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REVIEW

The role of P2 receptors in controlling infections by intracellular pathogens

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Abstract A growing number of studies have demonstrated the importance of ATP_e-signalling via P2 receptors as an important component of the inflammatory response to infection. More recent studies have shown that ATP_e can also have a direct effect on infection by intracellular pathogens, by modulating membrane trafficking in cells that contain vacuoles that harbour intracellular pathogens, such as mycobacteria and chlamydiae. A conserved mechanism appears to be involved in controlling infection by both of these pathogens, as a role for phospholipase D in inducing fusion between lysosomes and the vacuoles has been demonstrated. Other P2-dependent mechanisms are most likely operative in the cases of pathogens, such as *Leishmania*, which survive in an acidic phagolysosomal-like compartment. ATP_e may function as a "danger signal" that alerts the immune system to the presence of intracellular pathogens that damage the host cell, while different intracellular pathogens have evolved enzymes or other mechanisms to inhibit ATP_e-mediated signalling, which should, thus, be viewed as virulence factors for these pathogens.

Key words apoptosis · ATP · infection · inflammation · necrosis · purinergic receptors

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Abbreviations

ATP_e	Extracellular ATP
IL-1β	Interleukin-1β
IFN-γ	Interferon-γ
LPS	Lipopolysaccharide
PGE_2	Prostaglandin E2
PLD	Phospholipase D
ROI	Reactive oxygen intermediates
RNI	Reactive nitrogen intermediates
TNF-α	Tumour necrosis factor-α

Introduction

Intracellular pathogens invade, survive and replicate in mammalian cells, modulating host-cell membrane trafficking and cytoskeletal dynamics in order to establish persistent infection in the mammalian host [1–3]. Macrophages are a frequent target of microbial infections, and they respond to microbial invasion by producing factors such as reactive nitrogen and oxygen intermediates (RNIs and ROIs) that have strong microbicidal activity [4]. But many pathogens have evolved different strategies for avoiding destruction by the macrophage. Some intracellular pathogens, such as *Mycobacteria*, inhabit a compartment whose endocytic maturation is delayed, while *Chlamydiae* survive within a membrane-bound vacuole that avoids fusion with lysosomes and maintains a neutral pH [5, 6]. Unlike *Mycobacteria* and *Chlamydia*, the protozoan parasite *Leishmania* thrives in a parasitophorous vacuole that has an acidic pH and high hydrolytic activity, and *Trypanosoma cruzi* actively induces its uptake into lysosome-like host-cell vacuoles, from which, the parasite rapidly escapes into the cytosol [7–10].

At the end of their infection cycle, each of these intracellular parasites is released from the host cell, triggering macrophage death and inducing local inflammation, accompanied by the possible release of ATP, as shown for macrophages infected with *Mycobacterium tuberculosis* [11]. As extracellular ATP (ATP_e) can be used by neighbouring macrophages as ammunition to inhibit infection (described below), many intracellular pathogens—such as *Mycobacterium bovis* BCG, *Vibrio cholerae*, *M. tuberculosis*, *T. cruzi* and *Leishmania*—also secrete or express on their outer surface enzymes that degrade or synthesise nucleotides [12–15]; and microbial enzymes that consume or produce ATP are considered as virulence factors for *M. tuberculosis*, *Leishmania amazonensis* and *T. cruzi* [14, 16–18].

Macrophages activate microbicidal pathways and contribute to inflammation after the ligation of purinergic P2 receptors by ATP_e. This review will, therefore, describe some of the P2-dependent mechanisms used by macrophages to eliminate infection by intracellular bacteria and protozoan parasites, and, whenever possible, will correlate these findings with host susceptibility and resistance to infection.

