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P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation

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1. ABSTRACT

Inflammation is an important homeostatic response, which is managed by a complex network of interrelated pathways that determine the level, intensity and localization of inflammation. We now know that purinergic signalling is one of the pathways influencing the initiation, progression and down-modulation of the inflammatory response. Here, we review recent evidence on the role in inflammation of the purinergic signalling system, which is comprised of extracellular ATP, P2 receptors and ecto-enzyme cascades. Recent animal studies with a newly developed bioluminescent ATP probe (pmeLUC), enabling measurement of pericellular ATP *in situ*, have provided proof that ATP is present in inflamed tissues *in vivo* at extracellular concentrations sufficient for P2 receptor activation. Increased extracellular ATP levels amplify inflammation *in vivo* by promoting leukocyte recruitment and NALP3-inflammasome activation via $P2X_7$. Lowering extracellular ATP levels in inflamed tissues, for instance by stimulating its breakdown, inhibits the inflammatory response *in vivo*. In view of its important role in inflammation, the purinergic signalling system is bound to yield novel therapeutic opportunities for the treatment of inflammatory diseases.

2. INTRODUCTION

Inflammation is probably the most important homeostatic system that constantly tunes the level of adaptation of our body to the continuous challenges that come from the external as well as the internal environment. The classical view of inflammation is as a set of body defenses put in action when our organism is endangered by pathogens, and therefore reveals itself by the classical signs: tumor, rubor, calor, dolor, often accompanied by fever. However, this is a narrow view of this complex defense mechanism because we now clearly know that the inflammatory system is continuously active from birth to death, and 90% of noxious agents are effectively neutralized before we even realize their presence. To manage such a highly sophisticated system of interactions, living organisms have evolved a complex network of interrelated pathways that in the end set the level, intensity and localization of the inflammatory response.

One such pathway is the so-called purinergic pathway (Figure 1), comprising extracellular ATP and other purine and pyrimidine nucleotides as well as the nucleoside adenosine, which operate in concert to contribute to the regulation of a variety of biologic cell

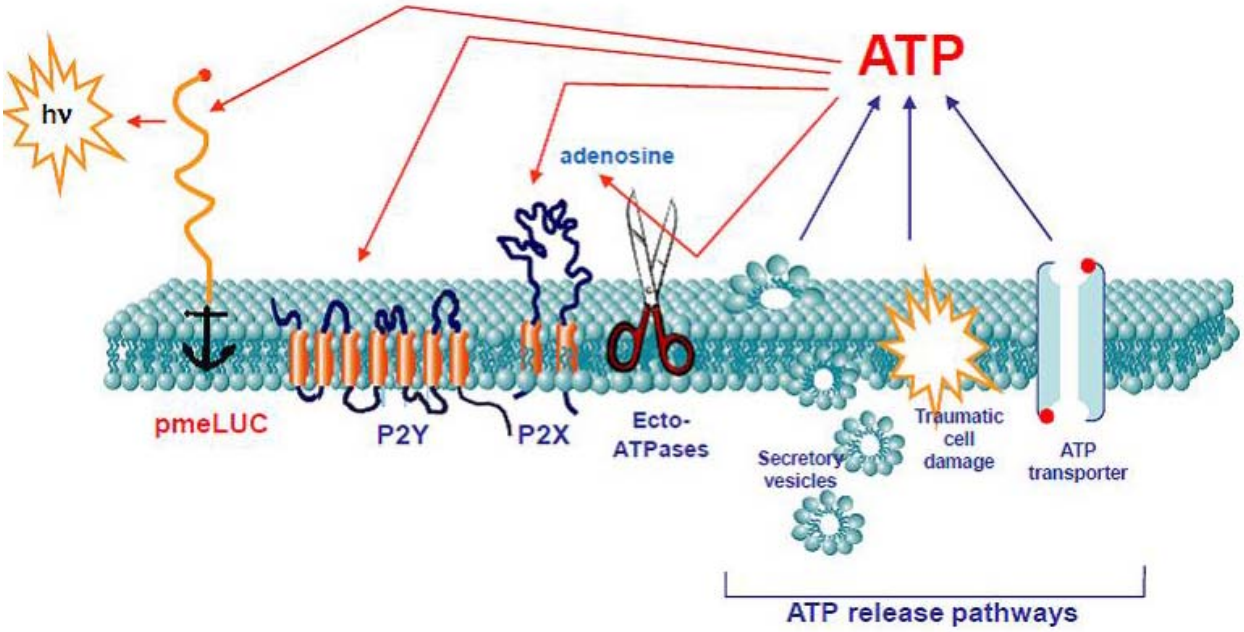

Figure 1. The rationale behind pmeLUC as a probe of the extracellular ATP concentration. Schematic rendition of the pathways for ATP release, ATP degradation and ATP interaction with P2 receptors and pmeLUC.

processes by signalling through a broadly expressed family of membrane-bound purinergic receptors. The purinergic receptor family consists of P1 receptors for adenosine (A_1, A_{2A}, A_{2B}, A_3 subtypes), and P2X ($P2X_{1\text{-}7}$ subtypes) and P2Y ($P2Y_{1,2,4,6,11\text{-}14}$ subtypes) receptors for ATP and related nucleotides, including ADP. Most cell types co-express several subtypes of P1 and P2 purinergic receptors, which can be activated in a concentration-dependent fashion. ATP can accumulate in the extracellular space through different mechanisms, such as vesicular release, active transport and massive release accompanying necrotic cell death, reaching high enough levels for purinergic receptor activation. Extracellular nucleotide and nucleoside concentrations, and thereby the nature and intensity of purinergic signalling, are controlled by a family of ecto-enzymes, including ecto-ATPase, ecto-5'-nucleotidase and adenosine deaminase.

