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Purine Release, Metabolism, and Signaling in the Inflammatory Response

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Abstract

ATP, NAD^+, and nucleic acids are abundant purines that, in addition to having critical intracellular functions, have evolved extracellular roles as danger signals released in response to cell lysis, apoptosis, degranulation, or membrane pore formation. In general ATP and NAD^+ have excitatory and adenosine has anti-inflammatory effects on immune cells. This review focuses on recent advances in our understanding of purine release mechanisms, ectoenzymes that metabolize purines (CD38, CD39, CD73, ENPP1, and ENPP2/autotaxin), and signaling by key P2 purinergic receptors (P2X7, P2Y2, and P2Y12). In addition to metabolizing ATP or NAD^+, some purinergic ectoenzymes metabolize other inflammatory modulators, notably lysophosphatidic acid and cyclic GMP-AMP (cGAMP). Also discussed are extracellular signaling effects of NAD^+ mediated by ADP-ribosylation, and epigenetic effects of intracellular adenosine mediated by modification of *S*-adenosylmethionine-dependent DNA methylation.

INTRODUCTION

Inflammation in response to tissue injury is initiated by the release of danger signals that stimulate the immune system. These include host-derived glycolipid mediators that activate invariant natural killer T (NKT) cells (1, 2); the transcription factor high-mobility group box 1 (HMGB1), which activates myeloid cells via Toll-like receptors (TLR) 2 and 4 (3); DNA, which activates innate immunity (4); and ATP, which is chemotactic to and activates neutrophils, macrophages, dendritic cells (DCs), and memory T cells (5). Oxidized nicotinamide adenine dinucleotide (NAD^+) also can influence inflammation by killing certain T cells (6).

Extracellular ATP and various other nucleotides activate P2X or P2Y receptors to stimulate inflammation. Over time ATP becomes less inflammatory or even anti-inflammatory due to the recruitment of regulatory cells and induction of ectoenzymes such as CD39 and CD73 that rapidly convert extracellular ATP to adenosine. This is accompanied by the induction of anti-inflammatory A_{2A} and A_{2B} adenosine receptors on immune cells. The role of adenosine receptors in immune cell regulation has been the subject of numerous recent reviews (7–18) and is discussed only briefly here. Rather, this review focuses on mechanisms of purine nucleotide release from cells, extracellular metabolism, and signaling by proinflammatory P2 purinergic receptors. We also discuss metabolism of ATP by ectoenzymes, and alternative substrates that influence inflammation: lysophosphatidylcholine (LPC) and 2′3′ cyclic GMP-AMP (cGAMP).

Receptors and enzymes that participate in purinergic signaling are summarized in **Tables 1** and **2**. We concentrate on a subset of purinergic receptors and ectoenzymes, indicated in red in **Tables 1** and **2**, that have been shown to have major impacts on signaling in immune cells. **Tables 3** and **4** show natural ligands and synthetic agonists and antagonists of purinergic receptors, as well as substrates, products, and inhibitors of ectoenzymes that metabolize purines and phospholipids.

Table 1 Purinergic receptors[a]

Purinergic receptor	Coupling	Natural ligands	Receptors
Adenosine (P1)	G proteins	Adenosine	A1, A2A, A2B, A3
P2X	Ligand-gated channels	ATP	P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7
P2Y	Gq coupled Gi coupled	ATP, ADP, UTP, UDP ADP ≫ ATP	P2Y1, P2Y2, P2Y4, P2Y6, P2Y11[b], P2Y12, P2Y13, P2Y14[c]

[a]The molecules discussed in this review are indicated in red.
[b]Coupled to Gq and Gs.
[c]Activated by UDP-glucose.

Table 2 Ectoenzymes[a]

Ectoenzyme family	Ectoenzyme name
Ectonucleoside triphosphate diphosphohydrolases	ENTPD1 (CD39), ENTPD2 (CD39L1), ENTPD3 (CD39L3), ENTPD5 (CD39L4), ENTPD6 (CD39L2), ENTPD8
Ecto-nicotinamide adenine dinucleotide glycohydrolase (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1)	CD38
NT5E (ecto-5-nucleotidase)	CD73
Ectonucleotide pyrophosphatase/phosphodiesterase	ENPP1, ENPP2 (autotaxin), ENPP3-7
Adenosine deaminases	ADA (ADA1), ADA2[b]

[a]The molecules discussed in this review are indicated in red.
[b]Absent in rodents.

Table 3 Purinergic receptors, and channels that are important for immune purinergic signaling

Receptor	Natural ligand	Agonist	Antagonist	Activities	Ref.
Adenosine receptors					
Adenosine A_{2A}	Adenosine	CGS21680	SCH58261	Anti-inflammatory in most immune cells, Gs coupled	155–157
Adenosine A_{2B}	Adenosine	NECA[a]; BAY 60–6583[b]	PSB-1115	Anti-inflammatory in myeloid cells (Gs), stimulates IL-6 production and angiogenesis (Gq)	158, 159
P2 purinergic receptors					
P2X7	ATP	BzATP	A-438079	Control Ca^{2+} entry into cells (1–10 μM), membrane pore formation (100 μM)	115
P2Y2	UTP > ATP	INS37217, PSB-1114	AR-C 118925XX	Macrophage chemotaxis	147, 160–162
P2Y12	ADP	2MeS-ADP	AR-C 66096 (ARL 66096), clopidogrel	Microglial activation, platelet aggregation	163

[a]NECA is a nonselective agonist that can be used in combination with A_1, A_{2A}, and/or A_3 blockers to study $A_{2B}R$ signaling.
[b]BAY60–6583 is a partial agonist of $A_{2B}R$ with greater Gs than Gq coupling.

ECTOENZYMES

CD39

Some of the enzymes that are involved in the extracellular metabolism of purines are illustrated in **Figure 1**. The most studied among these are CD39 and CD73, which together degrade extracellular ATP to adenosine. Since ATP and adenosine have opposing effects on inflammation, the local expression of CD39 and CD73 can shape the quality of immune responses. Upregulation of CD39 is a consistent consequence of activating conventional $CD4^+$ and $CD8^+$ T cells. Murine

Table 4 Ectoenzymes that are important for purinergic signaling in immune cells

Enzyme	Substrates	Products	Inhibitor	Activities	Ref.
CD38	NAD^+, cADPR	AMP, cADPR, AMP	CD38 inhibitor 78c	Highly expressed on tumor MDSCs	164
CD39 (ENTPD1)	ATP, ADP	ADP, AMP	ARL 67156	Converts ATP/ADP to AMP, upregulated in activated T cells	21
CD73 (NT5E)	AMP	Adenosine	PSB 12379	Converts AMP to adenosine	35–42
ENPP1	2′-3′-cGAMP, ATP	AMP, GMP	SK4A	Degradation of cGAMP controls innate immunity	28, 30
ENPP2 (autotaxin)	LPC, ATP	LPA, AMP, PP	HA130	Converts LPC to LPA	31
ADA	Ado, dAdo	Ino, dIno	EHNA, pentostatin	Non-receptor-mediated effects of purines on DNA methylation	150, 165–167

Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MDSC, myeloid-derived suppressor cell.

