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Purinergic signaling and infection by *Leishmania*: A new approach to evasion of the immune response

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ABSTRACT

Infection by protozoan parasites is part of the most common Tropical Neglected Diseases. In the case of leishmaniasis, several millions of people are at risk of contracting the disease. In spite of innumerous studies that elucidated the immune response capable of killing the parasite, the understanding of the evasion mechanisms utilized by the parasite to survive within the very cell responsible for its destruction is still incomplete. In this review, we offer a new approach to the control of the immune response against the parasite. The ability of the parasite to modulate the levels of extracellular ATP and adenosine either by directly acting on the levels of these molecules or by inducing the expression of CD39 and CD73 on the infected cell may influence the magnitude of the immune response against the parasite contributing to its growth and survival.

Leishmaniasis comprises a group of parasitic diseases caused by several species of the *Leishmania* genus. Depending on the combination of the parasite species and yet unknown factors of the host, the disease can manifest itself as a single cutaneous ulcer, multiple lesions throughout the body or even visceral infection which, when untreated, is often lethal [1]. The disease is transmitted by infected females of sand flies of the Phlebotominae family during blood feeding [2] and affects approximately 300,000 people worldwide annually, mainly in the tropical and subtropical areas [3].

The immune response against the parasite is dependent both on the innate as well as the adaptative responses (for recent reviews, see [4–7]). In this review we will focus primarily on the innate aspects of the immune response and the role of the purinergic signaling in the modulation of such response.

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Innate response to *Leishmania*

During blood feeding of infected phlebotomine females, neutrophils rapidly migrate to the site of infection and phagocytize metacyclic promastigotes that are injected together with the insect saliva [8,9]. The role of neutrophils during infection is contradictory. These cells have been reported to help eliminate the parasite via the production of ROS and the induction of extracellular traps [10,11]. However, they also may play a role in inhibiting macrophage activation, due to their death by apoptosis after infection by the parasite [12]. Neutrophils also secrete chemokines that recruit monocytes/macrophages as well as dendritic cells to the site of infection [4,13].

Dendritic cells (DC) are antigen presenting cells capable of producing IL-12 upon infection with some *Leishmania* species such as *Leishmania major* [14]. This production of IL-12 is crucial to the development of a specific Th1 response that is protective to the host [15,16]. However, *Leishmania amazonensis*, which causes a severe form of cutaneous leishmaniasis (diffuse cutaneous leishmaniasis) characterized by the lack of specific immune response in patients [17], inhibits DC activation and interfere with antigen presentation and development of T cell response [18] thus evading the immune response both in humans and in the mouse model [17–21].

Even though *Leishmania* can infect neutrophils and DC, macrophages are the cells in which these parasites live and multiply within the infected mammalian host. *Leishmania* gain access to macrophages via phagocytosis mediated by complement fragments that are deposited and inactivated on the surface of the parasite, due to the action of proteases present on the surface of the infective promastigote [22–25]. The entry of the parasite via complement receptor (CR3)-mediated phagocytosis interferes with macrophage activation allowing the parasite to prevent the initial respiratory burst that, otherwise, would destroy the parasite [26]. However, upon activation by IFN-γ [27,28], produced by both NK [29] and T cells (both CD4 [30] and CD8 [31]), macrophages are activated to produce ROS and NO which are the main effector mechanisms for parasite killing within these cells [32].

In spite of the potential ability of the host to control parasite growth, several mechanisms contribute to evasion of the immune response by *Leishmania*. These mechanisms include, in addition to those already mentioned above, escape from complement activation [26,33], inhibition of macrophage activation by apoptotic neutrophil [12], production of inhibitory cytokines such as IL-10 and TGF-β by macrophages and DC, and the induction of regulatory T cells capable of controlling the immune response [4,19,21]. Although these inhibitory mechanisms have been extensively studied, the evasion of the immune response by some parasite species, in special *L. amazonensis*, is not restricted to these mechanisms. For example, IL-10 deficient mice infected with *L. amazonensis* still develop lesions in spite of an increased Th1 response [34]. Thus, it is conceivable that other mechanisms of immune evasion are present during *Leishmania* infection and modulation of the immune response via purinergic signaling may be one of such mechanisms.

