including Panx1, expressed in various tissues and cells, Panx2 expressed in the central nervous system, and Panx3 primarily present in the skin and bone [33,34]. Interestingly, lipopolysaccharide was shown to induce ATP release via Panx in a TLR activation-dependent manner [35]. TLRs are also crucial to *Leishmania* infection [36], and we found that *L. amazonensis* phagocytosis by macrophages induced ATP secretion [22], suggesting possible participation of TLR in ATP release during *Leishmania* infection [Fig. 1].

When in the extracellular media, nucleotides are signaling molecules that activate P2 receptors. The low concentrations of ATP in the extracellular media are maintained by enzymes that rapidly metabolize ATP to adenosine, called ectonucleotidases. These enzymes consist of four large families of ectoenzymes that include ectonucleotide pyrophosphatase/phosphodiesterase, ectonucleoside triphosphate diphosphohydrolase (i.e., NTPDase1/CD39), alkaline phosphatases, and ecto-5'-nucleotidase (CD73) [37]. The adenosine formed by the action of these enzymes is known to activate P1 receptors [37].

The role of purinergic signaling in leishmaniasis has been explored over the last two decades [38–40]. Phagocytosis of *L.*

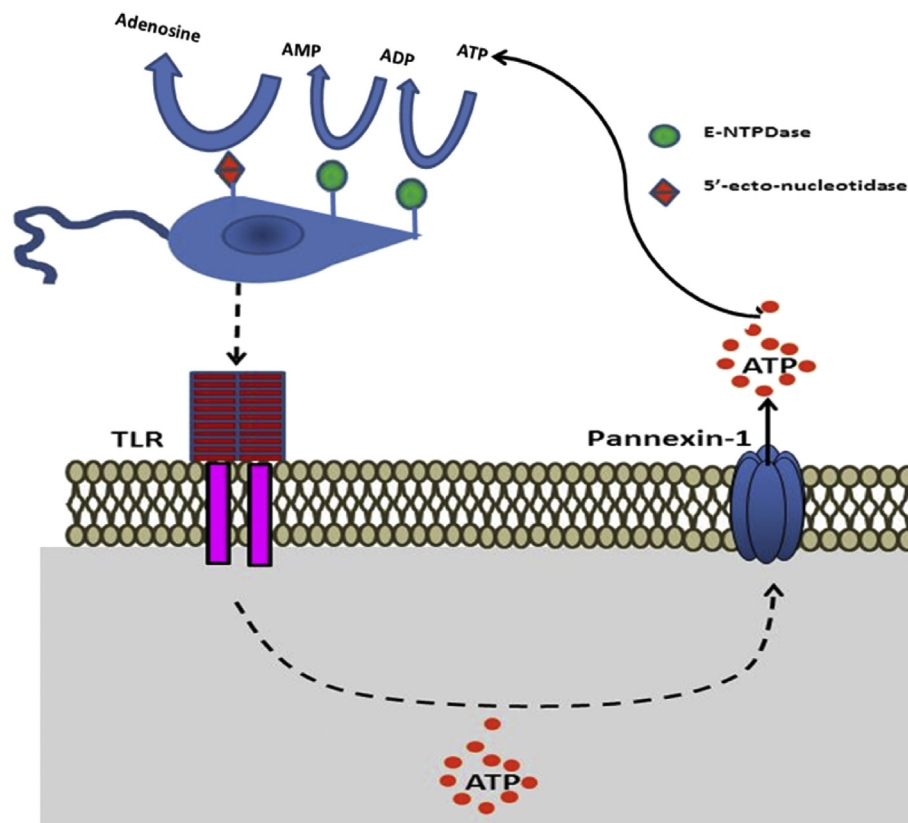


Fig. 2 *Leishmania* infection culminates in nucleotide release. *Leishmania* spp. is recognized by TLRs in the membrane of the host cell. The binding of parasites to these receptors induces ATP release to the extracellular environment through pannexin-1 channels. Once in the extracellular medium, ATP can be metabolized by ectonucleotidases such as ectonucleoside triphosphate diphosphohydrolase and ecto-5'-nucleotidase present in the *Leishmania* membrane, leading to Ado accumulation in the extracellular medium.