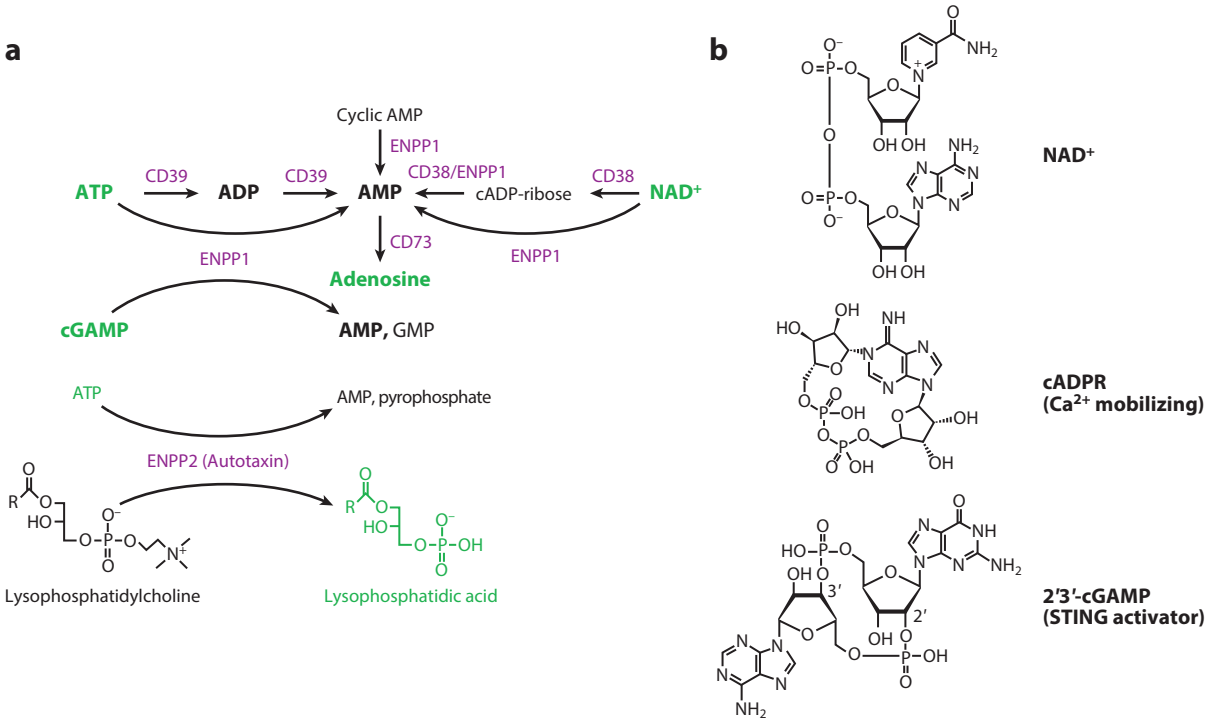

Figure 1

Ectoenzymes that catalyze the metabolism of ATP and other substrates. (*a*) Enzymes are shown in purple, and molecules with signaling activity are shown in green. Dual substrates for ENPP1 and ENPP2 are shown. (*b*) Chemical structures of some signaling nucleotides. Abbreviations: 2′3′-cGAMP, 2′3′-cyclic GMP-AMP; cADPR, cyclic ADP ribose; NAD^+, oxidized nicotinamide adenine dinucleotide.

regulatory T cells (Tregs) express both CD39 and CD73 and rapidly catalyze the conversion of ATP to adenosine to locally suppress T effector cell functions (19). Ablation of CD39 on microglial cells causes seizures in mice owing to antiseizure effects of adenosine in the brain (20). *CD39*$^{-/-}$ mice also exhibit improved control of *Listeria monocytogenes*, associated with increased production of inflammatory cytokines due to increased ATP and reduced adenosine signaling. In the late stage of infection, *CD39*$^{-/-}$ mice accumulate more *Listeria*-specific $CD8^+$ T cells in the spleen than wild-type animals, suggesting that CD39 activity limits the $CD8^+$ T cell response to infection (21).

CD39 expression on T helper 17 (Th17) cells is stimulated by unconjugated bilirubin (UCB), a product of heme oxidation that has known immunosuppressive properties. Upregulation of CD39 on Th17 cells is dependent upon ligation of the aryl hydrocarbon receptor by UCB (22). UCB produces immunoregulation in vivo by decreasing IL-17 expression and by increasing CD39 and IL-10 expression by $CD4^+$IL-17^+ cells. This produces protective effects in experimental colitis.

A percentage of tumor-infiltrating $CD8^+$ T cells recognize tumor antigens, which include overexpressed self-antigens, as well as tumor-specific neoantigens that arise as a consequence of tumor-specific mutations. Tumor-specific $CD8^+$ T cells are primed in tumor-draining lymph nodes and then migrate via the blood to the tumor, where they exert their cytotoxic function. $CD8^+CD103^+$ resident memory T (Trm) cells in highly inflamed tumors have increased expression of CD39 compared to less-inflamed tumors (23). In circumstances when extracellular ATP is moderate, CD39 blockade may facilitate immunotherapy by increasing ATP and reducing adenosine. However,

as discussed below, in circumstances of very high extracellular ATP, CD39 activity may protect immune cells from ATP-induced apoptosis.

CD38

CD38 is a cell surface glycoprotein that functions as an adhesion molecule as well as an ectoenzyme that hydrolyzes NAD^+ to cyclic ADP ribose (cADPR), an intracellular calcium-ion-mobilizing messenger. CD38 or ENPP1 hydrolyzes cADPR to AMP. The release of soluble enzyme and the ability of membrane-bound enzyme to become internalized suggest that CD38 has both extracellular and intracellular functions. CD38 is expressed on most thymocytes, some activated peripheral blood T cells and B cells, plasma cells, and DCs. In mice, CD38 is required for chemokine-mediated migration of mature DCs into secondary lymphoid tissues, and as a consequence, CD38 deficiency impairs humoral immunity to T cell–dependent antigens.