Effect of ATP_e on macrophage infection by *M. tuberculosis*

The first evidence for an involvement of ATP_e in the control of intracellular infections came from the laboratory of Kaplan, who demonstrated in 1994, that ATP_e-mediated apoptosis in *Mycobacterium tuberculosis*-infected macrophages is associated with the inhibition of mycobacterial infection [19]. This early study also showed that ATP_e-induced macrophage apoptosis, but not H_2O_2-induced necrosis, is associated with the killing of the intracellular mycobacteria. These findings were confirmed by Lammas et al., who proposed that the ATP_e-induced elimination of BCG-infected human macrophages are mediated by the purinergic receptor, $P2X_7$, through a mechanism independent of both RNIs and ROIs [20]. In comparison with other ligands that can trigger the lysis of macrophages, including complement-mediated cytolysis, Fas ligation and CD69 activation, only ATP_e treatment could stimulate the death of both host macrophages and intracellular mycobacteria. Subsequently, several laboratories confirmed that the $P2X_7$ receptor plays a role in limiting infection in murine and bovine macrophages infected with mycobacteria, and in human macrophages infected with BCG bacillus and virulent strains of *M. tuberculosis* [11, 21–23].

In addition to the $P2X_7$ receptor, other P2 receptor subtypes, possibly P2Y, are apparently involved in ATP_e-mediated bactericidal activity in macrophages [11, 21, 23]. Experiments using macrophages derived from $P2X_7$-deficient mice revealed that ATP_e stimulates the production of reactive species such as RNIs equally well in both wildtype and $P2X_7$-deficient macrophages [11]. Moreover, it was found that ATP_e induces bactericidal effects in the macrophages better than BzATP (the most potent known agonist for the $P2X_7$ receptor), suggesting that $P2X_7$ receptors are necessary, but not sufficient, for maximal ATP_e-dependent killing of intracellular *M. tuberculosis* by human and bovine macrophages [21, 22]. Lammas et al. have further observed that the ATP_e activity is potentiated by extracellular Zn^{2+} [20]. This effect was initially ascribed to the $P2X_7$ receptor, but now, $P2X_7$ activity is known to be blocked by extracellular Zn^{2+}, while the activity of another purinergic receptor, $P2X_4$, is potentiated by Zn^{2+} [24]. Since macrophages express functional $P2X_4$ receptors [25] and inflammatory mediators can upregulate this receptor on macrophages [26], it is likely that both $P2X_7$ and $P2X_4$ are involved in the ATP_e-induced killing of *M. tuberculosis* in macrophages.

More recent reports have also confirmed the predominant role of the $P2X_7$ receptor in mycobacterial clearance, extending these results to show that loss-of-function polymorphisms in human $P2X_7$ receptors lead not only to reduced ATP_e-induced apoptosis, but also to impaired ATP_e-induced killing of intracellular mycobacteria (BCG) by macrophages [27–29]. Nonetheless, more experiments will be required to elucidate the role that $P2X_7$, possibly in conjunction with other P2 receptors, may play in the killing of intracellular mycobacteria in vivo, since $P2X_7$-deficient mice control lung infection as well as wildtype mice after low-dose aerosol infection with virulent *M. tuberculosis* [30].

Cellular mechanisms of ATP_e-induced mycobacterial killing

ATP_e ligation of $P2X_7$ on macrophages results in a variety of different cellular effects, including the activation of phospholipase D (PLD), maturation and release of interleukin-1β (IL-1β), generation of macrophage polykarions, modulation of lipopolysaccharide (LPS) induced macrophage activation through modulation of iNOS expression and NO production, and the induction of macrophage death by necrosis and/or apoptosis [31–40]. The original findings from the Kaplan laboratory suggested that the apoptosis of macrophages is necessary for ATP_e-mediated killing of intracellular bacteria [19], but it was later established that the ATP_e-induced killing of mycobacteria in human and mice macrophages can occur without macrophage death, through a pathway requiring PLD activation, the acidification of phagosomes and phagosome–lysosome fusion [21]

(Fig. 1) [23, 41]. A more recent study showed that, when combined, two loss-of-function polymorphisms in human $P2X_7$ receptors impair ATP_e-mediated apoptosis, despite the normal killing of BCG bacillus [28], reinforcing the view that the apoptosis of macrophages is not necessary for the elimination of mycobacteria.