Purinergic signaling and inflammation

During infection or cell injury, ATP can be released to the extracellular milieu and has been described as a danger signal, alerting the immune system to alterations in cellular integrity [35–38]. ATP can also be released by intact cells through conexin and panexin channels and also via the $P2X_7$ receptor [39].

Extracellular ATP is a potent inducer of inflammation characterized by macrophage and DC activation and increased production of IL-12 and TNF-α by these cells [40–45]. ATP exerts its effects by binding to P2 receptors which are divided in two subtypes: P2X receptors, that are associated to ionic channels and protein G coupled-P2Y [46–48]. In immune cells, $P2X_7$ is the main ATP receptor and its activation accounts for most of the inflammatory effects of extracellular ATP [49–51].

In order to regulate the extracellular effects of ATP, its concentration is controlled by the action of extracellular enzymes of which the main players in immune cells are the ecto-NTPDase (CD39) that hydrolyses ATP to ADP and subsequently to AMP and the ecto-5′-nucleotidase (CD73) that removes the phosphate group of AMP leading to the production of adenosine. Adenosine is, then, deaminated to inosine by adenosine deaminase [52,53]. In addition, adenosine concentrations inside and outside the cell are kept relatively constant (between 30 and 300 nM) by the action of bidirectional nucleoside transporters. However, in pathophysiological conditions such as hypoxia, ischemia and cell injury, adenosine extracellular concentrations can peak at 10 μM [54–56].

By acting on P1 receptors, in particular A_{2A} and A_{2B}, adenosine counteracts the inflammatory effects of ATP [57]. Thus adenosine inhibits the production of inflammatory cytokines by macrophages and DC and the production of microbicidal effectors by neutrophils and macrophages. In addition, adenosine stimulates the synthesis of IL-10, one of the major regulatory cytokines [47,58–63].

Thus, the balance between extracellular ATP and adenosine can contribute to the control of inflammation by, respectively, stimulating or inhibiting the cells involved in the immune response. Next, we present data from the recent literature that show that the purinergic system may interfere with the establishment of infection by *Leishmania*.

The role of saliva

As mentioned above, *Leishmania* promastigotes are transmitted by the bite of an infected phlebotomine. During blood feeding, the insect regurgitates and a portion of saliva is inoculated in the host dermis. In order to facilitate the blood meal, phlebotomine saliva is endowed with several substances that prevent blood clotting formation. Thus, saliva from phlebotomine sand flies (*Phlebotomus papatasi* and *Phlebotomus argentipes*) is rich in adenosine and AMP [64–66]. In addition, transcriptome analysis indicated the presence of apyrases and 5′-nucleotidases in the saliva of *Lutzomyia longipalpis* [67], *Phlebotomus perniciosus*, *P. argentipes*, *Phlebotomus ariasi* [68] and *Phlebotomus duboscqi* [69]. AMP and adenosine

inhibit platelet aggregation [70]. The presence of nucleotidases in the saliva contributes to the hydrolysis of extracellular ATP, preventing platelet aggregation and blood clot formation [71].