CD38 has been implicated in the regulation of metabolism, and high expression is thought to contribute to pathologies due to aging, obesity, diabetes, heart disease, asthma, and inflammation. In addition, CD38 has been identified as a cell surface marker in hematologic cancer cells such as plasma cells in multiple myeloma. A cytotoxic anti-CD38 antibody (daratumumab) has been approved by the US Food and Drug Administration for use in multiple myeloma (24). Therapeutic anti-CD38 antibodies may have roles in the treatment of diseases beyond hematological malignancies, including solid tumors and autoimmune diseases (25). CRISPR/Cas9-based knockdown of CD38 in A549 human adenocarcinoma cells inhibited anchorage-independent cell growth, cell invasion, and xenograft growth in nude mice (26). CD38 mRNA and protein expression are elevated in some but not all human lung cancers, and human lung cancer cell lines have dramatically higher CD38 mRNA and protein expression than normal cells (26). One mechanism by which CD38 may protect tumors is by generating anti-inflammatory adenosine from extracellular NAD^+. However, as discussed below, degradation of extracellular NAD^+ may have mixed immune effects by protecting Tregs, NKT cells, and Trm cells.

ENPP1

Extracellular NAD^+ can serve as a source of AMP due to the activities of CD38 and ENPP1, also known as CD203a or PC-1 (27). However, a preferred substrate of ENPP1 is 2′3′-cGAMP (**Figure 1**), a potent activator of stimulator of interferon genes (STING) that can stimulate innate immunity and shrink tumors. The type I interferon response is induced by viral or mitochondrial DNA in the cytoplasm via activation of cyclic GMP-AMP synthase (cGAS) (28). The cytoplasmic domain of STING (an endoplasmic reticulum transmembrane protein) forms dimers, and cyclic dinucleotides bind at the dimer interface (29). cGAMP binding to STING promotes the aggregation of a STING/TANK-binding kinase 1 (TBK1)/interferon regulatory factor 3 (IRF3) complex. IRF3 is phosphorylated by TBK1 and then forms a dimer that induces the expression of type I interferons in the nucleus. cGAMP also enhances innate immune responses by inducing production of cytokines such as IFN-γ and by stimulating DC activation, which mediates cross-priming of $CD8^+$ T cells. ENPP1 inactivates cGAMP to GMP and AMP (30). The cGAMP hydrolytic activity of ENPP1 was confirmed using recombinant protein and was depleted in tissue extracts and plasma from *Enpp1*$^{-/-}$ mice (28). Cytoplasmic cGAMP can be transported from cells to encounter the ectoenzyme by an undefined mechanism. High levels of cGAMP possess significant antitumor activity in mice by triggering the STING-dependent pathway directly. Inhibitors of ENPP1 could have antitumor activity both by inhibiting the conversion of ecto-NAD^+ and ATP to adenosine and by inhibiting the degradation of cGAMP.

ENPP2 (Autotaxin)

ENPP2/autotaxin (ATX) is a tumor cell motility-stimulating factor, originally isolated from melanoma cell supernatants. It was initially thought that the effects of ENPP2 were due to its activity as a 5′-nucleotide pyrophosphatase/phosphodiesterase. Subsequently it was shown that in addition to dephosphorylating ATP, ENPP2 has more potent activity as a lysophospholipase D that catalyzes the production of the bioactive phospholipid mediator, lysophosphatidic acid (LPA), from LPC (**Figure 1**) and the production of sphingosine-1-phosphate (S1P) from sphingosylphosphorylcholine (31). LPA and S1P signal through G protein–coupled receptors to promote a variety of cellular responses, such as migration, proliferation, and survival of tumor cells, and they have proangiogenic properties. The K_m value of ENPP2 for LPC is 25-fold lower than that for nucleotide substrates (31). The ENPP2–LPA axis has been implicated in a wide range of physiological and pathological signaling, including tumor progression, inflammation, and multiple sclerosis (32). NFAT1 contributes to melanoma growth and metastasis in part by regulating the ENPP2 gene. There is a strong correlation between NFAT1 expression and metastatic potential in melanoma cell lines and tumors (33). Inhibitors of ENPP2 might exert antitumor activity by blocking the production of LPA and S1P, and by inhibiting extracellular ATP degradation.

CD73

Adenosine generated from AMP by CD73 (NT5E) inhibits the activation of most immune cells. It causes strong suppression of the immune response to bacterial infection (34), suppresses adaptive immune responses (19), and promotes immune escape of tumor cells. In a mouse model of collagen-induced arthritis, treatment with low doses of methotrexate increases CD73 enzyme expression and AMP hydrolysis, leading to an increase in adenosine production and immunosuppressive activity (35).

B16F10 mouse melanoma cells express CD73 and CD39. A significant amount of CD73 is detected on the B16F10 cell surface by immunofluorescence; however, its main localization appears to be intracellular. This supports the hypothesis that an extensive intracellular distribution of CD73 is present in a membrane-bound pool (i.e., lysosomes, Golgi apparatus, and transcytotic vesicles) and that CD73 undergoes continual exchange between the plasma membrane and internal membranes. CD73 is upregulated in melanoma patients progressing under adoptive T cell transfer or immune checkpoint blockade, arguing for an adaptive resistance mechanism (36). CD73 has prognostic value in high-grade serous ovarian cancer; high levels of CD73 are associated with shorter disease-free and overall survival (37). Melanoma growth in vivo is inhibited in CD73-deficient mice. Moreover, angiogenic responses of the host and melanoma are reduced upon CD73 inhibition [angiogenesis is stimulated by adenosine signaling (38–40)]. CD73 is also involved in melanoma cell invasion that probably occurs via direct CD73-mediated interactions with extracellular matrix constituents (41).

When activated by cytokines (IL-1β, IL-7, and IL-23) or LPS-activated DCs, respectively, γδ T cells express high or low amounts of CD73, which enables them to either inhibit or enhance adaptive immune responses. Low CD73 expression on γδ T cells is correlated with enhanced Th17-response-promoting activity. Hence, targeting CD73 expression on γδ T cells may influence their pro- or anti-inflammatory effects on Th17 responses (42).

PANNEXIN 1

In addition to serving as a danger signal early in the inflammatory process (43), extracellular ATP is the death knell for cells when they undergo apoptosis as a last-resort, sacrificial defense against

intracellular pathogens (44, 45). ATP is released in large amounts from necrotic cells, but additional mechanisms for controlling its release from apoptotic cells or activated cells have evolved. A major mechanism of channel-mediated ATP release is via pannexin 1 (Panx1). Six subunits of Panx1 form a plasma membrane channel termed pannexon. Although it is generally accepted that Panx1 functions as an ATP release channel, high ATP permeability of the channel is only observed with certain stimuli, including low oxygen, mechanical stress, and elevated extracellular potassium ion concentration (46).

