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Nucleotide signalling during inflammation

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Abstract

Inflammatory conditions are associated with the extracellular release of nucleotides, particularly ATP. In the extracellular compartment, ATP predominantly functions as a signalling molecule through the activation of purinergic P2 receptors. Metabotropic P2Y receptors are G-protein-coupled, whereas ionotropic P2X receptors are ATP-gated ion channels. Here we discuss how signalling events through P2 receptors alter the outcomes of inflammatory or infectious diseases. Recent studies implicate a role for P2X/P2Ysignalling in mounting appropriate inflammatory responses critical for host defence against invading pathogens or tumours. Conversely, P2X/P2Y signalling can promote chronic inflammation during ischaemia and reperfusion injury, inflammatory bowel disease or acute and chronic diseases of the lungs. Although nucleotide signalling has been used clinically in patients before, research indicates an expanding field of opportunities for specifically targeting individual P2 receptors for the treatment of inflammatory or infectious diseases.

Nucleotides—particularly ATP—are well known for their function as a universal energy currency[1]. Interestingly, ATP has a completely different role in the extracellular compartment, where it functions as a signalling molecule through the activation of nucleotide receptors[1]. These receptors are referred to as purinergic P2 receptors. In contrast to P1 receptors, which are activated by the ATP metabolite adenosine, P2 receptors are activated by ATP and/or other nucleotides (for example, UTP). On the basis of their signalling properties, P2 receptors can be further subdivided into metabotropic P2Y receptors (P2YRs) that are G-protein-coupled, and ionotropic P2X receptors (P2XRs) that are nucleotide-gated ion channels[2]. Although P2 receptors were originally described on the basis of their functional role in the central nervous system[3,4], more recent studies

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demonstrate their widespread expression throughout different tissues (Supplementary Table 1) and implicate them in innate or adaptive immune responses[2,5].

Cellular ATP release during inflammatory conditions

During certain conditions—for example inflammatory, ischaemic and hypoxic—several cell types release ATP from intracellular storage pools into the extracellular compartment[2,5,6]. Although ATP release can occur in an uncontrolled fashion (for example, during necrosis), many studies have examined molecular pathways that control extracellular ATP release[5]. For example, inflammatory cells can release ATP via pannexins or connexin hemichannels[2,7]. Pannexins—transmembrane protein channels that connect the intracellular with the extracellular space—have been implicated in the release of ATP from apoptotic cells[8], and other studies have implicated connexins in extracellular nucleotide release[6,7]. Connexins were originally described as gap junction proteins consisting of two hemichannels. However, isolated hemichannels (connexons) can function as conduits between the cytoplasm and the extracellular space, thereby controlling ATP release, for example from inflammatory cells[7] or vascular endothelia[9]. Other studies found that the release of uridine nucleotides such as UTP, UDP and UDP-glucose are increased during cystic fibrosis[10]. Together, these studies indicate that inflammatory disease conditions are associated with the extracellular release of nucleotides.

Molecular structure and signalling cascade of P2YR

P2YR belongs to the G-protein-coupled receptor (GPCR) family and contains an extracellular amino terminus, an intracellular carboxy terminus and seven transmembrane-spanning motifs (Fig. 1). At present, eight distinct mammalian P2YRs have been cloned and characterized ($P2Y_{1/2/4/6/11/12/13/14}R$). The missing numbers represent either non-mammalian receptors ($P2Y_3R$ is the chicken orthologue of human $P2Y_6R$) or other GPCRs that share some sequence homology with P2YRs but cannot be activated by nucleotides (for example, lysophosphatidic acid is a $P2Y_9R$ agonist)[11]. According to their phylogenetic and sequence divergence, two distinct P2YR subgroups have been proposed. The first group includes the $P2Y_{1/2/4/6/11}R$ subtypes, with a sequence homology of 35–52% in amino acid composition and the presence of a Y-Q/K-X-X-R defining motif in the transmembrane α-helix 7, thus affecting ligand-binding characteristics. The second group contains $P2Y_{12/13/14}R$, with members sharing a sequence homology of 47–48% and the presence of the K-E-X-X-L motif in transmembrane α-helix 7 (ref. 11). There is some evidence suggesting that the two P2YR subgroups also differ in their primary coupling to G proteins: the $P2Y_{1/2/4/6/11}R$ group is coupled to G_q/G_{11} (leading to calcium release via phospholipase C/inositol-1,4,5-triphosphate activation). By contrast, $P2Y_{12/13/14}R$ bind to $G_{i/0}$ proteins, which inhibit adenylate cyclase and modulate flow through ion channels[11,12]. However, there are several instances in which other signalling pathways have been identified. For example, the discrepancy between structural-group affiliation and functional characteristics is highlighted by $P2Y_{12}R$. Despite having only 20–25% sequence homology with $P2Y_{1/2/4/6}R$[12], there is considerable functional similarity. Indeed, $P2Y_{12}$ and $P2Y_2R$ can both activate monomeric G-proteins (such as Rac and/or RhoA)[13], and are the only P2YR subtypes that exhibit agonist-induced desensitization through GPCR kinases[14]. These

studies indicate that despite some sequence homology among P2YRs, there are marked differences between individual members of the P2YR family regarding their intracellular signalling cascades.

Endogenous ligands for P2YR

The most abundant and best-characterized endogenous ligand for P2YR is the nucleotide ATP. ATP binds to all P2YRs except $P2Y_6R$ and $P2Y_{14}R$[12]. Its binding characteristics exemplify the complexity of P2YR signalling: at low concentrations it is the only native agonist for $P2Y_{11}R$, but at higher concentrations it functions as a partial agonist for $P2Y_1R$ and $P2Y_{13}R$, or as an antagonist for human $P2Y_4R$ or $P2Y_{12}R$[11,12,15]. Other nucleotides, such as ADP, UTP, UDP or UDP-glucose, exhibit more specificity for individual P2YRs. For example, ADP activates $P2Y_1R$, $P2Y_{12}R$ and $P2Y_{13}R$, whereas UTP primarily binds to $P2Y_2R$ and $P2Y_4R$, and to a lesser extent to $P2Y_6R$, for which UDP is its preferred native ligand. $P2Y_{14}R$ is predominantly activated by UDP-glucose and other UDP-sugars, and to a lesser degree by UDP[11,12,15,16]. Indeed, the capacity of different nucleotides to bind specifically to individual P2YRs, or to act as either agonists or antagonists, highlights the complexity of the P2Y system and suggests non-redundant signalling pathways.

