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Role of extracellular nucleotides in the immune response against intracellular bacteria and protozoan parasites

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Abstract

Extracellular nucleotides are danger signals involved in recognition and control of intracellular pathogens. They are an important component of the innate immune response against intracellular pathogens, inducing the recruitment of inflammatory cells, stimulating secretion of cytokines, and producing inflammatory mediators such as reactive oxygen species (ROS) and nitric oxide (NO). In the case of extracellular ATP, some of the immune responses are mediated through activation of the NLRP3 inflammasome and secretion of the cytokine, interleukin-1β (IL-1β), through a mechanism dependent on ligation of the P2X7 receptor. Here we review the role of extracellular nucleotides as sensors of intracellular bacteria and protozoan parasites, and discuss how these pathogens manipulate purinergic signaling to diminish the immune response against infection.

Keywords

Intracellular pathogens; Danger signals; Extracellular ATP; Purinergic receptors; Inflammasome; Inflammation

1. Introduction

ATP and other nucleotides are released from cells at sites of inflammation of different tissues undergoing different disease states. The nucleotides can be released by active release or as a result of cell death [1–3]. Once in the extracellular space, the nucleotides behave as “danger signals” and can directly activate two families of membrane-bound nucleotide receptors named P2 receptors. The P2X receptors are non-selective cation ion channels [4], while P2Y receptors are G-coupled receptors [5]. Members of both families of P2R have been implicated in modulation of immunity by controlling cell migration [6–8], cytokine release [9,10], maturation of dendritic cells [11], and the immune response during infections with intracellular pathogens [12]. Thus, extracellular ATP can be secreted under a broad

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range of conditions including antigen presentation and macrophage interaction with bacteria or microbial products such as lipopolysaccharide (LPS) [13]. Once in the extracellular medium, ATP and UTP can mediate migration of macrophages and neutrophils towards inflamed organs [14,15], function as sensors for apoptotic cells and stimulate phagocytosis of apoptotic cells [14,16,17], and promote maturation of dendritic cells shaping the immune response [18].

P2R activation, in turn, is controlled by a family of ectonucleotidases that hydrolyze nucleotides and regulate the degradation of nucleotides into nucleosides, which can activate adenosine receptors named P1 receptors (P1R) [19,20]. Physiologically, the balance between extracellular nucleotide concentrations, and the expression of P2R, ectonucleotidases and P1R on the cell surface, are responsible for the extent to which purinergic signaling will contribute to the immune response [13] and host resistance to infection by microbial pathogens [21].

This review will summarize mechanisms whereby extracellular nucleotides are used to detect infection with intracellular pathogens (bacteria and protozoan) and stimulate an immune response against the infection.

2. The role of extracellular nucleotides during bacterial infection

Humphrey and Dubyak published in 1996 one of the first reports that LPS from bacteria can modify the sensitivity of macrophages to ATP. They demonstrated that pre-treatment with LPS and IFNγ enhances activation of phospholipase D (PLD), plasma membrane permeability, and cytolysis, as indicated by Ca^{2+} influx, ethidium bromide uptake, and lactate dehydrogenase release triggered by treatment with the P2X7 agonist benzoyl ATP (BzATP) [22]. Moreover, ATP ligation of P2X7 can modulate secretion of the pro-inflammatory cytokine, IL-1β, induced by LPS [23,24]. It was also shown that extracellular ATP could control mycobacterial infection in mice or human macrophages [25,26]. ATP-mediated inhibition of mycobacterial infection involves activation of PLD and increased phagosome-lysosome fusion [27–29].

Subsequently, a number of reports showed an association between gain-of-function or loss-of-function P2X7 single nucleotide polymorphisms (SNPs) with susceptibility or resistance to different diseases [30], including tuberculosis [31,32].

Chlamydiae are obligate intracellular bacteria that infect epithelial cells and macrophages. The bacteria survive within host cells by interfering with development of the vacuole harboring the bacteria (called an inclusion), blocking its fusion with lysosomes and inhibiting its acidification [33,34]. Using different *Chlamydia* species and strains, we have shown that extracellular ATP can decrease infection in macrophages and epithelial cells [35–38]. The effects are dependent on P2X7 ligation, PLD activation and phagolysosome formation, since PLD activation favors intracellular vesicle fusion [36]. P2X ligation also affects chlamydial infection in vaginally-infected mice [38]. Thus, vaginal infection was more severe in P2X7-deficient mice, which displayed a higher level of acute inflammation in the endocervix, oviduct, and mesosalpingeal tissues than infected wild-type mice [38]. These findings strongly suggest that purinergic signaling is involved in the immune response

against *Chlamydia* infection. More recently, we have expanded this concept by proposing that activation of PLD may be a general mechanism for elimination of parasites that normally reside within intracellular vacuoles that avoid fusion with lysosomes [37].