Depending on stimulus modality, pannexon can assume a large, ATP-permeable conformation or a small, chloride-selective and ATP-impermeable conformation (46). In either case, activation involves sequential stepwise modifications through multiple discrete open states, each with unique channel gating and conductance properties that reflect contributions of the individual subunits of the hexamer. Progressive channel opening occurs during both irreversible (caspase-mediated cleavage) and reversible (e.g., α1 adrenoceptor–mediated) forms of channel activation (47). In addition to using the pannexon channel, ATP can cross the cell membrane barrier in intact cells via other specialized membrane channels and/or via exocytotic release of ATP stored in cytoplasmic vesicles (48).

In uninjured tissues, relatively low amounts of ATP in the extracellular space are subject to rapid enzymatic degradation by ecto-ATPases (49). Thus, ATP activation of P2 receptors is best achieved if the release site is in close proximity to the receptor. This constellation is a given for vesicular ATP release in synapses, where ATP sometimes functions as a neurotransmitter and only has to diffuse a short distance across the approximately 20-nm-wide synaptic cleft. In cells where ATP has an autocrine function, the release channel and the receptor can be contiguous, thus avoiding excessive enzymatic breakdown of ATP. Another reason for close proximity of the release site and target is the relatively low affinity of some purinergic receptors for ATP. In particular, P2X7 requires up to millimolar concentrations of ATP for full activation (50). It is therefore not surprising that Panx1 and P2X7 were found not only to functionally interact but apparently also to be physically associated (51–53). Western blots of cell lysates immunoprecipitated with an anti-myc antibody against myc-Panx1 (52) or with an anti-Panx1 antibody (54) revealed the presence of both Panx1 and P2X7 in the precipitate. Furthermore, proximity ligation assays support an intimate interaction between Panx1 and P2X7 (55, 56). Functional associations have also been suggested for activation of the Panx1 channel by ATP through purinergic receptors of the P2Y type (57). Activation of Panx1 by P2Y receptors plays a key role in regenerative calcium wave propagation in various tissues including endothelial cells (58, 59). The functional interaction of the release channel with the receptor also allows for the phenomenon of ATP-induced ATP release (60), a positive-feedback loop. On the other hand, regenerative calcium waves allow for long-distance signaling by chemotactic ATP, as observed, for example, for the chemotaxis by ATP of microglia to a site of tissue injury (61–65).

P2X7 SIGNALING

As noted in **Table 1**, there are two families of P2 purinergic receptors: ATP-gated ion channels known as P2X receptors, and G protein–coupled P2Y receptors. P2X7 is a major regulator of the intensity and duration of many inflammatory responses (66–68). The receptor/channel is prominently expressed on monocytes, macrophages, microglia, and some T cells (NKT cells, Tregs, and Trm cells) and has been linked to diverse inflammatory and autoimmune diseases (69–74). On monocytes, macrophages, and microglia, gating of P2X7 for Ca^{2+} and K^+ induces cleavage and secretion of cytokines (67, 75). IL-1β production is tightly regulated since aberrant activation can lead to chronic inflammatory diseases (76, 77). Conversion to biologically active IL-1β requires

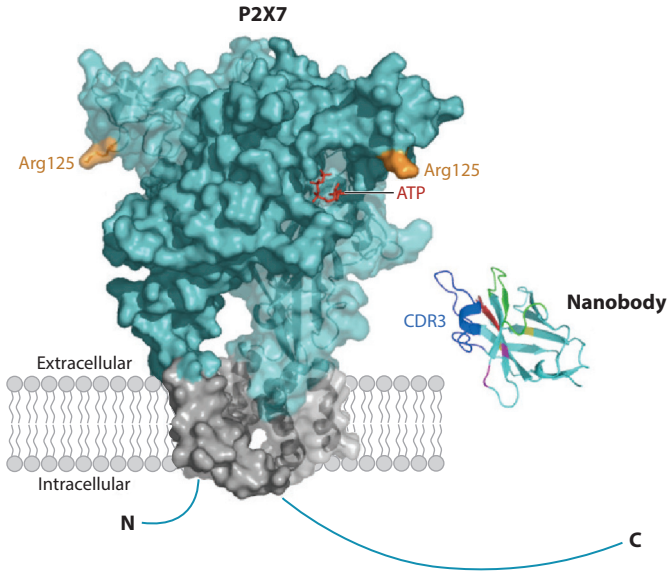

Figure 2

A model of P2X7. The P2X7-ion channel is gated by extracellular ATP or by NAD^+-dependent ADP-ribosylation. ATP bound to the ligand-binding site at the interface of two P2X7 subunits is depicted as a stick model in red. Arg125—the target for ADP-ribosylation by ARTC2.2—is indicated in orange. The extended CDR3 loop of a nanobody is depicted in dark blue. Assembled in PyMOL with models of mouse P2X7 (based on zebrafish P2X4, pdb file 4dw1) and an anti-lysozyme nanobody (pdb file 1mel3).

proteolytic processing by caspase-1, which itself is regulated by proteolytic cleavage following assembly of the nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) inflammasome complex (78, 79). Gating of P2X7 on pathogen-associated molecular pattern (PAMP)-primed macrophages triggers inflammasome assembly, cleavage of pro-IL-1β by caspase-1, and the release of large amounts of mature IL-1β by microvesicle shedding and other nonclassical secretion mechanisms (68, 80, 81).

Structurally, P2X7 comprises intracellular N and C termini, two transmembrane regions, and a large extracellular domain containing five disulfide bonds and four glycosylation sites—a topology similar to other ionotropic P2X receptors (66, 82) (**Figure 2**). The functional channel is composed of a stable trimer that traffics to the plasma membrane (83, 84). The 3D structures of zebrafish P2X4 in the closed and open ATP-bound conformations revealed a lever-like opening mechanism that links ATP binding to an expansion of lateral fenestrations in an iris-like opening of a pore lined by the transmembrane helices (85, 86). When gated to the open state by ATP binding, P2X7 ion channels produce a noninactivating influx of Ca^{2+} and Na^+ and efflux of K^+, resulting in rapid depolarization (50). Prolonged stimulation of P2X7 induces formation of a pore that allows passage of molecules up to 800 Da (52).

The ED_{50} for activation of P2X7 by ATP is about 100 μM, which is 10–100 times higher than the ED_{50}s of ATP for most other P2 receptors. This low affinity for ATP may limit activation of the receptor to areas of high inflammation. P2X7 can also be activated by 2′,3′-*O*-(benzoyl-4-benzoyl)-ATP (BzATP), an ATP analog, with a potency significantly greater than that of ATP, whereas the other P2X receptors either are insensitive or exhibit lower affinity for BzATP than ATP.