Pharmacological compounds that act on P2YR

Owing to the fact that P2YRs have crucial roles in regulating immune responses, they became an obvious pharmacological target for the treatment of inflammatory or infectious diseases. Interestingly the parasiticide suramin, which was widely used in the 1920s for the treatment of human onchocerciasis and trypanosomiasis[17,18], was later found to be a nonspecific inhibitor of P2YR and P2XR[11,15]. Although many P2YR-subtype-specific agonists or antagonists have been characterized in *in vitro* assays or animal studies of inflammatory disorders[11,12,15], presently only two types of P2YR-specific compounds are used in patients: antithrombotic $P2Y_{12}R$ antagonists (for example, clopidrogel) and the $P2Y_2R$ agonist denufosol, which was examined for the treatment of cystic fibrosis, but eventually failed in clinical trials[19]. One of the important future challenges for targeting PYR signalling in patients will include the development of highly selective P2YR antagonists, or specific combined P2R antagonists (for example, a $P2Y_2/P2Y_6/P2X_7R$ antagonist), which could be used for the treatment of chronic inflammatory disorders.

Functional roles of P2YR in unchallenged mice

Mice with genetic deletions for human P2YR homologous genes have been generated and characterized, with the exception of *P2RY11*, which is not expressed in mice[12]. Despite their widespread expression and their functional involvement in many diseases, mice with global deletions for individual P2YRs display only mild phenotypical alterations when maintained unchallenged in a germ-free environment. For instance, $P2ry2^{-/-}$ mice have slightly lower plasma concentrations of aldosterone, renin and potassium[20], whereas global deletion of *P2ry4* is associated with lower exercise capacity and reduced myocardial hypertrophy during a swimming exercise[21]. These findings indicate the likelihood of some redundancy in the signalling system, or compensatory mechanisms following global *P2ry* gene deletion.

P2YR signalling during inflammatory disease states

Several studies over the past decade have highlighted fundamental roles for P2YRs during inflammatory and infectious diseases. Particularly, signalling events through $P2Y_{2/6/12}R$ have shaped an ambivalent view of their function as either friend or foe during inflammation.

$P2Y_2R$

An early attempt at targeting P2Y signalling for the treatment of inflammatory disorders came from studies of $P2Y_2R$ agonists for the treatment of cystic fibrosis[22,23]. Cystic fibrosis is a life-shortening disease that affects over 30,000 children and adults in the United States[24]. The airways of patients with cystic fibrosis are susceptible to infection, characterized by neutrophilic inflammation. Although neutrophil proteases are critical for killing engulfed bacteria, neutrophil elastase accumulates in the airways of cystic fibrosis patients, impairing ciliary function, crippling bacterial clearance and degrading structural proteins[24]. From a molecular perspective, cystic fibrosis is characterized by a defect in the cystic fibrosis transmembrane conductance regulator gene, causing hyperabsorption of sodium leading to thickening of mucus, reduced mucociliary clearance and concomitant increases in susceptibility to bacterial infection[22,23]. Several studies have indicated that $P2Y_2R$ agonists can induce chloride secretion through inhibition of the epithelial sodium channel ENaC, activation of calcium-dependent chloride channels[25], stimulation of mucin production, surfactant secretion and ciliary beating (Fig. 2)[25]. These observations were followed by the development of the $P2Y_2R$ agonist denufosol for the treatment of patients suffering from cystic fibrosis[26]. In 2005 denufosol entered clinical trials, and a 28-day intervention study in a small cohort indicated a potential benefit in lung function of cystic fibrosis patients[27]. Unfortunately, long-term follow-up (48 weeks) in 466 patients was not associated with improved pulmonary function or reduction of pulmonary exacerbations[19]. These disappointing findings may be related to an inflammatory role for $P2Y_2R$ signalling (see below)[2].

In addition to a means for enhancing mucociliary clearance, $P2Y_2R$ agonists have been implicated in the promotion of wound healing[28]. In this context, $P2Y_2R$ signalling mediates the recruitment of leukocytes to the site of tissue damage as well as differentiation and proliferation of structural cells. Moreover, ATP release and concomitant $P2Y_2R$ signalling has been identified as a ‘find-me’ signal for leukocytes, promoting phagocytic clearance of apoptotic cells or bacteria by macrophages[29] and neutrophils[30,31], thereby contributing to the resolution of inflammation (Fig. 2).

Other studies have indicated that $P2Y_2R$ signalling contributes to fundamental leukocyte functions such as migration and mediator production by neutrophils, eosinophils, dendritic cells (DCs) and macrophages[32,33]. For example, migrating neutrophils can release ATP from their leading edge to amplify chemotactic signals and direct cell orientation by feedback signalling involving $P2Y_2R$ (Fig. 2)[31]. In addition, danger signals such as uric acid, complement factor 5a, Toll-like receptor ligands and interleukin (IL)-8 are stimulated by an autocrine ATP–$P2Y_2R$ loop to modulate migration and cytokine production of neutrophils or eosinophils[2,31,34–36]. Several studies point towards an ambivalent function of

$P2Y_2R$ in neutrophilic inflammation: although *P2ry2*$^{-/-}$ mice are less capable of containing bacterial infections[30,37], inappropriate activation of $P2Y_2R$ is associated with neutrophil-induced hyperinflammation and tissue damage during sepsis, chronic lung disease and hepatitis (Fig. 2)[38–41]. For example, neutrophils from patients with chronic obstructive pulmonary disease express higher levels of $P2Y_2R$, which is associated with higher elastolytic activity and migration capacity upon ATP stimulation compared to healthy controls[40]. In the context of $P2Y_2R$ agonists for the treatment of cystic fibrosis, these findings could explain why the long-term use of inhaled denufosol in cystic fibrosis patients failed to improve clinical outcomes, as it may have been associated with enhanced neutrophil activation and increased lung inflammation, thus overcoming the beneficial effects of improved mucociliary clearance.

