Purinergic signalling, DAMPs and inflammation

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Abstract

Danger sensing is one of the most fundamental evolutionary features enabling multicellular organisms to perceive potential threats, escape from risky situations, fight actual intruders and repair damage. Several endogenous molecules are used to "signal damage", currently referred to as "alarmins" or "Damage-Associated Molecular Patterns" (DAMPs), most being already present within all cells (pre-formed DAMPs), and thus ready to be released, and others neo-synthesized following injury. Over recent years it has become overwhelmingly clear that adenosine 5'-triphosphate (ATP) is a ubiquitous and extremely efficient DAMP (thus promoting inflammation) and its main metabolite, adenosine, is a strong immunosuppressant (thus dampening inflammation). Extracellular ATP ligates and activates the P2 purinergic receptors (P2Rs) and is then degraded by soluble and plasma membrane ecto-nucleotidases to generate adenosine acting at P1 purinergic receptors (P1Rs). Extracellular ATP, P2Rs, ecto-nucleotidases, adenosine and P1Rs are basic elements of the purinergic signalling network and fundamental pillars of inflammation.

ATP and adenosine accumulate at sites of inflammation. Thanks to the introduction of probes for in vivo semi-quantitative measurement of ATP in the tissue interstitium, the purinergic hypothesis has been validated beyond any possible doubt (11). We now know that ATP and adenosine are present at negligible amounts (nmoles/L) in the interstitium of healthy tissues, and accumulate to high levels in the inflammatory or tumor microenvironment (IME and TME, respectively) (6,13). Direct in vivo measurements show that ATP can rise to several tens or even hundreds of μmoles/L at sites of inflammation, while adenosine concentration is estimated to increase to a few μmoles/L. ATP, generated by glycolysis and oxidative phosphorylation, is normally present intracellularly in very high concentrations (mmoles/L), thus generating a strong outwardly directed gradient, both chemical and electrical, across the negatively charged plasma membrane (under physiological Ca^{2+} and Mg^{2+} concentrations ATP bears 2 negative charges). Therefore, any breach in plasma membrane integrity will cause a prompt ATP efflux. Due to its charge and low molecular mass, ATP will quickly diffuse through the aqueous extracellular milieu, until degraded by soluble or plasma membrane-bound ecto-nucleotidases. Besides passive efflux, ATP can also be released via active mechanisms involving different vesicle- and channel-mediated pathways such as constitutive or regulated exocytosis, connexin-43, pannexin-1, the calcium homeostasis modulator (CALMH) channel, and a member of the P2X receptor (P2XR) family, i.e. the P2X7 receptor (P2X7R) (16). Various pro-inflammatory stimuli are reported to trigger ATP release, although the pathway involved has not been identified in most cases. Early findings by our group showed that bacterial lipopolysaccharide (LPS) triggers ATP release from human macrophages and mouse microglial cells (14). More recent data suggest that accumulation of LPS in the macrophage cytosol also triggers ATP release via pannexin-1 activation. (33). Among other common proinflammatory factors, reactive oxygen species (ROS) are known to trigger large ATP release via multiple pathways (1). It is worth mentioning that hypoxia, a common condition at sites of inflammation, is another well-known condition that promotes ATP release (24). Importantly, infection by intracellular bacteria and protozoa triggers release of large amounts of ATP, a process

fundamental to promote an efficient immune response, or for the propagation of tissue damage (4; 27),(8). ATP acts as an extracellular messenger between cells of the same organism (i.e. in the same kingdom) as well as across kingdoms (i.e. prokaryotic versus eukaryotic), as shown by the beneficial effect exerted on gut follicular T helper cells by ATP derived from gut lumen bacteria (26).