On the basis of studies in mouse macrophages, activation of the NLRP3 inflammasome is thought to require two signals. The first signal can be provided by TLR stimulation and triggers the synthesis of pro-IL-1β and NLRP3. The second signal can be mediated by stimulation of P2X7 by ATP resulting in K^+ efflux, Ca^{2+} influx, and other signals that activate the inflammasome. Western blots of cell lysates revealed that Panx1 coimmunoprecipitates with various inflammasome components including caspases (54). Thus, activation may be facilitated by association of the Panx1/P2X7 complex with components of the inflammasome. The result is the release of mature cytokines including IL-1β (52) or under certain conditions apoptotic or pyroptotic cell death (53, 54).

An interaction between Panx1 and P2X7 suggests that even modest stimulation of this complex should result in feed-forward activation and inevitable cell death. However, the ATP release channel Panx1 self-inhibits at high extracellular ATP concentrations (**Figure 3**). This phenomenon is mediated by a series of amino acids in both extracellular loops of the Panx1 protein (87, 88). Thus, the ATP-binding site on Panx1 shares properties with P2X7 insofar as most ligands for the receptor, irrespective of whether they are activating or inhibiting, inhibit the Panx1 channel. In addition to the fast inhibition of Panx1 currents by ATP, desensitization of the Panx1/P2X7 complex also occurs by a slower endocytotic process (55, 89). Both processes safeguard cells from overstimulation-induced cell death. However, while the self-inhibition of the Panx1 channel is very effective in a normal extracellular milieu, it is attenuated in a dose-dependent way by increased extracellular K^+ concentration (90). At a level of 60 mM that can occur in highly inflamed tumors or the penumbra of a stroke (91), the self-inhibition of the channel is virtually eliminated.

Cell death in solid tumors or other inflamed tissues results in large amounts of ATP release and activation of P2X7s. A variety of other stimuli activate Panx1 channels to release ATP in the absence of cell death. These stimuli include mechanical stress (51) or low oxygen in erythrocytes (58, 92) and neurons (93), glutamate in neurons through NMDA (*N*-methyl-D-aspartate) receptors, and several other ligands through their cognate receptors (57, 94–99). Following $CD8^+$ T cell activation, P2X7 is stimulated by autocrine ATP release and maintains calcium influx initiated by T cell receptor engagement. Elevated intracellular Ca^{2+} increases mitochondrial metabolic activity, increases ATP release into the cytosol, and facilitates Trm cell expansion (5). Furthermore, increased extracellular K^+ serves as a stimulus for local Panx1-mediated ATP release in several cell types (54, 58, 100–102). Thus, K^+ not only interferes with the self-inhibition of the Panx1 channel but also is a powerful activator that changes the channel to the large, ATP-permeant conformation (101). Consequently, simultaneous increases in extracellular K^+ and ATP concentrations are particularly dangerous to cells (90).

Another stimulus capable of opening Panx1 channels is voltage clamp to positive potentials (103). However, in contrast to the stimuli listed above, voltage activation leads to a different open conformation of the Panx1 channel with high selectivity for chloride ions and no detectable ATP or cation permeability (101, 104, 105). Yet another stimulus, only observed in apoptotic cells, is cleavage by caspase-3 of the Panx1 tail consisting of 48 C-terminal amino acids, truncating the Panx1 protein to 378 amino acids and rendering it constitutively open (106). The caspase-activated Panx1 channel exhibits similar properties as the voltage-activated channel (47, 106, 107). The currents reverse at the same potential for both activation mechanisms. Similarly, no ATP release within a reasonable time frame is observed following either type of channel activation. Chloride selectivity and a lack of ATP permeability imply a similar channel conformation for both the voltage- and caspase-activated channels.

The Panx1 channel is linked to the inflammasome complex and to at least three apoptotic caspases through several pathways (108). **Figure 3** depicts three ways in which Panx1 appears to be linked to cell death:

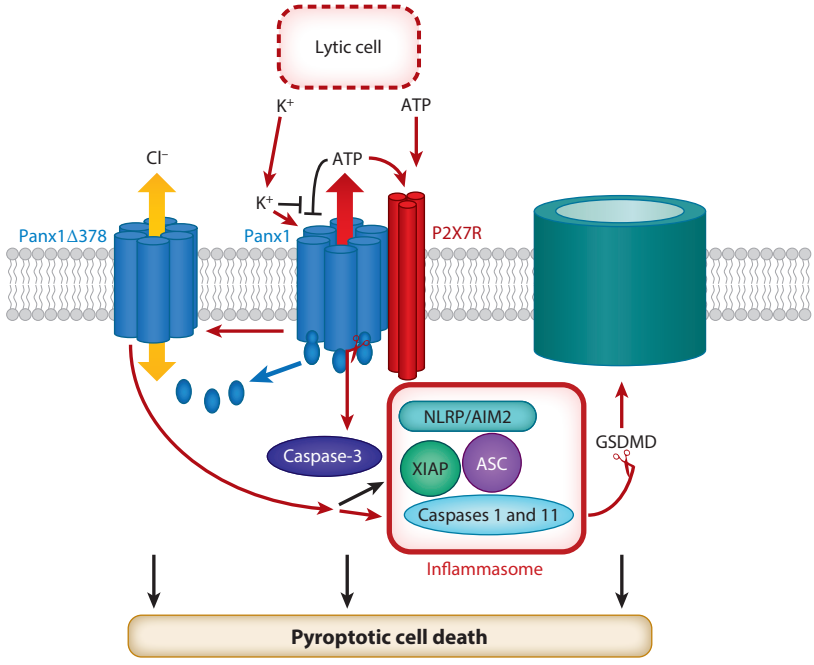

Figure 3

Involvement of Panx1 and P2X7 in cell activation and death. A high level of ATP released through Panx1 channels in response to activating stimuli or from dying cells activates P2X7, which in turn, in the presence of hypoxia or elevated K^+, triggers opening of the Panx1 channel to its large, ATP-permeant conformation. The resulting amplification of the ATP signal is self-limited by the accumulation of extracellular ATP, which can reach a high concentration in the channel's vestibulum and block the channel. The Panx1/P2X7 complex signals by protein-protein interaction to the inflammasome, initiating the caspase-mediated cleavage to form mature IL-1β and IL-18 and an inflammatory response by other cells. This tampered response to a modest stimulus results in a benign inflammatory defense and does not result in cell death. However, overstimulation of the Panx1/P2X7 complex will activate additional pathways and mediate cell death. For example, in secondary cell death due to neuronal ischemia, damaged cells, in addition to releasing ATP, also release glutamate, which activates Panx1 through NMDA receptors and K^+ accumulation, which is a potent stimulator of the large Panx1 channel conformation. Furthermore, K^+ interferes with the self-inhibition of Panx1 by ATP. Hyperactive Panx1/P2X7 complexes activate caspases that cleave the autoinhibitory C terminus of Panx1 to yield a truncated Panx1Δ378 and a 48–amino acid C-terminal peptide. Panx1Δ378 forms a constitutively open chloride-selective channel with no ATP permeability, which signals (without requirement for channel function) to the inflammasome either by directly activating it or by inhibiting the inflammasome inhibitor XIAP. Inflammasome caspases 3 and 11 also cleave gasdermin, and the N-terminal peptide assembles in the plasma membrane to form an ~14-nm pore. It appears that the combination of all these factors causes a fast demise of cells. However, in the absence of gasdermin, cell death can still be observed, albeit at a slower rate.