Studies in murine models of asthma or contact hypersensitivity demonstrate a contribution of $P2Y_2R$ to the induction and self-perpetuation of allergic diseases. Allergen provocation leads to ATP release and concomitant signalling through $P2Y_2R$, thus favouring the recruitment of immature myeloid DCs and eosinophils to the site of allergen exposures[42–44]. This is associated with the production of pro-allergic mediators (for example, IL-33, IL-8, eosinophil cationic protein) from different cellular sources (Fig. 2)[33,45,46]. Similarly, studies in humans indicate that $P2Y_2R$-induced migration and production of reactive oxygen species are enhanced in immature monocyte-derived DCs and eosinophils from allergic donors in the context of concomitant increases in $P2Y_2R$ expression[43].

Taken together, these findings suggest that ATP-elicited activation of $P2Y_2R$ can function as a ‘friend’ in the defence against bacterial infections, promotion of wound healing or in enhancement of mucociliary clearance mechanisms. By contrast, it can also lead to uncontrolled inflammation, attenuated resolution, promotion of chronic inflammatory disease states and fibrotic remodelling[47]. Indeed, $P2Y_2R$ antagonists—as opposed to $P2Y_2R$ agonists—could evolve as useful drugs for the treatment for chronic inflammatory diseases, such as chronic obstructive pulmonary disease (Fig. 2)[40,41].

$P2Y_6R$

$P2Y_6R$ is highly expressed on stromal cells and can be activated by UDP[48–50]. The ambivalent behaviour (‘friend or foe’) of $P2Y_2R$ signalling during inflammatory diseases also applies to $P2Y_6R$ signalling. Recent *in vivo* studies demonstrate a role of $P2Y_6R$ in innate immune responses against bacterial infection[51]. $P2Y_6R$ activation triggers chemokine release from monocytes, DCs, eosinophils and endothelial cells, thus promoting recruitment of inflammatory cells towards the site of inflammation or infection[11,48,51–53]. Similarly, one study demonstrated that injured neurons release UTP and UDP, causing the upregulation of $P2Y_6R$ expression on microglia, and concomitant enhancement of their phagocytic capacity for dying cells[54]. UDP signalling through $P2Y_6R$ can therefore function as an ‘eat-me’ signal for microglia, thereby initiating the clearance of dying cells or debris in the central nervous system.

By contrast, $P2Y_6R$ signalling is detrimental in models of endothelial[50] or epithelial[49] inflammation. Mucosal $P2Y_6R$ expression is increased during experimentally induced intestinal inflammation such as occurs during inflammatory bowel disease (IBD)[49]. IBD is a

heterogeneous group of disorders characterized by intestinal inflammation, including Crohn's disease and ulcerative colitis. Here, pharmacological inhibition or genetic deletion of *P2ry6* in murine models of intestinal inflammation is associated with improved disease outcomes[55]. Similarly, a functional role for $P2Y_6R$ signalling in promoting detrimental inflammation has been reported for chronic forms of lung disease, such as asthma[48]. Indeed, its functional role in promoting pathological airway inflammation during chronic lung disease is an important concern regarding potential considerations for the use of $P2Y_6R$ agonists for the treatment of cystic fibrosis as a means towards enhancing mucociliary clearance[25]. The idea that UDP-elicited $P2Y_6R$ activation can lead to self-perpetuating chronic inflammatory disorders is further supported by recent *in vivo* findings suggesting a functional role of $P2Y_6R$ in promoting atherosclerotic disease in murine models[56]. Taken together, these studies indicate that although activation of $P2Y_6R$ is important in initiating innate immune responses after infection, inappropriate $P2Y_6R$ signalling, predominantly on stromal cells, can drive detrimental immune responses in chronic inflammatory disorders such as atherosclerosis, chronic lung disease or IBD.

$P2Y_{12}R$

$P2Y_{12}R$, which is highly expressed on platelets, has fundamental roles in platelet activation and aggregation. Stimulation of $P2Y_{12}R$ inhibits adenylyl cyclase activity and increases phosphatidylinositol-3 kinase activity, resulting in the activation of the fibrinogen receptor (integrin αIIb β3), which is critical for platelet aggregation[5]. $P2Y_{12}R$ antagonists have been used successfully for antithrombotic therapy in patients[5]. Because platelets are a key source of inflammatory mediators, $P2Y_{12}R$ signalling has also been implicated in modulating inflammatory responses[57]. The fact that $P2Y_{12}R$ agonists trigger mediator release from platelets implicates inflammatory alternations in patients taking $P2Y_{12}R$ antagonists. Importantly, $P2Y_{12}R$ antagonists such as clopidogrel or ticagrelor are clinically used in patients as platelet inhibitors. Indeed, reduced levels of circulating inflammatory mediators (for example, tumour-necrosis factor-α, C-reactive protein, P-selectin) were found in patients receiving clopidogrel[58]. Preclinical studies confirm the proinflammatory role of $PY_{12}R$ signalling in models of vascular inflammation and asthma. For instance *P2ry12*$^{-/-}$ mice are protected in models of atherosclerosis[59,60]. Moreover, a surprising crosstalk involving leukotrienes and $P2Y_{12}R$ has been described during asthma. In brief, murine studies demonstrate that $P2Y_{12}R$ signalling on platelets is required for the pro-asthmatic action of leukotriene LTE4 (ref. 61). Furthermore, platelet-independent $P2Y_{12}R$ signalling events contribute to asthma, as $P2Y_{12}R$ antagonists can directly block cysteinyl leukotriene-induced release of eosinophil cationic protein from human eosinophils[62], and ADP-elicited $P2Y_{12}R$ activation enhances the capacity of DCs to activate allergen-specific T cells[63]. The clinical observation that single nucleotide polymorphisms of the *P2RY12* gene are associated with altered lung function in a cohort of asthmatic children provides additional evidence for a role of $P2Y_{12}R$ signalling in human asthma[64]. Taken together, these studies implicate $P2Y_{12}R$ signalling in promoting chronic inflammatory disorders such as asthma and atherosclerosis. However, additional clinical trials addressing the clinical efficacy of $P2Y_{12}R$ antagonists (such as clopidogrel or ticagrelor) for the treatment or prevention of chronic inflammation will be critical in establishing their clinical usefulness beyond current indications.