Plasma membrane receptors and degradation pathways. The P2R family is comprised of eight membrane-spanning, G-protein coupled receptors, named P2Y ($P2Y_{1,2,4,6,11-14}$) receptors (P2YRs,) , and seven intrinsic ion channels, named P2XRs ($P2X_{1-7}$) (7). ATP is not the preferred nucleotide agonist at most P2YRs, with the exception of P2Y11R and possibly P2Y2R (where ATP is equipotent with UTP), as ADP is preferred at P2Y1R, P2Y12R and P2Y13R, UTP at P2Y4R, UDP at P2Y6R, and UDP-glucose or UDP-galactose at P2Y14R. On the contrary, the P2XRs are much more "faithful" to ATP as this is the only physiological agonist at this receptor. Agonist affinity ranges from the low micromolar (most P2YRs) to the near millimolar level (the P2X7R). The number of P2R subtypes, the different nucleotide selectivity and the widely different affinity endow the purinergic system with a remarkable plasticity that enables these receptors with the ability to fulfill signalling functions in a multiplicity of pathophysiological settings. Incidentally, the different selectivity of P2YRs suggests that other nucleotides may also act as DAMPs (9).

Any extracellular messenger must be easily terminated to prevent overstimulation or desensitization, and purinergic signalling is no exception to this general rule as all cells are equipped with very potent ecto-nucleotidases grouped into four families: ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases), ecto-5′-nucleotidase (5′-NT), ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs), and alkaline phosphatases (APs) (16). The final result of ATP degradation by ecto-nucleotidases is the generation of adenosine, a potent immunosuppressant, thus fulfilling an additional key requirement of every homeostatic system, i.e. the production of negative feed-back signal to damp and revert activation (23). Although adenosine is also generated intracellularly and transported across the plasma membrane via concentrative and

equilibrative transporters, degradation of extracellular ATP is the primary mechanism of generation of extracellular adenosine. Adenosine effects are mediated by four receptors: the A1 (A1R), A2A (A2AR), (A2BR) and A3 (A3R) receptors, which are characterized by different coupling mechanisms and affinity (6).

Main pathophysiological functions of ATP and adenosine. Accumulation of ATP in IME and TME has multiple effects including: promotion of migration of inflammatory cells, redirection T helper cell differentiation, activation of the NLRP3 inflammasome, promotion of cytokine, chemokine and growth factor release, generation of oxygen and nitrogen radical formation, stimulation of growth of stromal (or tumor cells), potentiation of intracellular pathogen killing, and even direct cytotoxicity (29). Furthermore, ATP itself, depending on the dose, the receptor subtype engaged and the given cell type, may also suppress inflammation and induce tolerance. In inflammation, the primary P2Rs implicated are P2Y1R, P2Y2R, P2Y6R, P2X4R and P2X7R (18), while in the P1R subfamily most relevant are A2AR, A2BR and A3R (5). In the IME and TME, ATP release and degradation (and therefore adenosine generation) are closely correlated, thus the final effect is often a result of the stimulation of both P2Rs and P1Rs, as it is clearly shown in the case of polymorphonuclear leukocyte chemotaxis, which is dependent on both P2Y2R and A3R stimulation (20), or of myeloid-derived suppressor cell expansion and activation, which can be promoted by both adenosine, acting at A2BRs, and ATP acting at P2X7R (5,12). Furthermore, while the only physiological agonist at P1Rs is adenosine, different purine or pyrimidine nucleotides are agonists at different purinergic receptors, promoting different responses in inflammatory cells, e.g. microglial cell motility is strongly stimulated by ADP acting at P2Y12, while phagocytosis is enhanced by UDP acting at the P2Y6R (19). An as yet unexplored field is the interplay between P2YRs and P2XRs during parasitic infections and how this cross-talk affects host-parasite interaction (31).

At inflammatory sites ATP interacts with other pro-inflammatory factors

ATP is a potent stimulus for release of inflammatory factors, but very little is known of whether and how they may synergize to start or propagate inflammation. Anecdotal evidence suggests that some pro-inflammatory or bactericidal agents modulate P2R activation by ATP. This is the case of the neutrophil-derived bactericidal cathelicidin LL37 which potentiates ATP-dependent stimulation of the P2X7R, likely acting as a positive allosteric modulator (32). This is very interesting because due to the very low affinity of the P2X7R for ATP (in the near millimolar range) it has been often doubted whether this receptor was active under any conditions. However, LL37 data suggest that at sites of infection extracellular ATP may synergize at the P2X7R with other locally-released factors to drive inflammatory cell activation and cytokine release. The discovery of additional positive allosteric modulators of the P2X7R might provide important information on the pathophysiological function of this receptor and point to effective methods to modulate its activity and harness its full therapeutic potential.