Besides directly inhibiting infection, extracellular ATP contributes to the host immune response against intracellular bacteria, through its ability to stimulate P2X7-dependent IL-1β secretion [39,40]. Thus, chlamydial infection stimulates IL-1β or IL-18 secretion through activation of the NLRP3 inflammasome [41,42], and this mechanism may be important for host defense in a model of *Chlamydia pneumoniae* lung infection [43,44]. Since the activation of P2X7 could control microbial infection through activation of NLRP3 inflammasome and IL-1β or IL-18 secretion, it would not be surprising to observe both pathogen and host adapting to each other through evolution. In this context, it is interesting to note that *Chlamydia* renders infected cells resistant to ATP-induced host cell apoptosis [35].

Other intracellular bacteria, such as *Mycobacterium tuberculosis* and *Porphyromonas gingivalis*, also inhibit P2X7-mediated apoptosis of macrophages or primary gingival epithelial cells (GECs) apoptosis [45–47], which may be mediated by a homolog of nucleoside diphosphate kinase (Ndk) secreted by the bacteria [45,47]. It is possible that the *P. gingivalis* Ndk may also inhibits inflammation, since ATP treatment induces NLRP3-dependent caspase-1 activation and release of IL-1β in infected GECs cells [48].

Conversely, a leukotoxin, produced by the Gram negative bacterium *Aggregatibacter actinomycetemcomitans* that is mainly associated with severe forms of periodontitis [49], targets the P2X in human macrophages, inducing cell death through a mechanism known as pyroptosis and IL-1β release [50].

In future studies, it would be interesting to examine whether there is a correlation between the presence of SNPs in P2X7 and periodontal disease or pathogenesis due to vaginal infection with *Chlamydia*, such as pelvic inflammatory disease, as have been shown for tuberculosis and other inflammatory diseases [30,31].

3. Extracellular nucleotides as sensors for the presence of protozoan parasites

We had originally reported that *Trypanosoma cruzi* infection can increase the sensitivity of murine thymocytes to the effects of extracellular ATP, which correlates with the thymus involution phase that is typically observed in Chagas' disease [51]. Moreover, ATP induces P2X7-mediated increase in plasma membrane permeabilization, calcium signaling, and cellular death in CD4+/CD8+ double–positive thymocytes collected from infected mice only during the atrophy phase of disease [51]. However, in vivo assays showing thymus atrophy in P2X7-deficient mice suggested that P2X7 may not be centrally involved in this process [52]. Nonetheless, peritoneal macrophages from infected mice also show decreased expression of P2X7 [52]. There is still little information on how extracellular ATP or P2X receptors contribute to the immune response against *T. cruzi* infection, but recent studies describing in vivo infection in P2X7-deficient animals showed an effect of P2X7 on

migration of mast cells towards the inflamed heart [53], which may reduce the innate immune response. Our data showing reduced mast cell infiltration in the lungs of P2X7-deficient animals following LPS injection [54] support the analysis of *T. cruzi* infection and suggest that mast cells could play a major role in controlling infection by intracellular parasites through modulation of inflammation, which was not fully appreciated previously in the literature.

Purinergic signaling could also be involved in the immune response against leishmaniasis. Macrophage infection with *Leishmania amazonensis* positively modulates P2X7 expression, as shown by protein expression of the receptor, and an increase in ATP-induced apoptosis and plasma membrane permeabilization in infected cells [55]. The increase in ATP-induced permeabilization has also been observed in spleen macrophages from *Leishmania donovani*-infected mice [56]. Furthermore, ligation of P2X7 can control the levels of intracellular *Leishmania* in macrophages, through a mechanism that is independent of NO secretion but involves ROS production and IL-1β secretion ([55] and unpublished data).