P2XR signalling during inflammation

Molecular structure and signalling cascade of P2XR

P2XRs are plasma membrane channels selective for monovalent and divalent cations (Na^+, K^+, Ca^{2+}) which are directly activated by extracellular ATP (Fig. 1)[65]. Seven different subunits have been identified so far ($P2X_{1-7}R$)[65]. The primary sequence of P2XRs has no important sequence homology with other ligand-gated ion channels, ATP-binding proteins or other known proteins[66]. P2XRs share a common topology with two transmembrane domains (TM1 and TM2), a large extracellular loop responsible for ligand binding, and an intracellular N and a longer C terminus[67]. The extracellular 'loop' starts in proximity of position 52, and ends near proline 329, therefore most of the P2XR protein protrudes from the plasma membrane. Evidence suggests that functional P2XRs are trimers, with three peptide subunits arranged around an ion-permeable channel pore, where ATP binding promotes subunit rearrangement and ion channel opening[68]. Three molecules of ATP seem to bind to the extracellular portions of P2XR[67]. Channel opening induces transmembrane ion fluxes, that is, Na^+ and Ca^{2+} influx and K^+ efflux, leading to plasma membrane depolarization, and—due to the increase of intracellular Ca^{2+} levels—activation of Ca^{2+} signalling cascades, such as p38 MAPK or phospholipase A_2 activation[67]. Interestingly, $P2X_7R$ is capable of activating the NOD-like-receptor-mediated inflammasome assembly with pro-caspase 1 proteolytic activation and subsequent pro-IL-1 and pro-IL-18 cleavage and release of their biologically active forms[65]. The long C-terminal 'tail' of $P2X_7R$ allows it to undergo a conformational change resulting in the so called 'permeability transition'; that is, $P2X_7R$ changes from a cationic channel to a wider pore, allowing transmembrane fluxes of small hydrophilic molecules (including ATP) with a molecular mass of approximately 900 (refs 69, 70).

In addition, some P2XRs, for example $P2X_1R$, can form heterotrimers with $P2X_2R$, $P2X_4R$ and $P2X_5R$ subunits, whereas $P2X_2R$ forms heterooligomers with $P2X_3R$[66]. Together these findings indicate that despite the fact that all P2XRs are activated by ATP and share significant sequence homology with each other, highly distinct functions are unique for individual members of the P2XR family.

Endogenous and pharmacological ligands for P2XR

In contrast to P2YR signalling, for which more than one native agonist exists, human P2XRs share ATP as their main endogenous agonist[71]. Owing to the central role of $P2X_7R$ during inflammatory disorders, great efforts have been made to develop selective antagonists. For example, the $P2X_7R$ antagonist AZ9056 was used in patients with rheumatoid arthritis. Although initial studies were promising, Phase IIb/III studies with different $P2X_7R$ antagonists failed to improve long-term clinical outcomes[72]. In addition, there are ongoing PhaseII studies with $P2X_7R$ and $P2X_3R$ antagonists in chronic pain and chronic cough (http://www.clinicaltrials.gov/)[72], highlighting the potential for P2XRs as drugable targets in the treatment of inflammatory disorders.

Functional roles of P2XR in unchallenged mice

Physiological roles of P2XRs are indicated by phenotypic manifestations in mice lacking individual P2XR subtypes. Although all single *P2rx* knockout mice are viable and survive to adulthood, some of them reveal unexpected phenotypes, suggesting that functional roles of individual receptor subunits cannot be compensated for by others. For instance, *P2rx1*$^{-/-}$ mice show reduced vas deferens contraction and are infertile[73], whereas *P2rx2*$^{-/-}$ mice develop severe progressive hearing loss[74], and *P2rx3*$^{-/-}$ mice experience urinary bladder hyporeflexia[75]. Mice lacking both *P2rx2* and *P2rx3* have enlarged spleens and increased numbers of immune cells[76]. These findings suggest P2XR-subtype-specific signalling functions under physiological conditions, including immune functions.