Over the last decade we have learnt that purinergic signalling has a pivotal role in the context of inflammation, at all levels of integration and activity: from the very initial generation and detection of danger signals, up to the final presentation of antigens to effector T cells and to their stimulation, and importantly even in the resolution of inflammation, as P2 receptors and extracellular ATP are also involved in immunosuppressive pathways (1, 2). Awareness of the role of the purinergic axis in host defense opens an exciting new avenue for the development of anti-inflammatory and immunoregulatory drugs.

In the present review, we provide an overview of recent evidence on the role of ATP in inflammation, focusing on the presence of ATP in the extracellular microenvironment at inflammatory foci, as well as on the influence of ATP metabolism and P2 receptor stimulation on inflammatory processes.

3. IS EXTRACELLULAR ATP PRESENT AT INFLAMMATORI FOCI?

After almost a century from its discovery by Karl Lohmann (3), ATP, the most fundamental intracellular energy currency, is enjoying a second life in an unanticipated role as a mediator of inflammation. In fact countless reports now prove beyond any reasonable doubt that this nucleotide is a strong activator of inflammatory cells acting at plasma membrane P2 receptors (1, 4, 5). A wealth of ATP-triggered *in vitro* responses have been described so far, stemming from cytokine release to superoxide generation, from stimulation of chemotaxis to intracellular pathogen killing. However, despite the unequivocal *in vitro* evidence for the potent activity of ATP as a stimulant for the release of inflammatory mediators, *in vivo* evidence is much weaker. In particular, there has been so far no direct demonstration that ATP indeed accumulates at inflammatory foci, and without such a proof the overall hypothesis is unsupported. This is a difficult issue to tackle. To be able to measure ATP in inflammatory exudates, one should be able to sample the tissue interstitium in such a way that cell damage is avoided since, given the high intracellular ATP concentration, any cell damage or activation could easily cause a discharge of ATP. This risk is present in all procedures that involve the physical perturbation of the tissue microenvironment, such as sampling of inflammatory foci with needles or dialysis tubes, or probing the tissue with microelectrodes (6): any procedure will perturb tissue homeostasis and cause an artifactual ATP release. For this reason, a few years ago we set to design and develop a probe that allowed *in situ* measurement of extracellular ATP causing minimal tissue perturbation (7, 8). To this aim we modified the well

known bioluminescent ATP probe firefly luciferase to obtain a recombinant probe targeted to and stably expressed on the cell plasma membrane. The anticipation was that such a probe should allow non-invasive measurement of pericellular ATP. The chimeric probe was made by appending a luciferase cDNA to targeting sequences (leader sequence and GPI anchor) derived from the folate receptor. This plasma membrane-expressed luciferase was named pmeLUC (Figure 1). The pmeLUC cDNA was transfected into HEK293 cells to generate stable clones (HEK293-pmeLUC cells). *In vitro* test showed the absolute selectivity of pmeLUC for ATP over all other nucleotides (ATP analogues included). Affinity for ATP of the chimeric membrane-attached luciferase was lower compared to the soluble enzyme, as lowest detectable ATP concentration ranged between 5 and 10 micromolar. In subsequent experiments we used HEK293-pmeLUC cells as *in vivo* probes of the interstitial ATP concentration. To this aim, we first investigated tumour-associated inflammation as a well defined disease condition in which ATP is postulated to accumulate in the tumour microenvironment. Mice were inoculated either intra peritoneally (i.p.) or sub-cutaneously (s.c.) with human ovarian carcinoma (OvCar) cells or human melanoma MZ2-MEL cells, respectively. After engrafting and full development of the tumour, HEK293-pmeLUC were injected either i.p. (OvCar) or intratumorally (MZ2-MEL), and the mice analyzed with a Caliper-Xenogen X100 total body luminometer. Light emission was very intense and localized at tumour foci. Injection of HEK293 cells into healthy animals produced no light emission, except for a weak and transient luminescence very likely due to acute ATP release from injured cells at the site of injection. Quantification of light emission indicated that at tumour foci the extracellular ATP concentration is in the hundred micromolar range. Rather interestingly, shortly after i.p. injection of HEK293-pmeLUC cells into OvCar-bearing mice, a large and diffuse peritoneal luminescence was recorded, a direct indication that cancer-associated peritonitis causes substantial release of ATP into the peritoneal cavity. Recently, Idzko and co-workers examined with HEK293-pmeLUC cells two additional inflammation models: graft-versus-host reaction (GvHR) and contact hypersensitivity. In the first model, inoculation of allogenic hematopoietic cell transplantation causes a typical inflammation in the gastro-intestinal tract which was paralleled by a large luminescence signal located within the gastrointestinal mucosa and the peritoneal cavity, to witness the large accumulation of extracellular ATP triggered by the inflammatory process (9). In the second model, contact hypersensitivity, skin application of the contact allergen trinitrochlorobenzene causes a local but very large ATP release resulting in a strong bioluminescence emission from locally-injected HEK293-pmeLUC cells (10). Thus, the most recent and technically advanced experiments all concordantly demonstrate that ATP at inflammatory foci not only is present, but reaches a surprisingly high concentration (in the hundred micromolar range). Such a high concentration is sufficient to activate all P2 receptor subtypes and at the same time generate large amounts of the anti-inflammatory agent adenosine (Figure 1).

4. DOES REDUCED EXTRACELLULAR ATP AFFECT INFLAMMATION?

As discussed in the previous section, extracellular ATP concentrations can rise up to high micromolar levels *in vivo* under inflammatory conditions. *In situ*ations of acute tissue stress or damage, the prompt rise in extracellular ATP is more than just a side effect of tissue injury, that is, it is exploited by the immune system to aid in the initiation of an acute inflammatory reaction. The high ATP levels that can be found in the extracellular space following cellular damage or stress are recognized by the innate immune system as a danger signal. Accordingly, ATP is part of the family of damage-associated molecular patterns (DAMPs) that trigger an immediate innate immune response through the activation of innate immune cells, including neutrophils, monocytes and macrophages, and dendritic cells. Extracellular ATP concentrations are continuously being controlled by a cascade of ecto-enzymes that are present on exterior cell surfaces, among which the E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases) are of considerable importance, especially E-NTPDase1 (CD39, ecto-apyrase) (11, 12).