In fact, it was recently shown [42] that cyclosporine A, an inhibitor of the mitochondrial permeability transition, increases the survival of human monocyte-derived macrophages infected with *M. tuberculosis*, restores $P2X_7$ function and enhances antimycobacterial activity. Conversely, *M. tuberculosis* has developed mechanisms to evade $P2X_7$-triggered mechanisms, since it can secrete a nucleoside diphosphate kinase that produces ATP and kills macrophages through a $P2X_7$-dependent mechanism [14]. New experiments are needed to clarify the role played by ATP_e in killing intracellular pathogens and inducing host-cell death.

Inhibition of chlamydial infection in macrophages

The *Chlamydia* species are obligate intracellular bacteria that infect mainly epithelial mucosa, where they survive within intracellular vacuoles that avoid fusion with host-cell lysosomes [6, 43]. Different strains of *C. trachomatis* are responsible for the infection of genital and ocular tissue in humans [44–48]. *C. pneumoniae* is a common cause of community-acquired pneumonia in humans and is associated with an increased risk for atherosclerosis [44, 49]. Both *C. trachomatis* and *C. pneumoniae* can invade epithelial cells and macrophages in vitro and in vivo [49, 50].

It was recently shown that ATP_e inhibits the infection of macrophages by the murine species of *C. trachomatis*, *C. muridarum*, through a mechanism that required PLD activation and fusion between lysosomes and the *Chlamydia* vacuoles [51]. The effect of ATP_e was dependent on the presence of the $P2X_7$ receptor, since there was no PLD activation nor killing of chlamydiae in infected macrophages that had been isolated from $P2X_7$-deficient mice. Although $P2X_7$ ligation also led to macrophage death, the inhibition of PLD prevented chlamydial killing but had no effect on macrophage death, suggesting that PLD activation was directly responsible for the inhibition of chlamydial infection (Table 1). Moreover, fusion between lysosomes and chlamydial vacuoles preceded macrophage death, further strengthening the conclusion that the killing of chlamydiae is independent of host-cell death [51].