P2X$_7$R signalling during inflammatory disease

Compelling evidence implicates P2XR during inflammation and immune response against microbes[72,78]. Although several other P2XRs are functional during inflammation (for example, P2X$_4$R), P2X$_7$R in particular has been shown to affect the outcomes of inflammatory or infectious diseases. This may be due to the fact that P2X$_7$Rs are predominantly expressed on immune cells such as mast cells, macrophages, microglia and DCs (s)[2]. Indeed, functional studies implicate P2X$_7$R in immune responses against bacterial and parasitic infection. For example, activation of P2X$_7$R is involved in the formation of macrophage multinucleated giant cells, an important step for the control of tuberculosis (Fig. 3)[77]. Other studies implicate P2X$_7$R signalling in the elimination of intracellular microbes—such as *Mycobacterium tuberculosis*, *Chlamydia psittaci*, *Leishmania amazonensis* or *Toxoplasma gondii*—by either killing of the pathogen or by inducing cell death of infected macrophages (Fig. 3)[78]. Human studies indicate that loss-of-function mutations in the *P2RX7* gene are associated with increased susceptibility to tuberculosis or toxoplasmosis[78]. Owing to its proinflammatory role via activation of the inflammasome, and its direct cytotoxic or pro-apoptotic function, many reports implicate a role for the ATP–P2X$_7$R axis in tumour suppression. P2X$_7$R expression is lower in some types of cancer and loss-of-function mutations in the *P2RX7* gene have been linked to the pathogenesis of chronic lymphatic leukaemia[79]. Additional evidence comes from studies demonstrating that loss-of-function mutations of the *P2RX7* gene in patients with breast cancer are associated with an increased risk of progression to metastatic disease states[80]. This study specifically implicated DC–P2X$_7$R signalling in resistance to chemotherapy (Fig. 3). DCs present antigens from dying cancer cells to prime tumour-specific interferon-γ (IFN-γ)-producing T cells. Dying tumour cells release ATP, which activates P2X$_7$R expressed on DCs, which in turn causes inflammasome assembly and subsequent secretion of IL-1β (Fig. 3). Accordingly, anticancer chemotherapy was shown to be inefficient against tumours established in purinergic receptor P2rx7$^{-/-}$ hosts[80]. Together, such findings implicate ATP signalling through the P2X$_7$R in host-defence mechanisms against intracellular pathogens and cancers.

It is particularly interesting that P2X$_7$R signalling on DCs can have very different effects on T-cell priming, depending on the specific context, including CD8$^+$ and CD4$^+$ T-cell differentiation (Fig. 3). In contrast to its essential role in immune priming for response against tumours or pathogens, the involvement of P2X$_7$R in the polarization of antigen-

specific effector T cells by DCs contributes to the induction and maintenance of chronic inflammation. $P2X_7R$ signalling on DCs is involved in the sensitization phase of allergic disorders such as contact hypersensitivity ($CD8^+$ T-cell priming)[44] and asthma ($CD4^+$ T-cells, T_H2 response)[81], and contributes to transplant rejection ($CD4^+$ T cells, T_H1 response; Fig. 3). For example, recent studies implicate $P2X_7R$ signalling in graft-versus-host disease, a common complication following an allogeneic tissue transplant, in which immune cells in the tissue recognize the recipient as 'foreign', leading to an immunological reaction of transplanted immune cells against the host. Indeed, $P2X_7R$ inhibition or deficiency on DCs is associated with reduced severity of graft-versus-host disease[82]. As such, $P2X_7R$ signalling of antigen-presenting DCs led to an increased expression of CD80 and CD86 *in vitro* and *in vivo* and activated a cascade of proinflammatory events, including signal transducer and activator of transcription 1 (STAT1) phosphorylation, IFN-γ production and donor T-cell expansion[82]. Again, other studies report that $P2X_7R$-mediated priming can contribute to T_H17-driven autoimmune diseases, such as psoriasis (Fig. 3)[83]. By triggering the production of pro-allergic mediators from eosinophils, mast cells, macrophages and DCs, $P2X_7R$ signalling also contributes to the effector phase and chronification of allergic disorders[42,81]. Furthermore, increased expression of $P2X_7R$ can be found on eosinophils and macrophages in asthma (Fig. 3)[81], and loss-of-function mutations in the *P2rx7* gene have been associated with attenuated risk of allergen sensitization and asthma[84].

In line with these findings, other studies report a detrimental role of $P2X_7R$ in promoting excessive inflammation during IBD, by showing that ATP derived from commensal bacteria activates a unique subset of lamina propria cells ($CD70^{high}$ $CD11c^{low}$ cells), leading to T_H17 cell differentiation (Fig. 3)[85]. Indeed, germ-free mice exhibit lower concentrations of luminal ATP, accompanied by fewer lamina propria T_H17 cells[85]. $P2X_7R$ also participates in IBD pathogenesis by mediating enteric neural death (Fig. 3)[85,86]. Finally, mast-cell-dependent mechanisms of intestinal inflammation are under the control of $P2X_7R$, as increased $P2X_7R$ expression can be found in mast cells from Crohn's patients and inhibition of $P2X_7R$ on mast cells dampens intestinal inflammation (Fig. 3)[87].

Together, these findings expose $P2X_7R$ signalling during inflammation as a double-edged sword: these receptors have a critical role in mediating appropriate inflammatory and immunological responses against invading pathogens orcancer cells, respectively, but contribute to chronic inflammatory disease states in a wide range of inflammatory disorders, such as chronic lung disease[40,88], asthma[81,84] or IBD[86,87], when activated inappropriately.

Termination of ATP signalling

Termination of P2R signalling involves the conversion of ATP/ADP to adenosine within the extracellular compartment by the activity of ectonucleotidases. The four main groups of ectonucleotidases are the ectonucleoside triphosphate diphosphohydrolases (NTPDases), ecto-5′-nucleotidase (CD73), ectonucleotide pyrophosphatase/phosphodiesterases and alkaline phosphatases[89]. NTPDases represent a family of ubiquitously expressed membrane-bound enzymes. The catalytic sites of plasma membrane-expressed NTPDases 1–3 and 8 are oriented towards the extracellular milieu[90]. Owing to its high expression in many tissues and its ability to catalyse the conversion of ATP (and ADP) down to AMP, many studies have

found a functional role for NTPDase1 (CD39) in the termination of P2R signalling[90,91]. Next, extracellular AMP is converted to adenosine by CD73 (Fig. 1)[5]. Therefore, termination of ATP signalling is closely linked to the generation of extracellular adenosine. In many instances, adenosine-elicited P1R signalling dampens acute inflammation and tissue injury[92,93], thus opposing inflammatory functions of P2Rs (Fig. 1)[94,95].