amazonensis by macrophages induces the release of ATP into the extracellular space [22]. Once released, ATP and other nucleotides activate microbicidal mechanisms via P2 receptors. These nucleotides can also be degraded by ectoenzymes generating adenosine, which has anti-inflammatory effects. In this review, we discuss the role of purinergic signaling in leishmaniasis, highlighting mechanisms that contribute to parasite control and contribute to a better understanding of the infection physiology [see Fig. 2].

P2 receptors and leishmaniasis

The family of P2 receptors is expressed in almost all cells in the body [41]. This family of receptors is subdivided into P2X and P2Y. The P2Y receptor family includes metabotropic G-coupled protein receptors. These receptors have seven transmembrane domains [Fig. 1A], and eight members have been cloned in mammals: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄ [42]. P2Y receptors can be differentiated according to the type of G-coupled protein: while P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors bind to Gq protein, P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors are linked to Gi protein. The P2Y₁₁ receptor, on the other hand, can bind both Gq and Gs proteins, being the only one with this capacity [42]. The family of P2Y receptors triggers cellular events such as activation and differentiation of human pro-monocytic cells [43]. They also mediate the production of IL-8 in inflammatory bowel diseases [44]. Moreover, P2Y receptors are involved in chemotaxis and phagocytosis of macrophages [45,46], chemotaxis of

neutrophils [47], and the clearance of damaged and apoptotic cells [48].

P2X are ionotropic receptors that form non-selective cationic channels permeable to Na⁺, K⁺, and Ca²⁺. Seven subtypes of the P2X family have been cloned: P2X1 to P2X7. The P2X5 receptor subtype is an exception, being permeable to Cl⁻ [49]. The most studied of the P2X receptors is the P2X7 subtype. Their activation leads to the opening of non-selective pores in the cell membrane, allowing the passage of molecules up to 900 Da [50]. In addition, as a consequence of P2X7 receptor activation, several cellular mechanisms are triggered, including apoptosis, exosome release, and cytokine secretion (i.e., IL-1 β and IL-18), along with inflammatory lipid mediators such as LTB₄ [22,49,51]. P2X7 also triggers the activation of microbicidal mechanisms, including reactive oxygen species (ROS) and nitric oxide (NO) production [40]. Thus, the relevance of the P2X7 receptor in the control of several diseases caused by protozoan parasites has already been demonstrated [52]. Polymorphisms in the P2X7 receptor gene have also been associated with susceptibility to certain infectious diseases [53].

The importance of extracellular nucleotides and the activation of purinergic receptors during infection by *Leishmania* has been characterized [38,52]. ATP can lead to the elimination of *L. amazonensis* in infected macrophages via the P2X7 receptor, with an increased expression and functionality of this receptor during *in vitro* infection [54]. ATP controls *L. amazonensis* infection by activating the P2X7 receptor, which induces the release of LTB₄ in peritoneal macrophages [22]. In addition, P2X7-deficient mice are more susceptible to *L.*