In addition, macrophage infection with *L. amazonensis* can differentially modulate P2X7-associated dye uptake. While membrane permeabilization for anionic dyes is upregulated, the uptake of cationic dyes is strongly decreased, and the decrease is dependent on active manipulation of the host cell by *Leishmania* [57]. The positive modulation of anionic dye flow correlates with host defense against parasite infection. P2X7-dependent ion fluxes have been previously associated with pannexin pore formation and NLRP3 inflammasome activation [58,59]. It is possible that the positive modulation of anionic dyes allows pathogen-associated molecular patterns (PAMPs) from the parasite to gain access to the cytoplasm, thereby triggering NLRP3 inflammasome assembly, inducing IL-1β secretion, and culminating with parasite control (Fig. 1) [60].

Interestingly, dead Leishmania also positively modulates uptake of anionic dyes, while having no effect on cationic permeabilization. Therefore, uptake of cationic dyes may not be involved with the host cell response against infection, but it is unclear why the protozoan infection actively down modulates cationic dye fluxes.

In addition to positive modulation of P2X receptor expression, *L. amazonensis* infection modulates P2Y receptors in infected macrophages. Thus, infected macrophages are more sensitive to UTP-induced intracellular calcium mobilization than uninfected macrophages, and UTP and UDP induce apoptosis only of infected macrophages through a pathway requiring caspase-3 activation. Finally, treatment with the nucleotides reduces the level of parasite infection (Fig. 2), through a mechanism involving production of ROS and NO inflammatory mediators [61].

Unlike the effect of P2 receptors, activation of the A2B adenosine receptor seems to contribute to establishment of infection by *Leishmania*. Thus, inhibition of A2B leads to lower levels of parasitism and fewer lesions in *Leishmania*-infected mice [62]. In addition, *Leishmania* infection leads to increased expression of CD39 (ectonucleoside triphosphate diphosphohydrolase) and CD73 (ecto-5′-nucleotidase) on dendritic cells, suggesting that A2B receptor activation may be used by parasites to inhibit dendritic cell function [63].

L. amazonensis infection upregulation of P2X and P2Y receptors in macrophages can therefore be viewed as novel "weapons" used by the host in its fight against infection. While ATP can mediate host immune responses through P2X7-dependent inflammasome activation, the pyrimidinergic nucleotides, through ligation of P2Y, can contribute to host defenses using the well-known inflammatory mediators, ROS and NO [64].

Toxoplasma gondii is an obligate intracellular parasite that infects virtually all nucleated cells. Once inside the host cell, the parasite avoids acidification of the entry vacuole. Recently we and others have reported that ATP ligation of P2X7 leads to fusion between lysosomes and the parasitophorous vacuole, and subsequent elimination of *T. gondii* tachyzoites in murine or human macrophages [65,66]. Interestingly, ATP has no effect on the parasite burden in infected human macrophages with the 1513A > C loss-of-function polymorphism in P2X7 or murine macrophages from P2X7-deficient mice [66]. Conversely, a gain-of-function polymorphism in P2X7 is associated with resistance to both congenital and ocular toxoplasmosis [32,67]. ATP-induced tachyzoite elimination is NO independent, while ATP induces ROS production and apoptosis in infected macrophages [65,66]. ROS production is an important host defense mechanism against intracellular parasites in general [68], and low levels of ROS lead to NLRP3 inflammasome-mediated caspase-1 activation [40,40,69]. Therefore it is tempting to speculate that ATP-induced ROS production in *T. gondii*-infected macrophages correlates with NLRP3 inflammasome-mediated caspase-1 activation and IL-1β secretion, as has been observed for other intracellular parasites [68]. Moreover, studies have demonstrated the protective role of IL-1β in mice against infection with intracellular pathogens, including *Chlamydia muridarum*, *Mycobacterium avium* and *T. gondii* [70–72].

4. Concluding remarks

Virtually every known multicellular or unicellular organism has the ability to detect changes in extracellular ATP concentrations, and purinergic signaling represents a primordial form of chemical intercellular signaling [73]. It is therefore reasonable that, during the co-evolution of parasites and humans, the latter have developed different mechanisms for detecting the presence of pathogens through the use of this ancient sensor of nucleotides. Not surprisingly, extracellular nucleotides can rally efficiently various cells of the innate immune system against intracellular parasites, such as macrophages, neutrophils, eosinophils, and mast cells [7,8,14,15,54,74].