Consistent with a protective role for the CD39/CD73 pathway in terminating inflammatory P2R signalling, and concomitantly increasing extracellular adenosine levels and signalling events, several studies show that *Cd39*$^{-/-}$ or *Cd73*$^{-/-}$ mice are prone to tissue injury during inflammatory conditions such as acute lung injury or intestinal inflammation[96,97]. For example, patients with a single nucleotide polymorphism associated with low levels of CD39 expression have increased susceptibility to Crohn's disease, suggesting that deficiency in CD39 could be associated with IBD in humans[96]. Other reports suggest that CD39 exerts a protective thromboregulatory function in stroke by preventing P2R-mediated thrombosis[91]. Moreover, several studies implicate the CD39/CD73 pathway in the immunosuppressive roles of regulatory T cells (T_{reg}). These are a group of $CD4^+$ lymphocytes that suppress T-cell responses against a variety of pathogens and control inappropriate immune activation, thus limiting collateral tissue damage but allowing pathogen persistence[98]. As such, T_{reg} cells from *Cd39*$^{-/-}$ mice demonstrate attenuated suppressive functions *in vitro* and fail to block rejection of allografts *in vivo*[99]. Similarly, *Cd73*$^{-/-}$ mice fail to resolve lung injury induced by lipopolysaccharide inhalation due to impairment of T_{reg} functions[100].

The ambivalence of CD39/CD73-mediated control of T_{regs} is further exemplified during infections with human immunodeficiency virus (HIV), the retrovirus known to cause AIDS in humans. HIV infections are characterized by a progressive CD4 lymphopenia in conjunction with defective HIV-specific CD8 responses that are critical for the control of viral replication[98]. As such, the consequences of T_{reg} expansion, as seen during HIV infection, could have either a beneficial function by suppressing generalized T-cell activation, or could be fatal owing to attenuated HIV-specific responses and thus promoting viral persistence[98]. For example, studies demonstrate that HIV-1-positive patients have an increase of T_{reg}-associated expression of CD39. These findings indicate that the CD39/CD73 pathway is involved in T_{reg} suppression in HIV infection[101]. A genetic association study demonstrated that a polymorphism in the *CD39* gene is associated with attenuated CD39 expression and slower progression to AIDS in HIV-infected patients[101]. Thus, it can be speculated that $CD39^+$ T_{regs} are the most potent T_{reg} subset to inhibit HIV-specific T-cell responses. This could at least in part account for their association with disease progression. Other examples for a detrimental role of CD39-dependent ATP breakdown come from studies of autoimmune hepatitis in which natural killer T cell dysfunction in *Cd39*$^{-/-}$ mice protects against concanavalin A-induced hepatitis. Heightened levels of apoptosis of *Cd39*$^{-/-}$ natural killer T cells *in vivo* and *in vitro* appear to be driven by unimpeded activation of $P2X_7R$[102]. Similarly, enzymatic removal of ATP by apyrase (conversion of ATP/ADP to AMP) or ectopic CD39 expression attenuates clearance of apoptotic cells, indicating a detrimental role for CD39-dependent ATP phosphohydrolysis in dampening efficient corpse clearance via $P2Y_2R$ signalling[29]. Other studies demonstrate the existence

of bacterial ectotriphosphate diphosphohydrolases—similar to human CD39—which are critical for the intracellular multiplication of *Legionella pneumophila* by preventing P2R-elicited immune responses. As such, these findings implicate bacterial ectotriphosphate diphosphohydrolases in virulence[103]. Together these studies exemplify an ambivalent role for the termination of P2R signalling via enzymatic phosphohydrolysis. Although this pathway is critical in preventing excessive P2R-dependent inflammation in a sterile environment[104], CD39 function can become detrimental for the appropriate clearance of apoptotic debris[29], inflammation directed against bacterial infections[103] or by generating an immunosuppressive environment, which promotes the development or progression of cancer[5].

Functional role of P1 signalling during inflammation

Extracellular AMP generated by phosphohydrolysis of precursor nucleotides (for example, ATP or ADP) has no clearly characterized signalling function (for example, through specific AMP receptors). However, extracellular AMP serves as the metabolic substrate for the extracellular generation of adenosine via CD73 (Fig. 1)[5]. Once generated within the extracellular compartment, adenosine can function via activation of four distinct P1 receptors: ADORA1, ADORA2A, ADORA2B or ADORA3. Adenosine signalling is terminated via uptake of adenosine from the extracellular towards the intracellular compartment through equilibrative nucleoside transporters and is metabolized to inosine via adenosine deaminase[105], or to AMP via adenosine kinase[106]. Several studies implicate adenosine signalling in dampening excessive inflammation[92]. For example, *Adora2a*$^{-/-}$ mice experience increased inflammation including extensive tissue damage, more prolonged and higher levels of proinflammatory cytokines, and mortality when exposed to sub-threshold doses of inflammatory stimuli[93]. Other studies demonstrate that ADORA2B signalling dampens excessive inflammation during acute lung injury[107], promotes ischaemia tolerance and improves anaerobic carbohydrate metabolism[108,109]. Similarly, genetic deletion or pharmacological blockade of equilibrative nucleoside transporters is associated with increased adenosine levels and improved outcomes during inflammatory disease states[110,111]. In most instances, the anti-inflammatory signalling effects of adenosine are associated with improved outcomes during inflammatory diseases such as IBD[112], or during sepsis induced by caecal ligation and puncture[113]. However, other studies indicate that the anti-inflammatory effects of adenosine signalling can be detrimental in containing an infection with live bacteria. For example, a recent study demonstrates that antagonism of P1 receptors (for example, ADORA2B) can be useful in enhancing macrophage-mediated bacterial phagocytosis and improving polymicrobial sepsis survival in mice[114]. Together, these studies highlight that P1 and P2 receptors frequently have opposing effects in biological systems, and that shifting the balance from purinergic P2YR and P2XR signalling towards adenosine-mediated P1 signalling is an important therapeutic concept in efforts to dampen pathological inflammation and promote healing[5].

Conclusions

The field of extracellular nucleotide signalling and metabolism is a dynamic area of research with important opportunities for novel treatments for inflammatory or infectious diseases.