Evidence for a pro-inflammatory role of high ATP levels in acute inflammation derives from a number of studies reporting that targeting ATP-hydrolyzing pathways, i.e. promoting breakdown of ATP in the extracellular compartment, affects early-stage inflammatory processes. Upon initiation of an acute inflammatory response as a result of tissue damage or infection, neutrophils are amongst the first innate immune cells to accumulate at inflammatory foci. Neutrophils have potent pro-inflammatory and bacteria-killing potential, which however often comes at the cost of damage to healthy tissues when their activity is not tightly regulated (13). ATP has been shown to prime neutrophils for enhanced inflammatory responses, such as ROS (reactive oxygen species) production and degranulation of anti-microbial molecules (14-17). Besides enhancing inflammatory activity of neutrophils, ATP also promotes the recruitment of neutrophils. Chen *et al.* (2006) demonstrated that human neutrophils released ATP in response to treatment of cells with FMLP (N-formyl-Methionyl-Leucyl-Phenylalanine), a potent neutrophil chemoattractant (18). ATP appeared to be released mainly at the leading edge of migrating cells. Upon addition of ATP-hydrolyzing enzymes (apyrase, ATPase, alkaline phosphatase), FMLP-induced chemotaxis of neutrophils was almost completely inhibited, as was superoxide production (18). The predominant ecto-enzyme expressed by human neutrophils that catalyzes ATP hydrolysis was shown to be E-NTPDase1, which was localized at the leading edge of migrating neutrophils (19). The presence of this enzyme seems to be essential for the process of chemotaxis of neutrophils promoted by chemoattractants such as FMLP and IL-8, in which ATP and its breakdown product adenosine dually regulate the speed and direction of migration of neutrophils via activation of $P2Y_2$ and A_3 receptors, respectively (18, 19).

More recently, it was suggested that ATP also plays an indirect role in bacterial-induced neutrophil migration through effects on production of IL-8 by monocytes (20). Using a murine air pouch model, injection of a TLR2 agonist elicited neutrophil migration *in vivo*, which was inhibited upon administration of soluble apyrase. Furthermore, supernatants of monocytes stimulated with a TLR2 agonist recruited significantly less neutrophils when apyrase was present during stimulation *in vitro*. The reduced neutrophil migration was due to an inhibitory effect of apyrase on IL-8 production by TLR2-stimulated monocytes. It was concluded that ATP was responsible for the observed effects, presumably through the activation of $P2Y_2$ or $P2Y_6$ receptor subtypes by ATP released following TLR2 stimulation (20)

Thus, as an early endogenous cue of cellular stress or damage, ATP is likely to induce neutrophil recruitment at stressed or damaged sites at the very early stages of inflammation. An important role for CD39 in controlling inflammatory cell trafficking in response to ATP has also been demonstrated using animal models with deficient CD39 function. Hyman *et al.* (2009) showed in a murine model of stroke induced by permanent cerebral ischemia that infarct volume was exacerbated in CD39-deficient mice compared to wild-type controls. The cerebral infarct was reduced by treatment of CD39-deficient mice with apyrase, which was partly due to decreased infiltration of neutrophils and macrophages in the ischemic tissue (21). Similar findings had been reported previously in other models of ischemic tissue damage (22-24). Apyrase treatment reduced the number of leukocytes expressing $alpha_Mbeta_2$-integrin (MAC-1, CD11b/CD18) in CD39-deficient mice (21). Up-regulation of $alpha_Mbeta_2$-integrin is important for leukocyte adhesion and transmigration. It has been previously shown that ATP is able to up-regulate the expression of $alpha_Mbeta_2$-integrin by monocytes as well as by neutrophils (25-27). In CD39-deficient mice, up-regulated $alpha_Mbeta_2$-integrin contributed to the enhanced sequestration of neutrophils and macrophages in cerebral ischemic tissue (21). The authors concluded that control of extracellular ATP levels by CD39 expressed by leukocytes is involved in the regulation of adhesive properties of leukocytes for inflamed vasculature and circulating cells, such as platelets.

Control of extracellular ATP levels by CD39 also affects inflammation in the skin (dermatitis). CD39-mediated ATP hydrolysis by Langerhans cells (i.e. epidermal dendritic cells) has been shown to play an important role during skin inflammation initiated in response to skin irritants (28). Keratinocytes exposed to several skin-irritant chemicals released ATP as a result of cellular injury. In CD39-deficient mice, exacerbated inflammation in response to these irritant chemicals was observed compared to wild-type mice. Injections of apyrase diminished the early inflammatory response in the skin of CD39-deficient mice as well as of wild-type mice (28), suggesting that the amplified inflammatory response triggered by irritant chemicals was probably due to a reduced ability of Langerhans cells to degrade ATP released in excess. Interestingly, in this study it was also demonstrated that antigen-presenting function and T-cell stimulatory capacity of Langerhans cells was impaired in CD39-deficient mice during an allergic contact hypersensitivity reaction. This was likely due to P2 receptor desensitization, since responsiveness of $CD39^{-/-}$ dendritic cells to ATP was restored by apyrase treatment (28). Additionally, intradermal administration of the non-hydrolysable P2 receptor agonist ATP-gamma-S has been shown to augment the immune responsiveness of mice *in vivo*, and to enhance Ag-presenting function of Langerhans cells *in vitro* (29).