1. Stimulation of oocytes coexpressing Panx1 and P2X7 with high ATP concentrations leads to cell death within ~5 min (53). Oocytes undergo prolific apoptotic pruning for quality control of the cells to be fertilized (109). The Panx1/P2X7 complex is probably able to engage the oocyte's highly capable endogenous apoptotic machinery to mediate ATP-induced apoptosis in this expression system.
2. Cleavage of Panx1 by caspase-3 renders the channel constitutively active and is exclusively observed in apoptotic cells (106). A truncation mutant, Panx1Δ378, replicating the caspase-cleaved protein exhibits the same biophysical properties and leads to cell death (47, 90,

107). Since Panx1Δ378 does not yield the C-terminal peptide that is generated by caspase-3 cleavage, it is unlikely that the free peptide is responsible for the death signal. Even the chloride permeability of the channel may not be required since Panx1Δ378-mediated cell death was observed with the channel blocked by a chloride ion flux inhibitor, carbenoxolone (90). In addition to being upstream of its target, Panx1, caspase-3 is downstream of Panx1, since inhibition of the Panx1 channel was observed to also inhibit activation of caspase-3 (90).

3. Another membrane channel involved, and probably mediating cell death directly, is formed by gasdermin proteins in response to cleavage by caspase-1 (110, 111) (**Figure 3**). This mechanism is associated with lymphocyte depletion and immunosuppression in response to viral infection (112). After cleavage, the N-terminal peptides of gasdermins assemble as complexes of 16 homomers in the plasma membrane to form ~14-nm-wide pores (113, 114). Since caspase-1 activation is downstream of the Panx1/P2X7 complex, gasdermin appears to be the final executioner in inflammatory cell death.

The Panx1/P2X7 complex evidently is on the very top of the signaling chain of command for apoptotic cell death. While this process is an essential part of the host defense mechanism, it is also responsible for an exasperating consequence of tissue trauma. Following injury to most tissues, many more cells undergo secondary cell death than are acutely killed by the original trauma. In these circumstances, targeting the Panx1/P2X7 complex may lead to an improved outcome by limiting secondary cell death.

CELLULAR RESPONSES TO P2X7 ACTIVATION

Th17 Cells

P2X7 signaling is important for the development of Th17 cells, which are major effector cells in the pathogenesis of inflammatory diseases (115). In a DC/$CD4^+$ T cell coculture system, pretreatment with an antagonist of P2X7 (A-438079) causes inhibition of Th17-promoting cytokines (IL-1β, TGF-β1, IL-23p19, and IL-6). Blockade of P2X7 inhibits hind-paw swelling and ameliorates pathological changes in ankle joints in the collagen-induced arthritis model associated with Th17 cell activation (115).

$CD8^+$ Cells

Extracellular ATP stimulates central memory and $CD8^+$ Trm cells that are involved in various chronic inflammatory diseases (116, 117). Blockade of P2X7 reduces formation and maintenance of $CD8^+$ memory T cells.

Macrophages

The activation of P2X7 on M1 polarized macrophages induces the assembly of the NLRP3 inflammasome, leading to the release of proinflammatory cytokines. However, P2X7 signaling to the inflammasome is uncoupled in M2 macrophages. P2X7 stimulation in macrophages also is able to release potent anti-inflammatory proteins, such as annexin A1, independently of their polarization state, suggesting a potential role for P2X7 during resolution of inflammation that is not linked to the release of proinflammatory cytokines (118). Adenosine also regulates inflammasome activity, surprisingly increasing the duration of the inflammatory response via activation of the A_{2A} receptor. Adenosine does not replace signals provided by stimuli such as endotoxin or ATP

but sustains inflammasome activity via a cAMP/PKA/CREB/HIF-1α pathway. In the setting of a lack of IL-1β responses after previous exposure to LPS due to TLR4 desensitization, adenosine can supersede a tolerogenic state and drive IL-1β production. Hence, despite the generally anti-inflammatory effects of adenosine, inflammasome activity can be sustained in macrophages by A_{2A} receptor–mediated signaling (119).

Dendritic Cells

Treatment of mouse bone marrow–derived DCs with exogenous ATP stimulates release of high levels of IL-1β and IL-18, and this release is significantly inhibited by apyrase, which degrades ATP, or the P2X7 blocker A-438079. Cytokine release is mediated by caspase-1-dependent processing of pro-cytokines (120). ATP also stimulates DC motility via an autocrine signaling loop, which is initiated by the activation of P2X7 and further amplified by Panx1 channels by increasing ATP release. In the absence of Panx1, DCs fail to increase their speed of migration in response to ATP, despite exhibiting a normal P2X7–mediated Ca^{2+} response. In addition to DC migration, Panx1-channel- and P2X7-dependent signaling are required to stimulate the reorganization of the actin cytoskeleton. In vivo, functional Panx1 channels are required for the homing of DCs to lymph nodes, although they are dispensable for DC maturation. Thus, P2X7 and Panx1 channels are major players in the regulation of DC migration and cytokine maturation (121).

Mast Cells

Mast cells contribute to an inflammatory milieu in response to endotoxin and ATP by secreting mature cytokines in response to NLRP3 inflammasome activation. CD39 sets an activation threshold for P2X7-dependent mast cell death and concomitant IL-1β release. Deletion of CD39 or stimulation with nonhydrolyzable ATP leads to a lower activation threshold for P2X7-dependent responses. Intriguingly, stimulation with low ATP concentrations augments the production of IL-1β in endotoxin-primed mast cells in a P2X7-independent but caspase-1-dependent manner. This may be due to the involvement of other high-affinity P2 receptors. Thus, in mast cells there appears to be a fine-tuned interplay between ATP/purinergic receptors and enzymes that can control inflammation and cell death decisions (122).