In addition to prevention of blood clotting, the presence of adenosine, AMP and ectonucleotidases may also influence the host immune response and facilitate the establishment of the infection. It has been demonstrated that addition of salivary gland extract together with the parasite exacerbates lesion development in the murine model of leishmaniasis [72–75]. Recent studies by Carregaro and colleagues [76] demonstrated that adenosine and AMP present in the saliva of *P. papatasi* exacerbate lesion development in *L. amazonensis* infected mice, probably due to increased IL-10 production and induction of tolerogenic dendritic cells and regulatory T cells. These effects seem to be mediated by the A_{2A} adenosine receptor. Corroborating the role of nucleotides and nucleosides of the insect saliva in the immunomodulation of the host response Katz and colleagues [64] demonstrated that *P. papatasi* saliva decreases NO production by activated macrophages. The same was not observed when the saliva of *L. longipalpis* is used. Interestingly, the levels of AMP are smaller in the saliva of *L. longipalpis* than in the saliva of *P. papatasi*. In addition, adenosine deaminase activity has been observed in the saliva of *L. longipalpis* [77] and *P. duboscqi* [78], but not in the saliva of *P. papatasi* [78]. The presence of adenosine deaminase could contribute to a decrease in adenosine concentration at the bite site thus inhibiting the modulatory effect of the saliva.

Altogether, these results indicate a strong involvement of the purinergic signaling pathway during the first moments of the inoculation of the parasite in the host dermis.

Leishmania ectonucleotidases

In addition to the effects promoted by the insect saliva, ectoenzymes present on the surface of the promastigote may also contribute to the establishment of the infection by *Leishmania*.

Leishmania and other trypanosomatids are incapable of de novo synthesis of the purine ring and thus depend on salvage pathways to synthetize purine nucleotides [79,80]. In order to obtain nucleosides from the external medium these parasites express ectonucleotidases thus enabling the external hydrolysis of nucleotides to the respective nucleosides.

Amongst the several ectonucleotidases the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase – EC 3.6.1.5) and the ecto-5′-nucleotidase (EC 3.1.3.5) have been shown to exert important roles in the infection by *Leishmania*. E-NTPDase has been reported on *Leishmania tropica* [81] and *L. amazonensis* [82,83]. In addition to E-NTPDase, we also demonstrated that *L. amazonensis* presents ecto-5′-nucleotidase activity [84].

Several studies have associated the presence of E-NTPDase and ecto-5′-nucleotidase activities to the virulence of different *Leishmania* species [82,84–86]. Thus, parasites with increased ectonucleotidase activity are more capable of infecting macrophages [87] and induce larger lesions in mice [84,86]. Furthermore, we have demonstrated a positive correlation between the ATP, ADP and AMP hydrolytic activity in *Leishmania braziliensis* isolates and their ability to control the establishment of the immune response in mice. More importantly, a positive correlation between the nucleotide hydrolytic activity and the severity of the clinical manifestation was also observed [85]. Similarly, *L. amazonensis* isolates with different ectonucleotidase activity show distinct capacity to survive in infected macrophages and to down-modulate nitric oxide production [87]. Interestingly, the association of ectonucleotidase activity and parasite virulence seems not to be restricted to *Leishmania*, given that it has been reported also in *Trypanosoma cruzi* [88], *Toxoplasma gondii* [89] and *Legionella pneumophila* [90]. These studies suggest that the hydrolysis of extracellular ATP to adenosine, in addition to fuel the purine salvage pathway, may also contribute to the modulation of the host immune response. In fact, we have shown that inhibition of adenosine receptors during infection by *L. braziliensis* increase the production of inflammatory cytokines and decreases tissue parasitism [84]. The fact that extracellular adenosine can modulate the immune response against the parasite suggests a potential role of ectonucleotidases in the virulence of the parasite. Corroborating this hypothesis, we and others have shown a correlation between the level of ectonucleotidase activity and expression and the virulence of the parasite, for both *Leishmania* and *T. cruzi* [86,88,91]

Interestingly, in addition to the production of extracellular adenosine, it has also been demonstrated that E-NTPDases of *T. cruzi* and *L. amazonensis* are involved in the attachment of the parasite to the host cell [83,92].

Finally, it has been shown that, although *L. amazonensis* triggers the release of neutrophil extracellular traps [10], the parasite's 3′-ectonucleotidase is capable of degrading these traps [93], enhancing even more the contribution of ectonucleotidases to the control of the innate immune response at the first moments of interaction between the parasite and the host cells.