Recent data from several studies indicate that ATP is involved in allergic airway inflammatory reactions *in vivo*. In an elegant study by Idzko *et al.* (2007), it was shown that when extracellular ATP in the airways of allergen-challenged asthmatic mice was neutralized by apyrase, inflammation was inhibited (30). Furthermore, ATP levels were increased in bronchoalveolar lavage fluid (BALF) from asthmatic subjects 24 hours after allergen but not saline challenge. The elevated ATP levels were highly correlated with increased BALF eosinophil numbers, a cardinal feature of airway inflammation. Similar results were found in BALF of ovalbumin(OVA)-sensitized asthmatic mice, but not in saline-sensitized mice. After neutralizing extracellular ATP levels by administering apyrase before allergen challenge, a reduction in the number of BALF inflammatory cells (eosinophils, macrophages and lymphocytes), goblet-cell hyperplasia and peribronchial inflammation was observed as well as reduced production of Th_2 cytokines IL-4, IL-5 and IL-13 in mediastinal lymph node cell cultures. The effect of ATP on eosinophilic inflammation appeared to be mainly due to effects of ATP on maturation and activation of myeloid dendritic cells involved in airway allergen sensitization (30). In a more recent study, the role of ATP in asthmatic airway inflammation was further elucidated by demonstrating that $P2X_7$ receptor signalling contributed to the pathogenesis of allergic airway inflammation (31). Expression of the $P2X_7$ receptor was up-regulated in lung tissue and on BALF cells of OVA-sensitized mice, as well as on BALF macrophages and blood eosinophils from asthmatic humans (31). Moreover, cardinal features of inflammation in response to allergen challenge were found to be reduced in $P2X_7$-deficient mice or in animals treated with the $P2X_7$ receptor antagonist KN-62. Blocking $P2X_7$ receptor signalling impaired the T-cell priming capacity of dendritic cells, which resulted in the attenuation of allergic airway inflammation (31).

In addition to the key findings reported by Idzko and colleagues, several other reports have provided additional evidence for a role of ATP in airway inflammation. Riteau *et al.* (2010) showed that administration of apyrase to mice, in which lung inflammation and fibrosis had been induced by nasal instillation of bleomycin, led to reduced neutrophil recruitment and activity in the lungs, and to decreased lung IL-1-beta and tissue-inhibitor of metalloproteinase-1 (TIMP-1; a factor involved in tissue remodelling) levels. Diminished signs of bleomycin-induced lung inflammation and remodelling processes were also observed in $P2X_7$-

deficient mice (32). These authors also found increased BALF ATP levels in patients with pulmonary fibrosis compared to healthy controls (32). In studies using two other mouse models of lung inflammation, pulmonary accumulation of neutrophils and pulmonary edema during acute lung injury induced by mechanical ventilation or LPS inhalation were shown to be attenuated by pre-treatment of animals with soluble apyrase, nucleotidase, or both (33, 34). Enhanced inflammatory responses to mechanical ventilation or aerosolized LPS in CD39-deficient as well as in ecto-5'-nucleotidase (CD73)-deficient animals were indicative of a critical role for extracellular nucleotide phosphohydrolysis by CD39 and CD73 in alleviating inflammation associated with lung injury (33, 34). Even though the authors suggested that mainly failure to generate extracellular adenosine would be responsible for the enhanced inflammatory response in $CD39^{-/-}$ and $CD73^{-/-}$ animals, involvement of ATP is equally plausible, that is, failure to breakdown ATP present at increased extracellular levels in acutely injured lung tissue compartments likely contributed to the enhanced inflammatory response. Chances are that a mechanism of combined ATP and adenosine action operates *in vivo*. The ecto-enzyme cascade functions as an important negative feedback pathway whereby elevated levels of extracellular ATP contribute to the initiation of acute inflammation, which is subsequently inhibited by breakdown of ATP and generation of adenosine.

Using a mouse model of smoke-induced lung inflammation and emphysema, it has been shown that chronic exposure to smoke resulted in increased BALF ATP levels (35). Exposure of neutrophils to cigarette smoke induced release of ATP as well as release of IL-8 and elastase, which was inhibited by co-incubation of smoke-exposed neutrophils with apyrase (35). The authors concluded that ATP may be involved in the pathogenesis of COPD by affecting neutrophil-mediated inflammatory responses (35, 36). Further *in vivo* evidence for a putative role of ATP in COPD was provided by Cicko *et al.* (2010) who demonstrated that ATP neutralization by apyrase reduced the degree of smoke-induced lung inflammation in mice (37). Exposure of animals to cigarette smoke led to lung inflammation and also to a strong increase in BALF ATP levels, which correlated with the number of BALF neutrophils and macrophages. Treatment of animals with apyrase lowered ATP levels, reduced BALF neutrophil and macrophage counts, and also diminished concentrations of the proinflammatory cytokines IL-1-beta, IL-6, IFN-gamma and MIP-2 compared to vehicle-treated animals (37). A role for $P2X_7$ receptor signalling in the smoke-induced lung inflammation was demonstrated in mice (38). The effects of cigarette smoke on the production of proinflammatory cytokines and tissue-degrading enzymes by neutrophils and macrophages in the airways has also been confirmed in humans as well as the involvement of ATP release in these early inflammatory processes (39).

Taken together, an intervention aimed at lowering extracellular ATP levels *in vivo* may prove to be an effective therapeutic strategy to alleviate conditions that are associated with excessive inflammation by abolishing pro-inflammatory P2 receptor signalling. As an indirect consequence of interventions aimed at breaking down extracellular ATP, adenosine may accumulate in the extracellular space, which is known to have strong anti-inflammatory effects, mainly through activation of the adenosine A_2 and A_3 receptor subtypes (40, 41).

5. DOES P2 RECEPTOR STIMULATION INFLUENCE INFLAMMATION?

Although many reports in the 1990s and early 2000s had already demonstrated effects of ATP on various inflammatory processes via purinergic P2 receptor pathways (1, 4), it was not until the fairly recent discovery of the $P2X_7$ receptor being central in the activation of the NALP3 inflammasome that a role for ATP-mediated P2 receptor signalling in the innate immune system has become appreciated.