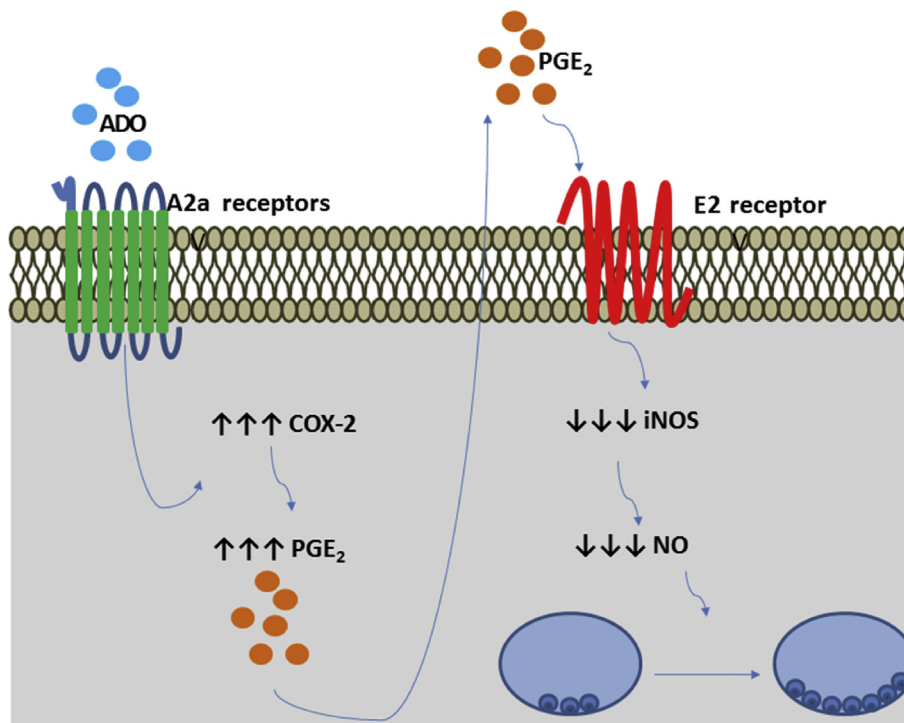


Fig. 3 P1 receptor activation and *Leishmania* survival. Ado activates A_{2A} receptors in the membranes of host cells. The activation of A_{2A} receptors induces COX-2 expression and consequent production and release of PGE₂ to the extracellular environment. PGE₂ activates prostaglandin E₂ receptors in an autocrine or paracrine manner, leading to the decrease of iNOS expression and NO production, resulting in the growth and establishment of *Leishmania* spp. Infection.

amazonensis infection [54]. The absence of P2X7 receptor during *L. amazonensis* infection resulted in higher IFN- γ and IL10 levels and lower IL-12p40, IL-4, IL-17, and TGF- β levels than in wild-type animals [54]. These data suggest that the lack of P2X7 receptors in *L. amazonensis*-infected mice causes intense inflammation associated with a Th1 response.

The P2X7 receptor is a well-known second signal for the full activation of inflammasome NLRP3, culminating in the conversion of pro-caspase-1 to its active form [55,56]. The caspase-1 enzyme is involved in the processing and release of cytokines such as IL-1 β and IL-18, with subsequent systemic effects on the immune system. NLRP3 inflammasome assembly is essential for initiating and progressing inflammatory responses by activating macrophages, monocytes, and dendritic cells [57]. Moreover, inflammasome activation plays an essential role during intracellular protozoa infections, including leishmaniasis [58]. Lima-Junior and collaborators (2013) demonstrated the role of NLRP3 in inflammasome and NO production in the control of CL [59]. Furthermore, NLRP3 inflammasome and IL-1 β have crucial roles in the visceralization of *L. donovani* soon after transmission by the natural vector [60]. Based on these findings, several reports demonstrated the ability of *Leishmania* to inhibit or evade NLRP3 inflammasome expression and activation [61,62]. Recently, we found that *L. amazonensis* infection control via P2X7 receptor and LTB₄ release depends on NLRP3 inflammasome and caspase-11 activation [63] [Fig. 3].

In addition to P2X receptors, P2Y₂ and P2Y₄ receptors are overexpressed and upregulated during infection. Uridine triphosphate (UTP) in the extracellular media can induce apoptosis of infected macrophages and, consequently, eliminate the parasite [64]. The role of UTP was also described *in vivo* during *L. amazonensis* infection, shifting the immune responses to a Th1 profile and increasing host resistance via ROS production [39]. Subsequential studies revealed that P2Y₂ and P2X₇ receptors cooperate to trigger potent innate immune responses against *L. amazonensis* infection, which involves Panx-1 and LTB₄ release [65] [Fig. 3]. Activation of P2Y receptor by UTP induced ATP release with consequent P2X₇ receptor activation and NLRP3 inflammasome assembly [66].