On the one hand, P2R signalling functions to coordinate appropriate immune responses against invading pathogens or tumours. Indeed, pharmacological approaches that amplify extracellular ATP signalling hold promise as therapies for the treatment of cancer or during uncontrolled infections with live pathogens. Such strategies could include inhibition of ATP breakdown (for example, via nucleotidase inhibitors) or treatment with P2 receptor agonists. Conversely, inadequate P2R signalling has been associated with excessive inflammation, chronification and inappropriate resolution and fibrosis in a wide range of inflammatory diseases. In this context, treatment strategies that block P2R signalling, promote extracellular conversion of ATP to adenosine and activate adenosine receptors have been implicated in the treatment of acute or chronic inflammatory diseases. We therefore anticipate that compounds targeting these pathways will be further exploited in the treatment of inflammatory conditions in human patients in the near future.

Note added in proof

Two reports appeared online regarding the atomic structure of the $P2Y_{12}R$ while the current review was in press. The first report provides a 2.6 Å resolution crystal structure of the human $P2Y_{12}R$ in complex with the non-nucleotide antagonist AZD1283 (ref. 115), thus providing important insights for the development of $P2Y_{12}R$ ligands and allosteric modulators as drug candidates. The second report provides the structures of the human $P2Y_{12}R$ in complex with a full agonist (2-methylthio-adenosine-5′-diphosphate) at a resolution of 2.5 Å, and the corresponding ATP derivative 2-methylthio-adenosine-5′-triphosphate at 3.1 Å resolution[116]. The agonist-bound P2Y12R structure answers ambiguities surrounding P2Y12R-agonist recognition, and suggests unexpected interactions with several residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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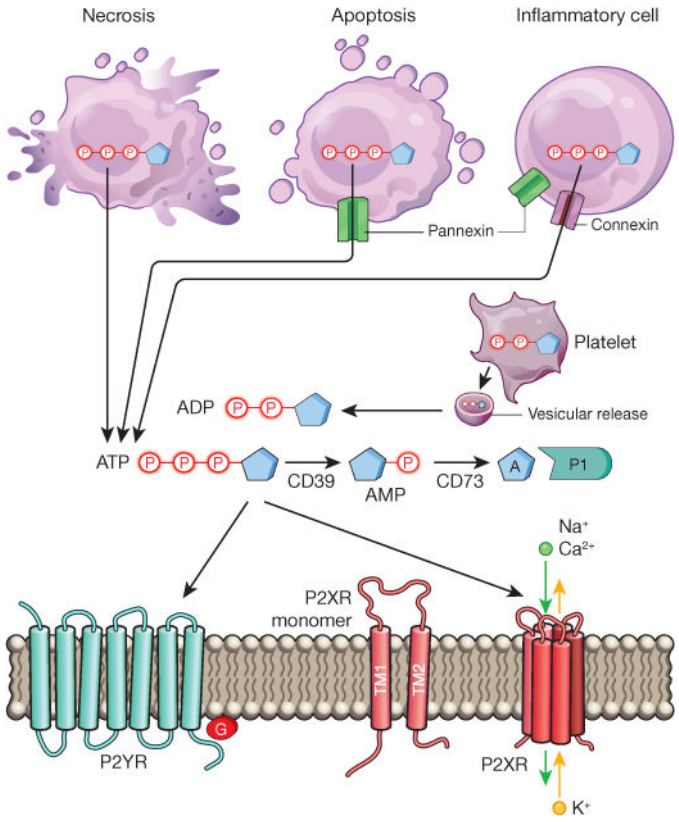

Figure 1. Extracellular nucleotide release and signalling during inflammation

During inflammation, multiple cell types release nucleotides, for example ATP or ADP, from their intracellular compartments into the extracellular space. Nucleotides can be released during mechanical injury, necrosis, apoptosis or inflammatory cell activation. Several molecular pathways have been implicated in this process, such as vesicular ADP release from platelets, pannexin-mediated ATP release during apoptosis, and connexin-or pannexin-mediated ATP release from inflammatory cells, such as neutrophils. Extracellular nucleotides function as signalling molecules through the activation of purinergic P2 receptors. These receptors can be grouped into metabotropic P2Y receptors (P2YRs; GPCRs with seven transmembrane-spanning motifs) or ionotropic P2X receptors (P2XRs), which are nucleotide-gated ion channels. Each P2XR is formed by three subunits (P2XR monomers), each of which consists of two transmembrane regions, TM1 and TM2. Binding of three molecules of ATP to the assembled P2X channel causes opening of a central pore. These conformational changes allow for flux of ions such as sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) across the membrane. ATP signalling is terminated by the enzymatic conversion of ATP to adenosine through the ectonucleoside triphosphate diphosphohydrolase CD39 (conversion of ATP/ADP to AMP) and the ecto-5′-nucleotidase CD73 (conversion of AMP to adenosine). Similar to ATP, adenosine (A) functions as an extracellular signalling molecule through the activation of purinergic P1 adenosine receptors.