On the other hand, different pathogens have evolved the ability to interfere with this important signaling pathway in order to favor infection. Thus, pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Bacillus anthracis* have developed toxins that target P2X7 and induce cell death of macrophages or lysis of erythrocytes [75–78]. While others, such as *M. tuberculosis*, *Mycobacterium bovis*, *L. amazonensis*, *P. gingivalis*, and *T. gondii* secrete Ndk, which scavenge extracellular ATP and act as a virulence factor [45,79–81] (Table 1). Thus, identification of strategies used by different intracellular pathogens to manipulate purinergic signaling, or defense mechanisms relying on purinergic receptors and used by the immune system to eliminate infection, could lead the way to development of future therapeutic interventions targeting purinergic receptors and downstream mediators.

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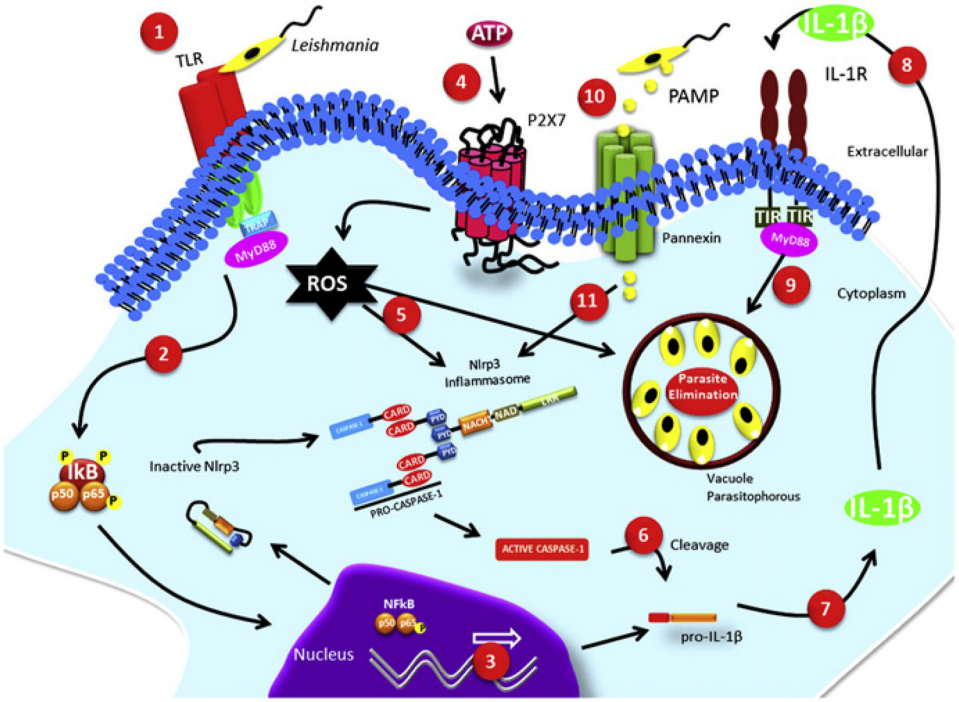

Fig. 1.

Schematic overview of intracellular parasite elimination following ATP ligation of P2X7. (1) The presence of the parasite triggers a signaling cascade by members of the Toll-like receptor (TLR) family, (2) dephosphorylation of IκB allows NFκB translocation to the nucleous, (3) and expression of genes such as pro-IL-1β. (4) Ligation of P2X7 by extracellular ATP induces ROS production, (5) which can either directly stimulate parasite elimination or activate the NLRP3 inflammasome. (6) Activated caspase-1 cleaves pro-IL-1β, (7) generating the biologically active IL-1β, (8) which acts on its receptor and (9) contributes to the elimination of the parasites. (10) Additionally, PAMPs from parasites can enter the cell through the pannexin pores activated by extracellular ATP, (11) which in turn can also activate the NLRP3 inflammasome.

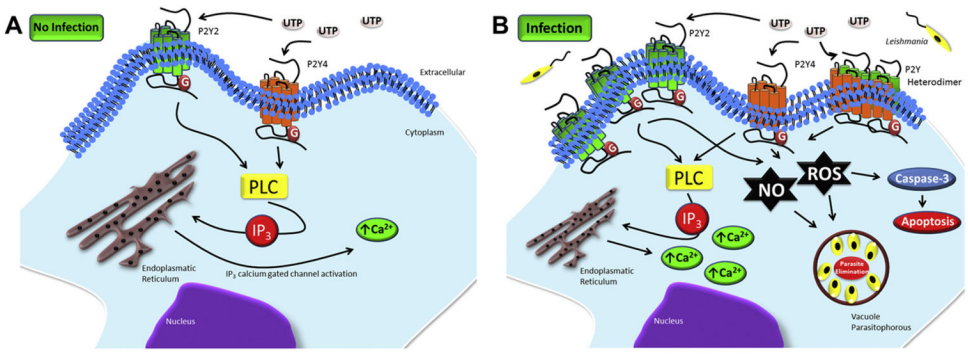

Fig. 2.