According to a simplified Yin/Yang vision of the purinergic system, P2R stimulation is understood to promote, while P1R stimulation is understood to suppress inflammation (see Fig. 1). This simplified picture has prompted many Pharma and Biotech Companies to develop a large number of reagents, small molecule drugs or antibodies, targeting the P2Rs most likely involved in inflammation and cancer, i.e. the P2X7R and the adenosine A2AR and A2BR (25,30,6). Along this same line of thinking, reagents targeting ecto-nucleotidases to slow down ATP hydrolysis and adenosine generation have also been developed (2).

Despite much excitement and the very promising pre-clinical data, results of Phase I and II Clinical Trials aimed at testing the efficacy of P2X7R blockade in chronic inflammatory diseases (rheumatoid arthritis, Crohn disease, osteoarthritis) fell short of expectations. A similar mixed picture has emerged from Phase I to III clinical tests of P1R agonists and antagonists, although last year US Food and Drug Administration has approved istradefylline, a selective antagonist of the A2AR, for the treatment of Parkinson's disease. Best hopes rest on therapeutic A2AR targeting in cancer where administration of A2AR inhibitors, alone or together with immune check point

blockers, seem to be effective (3) (30). An alternative avenue to interfere with the adenosinergic pathway is to target the ecto-enzymes responsible for extracellular ATP degradation, primarily CD39 and CD73. Oleclumab is an anti-CD73 monoclonal antibody that is currently being tested in combination therapy in cancer in over 17 Clinical Phase 1-II trials (e.g. NCT03773666), while TTX-030 is an anti-CD39 monoclonal currently in Phase I Clinical Trial (NCT03884556) as the sole agent or in combination with immunotherapy or chemotherapy (21).

Rationale for targeting P1Rs and P2Rs in inflammation and cancer include, 1) blockade of adenosinergic receptors (chiefly A2AR) which removes adenosine-mediated immunosuppression, thus promoting "healthy" inflammation and restoring an efficient anti-tumor immune response, and 2) blockade of P2Rs (chiefly P2X7R) to inhibit excessive release of pro-inflammatory cytokines, such as IL-1β and IL-18, thus dampening "uncontrolled/pathological" and possible detrimental inflammation. However, in perspective modulation of purinergic signalling might be helpful in the control of release of additional agents which are not considered "sensu strictu" inflammatory mediators. For example, A2ABR, and more so P2X7R, stimulation promotes vascular endothelial growth factor (VEGF) release in cardiac, retinal, skin cells as well as in several tumor cell types (6) (12). Thus, A2ABR or P2X7R inhibition might be useful to counteract pathological angiogenesis, as for example in tumors, in arthritic joints or in the retina. Such a therapeutic application is supported by the recent demonstration that systemic administration of small molecule drug P2X7R blockers to diabetic rats fully reverts intra-retina VEGF and IL-6 expression and increased retina vessel permeability (10). A deeper knowledge of the purinergic network might also help to support a rational repurposing of well-known and widely diffused drugs primarily targeting molecules unrelated to purinergic signalling. In this regard, recent data suggest that the widely-diffused anti-histamine drug clemastine could be used to potentiate anti-mycobacterial therapy thanks to its (known but neglected) positive allosteric effect at the P2X7R (22). On the same line, the discovery that dihydropiridine Ca^{2+} channel blockers have an anti-inflammatory activity due to interference with P2X7R-dependent pathways could provide novel tools for fighting neuroinflammation (28).

More recently, development of high affinity P2X7R PET tracers has demonstrated the suitability of this receptor as a biomarker in neuroinflammation (17), while serum P2X7R levels might be used as a systemic marker of inflammation (15).

In conclusion, a thorough investigation of purinergic signaling is likely to unveil novel important inflammatory check-point molecules, provide hints for repurposing old therapeutics, and designing novel drugs and diagnostic tools.