Ectonucleotidases and leishmaniasis

The ectonucleotidases play significant roles in nucleotide metabolism, preventing interaction of nucleotides with their

receptors and consequent activation of the immune system and inflammation in mammals. NTPDases modulate ATP degradation. Several subtypes have already been described: NTPDase 1, 2, 3, and 8 are expressed on the cell surface, NTPDase 4, 5, 6, and 7 are located inside cells [67]. These enzymes possess different catalytic properties: NTPDase 1 or CD39 hydrolyzes ATP and adenosine diphosphate equally, while NTPDase 2, 3, and 8 have ATP as the preferential substrate [68].

CD73/ecto-5'-nucleotidase hydrolyzes 5'-adenosine monophosphate, generating adenosine (Ado) and inorganic phosphate [69]. Ado may be regulated by adenosine deaminase, which modulates adenosine levels by deaminating this nucleoside into inosine [37].

The principal member of the NTPDase family in mammals is CD39; its role is to limit ATP concentrations in the extracellular medium and catalysis of ATP to ADP and ADP to AMP, subsequently converted into Ado by CD73 [37]. Outside cells, Ado has a half-life of a few seconds and can activate P1 purinergic receptors [39]. In humans, CD39 and CD73 are constitutively expressed in innate and adaptive immune cells such as monocytes, granulocytes, B-cells, and T-cell subsets [70]; it activates predominantly anti-inflammatory responses.

However, NTPDases were not described only in mammals. Several species of Trypanosomatids, including *Leishmania*, including *Leishmania infantum*, *Leishmania braziliensis*, *L. donovani*, *Leishmania mexicana*, *L. major*, *L. amazonensis*, and *Leishmania tropica*, express two predicted NTPDases [71,72]. Ecto-ATPase activity was demonstrated in *L. amazonensis*, *L. tropica*, and *L. infantum* [72–74] [Fig. 1].

L. amazonensis infection increases the number of CD39⁺CD73⁺ DC with consequent deactivation of these cells, promoting the establishment of the disease [75]. In *L. donovani* and *L. infantum* infections, ectonucleotidase inhibition in host cells decreased parasite survival and increased host-favorable cytokine production [76,77]. These findings demonstrated the role of these enzymes in *Leishmania* infection pathophysiology. These findings also corroborate the observation that patients with VL showed higher serum Ado levels than healthy individuals [78], suggesting that Ado is an essential regulator in VL.

Moreover, sandfly saliva exhibits ATPase activity which can hydrolyze ATP [79]. This saliva possesses high levels of Ado which modulates the inflammatory microenvironment in the skin, causing NO inhibition and macrophage inactivation,

Table 1 Adenosine receptor signaling and leishmaniasis.

Adenosine receptor	G-coupled protein	Second messengers	Effector enzymes	Effect in leishmaniasis
A _{2A}	Gs → ↑ Adenylate cyclase	cAMP ↑	↑ PKA	Favors leishmaniasis [95]
A _{2B}	Gs → ↑ Adenylate cyclase Gq → ↑ PLC γ	cAMP ↑ IP ₃ + DG	↑ PKA ↑ PKC	Favors leishmaniasis [96]
A ₁	Gi → ∅ Adenylate cyclase Go → ↑ PLC β	cAMP ↓ IP ₃ + DG	∅ PKA ↑ PKC	Unknown
A ₃	Gi → ∅ Adenylate cyclase Gq → ↑ PLC γ	cAMP ↓ IP ₃ + DG	∅ PKA ↑ PKA	Unknown

Abbreviations: PLC: Phospholipase C; IP₃: Inositol 1,4,5-trisphosphate; DG: Diacylglycerol; PKA: Protein kinase A; PKC: Protein kinase C; ∅: inhibition.

consequently favoring parasitic growth in host cells such as macrophages and neutrophils [80,81].