The innate immune system is able to detect infection by sensing microbial determinants (pathogen-associated molecular patterns; PAMPs) that are recognized by innate pattern-recognition receptors, including Toll-like, NOD-like and RIG-I-like receptors. These innate receptors are broadly expressed in different cellular compartments and, upon pathogen recognition, initiate an innate immune and inflammatory response to eliminate pathogens and instruct pathogen-specific adaptive immune responses (42, 43). As an intracellular complement of the extracellular Toll-like receptor family, NOD-like receptors are a family of proteins that form cytoplasmic multiprotein complexes known as inflammasomes, which lead to the activation of inflammatory caspases and production of pro-inflammatory cytokines. Importantly, inflammasomes can sense microbial as well as non-microbial danger signals (DAMPs), and are therefore essential for initiating inflammation and innate immunity when signs of tissue injury are detected (44). Among three prototypes of inflammasomes that can be distinguished (i.e. NALP1, NALP3 and IPAF), the NALP3 inflammasome is activated by a variety of ligands that can be either of exogenous or endogenous origin, leading to caspase-1-mediated production of IL-1-beta and IL-18 (44).

Mariathasan and co-workers provided formal proof that extracellular ATP induced activation of the NALP3 inflammasome (45, 46), albeit the role of this nucleotide in caspase-1 activation had previously been clearly demonstrated by Perregaux and Gabel (47) and Ferrari and co-workers (48). In the meantime, it has been established that ligation of the $P2X_7$ receptor by extracellular ATP leads to activation of the NALP3 inflammasome and release of mature IL-1-beta and IL-18 by monocytes/macrophages and dendritic cells, via a mechanism probably involving pannexin-1, potassium efflux and ROS production (49-53). $P2X_7$ receptor-mediated activation of the NALP3 inflammasome is induced by extracellular ATP either in an autocrine fashion upon its release induced by other danger signals (52), or in a paracrine fashion upon ATP release from distressed or injured bystander cells (54). The importance of the innate ATP-$P2X_7$-NALP3 inflammasome pathway in the

induction of inflammation in humans has also been demonstrated in patients with autoinflammatory diseases, which are rare disorders involving NALP3 inflammasome dysfunction characterized by recurrent inflammatory bouts and spontaneous IL-1-beta release (55). Increased expression and responsiveness of the $P2X_7$ receptor as well as elevated plasma ATP levels were shown in patients suffering from Schnitzler's syndrome or the syndrome of synovitis acne pustulosis hypertosis osteitis (SAPHO), in whom clinical symptoms could be abated by anti-inflammatory therapy with prednisone or anakinra (IL-1 receptor antagonist) (56, 57). In addition, a link between ATP, IL-1-beta secretion and mutations in the NALP3 gene was observed also in patients with two other auto-inflammmatory diseases, i.e. chronic infantile neurologic, cutaneous, articular syndrome (CINCA) and Muckle-Wells syndrome (MWS) (58).

Thus, through the NALP3 inflammasome, $P2X_7$ receptor activation by ATP appears to be essential for triggering or amplifying innate immunity and inflammation *in vivo*. This is also supported by strong evidence deriving from studies with $P2X_7$ receptor knock-out mice, which display a reduced inflammatory and immune response associated with decreased production of proinflammatory cytokines (IL-1-beta, IL-6) and reduced severity of histological signs of inflammation (59, 60). Taken together, the above data indicate that the ATP-$P2X_7$-NALP3 inflammasome pathway is part of the innate arsenal that the immune system requires to mount an effective protective inflammatory response following pathogen invasion or exposure to injurious agents.

Activation of the NALP3 inflammasome by ATP also appears to be important for the progression of inflammation and the initiation of an adaptive immune response. Recently, it was demonstrated that NALP3 inflammasome-dependent production of IL-1-beta by dendritic cells was a key step in the priming of $CD8^+$ cytotoxic T cells, linking the innate and adaptive immune response (53, 61). Tumor-bearing mice lacking components of the NALP3 inflammasome or the $P2X_7$ receptor failed to respond to chemotherapy. The mechanism responsible was inability of ATP, which was released by dying tumor cells, to activate the NALP3 inflammasome and stimulate production IL-1-beta by dendritic cells, resulting in failure of these cells to induce an antitumor response by IFN-gamma-producing $CD8^+$ T cells. $CD4^+$ T cell production of IL-2 and IFN-gamma was also decreased in this experimental mouse model (53). These results suggest that stimulating the ATP-$P2X_7$-NALP3 inflammasome pathway may be beneficial in the treatment of cancer.

In other progressive chronic inflammatory diseases, inhibition of $P2X_7$ receptor pathways appears to be beneficial. As mentioned before, ATP promotes early-stage inflammatory processes in the airways by enhancing leukocyte recruitment and increasing proinflammatory cytokine production through a mechanism involving $P2X_7$ receptor signalling. A role for $P2X_7$ receptor signalling has also been observed in later stages of lung inflammation. A decreased number of macrophages and lymphocytes as well as reduced production of IL-1-beta and the tissue-remodelling enzymes matrix metalloproteinase-1 and TIMP-1 were observed in $P2X_7$-deficient mice two weeks after induction of lung injury by bleomycin (32). Furthermore, $P2X_7$-deficient mice tended to recover faster than wild-type animals after lung injury, and less lung fibrosis was observed in $P2X_7$-deficient mice (32). In another recent study, mice that had been exposed to cigarette smoke for 4 and 7 months showed reduced destruction of lung parenchyma and less signs of lung emphysema when treated with the $P2X_7$ receptor antagonist KN-62 (38). In addition, chronic allergic airway inflammation is also affected by $P2X_7$ receptor activation. Treatment of mice with KN-62 one month after induction of allergic lung inflammation led to a reduction in BALF eosinophil and lymphocyte numbers, decreased BALF levels of IL-1-beta, IL-5, IL-6 and IL-13 as well as reduced tissue signs of inflammation (31). These results indicate that chronic airway inflammation was less pronounced when the $P2X_7$ receptor pathway was blocked, suggesting that $P2X_7$ antagonism may be an effective therapeutic strategy in the treatment of lung diseases such as asthma and COPD. Even though inhibition of $P2X_7$ receptor signalling in humans *in vivo* by administration of selective antagonists could be a promising treatment strategy in certain (chronic) inflammatory conditions, currently available antagonists, such as KN-62 and oxATP, show disparate potency between species and are not entirely specific for the $P2X_7$ subtype, thus also capable of antagonizing the function of other P2 receptor subtypes. Newly developed compounds with high potency and selectivity for the $P2X_7$ receptor, such as AZD-9056 from AstraZeneca, are currently being evaluated in human phase I and II clinical trials of COPD, osteoarthritis, rheumatoid arthritis and inflammatory bowel disease (62-64).