SIGNALING BY NAD^+

ADP-ribosylation is a posttranslational modification regulating protein function in which ADP-ribosyltransferases (ARTs) covalently transfer ADP-ribose from NAD^+ onto specific target proteins. P2X7 is activated by ADP-ribosylation on an extracellular domain. As discussed above, P2X7 can be gated directly by extracellular ATP. ADP-ribosylation of R125 of P2X7 is catalyzed by the glycosylphosphatidylinositol-anchored ARTC2 ectoenzyme (123) and positions the ADP-ribose to fit into the nucleotide-binding site of P2X7, thereby gating the channel (124–126) (**Figure 2**).

On T cells, gating of P2X7 by ATP or by ADP-ribosylation induces shedding of CD62L and cell death (124, 127). Tregs, NKT cells, and Trm cells all express high levels of P2X7 and are particularly sensitive to NAD^+-induced cell death (128–132). During the routine preparation of leukocytes from lymphatic organs, sufficient amounts of NAD^+ are released from stressed cells to initiate ADP-ribosylation of P2X7 on these cells even when they are prepared at 4°C (123). This impairs the function and vitality of these important T cell subsets in subsequent in vitro and in vivo assays (128–130). NAD^+-dependent ADP-ribosylation of P2X7 during cell preparation can

be effectively blocked by systemic injection of the ARTC2-antagonizing nanobody s+16a shortly before harvesting tissues and cells (130). This improves recovery of functional Tregs, NKT cells, and Trm cells (128–130).

Dampening of Treg function via NAD^+-induced gating of P2X7 can be employed in vivo as a strategy to promote the antitumor response of effector T cells. Systemic injection of NAD^+ results in the selective depletion of Tregs via NAD^+-mediated activation of P2X7, which enhances anticancer immune responses in several mouse tumor models (6, 129).

P2X7 as a Therapeutic Target

Given its central role in activating and sustaining inflammatory reactions, P2X7 itself has attracted considerable attention as a potential therapeutic target (66, 73, 133–135). Small-molecule antagonists of P2X7, including brilliant blue G (BBG), oxidized ATP, KN-62, and A-438079, have shown therapeutic benefit in several preclinical models of inflammatory diseases, including endotoxin-induced fever, inflammatory pain, contact hypersensitivity, and antibody-induced nephritis (69–72). However, these small molecules are fraught with lack of specificity and undesired side effects. Recently, highly specific nanobodies have been developed that effectively block ATP-induced and NAD^+-induced gating of P2X7 (136) (**Figure 2**).

Nanobodies are single-domain antibodies derived from heavy-chain antibodies naturally occurring in camelids (137–140). These biologics exhibit the unusual propensity to bind to functional clefts on protein surfaces such as the active site of enzymes that usually are not accessible to conventional antibodies (141). In vivo, nanobodies show excellent tissue penetration and little if any toxicity (142). To date more than 1,000 patients and healthy subjects have received nanobodies in clinical studies without any obvious off-target side effects (140, 143). Systemic injection of P2X7-specific nanobodies into mice resulted in complete occupation and functional modulation of P2X7 on T cells and macrophages in spleen, lymph nodes, liver, and the peritoneal cavity within minutes after injection (136). Systemic injection of the P2X7-blocking nanobody 13A7 into mice ameliorated experimental glomerulonephritis, while injection of the P2X7-potentiating nanobody 14D5 made disease significantly worse (**Figure 4**). In a surrogate inflammation model with endotoxin-treated human blood samples, anti-P2X7 nanobody Dano1 effectively blocked ATP-induced release of IL-1β with 10,000-fold higher potency than benchmark small-molecule inhibitors (136). These results underscore the utility of the nanobody technology to generate antibody therapeutics against ion channels and confirm P2X7 as a therapeutic target for inflammatory disorders.

P2Y RECEPTORS

P2Y2

The purinergic receptor P2Y2 binds ATP and stimulates chemotaxis of various cells including macrophages, neutrophils, eosinophils, and others (144, 145). In neutrophils, mTOR signaling contributes to chemotaxis by regulating ATP production and purinergic signaling. Blocking mTOR signaling with rapamycin or blocking mitochondrial ATP production with CCCP impairs cellular ATP release and neutrophil chemotaxis. Autocrine stimulation with adenosine produced by local ATP degradation stimulates A_{2A}Rs and causes cAMP accumulation at the trailing side of migrating cells. Protein kinase A activation inhibits mTOR signaling and inhibits mitochondrial Ca^{2+} uptake, therefore resulting in uropod retraction (146). Global P2Y2 knockout mice exhibit impaired myeloid cell chemotaxis and are protected in various models of acute inflammation (147).