P1 receptors and leishmaniasis

Ado is an agonist for the P1 receptor family that includes four metabotropic receptors. All are G-coupled protein receptors, named A_1 , A_{2A} , A_{2B} , and A_3 [82]. Depending on the type of G-protein, these receptors are coupled and can generate inhibitory or stimulatory effects concerning the production of cyclic AMP (cAMP). The activation of A_1 and A_3 has inhibitory effects via G_i/G_o , and G_i/G_q proteins, respectively. By contrast, A_{2A} and A_{2B} receptors stimulate cAMP production and activate adenylyl cyclase via G_s and G_s/G_q , respectively [82,83]. Moreover, the activation of A_{2B} receptors may also induce phospholipase C activation via G_q [83] [Table 1]. In general, the activation of P1 receptors can generate several effects, including inflammatory reactions [83,84].

Ado possesses immunosuppressive properties; it suppresses M1 macrophage functions and favors polarization to the M2 phenotype [85]. In addition, the activation of Ado receptors may impair the differentiation of monocytes into macrophages [86].

Macrophages produce nitric NO through inducible NO synthase (iNOS) with an anti-leishmanial effect [87]. Interestingly, Ado can negatively regulate the release of NO by impairing the expression of iNOS [88]. Moreover, A_{2B} receptor signaling prevents $IFN-\gamma$ production by Th1 cells and, consequently, $IFN-\gamma$ -induced macrophage activation [89]. *L. infantum* can subvert the host response by inducing A_{2A} receptor signaling to promote the establishment of the infection [90] [Fig. 3]. Moreover, A_{2B} receptor activation can impair DC activation [91]. The anti-inflammatory effects of PGs have been demonstrated during the resolution phase of inflammation [92] [Fig. 3]. PGE_2 suppresses macrophage inflammatory responses and allows *Leishmania* establishment [93,94]. PGE_2 impairs nitric oxide synthase expression and, consequently, downregulates NO production [92,95], combining with PGE_2 to favor infection [Fig. 3]. These findings suggest that Ado may induce the formation of an anti-inflammatory environment that facilitates the parasite growth and consequent establishment of infection.

Conclusion

In summary, studies of purinergic signaling during *Leishmania* infection are crucial for understanding the disease's resistance and susceptibility. Consequently, new therapeutic methods could be developed using these mechanisms. As described here, the parasites have developed strategies to evade control pathways. The host triggers the purinergic signaling as an attempt to eradicate the parasite. P2 receptors, when activated during infection, stimulate the production and release of several microbicidal molecules. However, to perpetuate itself, *Leishmania* built pathways that can prevent its death, using purinergic mechanisms such as ectonucleotidase overexpression and P1 receptor activation.

In this context, the development or discovery of specific P2Y₂ or P2X7 agonists could contribute to parasite control boosting the immune response in leishmaniasis. Interestingly, natural compounds have been explored as modulators of P2 receptors [96,97]. Clemastine fumarate, a synthetic ethanolamine that potentially acts as a P2X7 receptor positive allosteric modulator, showed antileishmanial effects [98]. Diquafosol (INS365), a P2Y₂ agonist, has already been approved as a topical treatment for dry-eye disease could be repurposed for cutaneous leishmaniasis treatment [99]. In addition to P2 agonists, the administration of CD39/CD73 inhibitors or neutralizing antibodies that limit adenosine generation could also be an interesting approach to inhibit parasite growth in host cells [70]. Finally, adenosine A_{2A} and A_{2B} antagonists could also inhibit the formation of an immunosuppressive environment that contributes to parasite spreading, as previously proposed for the treatment of some types of cancer [70].

Development of more severe forms of leishmaniasis and even asymptomatic disease may be caused by host defect in the signaling triggered by the purinergic receptor; nevertheless, this has not yet been demonstrated in *Leishmania*-infected patients and would be of great importance for understanding the disease and its worsening. Such processes have been shown to occur in other infections, such as *Mycobacterium tuberculosis* that causes tuberculosis [53].

Conflicts of interest

The authors declare no competing or financial interests.

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