Besides the well-recognized proinflammatory effect due to $P2X_7$ receptor stimulation in the presence of high ATP concentrations, the role of extracellular ATP as a direct down-modulator of inflammation has received comparatively little attention so far (2, 65). Pelegrin and Surprenant (2009) recently reported intriguing data that suggest a changing role for ATP at high extracellular concentrations during the process of macrophage polarization in the course of ongoing inflammation (66), i.e. the phenotypic switch of classically activated, proinflammatory M1-type macrophages during the initial stages of inflammation to alternatively activated, anti-inflammatory M2-type macrophages during the resolution phase of inflammation (67). They showed that, whereas ATP induced IL-1-beta production by M1-type mouse macrophages via $P2X_7$ receptor activation, IL-1beta production by M2-type cells was inhibited when cells were stimulated with ATP at high extracellular levels (up to 5 mM). It was found that this anti-inflammatory effect of ATP was not receptor-mediated, but instead the PPi group of ATP induced uncoupling of the $P2X_7$-NALP3-inflammasome pathway, probably through inhibition of ROS production and intracellular clustering of actin, which blocked the activation of caspase-1 and release of IL-1-beta (66).

Moreover, it has been shown that ATP at low extracellular concentrations is able to down-modulate inflammatory responses by inhibition of pro-inflammatory cytokine production by macrophages (68, 69) and by inducing semi-maturation of dendritic cells, thereby inhibiting their T-cell stimulatory capacity (70-72). P2Y receptors are probably responsible for mediating the anti-inflammatory effects of ATP, the most likely candidate being the $P2Y_{11}$ subtype (73, 74). Although early animal studies in the 1980s and 1990s by Chaudry and co-workers had already suggested that ATP-$MgCl_2$ administration *in vivo* could aid in the restoration of immune dysfunction after hemorrhagic shock and sepsis (75-79), convincing *in vivo* evidence on anti-inflammatory effects of ATP- mediated purinergic P2 receptor signalling is still lacking. Swennen and co-workers (80-82) have demonstrated in an *ex vivo* whole blood assay that ATP is able to inhibit inflammation. Administration of ATP at concentrations up to 300 micromolar led to inhibition of NF-kappa-B activation and decreased production of TNF-alpha and IFN-gamma in response to stimulation of human whole blood samples with LPS (lipopolysaccharide) and PHA (phytohemagglutinin). Additionally, IL-10 production was increased by ATP under these conditions (80-82). It was shown pharmacologically that the $P2Y_{11}$ subtype mediated the inhibition of TNF-alpha production by ATP, whereas the $P2Y_{12}$ subtype mediated the stimulation of IL-10 production (83). Recently, we have published a case report showing that administration of ATP by intravenous infusion to a patient with rheumatoid arthritis ameliorated cardinal signs of disease (84), suggesting that low-level ATP treatment may exert anti-inflammatory effects *in vivo*.

Taken together, targeting P2 receptor signalling pathways *in vivo* may prove to be an effective approach to treat chronic inflammatory conditions that are associated with up-regulated inflammatory processes, such as rheumatism, cancer, lung diseases and bone disorders.

6. DO P2 RECEPTORS PLAY A ROLE IN "OSTEOIMMUNOLOGY"?

The "purinergic hypothesis" saw the light in the scientific context of neuropharmacology (85), and even among neuropharmacologists was looked upon with scepticism, until recently. Outside its "birthplace" purinergic transmission was considered by default with even less attention and stronger scepticism, immunologists were no exception to this rule. During the eighties, a few brave investigators started to rigorously investigate the participation of extracellular ATP and purinergic receptors to immunomodulation, providing experimental evidence for a role of this signalling pathway (86-88) but these findings were never allowed full citizenship in the immunology community. However, over the last fifteen years there was a slow but steady change of attitude, supported by the unequivocal evidence that selected P2Y and P2X receptor subtypes play a central role in immune cell chemotaxis, proliferation, pain sensation, antigen presentation and cytokine release (47, 48, 89-93). Nowadays, there are few doubts that purinergic signalling is increasingly recognized as a key pathway in the modulation of both innate and adaptive immunity.