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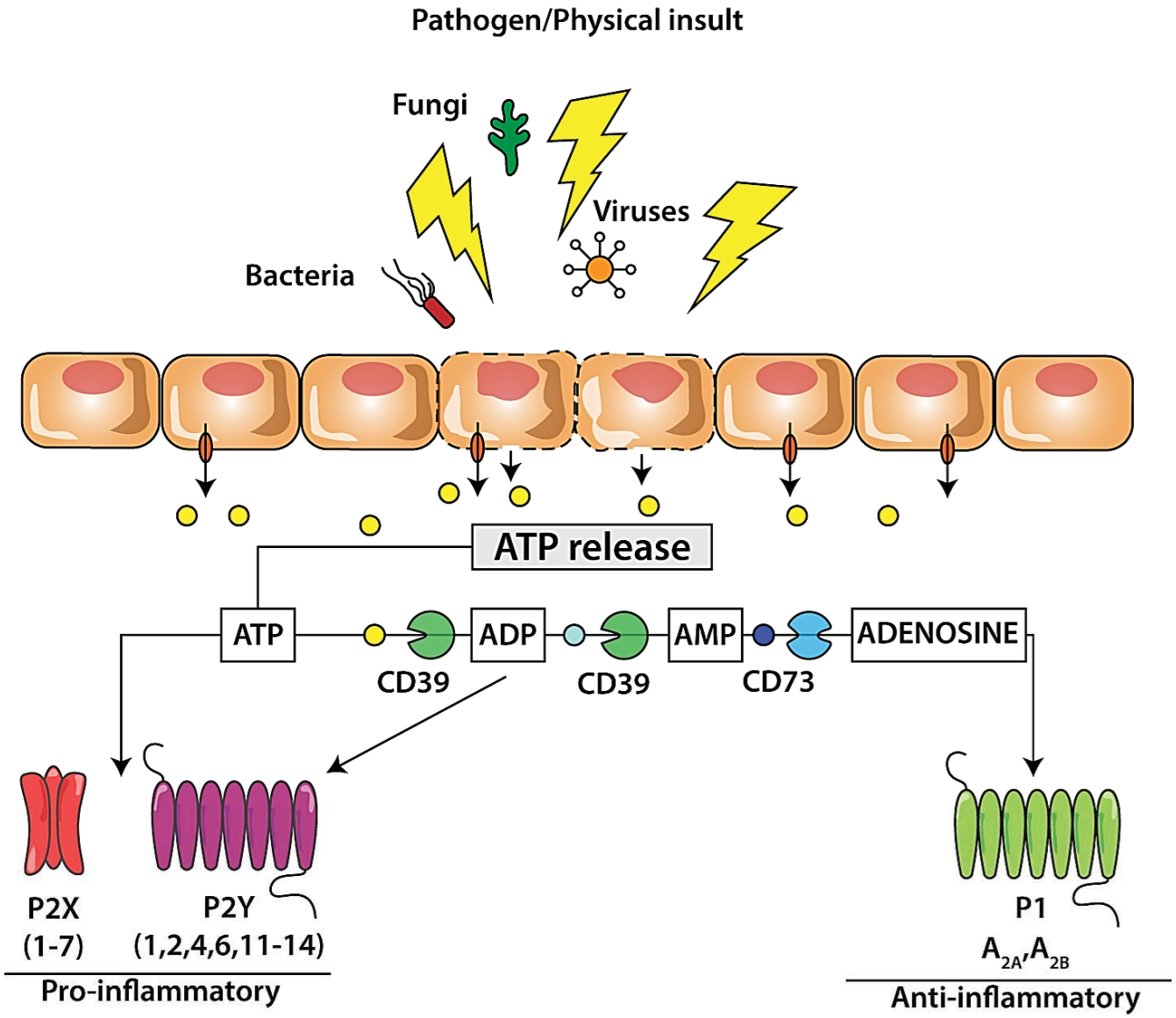
Legend to Figure 1

Simplified rendition of the opposite effects of extracellular ATP and adenosine on inflammation. Different noxious agents, whether of physical or biological origin, trigger ATP release due to lesions to the plasma membrane or to activation of non-lytic release mechanism. In the pericellular space ATP is degraded thanks to the sequential activity of CD39 and CD73 ecto-nucleotidases, thus generating adenosine. Extracellular ATP has primarily a pro-inflammatory activity, while adenosine is a potent immunosuppressant.


Pathogen/Physical insult
Fungi
Viruses
Bacteria
ATP release
ATP
ADP
AMP
ADENOSINE
CD39
CD39
CD73
P2X
(1-7)
P2Y
(1,2,4,6,11-14)
Pro-inflammatory
P1
A_{2A},A_{2B}
Anti-inflammatory