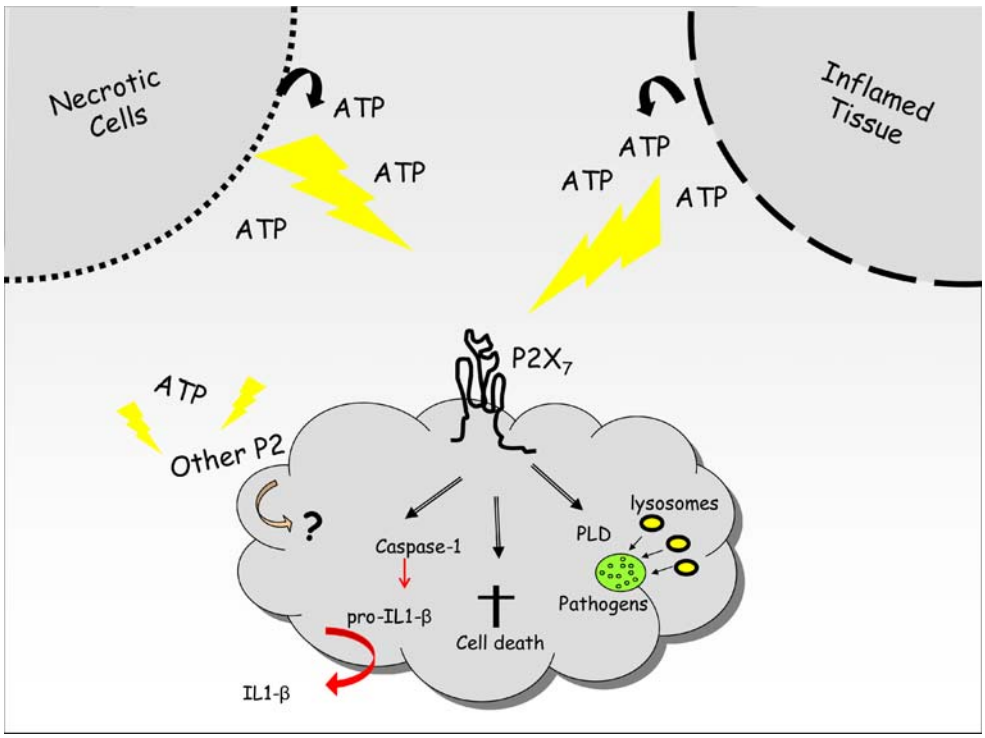

Fig. 1 ATP released from infected cells undergoing necrosis or sites of inflammation can bind to the $P2X_7$ receptor on neighbouring macrophages and other cells. Ligation of the $P2X_7$ receptor initiates signalling through several pathways, which result in the activation of caspase-1, activation of phospholipase D (PLD) and, ultimately, cell death. Caspase-1 activation stimulates the inflammatory response by the cleavage of pro-IL-1β and secretion of the mature cytokine. PLD activation modifies membrane trafficking in the cell, which can induce fusion between lysosomes and vacuoles, harbouring intracellular pathogens such as mycobacteria and chlamydiae. The death of macrophages is partly necrotic, which may amplify inflammation even further, through the ligation of $P2X_7$ receptors on other cells. Some extracellular ATP may also bind to other P2 receptors, which may inhibit infection by activating as-yet-uncharacterised pathways

Table 1 Effect of extracellular ATP on intracellular pathogens

Cell type	Pathogen	Effect described	P2 receptor subtype involved	References
Human macrophages	BCG	–Mycobacterial clearance	Not determined	[19]
Human macrophages	BCG	–Mycobacterial clearance	$P2X_7$, P2Y	[20], [23]
	BCG—*M. tuberculosis* H37Ra	–Acidification of mycobacteria-containing phagosomes –Inhibition of $P2X_7$-associated permeabilsation	$P2X_7$	[42]
Mouse macrophage	BCG	Production of NO and ROI	P2Y(?)	[11]
Human macrophages	*M. tuberculosis* (H37Rv, Erdman and CSU#93)	Mycobacterial clearance; PLD activation	$P2X_7$+P2(?)	[21]
Mouse macrophages and J774 cell line	BCG	Mycobacterial clearance, PLD activation, phagosome–lysosome fusion	$P2X_7$	[41]
Bovine macrophages	BCG	Mycobacterial clearance, increase $P2X_7$ mRNA	$P2X_7$	[22]
J774 macrophage	*C. caviae*	– Chlamydial clearance– Inhibition of $P2X_7$-mediated apoptosis	$P2X_7$	[52]
Mouse macrophages	*C. trachomatis*	Chlamydial clearance, PLD activation, phagosome–lysosome fusion	$P2X_7$	[51]
Mouse thymocytes	*T. cruzi*	Modulation of thymocyte death	$P2X_7$ (?)	[64]
Mouse macrophages	*T. cruzi*	Inhibition of $P2X_7$-mediated permeabilisation	$P2X_7$	[65]
Mouse macrophages	*L. amazonensis*; *L. donovani*	Increase of $P2X_7$-mediated permeabilisation	$P2X_7$	[54]

PLD=phospholipase D; ROI=reactive oxygen intermediates

Conversely, as the activation of $P2X_7$-dependent pathways is deleterious for *Chlamydia*, both directly and through the demise of the macrophage, the intracellular pathogen has also evolved a mechanism for protecting its host cell. Thus, the infection of macrophages with a related species, *C. psittaci* (also known as *C. caviae*), inhibits partially ATP_e-mediated macrophage death [52]. While the molecular basis for host-cell protection remains to be investigated, chlamydial infection decreases partially the ability of ATP_e to induce plasma–membrane permeabilisation and calcium fluxes [52]. Chlamydiae, therefore, resemble other pathogenic bacteria and protozoan parasites that attempt to protect themselves and the host cell by degrading nucleotides or hydrolysing ATP.