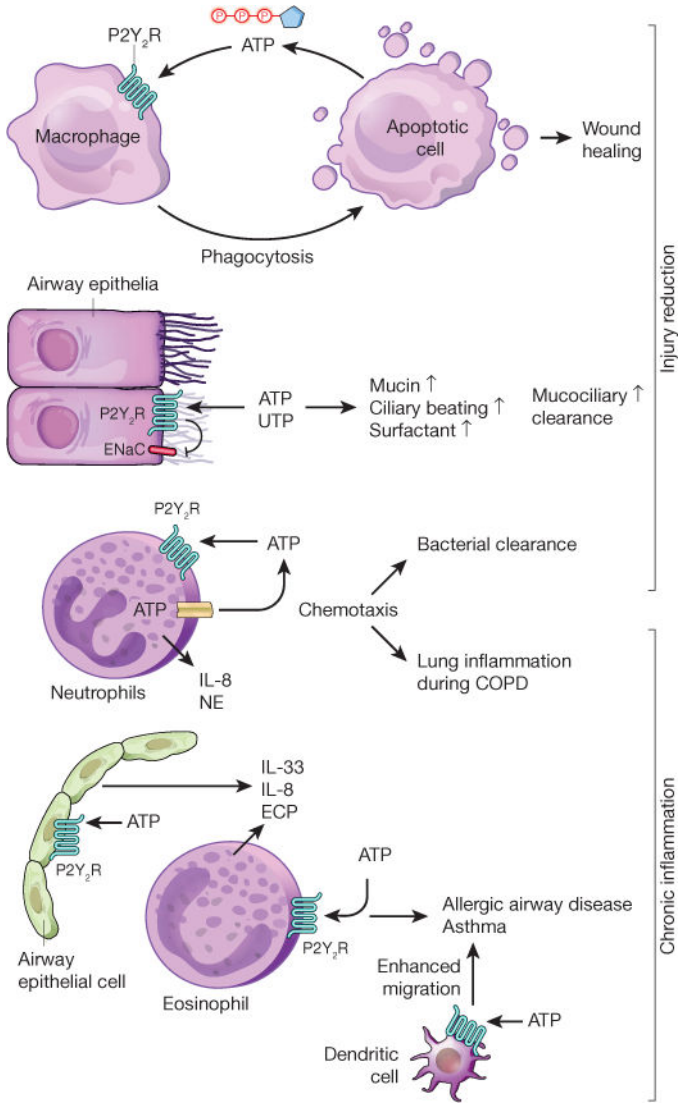

Figure 2. P2Y$_2$R signalling during injury resolution and chronic inflammation

P2Y$_2$R signalling on phagocytes, such as macrophages, contributes to the clearance of apoptotic cells, which release the P2Y$_2$R agonist ATP as a 'find-me' signal. P2Y$_2$R-mediated clearance of apoptotic cells and debris contributes to wound healing. Activation of P2Y$_2$R by UTP or ATP promotes mucociliary clearance in the airways via inhibition of the epithelial sodium channel (ENaC), which is associated with concomitant increases in mucin production, surfactant-secretion and ciliary beating. Neutrophil-dependent ATP release and autocrine activation of P2Y$_2$R contributes to purinergic chemotaxis, thereby enhancing bacterial clearance during pneumonia. On the other hand, P2Y$_2$R-mediated release of IL-8 and neutrophil elastase (NE) from neutrophils contributes to the pathogenesis of chronic obstructive lung disease (COPD). ATP-elicited P2Y$_2$R signalling on alveolar epithelial cells or eosinophils causes production of pro-allergic mediators (for example, IL-33, IL-8, eosinophil cationic protein) during allergic airway disease. Similarly, P2Y$_2$R signalling on dendritic cells has a role during the induction and self-perpetuation of asthma.

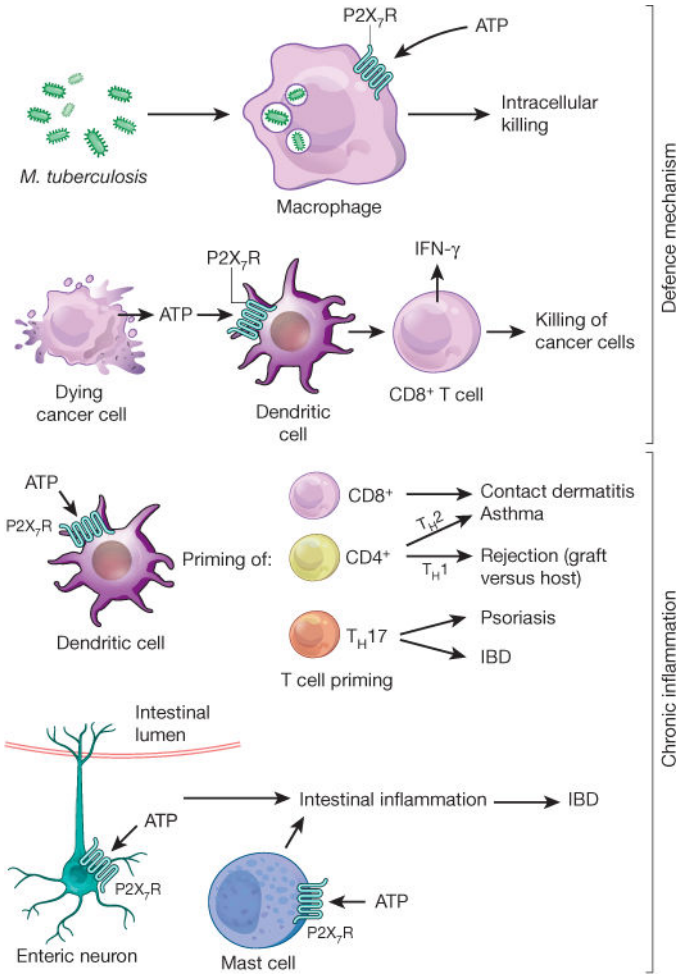

Figure 3. P2X$_7$R signalling during infection and inflammation

P2X$_7$R is required for mounting an appropriate inflammatory response to defend against invading pathogens, for example during intracellular killing of *Mycobacterium tuberculosis* by macrophages. Dying tumour cells release ATP, which activates P2X$_7$R expressed on DCs, which in turn promotes the priming of IFN-γ-producing cytotoxic CD8$^+$ T cells that kill cancer cells. On the other hand, P2X$_7$R signalling on DCs and concomitant T-cell priming contributes to allergic disease states, such as CD8$^+$ T-cell-elicited contact dermatitis. DC-mediated T-cell priming under the control of P2X$_7$R signalling has also been shown to promote T$_H$1 responses that are implicated in graft-versus-host disease, which contributes to the rejection of a transplanted organ. Similarly, P2X$_7$R-mediated T-cell priming towards a T$_H$2 response promotes allergic airway disease during asthma. Priming of T$_H$17 cells is critical during psoriasis and contributes to intestinal inflammation as occurs during IBD. P2X$_7$R signalling on enteric neurons or mast cells has been implicated in promoting intestinal inflammation during IBD.