This is having unexpected and far reaching implications for our comprehension of the pathophysiology of multiple organ systems, bone included. There are few doubts that bone, far from being a mere scaffold to sustain soft tissue and allow us to move around, is a highly integrated metabolic apparatus which regulates the hematopoietic, endocrine and immune systems. The links between bone and immunity are so close that the definition "osteoimmunology" has been coined to identify this burgeoning field of investigation (94). Bone cells (chiefly osteoclasts and osteoblasts) synthesize and release a wealth of immunoregulatory factors and conversely are the target of immune cells and soluble immune mediators (95). In this scenario P2 receptors, albeit underappreciated and underinvestigated, play a major role. In fact, P2Y receptor stimulation has multiple actions on bone metabolism and turnover, as well as on the release of soluble mediators. Stimulation of $P2Y_6$ receptors triggers the activation of the key pro-inflammatory transcription factor NF-kappa-B (96), while $P2Y_1$ receptor activation induces RANKL expression in periodontal cells (97). Basically no information is currently available on the role of bone P2X receptors in driving release of cytokines or of other pro-inflammatory mediators by bone cells. This is really surprising, as it is well known that bone cells are the target of key pro-inflammatory cytokines such as TNF-alpha, IL-1 and IL-6, and yet no attention has been paid to the bone cells themselves as source of these same cytokines upon P2 receptor stimulation. Several reports have now proven beyond any possible doubt that ATP release into the bone microenvironment occurs under several circumstances, in health and disease, and yet such an increased purinergic stimulation has never been associated to the activation of local release of inflammatory cytokines by bone cells. Available evidence demonstrates that osteoblasts and osteoclasts posses the full machinery for production of all the major cytokines in response to P2 receptor stimulation (98), thus it is highly probable that changes in the ATP concentration within bone microenvironment might cause local cytokine release. This is of particular relevance in reference to those cytokines with a strong bone-resorbing activity such as TNF-alpha and IL-1-beta. Activity of these cytokines is currently implicated in the pathogenesis of osteoporosis associated to rheumatologic diseases or to menopause, their source being identified in bone-infiltrating immune cells (95). Yet, operation of the bone purinergic signalling pathway which is now being unveiled provides an unanticipated local source of inflammatory cytokines in the absence of recruitment of inflammatory cells.

Bone is a target in chronic inflammatory diseases, autoimmune disorders and cancer. Consequences are normally osteoporosis, osteomalacia, increased fracture risk, and pain. It is clear that bone involvement is not the mere consequence of the general systemic inflammatory status but rather the effect of the local cytokine release. In any case, pharmacological modulation of the pathways mediating release of inflammatory mediators is anticipated to counteract bone loss in inflammation. So far, the P2 receptor that has the best chances for pharmacological intervention is $P2X_7$. This receptor is primarily coupled to

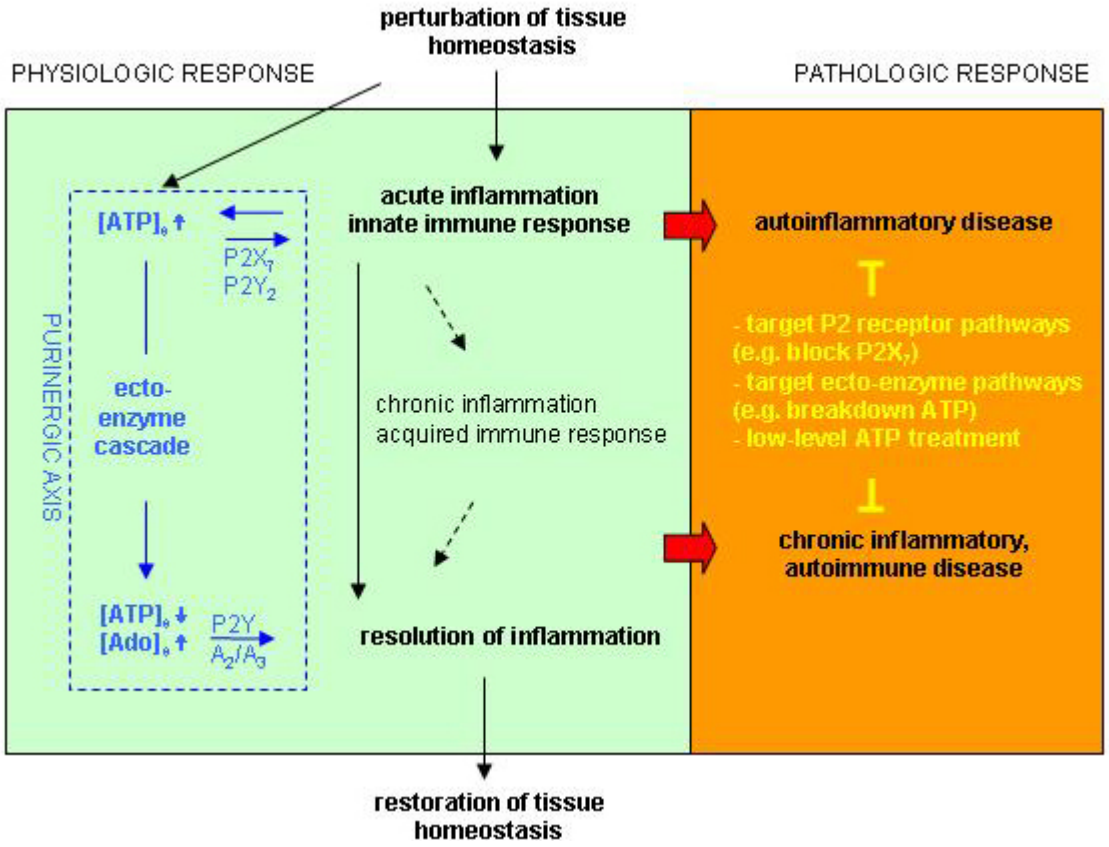

Figure 2. The role of the purinergic axis in the inflammatory response following perturbation of tissue homeostasis. As a physiologic response, inflammation is initiated upon perturbation of tissue homeostasis, for instance due to infection or injury. This response is accompanied by an accumulation of extracellular ATP ($[ATP]_e$), which is desequestered as a result of cell lysis or is released by stressed and activated cells. High-level ATP signalling, mediated in part by $P2Y_2$ and $P2X_7$ receptors, contributes to the acute inflammatory and innate immune response by promoting leukocyte recruitment and NALP3-inflammasome activation. Upon progression of inflammation, ecto-enzymes catalyzing the breakdown of ATP cause a decrease in extracellular ATP concentrations as well as an increase in extracellular adenosine concentrations ($[Ado]_e$). The lowered ATP and elevated adenosine levels then contribute to the down-modulation and resolution of inflammation, putatively through P2Y and A_2/A_3 receptor signalling, respectively. The ultimate goal of this physiologic response is the restoration of tissue homeostasis. Exploitation of the purinergic axis, comprised of extracellular ATP, purinergic receptors and ecto-enzyme cascades, might be an attractive approach for the development of therapeutic interventions to treat conditions that are associated with a pathologic response, such as autoinflammatory or chronic inflammatory, autoimmune diseases.

generation of the potent osteolytic cytokine IL-1, but TNF-alpha and IL-6 are also induced following its activation, and $P2X_7$ is expressed by osteoblasts and osteoclasts. Direct evidence that osteoblast/osteoclast $P2X_7$ stimulation triggers cytokine release is lacking, but on the basis of previous data from mesenchimal and monocyte-derived cells we believe that it would be very unlikely that the $P2X_7$ receptor is silent in these cells.