Purinergic signaling cascade due to nucleotide activation of P2Y2 and P2Y4 during *Leishmania amazonensis* infection. A) There is little signaling through P2Y receptors in uninfected cells. B) In infected cells, enhanced PLC activation and NO and ROS production leads to intracellular calcium mobilization, NO and ROS production, and caspase-3-dependent host cell apoptosis. Taken together, these mechanisms eliminate the parasites or help to contain the infection.

Table 1

Effects of extracellular nucleotides on intracellular pathogens, and effects of pathogens on purineric receptor signaling.

Cell type	Pathogen or pathogen toxin	Effect described	Purinergic receptor subtype involved	References
Human macrophages	BCG	Mycobacterial clearance	P2X7, P2Y	[25,26]
Human macrophages	BCG, *M. tuberculosis*	Mycobacterial clearance, inhibition of ATP-induced permeabilization	P2X7	[82]
Human macrophages	*M. tuberculosis*	Mycobacterial clearance, PLD activation	P2X7 + P2(?)	[28]
Murine macrophages and J774 cell line	BCG	Mycobacterial clearance, PLD activation, phagosome-lysosome fusion		[27]
Murine macrophages and cell line J774	*Chlamydia caviae*	Inhibition of apoptosis induced by ATP, down regulation of P2X7, chlamydial clearance	P2X7	[35]
Murine macrophages	*Chlamydia trachomatis*	Chlamydial clearance, PLD activation, increase in phagolysosome formation	P2X7	[36]
Human cervical epithelial cells	*Chlamydia trachomatis*	Chlamydial clearance, PLD activation	P2X7	[38]
Human cervical epithelial cells	*Chlamydia trachomatis*	Chlamydial retarded growth, increase of cAMP	A_{2B}	[83]
Gingival epithelial cells	*Porphyromonas gingivalis*	Inhibition of apoptosis induced by ATP, *P. gingivalis* clearance	P2X7	[45]
Gingival epithelial cells	*Porphyromonas gingivalis*	Caspase-1 activation and IL-1β secretion, *P. gingivalis* clearance	P2X7	[48]
Human macrophages	*Aggregatibacter actinomycetemcomitans* (leukotoxin)	Caspase-1 activation and IL-1β secretion (pyroptosis)	P2X7	[50]
Murine macrophages and RAW274.6 macrophages	*Bacillus anthracis* (lethal toxin)	Pannexin pore opening, caspase-1 activation and IL-1β secretion, P38 MAPK inhibition, macrophage death	P2X7	[75,76]
Equine, murine and human erythrocytes	*Escherichia coli* (α-Hemolysin)	Pannexin pore opening, (hemolysis)	P2X1, P2X7	[77]
Murine and equine erythrocytes	*Staphylococcus aureus* (α-toxin)	Pannexin pore opening, (hemolysis)	P2X1, P2X7	[78]
Murine thymocytes	*T. cruzi*	Modulation of thymocytes death	P2X7(?)	[51,52]
Murine macrophages	*T. cruzi*	Inhibition of ATP-induced permeabilization	P2X7	[52]
Murine macrophages	*L. amazonensis, L. donovani*	Parasite clearance, increased apoptosis and permeabilization induced by ATP, reduced cationic permeabilization	P2X7	[55,57]

Cell type	Pathogen or pathogen toxin	Effect described	Purinergic receptor subtype involved	References
Murine macrophages	*L. amazonenis*	Parasite clearance, increased Ca2+, apoptosis, ROS and NO production	P2Y2, P2Y4, P2Y2/P2Y4 hetero-oligomerization (?)	[61],
Murine dendritic cells	*L. amazonensis*	Modulation of dendritic cell function (reduce T cell proliferation)	A2B	[63]
Murine macrophages	*L. amazonensis*	Increase in parasite and load lesions	A2B	[62]
Human and murine macrophages	*T. gondii*	Parasite clearance, phagolysosome formation, ROS production, host cell apoptosis	P2X7	[65,66]

PLD = phospholipase D; ROS = reactive oxygen species; cAMP = cyclic AMP; IL-1β = interleukin-1β; NO = nitric oxide.