Collectively, these results indicate that, in addition to their role in the insect saliva, ectonucleotidases from the parasite are important tools to evade the innate immune response and should be considered as potential targets for vaccine development against *Leishmania*.

Purinergic signaling and immune response

Once inoculated in the dermis of the host *Leishmania* promastigotes are phagocytized by cells present at the site of infection. Although the macrophage is the cell in which the parasite proliferates and persist to establish the infection, it has been demonstrated that *Leishmania* are also phagocytized by neutrophils and dendritic cells and this interaction can modulate the immune response to the parasite.

Neutrophils rapidly infiltrate the site of infection and phagocytize the inoculated promastigotes [8,9]. It has been proposed that interaction of the parasite with these cells induces neutrophil apoptosis. The interaction of the apoptotic neutrophil loaded [12] or not [8] with the parasite would then deactivate the macrophage, favoring parasite survival within the phagolysosome. As mentioned above, *Leishmania* also induces the release of NET (neutrophil extracellular traps) by neutrophils and may also degrade the nucleic acids present in

this structure via the action of a 3′-nucleotidase [10,93]. Although no direct proof has been yet provided, it is conceivable that nucleic acid hydrolysis may release adenosine which, by acting on the adenosine receptors A_{2A} and/or A_{2B}, could modulate the host immune response.

Dendritic cells have also been shown to be infected by *Leishmania* promastigotes [94]. This infection is capable of modulating the dendritic cell ability to present antigen to T cells thus affecting the establishment of the adaptative immune response [18,95–98]. Our group has shown that, at least part of the modulatory effect of the infection on the activation of dendritic cells (expression of CD40) is dependent on the activation of the A_{2B} adenosine receptor, mediated by the production of extracellular adenosine originated by the increased ectonucleotidase expression (CD39 and CD73) on the surface of infected cells [18]. In addition, this study also shows that inhibition of dendritic cell activation interferes with T cell proliferation which can be reverted by the blockade of the A_{2B} adenosine receptor.

Infection of macrophages by *Leishmania* is dependent on the phagocytosis of the parasite. Interestingly, an increased expression of $P2X_7$ receptors is observed in infected macrophages [99,100]. Due to the upregulation of the $P2X_7$ receptors, addition of extracellular ATP to infected macrophage cultures reduces the percentage of infected macrophages, suggesting a possible role of these receptors in the control of the parasite. The control of parasite growth by activated $P2X_7$ receptors seems to be mediated by the production of LTB4 [101].

In spite of the expression of $P2X_7$ receptor by infected macrophages and the ability of added extracellular ATP to control parasite proliferation, *Leishmania* survives within these cells, indicating that other purinergic signaling mechanisms may exist in the infected macrophage. In fact, we have observed that macrophages infected by *L. amazonensis* express high levels of CD39 and CD73 and that inhibition of the A_{2B} adenosine receptor during infection favors the survival of the parasite (unpublished observations). Furthermore, we have demonstrated that activation of the adenosine A_{2B} receptor inhibits the production of NO and IL-12 by infected macrophages even in the presence of activating stimuli such as IFN-gamma and LPS [87], allowing for the enhanced survival of the parasite. Corroborating these findings, a recent study demonstrated increased expression of the A_{2B} in monocytes from patients with visceral leishmaniasis and the possible involvement of the activation of this receptor with IL-10 production by infected cells [102].

Conclusion

Several lines of evidence indicate that purinergic signaling interferes with many aspects of the immune response against *Leishmania*. These findings provide a different approach to mechanisms of immune response evasion by the parasite similar to those being investigated in several types of tumors. Although in its initial phases, the field seems promising and may contribute to the development of new therapeutic approaches to the control of parasite progression via the activation of $P2X_7$ receptors or inhibition of adenosine receptors.

Conflict of interest

The authors declare that they have no competing interests.

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