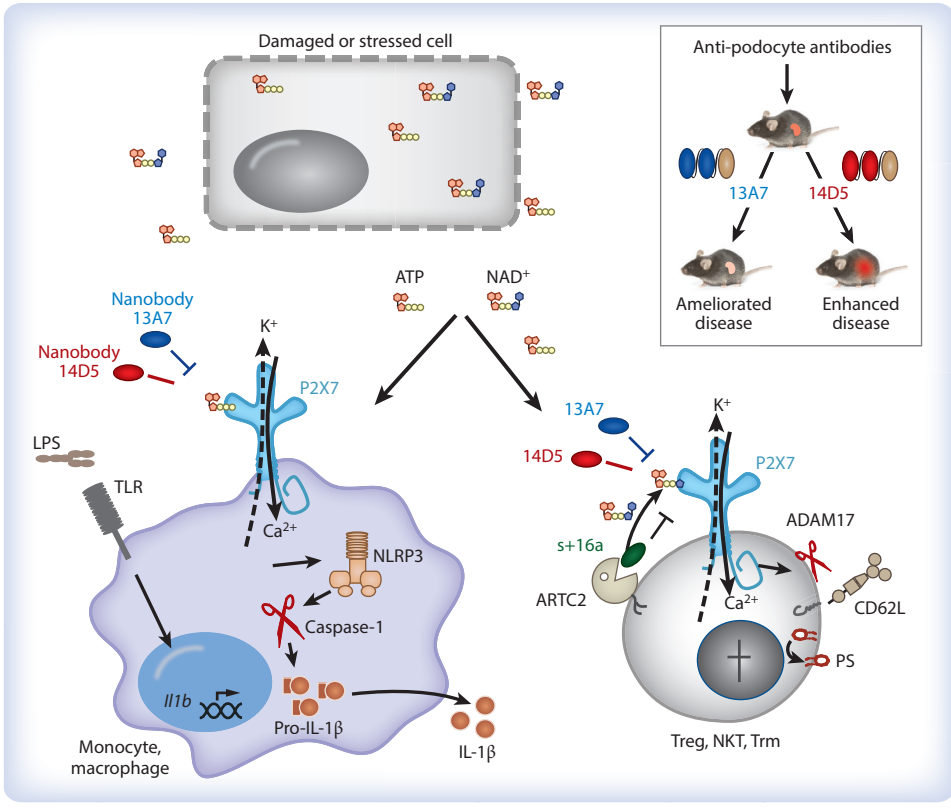

Figure 4

Consequences of NAD^+ and ATP release from injured cells. In LPS-activated monocytes and macrophages, gating of P2X7 by extracellular ATP induces assembly of the NLRP3 inflammasome, caspase-1-mediated cleavage of pro-IL-1β, and release of the mature proinflammatory cytokine. On T cells, gating of P2X7 by ARTC2-catalyzed NAD-dependent ADP-ribosylation triggers externalization of PS, ADAM17-mediated shedding of CD62L, and ultimately cell death. P2X7-specific nanobodies effectively block (13A7) or potentiate (14D5) gating of P2X7 by ATP and by NAD-dependent ADP-ribosylation. In a mouse model of anti-podocyte-induced nephritis, P2X7-blocking and P2X7-potentiation nanobodies ameliorate and enhance disease, respectively. Systemic injection of the ARTC2-blocking nanobody s+16 reduces killing of Tregs, NKT cells, and Trm cells by blocking ADP-ribosylation of P2X7 in response to NAD^+ released from stressed cells. Abbreviations: NAD^+, oxidized nicotinamide adenine dinucleotide; NKT, natural killer T cell; PS, phosphatidylserine; TLR, Toll-like receptor; Treg, regulatory T cell; Trm, tissue-resident memory cell.

Blockers of P2Y2 receptors are candidates for treatment of cystic fibrosis, asthma, and lung viral infection (148).

P2Y12

P2Y12 is best known as an ADP receptor that stimulates platelet aggregation and blood clotting. The receptor couples to Gαi2, inhibits cAMP accumulation, and stimulates phosphatidylinositol-3-kinase and Rap1b. The nucleoside analog ticagrelor and active metabolites of the thienopyridine compounds ticlopidine, clopidogrel, and prasugrel block P2Y12 and thereby inhibit ADP-induced platelet aggregation. P2Y12 is also found on microglial cells, where it functions as a sensor for adenine nucleotides. Its activation induces rapid chemotaxis in response to tissue injury (149).

Recent studies suggest the involvement of microglial P2Y12 in activity-dependent neuronal plasticity. Interestingly, there is evidence for changes in P2Y12 expression in central nervous system pathologies including Alzheimer disease and multiple sclerosis.

PURINERGIC REGULATION OF DNA METHYLATION

Emerging evidence indicates that adenosine exerts epigenetic effects that are not mediated by adenosine receptors. *S*-adenosylmethionine (SAM) is a methyl donor for cellular transmethylation reactions that modify histones and DNA. DNA methyltransferases catalyze the transfer of a methyl group from SAM, resulting in the production of *S*-adenosylhomocysteine and methylation of DNA cytosines. Whereas acute stimulation of cells with SAM causes global DNA hypermethylation, increasing intracellular levels of adenosine and certain other purines inhibits SAM-dependent transmethylation reactions, resulting in global DNA hypomethylation. These changes in DNA methylation produce epigenetic modulation of many genes. Interestingly, adenosine levels are high in solid tumors and may contribute to global hypomethylation of DNA, a consistent feature of neoplastic transformation.

Expression of the adenosine $A_{2A}R$ is reduced after treatment of rats with SAM to enhance DNA methylation. Two weeks of SAM treatment produced a reduction in rat striatal $A_{2A}R$ mRNA and protein content and reduced $A_{2A}R$-mediated signaling. 5-Methylcytosine levels in the 5′ untranslated region of *Adora2a* mRNA were significantly increased in the striatum of SAM-treated animals, suggesting a correlation between SAM-mediated hypermethylation and reduced $A_{2A}R$ expression (151). A key enzyme that controls intracellular adenosine levels is adenosine kinase (ADK), which catalyzes AMP formation from adenosine. ADK activity is important not only for adenosine salvage but also for maintaining SAM-dependent transmethylation processes (152). Hypoxia lowers the expression of ADK and increases intracellular adenosine. Knockdown of ADK elevates intracellular adenosine and promotes endothelial cell proliferation, migration, and angiogenic sprouting that is associated with hypomethylation of the promoters of proangiogenic genes including VEGFR2. Methylation-specific PCR, bisulfite sequencing, and methylated DNA immunoprecipitation confirmed hypomethylation in the promoter region of VEGFR2 in ADK-deficient (adenosine-high) endothelial cells. These findings suggest that ADK downregulation and elevation of intracellular adenosine in endothelial cells promote angiogenesis (153). In addition to adenosine, deoxyadenosine and its analogs and metabolites have been found to influence transmethylation-dependent transcription of promoters that trigger a robust innate immune type I interferon response, similar to what has been noted in response to the DNA methyltransferase inhibitor 5-azacytidine (154). These findings suggest that intracellular purines that regulate SAM-dependent transmethylation reactions may exert epigenetic effects.

SUMMARY AND CONCLUSIONS

Extracellular purines play a pivotal role in controlling the chemotaxis, activation, proliferation, and differentiation of immune cells. Inflammation is controlled in part by the tightly regulated release and extracellular metabolism of purines, which signal via a large family of purinergic receptors as well as by extracellular ADP-ribosylation with NAD^+ as a substrate, and DNA methylation that is controlled by intracellular adenosine and other purines. The expression and function of molecules involved in purine release, metabolism, and signaling are often induced in activated immune cells, and their activities are controlled by factors in the local environment such as bacterial toxins, hypoxia, and K^+ concentration. Of interest is the observation that some of the enzymes that degrade ATP, NAD^+, and other purine nucleotides also degrade other factors that control

inflammation, LPC, and 2′3′-cGAMP. Inhibitors of ENPP1 or ENPP2 may influence the progression of inflammatory diseases, innate and adaptive immunity, and cancer by simultaneously influencing signaling by ATP, NAD^+, adenosine, LPA, and 2′3′-cGAMP. As our understanding of purine release, metabolism, and signaling grows, we will gain the ability to modify immune responses by targeting purinergic receptors, enzymes, and epigenetic regulators.

DISCLOSURE STATEMENT

F.K.-N. receives a share of antibody and nanobody sales via MediGate GmbH, a wholly owned subsidiary of the University Medical Center Hamburg-Eppendorf. F.K.-N. is co-inventor on patent applications on P2X7-specific nanobodies.

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