7. CONCLUSION AND THERAPEUTICAL PERSPECTIVES

The data presented in this review indicate that purinergic signalling plays an important role in inflammation (Figure 2). The purinergic axis is comprised of extracellular ATP and P2 receptors as well as the ecto-enzymes that control extracellular ATP levels and, thus, the magnitude of ATP signalling. Following perturbation of tissue homeostasis, for example by infection or injury, inflammation is initiated as a protective physiologic response ultimately aimed at restoring tissue integrity and homeostasis. In the early stages of the inflammatory response, an immediate and robust rise in extracellular ATP at inflammatory foci occurs due to massive release of ATP into the extracellular microenvironment via both lytic (i.e. desequestration of intracellular ATP following cell lysis) and non-lytic (i.e. secretion of ATP by activated immune cells) mechanisms. In this way, ATP accumulates at high extracellular and pericellular levels, and contributes to the initiation and progression of acute inflammatory and innate immune responses by promoting the local recruitment and activation of inflammatory cells, in part through $P2Y_2$ receptors, and by activating the NALP3 inflammasome through $P2X_7$ receptors.

In the course of resolution of inflammation, extracellular ATP levels, which are elevated during the initial phases, decline due to the activity of ecto-enzymes, including E-NTPDases. Low extracellular ATP levels, together with the elevated extracellular adenosine concentration, are thought to be involved in the down-modulation and resolution of inflammation, putatively through P2Y and A_2/A_3 receptors, respectively. The purinergic axis thus appears to play a role during the different stages of inflammation in response to perturbed tissue homeostasis, probably as one of the interrelated physiologic signalling pathways that help restore tissue homeostasis (Figure 2).

When the normal physiologic response is disturbed at any stage, inflammation may become dysregulated and health is compromised, as is the case in autoinflammatory or chronic inflammatory, autoimmune diseases. In case of such inflammatory conditions, the purinergic system provides promising avenues for therapeutic intervention. First, targeting ATP-hydrolyzing ecto-enzyme pathways may prove to be an effective strategy to inhibit proinflammatory high-level ATP signalling in conditions characterized by recurring episodes of acute inflammation, for instance in asthmatic disease and COPD.

Second, targeting specific P2 receptor pathways may also prove to be an effective treatment strategy, with $P2X_7$ as the most attractive candidate. Blocking $P2X_7$ signalling would lead to diminished NALP3 inflammasome activation and IL-1-beta production, and may therefore be beneficial in autoinflammatory diseases as well as rheumatic conditions that are associated with autoinflammation, such as gout. On the contrary, stimulating the $P2X_7$-NALP3-inflammasome pathway may be a beneficial approach for the treatment of cancer, that is, a way to promote the killing of tumour cells.

A final approach to exploit the purinergic signalling system could be the use of its natural ligand ATP. Indeed, several reports have suggested that administration of ATP via intravenous infusion has anti-inflammatory potential in cancer and rheumatoid arthritis (84, 99, 100). In a randomized clinical trial in patients with advanced non-small-cell lung carcinoma, we administered a regular low-dose ATP infusion ($\leq$75 mcg/kg.min^{-1} for 30 hours) once per 2-4 weeks over a maximal period of 24 weeks. In addition to marked clinical effects of ATP infusions, including inhibition of loss in weight, fat mass, muscle mass, muscle strength and fatigue, and improved functional status and overall quality of life (99), we observed significant anti-inflammatory effects of ATP treatment, as demonstrated by stabilization of plasma C-reactive protein concentrations, which increased in the untreated control group (100). Moreover, ATP treatment completely annihilated the decrease in albumin concentrations observed in the control group (99). Recently, we have published a case report showing that administration of low-dose ATP by intravenous infusion to a patient with rheumatoid arthritis markedly ameliorated both the inflammatory status (C-reactive protein, TNF-alpha, IL-12) and cardinal disease symptoms (disease activity score (DAS), pain, fatigue, functional status, and quality of life) (84).

The precise mechanism of the anti-inflammatory effect of ATP *in vivo* has not been fully elucidated. The administered ATP has been shown to induce only mild side effects due to breakdown of ATP to adenosine in the vascular bed (101). The large majority of ATP (estimated at 50-90% depending on the administered dose) was recovered with high efficiency by erythrocytes, leading to a 50-60% rise in intracellular ATP concentrations in erythrocytes with a half-life of 6 hours. Apparently, ATP is slowly released by erythrocytes into the extracellular compartment where it plays a role in vasodilation (102-104); such a slow ATP release by erythrocytes could therefore also influence immune cells, thereby inducing the anti-inflammatory effects of low-level ATP that have been observed in humans *in vivo*.

In conclusion, purinergic signalling appears to play an important role in fine-tuning the homeostatic pathway in inflammation. Therefore, exploitation of this system is bound to yield novel therapeutic opportunities for the treatment of diseases associated with deregulated, pathologic inflammatory responses. Recent studies indicate that pulmonary diseases, cancer and bone diseases are promising research areas. Combined *in vitro* and *in vivo* research will be necessary to fully appreciate and elaborate purinergic treatment options and their underlying mechanisms.

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