Effects of ATP_e on leishmaniasis

Leishmaniasis is used to describe several diseases caused by the obligate intracellular protozoan parasite *Leishmania*, which infects mainly macrophages [53]. The diseases range from self-healing cutaneous lesions to visceral and potentially fatal disseminating infection. *Leishmania* infections are found in 80 countries, with a prevalence of 12 million human cases. The development of different clinical forms is associated with both the immunological status of the host and the parasite species [53]. The expression of the $P2X_7$ receptor has recently been examined during *Leishmania* infection, revealing upregulation of the receptor during both in vivo and in vitro infection with *L. amazonensis* [54]. These changes were correlated with functional responses, as reflected by an increase in ATP_e-mediated plasma–membrane permeabilisation and host-cell apoptosis ([54] and Chaves et al. (manuscript in preparation)) (Table 1). The increase in ATP_e-induced membrane permeabilisation was also observed in spleen macrophages isolated from mice infected with *L. donovani*, suggesting that this may be a general phenomenon relevant for all *Leishmania* infections.

It has been proposed that intracellular infection by *Leishmania donovani* inhibits macrophage apoptosis induced by growth factor deprivation [55]. In contrast, there is an increase in the ATP_e-mediated apoptosis of macrophages infected with *Leishmania* (our unpublished data), consistent with the increases in ATP_e-mediated membrane permeabilisation. Thus, despite the production of ecto-ATPases by *Leishmania* [16], this strategy is not sufficient to protect the host cell against the infection-dependent upregulation in $P2X_7$ expression.

We have observed that the presence of ATP_e during *L. amazonensis* infection does not interfere with *Leishmania* invasion, but the ATP_e treatment of macrophages that are already infected with *L. amazonensi* leads to a decrease in *Leishmania* survival (our unpublished observations). In addition, we observed that ATP_e has no effect on the

viability of extracellular *Leishmania* promastigotes. In fact, some nucleotides, such as UTP, stimulate the proliferation of promastigotes. It is also worthwhile noting that the more infective forms of *L. amazonensis* express more magnesium-dependent ecto-ATPase on their membranes than less virulent *Leishmania*, leading to the proposal that the ecto-ATPase should be viewed as a virulence factor of the parasite [16].

The cellular pathway allowing ATP_e to decrease *Leishmania* infection remains to be determined, but must be different from the PLD activation observed during *Chlamydia* and *Mycobacterium* infection [41, 51], since both *Chlamydia* and *Mycobacterium* inhibit phagolysosome formation and acidification [4, 6, 8], while *Leishmania* survives well in acidic phagolysosomes [56]. Interestingly, *Leishmania* lipophosphoglycans, which promote parasite survival, act by perturbing MAPKinase signalling in macrophages to inhibit macrophage IL-1β [57]. This might be relevant for the involvement of P2 receptors in the escape mechanisms used by *Leishmania*, since several P2 receptors are connected to MAPKinase pathways [58].

The involvement of ATP_e in Chagas' disease

Chagas' disease is caused by the facultative intracellular protozoan pathogen, *T. cruzi*. The disease is a chronic inflammatory condition characterised by cardiomyopathy and digestive disorders [59, 60]. *T. cruzi* infection affects over 17 million people in endemic areas of Latin America, leading to 45 thousand deaths per year [61]. The involvement of ATP_e signalling was recently examined during the acute phase of *T. cruzi* infection. Thymus atrophy occurs during the acute phase of infection but the thymus recovers weight and cellularity during the chronic phase [62]. These alterations do not appear to be associated with stress or glucocorticoid release [63]. It has recently been observed that thymocytes are sensitive to ATP_e-induced membrane permeabilisation and host-cell death only during the atrophy phase of infection, with $CD4^+/CD8^+$ double-positive thymocytes being the most sensitive subpopulation of thymocytes [64] (Table 1). Since the phenomenon of ATP-induced permeabilisation can be blocked by the $P2X_7$ inhibitors, oxidised ATP and Mg^{2+}, and the $P2X_7$ agonist, BzATP, was more potent than ATP, we proposed that the increased sensitivity to ATP_e may be responsible, at least in part, for the thymocyte clearance and thymic atrophy observed during the acute phase of Chagas's disease [64].

It is, therefore, reasonable to suppose that ATP_e plays an important role in the *T. cruzi* infection cycle. In this context, it is worth noting that the ecto-ATPases produced by these parasites have been associated with strain virulence [17].

$P2X_7$ receptors and inflammation

The stimulation of $P2X_7$ by ATP_e leads to caspase-1 activation, cleavage of pro-IL-1β and the secretion of mature IL-1β [66] (Fig. 1). The $P2X_7$ receptor is also upregulated in macrophages by inflammatory cytokines, such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), and lipopolysaccharide (LPS). LPS, a surface component of most Gram-negative bacteria, and IFN-γ act synergistically to upregulate $P2X_7$ receptor function and $P2X_7$ mRNA in the human monocyte cell line, THP-1, and human macrophages [34, 37]. In fact, $P2X_7$ receptors contain several motifs that are homologous to motifs from other receptors known to be involved in protein–protein interactions and LPS binding [67]. The upregulation of $P2X_7$ expression in monocytes by TNF-α, LPS and IFN-γ is consistent with the ability of these cytokines to act as inflammatory mediators, and these effects are markedly attenuated by coincubation with prostaglandin E2 (PGE_2) or the membrane-permeable cAMP analogue, dibutyril cAMP [68]. It is tempting to speculate that the temporal sequence of macrophage exposure to pro-inflammatory activators and anti-inflammatory stimuli (such as PGE_2) might regulate not only receptor expression, but also downstream signalling by the $P2X_7$ receptors.

It is known that macrophages express adenosine receptors, which are expressed during the differentiation of monocytes to macrophages and may influence phagocytosis [69]. Moreover, the treatment of macrophages with IFN-γ upregulates expression of the adenosine receptor, A_{2B}, and the activation of A_{2B} receptors is involved with the deactivation of macrophages, possibly through an increase of cAMP [70]. Therefore, the extracellular nucleotides may be involved with activation and a feedback mechanism for macrophage deactivation, depending on the timing and type of nucleotide released during infection. In this context, \studies with $P2X_7$-deficient mice have reinforced the view that $P2X_7$ receptors are involved in inflammation. Thus, disruption of the $P2X_7$ receptor gene is associated with less severe disease in an arthritis model [71] and studies of chronic inflammation and neuropathic pain [72]. These results led to the hypothesis that the $P2X_7$ receptor, through the regulation of IL-1β production and secretion, plays a common, early role in the development of pain of neuropathic and inflammatory origin [71, 72]. Additionally, a recent study demonstrates that the inhibition of the $P2X_7$ receptor attenuates fever and cytokine responses induced by LPS in rats [73]. Finally, the $P2X_7$ receptor is present and its expression is modulated by inflammation in sites of chronic inflammation [74].

All of these findings reinforce the conclusion that $P2X_7$-dependent signalling plays a significant role in host

responses during various types of inflammatory disease. However, the involvement of $P2X_7$ during disease is complicated by the presence of other P2 receptors, which may also contribute to either pro- or anti-inflammatory immune responses. Moreover, ATP can negatively regulate Toll-like receptor signalling, suppress LPS-induced MCP-1 and TNF-α, and augments IL-10 production in human monocytes [75]. Many infectious agents that survive in these immune effector cells may have evolved complex nucleotide-based strategies to evade the immune system. Dissection of these strategies may lead us to a better understanding of the role of nucleotide signalling in the immune response and to the development of new approaches to combat infectious diseases.

Concluding remarks

As large concentrations of ATP are present outside of the cell only when the cell is damaged or is part of an inflamed tissue (Fig. 1), it has been proposed that ATP_e may function as a generic "danger signal," which could alert the immune system to the presence of any type of intracellular pathogen that induces host-cell death [76–78]. Different intracellular pathogens, such as bacteria and protozoan parasites, express ecto-ATPases or other mechanisms to either inhibit or enhance ATP_e-mediated death of their host cell, suggesting that ATP_e may have been used by the host as an ancient danger signal, to which, intracellular pathogens have been exposed since the early evolution of the immune system. In this context, the ability of some intracellular pathogens to inhibit the ATP_e-dependent response mediated by P2 receptors could be an example of adaptation of the pathogenic invaders to the immune response, and may help to explain why the most virulent pathogens express high ecto-ATPase levels on their surface [14–17]. Given the availability of animals that are deficient in $P2X_7$ and other P2 receptors, further research in the future will, thus, need to address the relevance of P2-dependent immune mechanisms in controlling infections in whole organisms.

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