Targeting hypoxia–A_{2A} adenosine receptor-mediated mechanisms of tissue protection

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Despite inflammation having beneficial effects, the action of toxic proinflammatory molecules can result in excessive tissue damage that subsequently contributes to the pathogenesis of many major diseases. The development of novel drugs and therapeutic strategies for the treatment of inflammation requires an improved understanding of the molecular mechanisms that terminate inflammation. The physiological hypothesis proposes that excessive levels of inflammatory tissue damage result in local hypoxia and accumulation of extracellular adenosine. The A_{2A} adenosine receptor and hypoxia-inducible factor play important roles in the attenuation of proinflammatory processes in a delayed, negative-feedback manner and thus protect organs from excessive damage. Targeting individual stages of the hypoxia–A_{2A} receptor signaling pathway represents an attractive strategy for the modulation of inflammation.

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▼ Inflammation represents a host defense response to a variety of harmful stimuli and is crucial to the survival of an organism. Inflammatory processes are mediated by the actions of a variety of proinflammatory cytokines and cytotoxic molecules that are secreted by activated immune cells to destroy pathogens and virus-infected cells. Lymphotoxin-α, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), as well as other cytokines and cytotoxic molecules, are thought to play major roles in the initiation of inflammation. The complex interplay of cytokines and cytokine-induced chemokines results in the attraction and activation of macrophages, lymphocytes and neutrophils, and is responsible for the recruitment and migration of these cells to the site of inflammation [1]. In addition to the beneficial effects of pathogen destruction, the action of toxic proinflammatory molecules might result in collateral tissue damage or prolonged or inappropriate inflammation, which is implicated in the etiology and pathogenesis of major diseases, including cancer, heart disease and atherosclerosis [2].

These considerations emphasize the need for a better understanding of the processes that control and terminate inflammation. Various molecules have been shown to be capable of blocking inflammation [3]. However, vigorous testing was required to determine which of these molecules participate in the physiological mechanism that 'senses' the need to downregulate inflammation and hence protects against excessive inflammatory tissue damage.

Consider the possibility that when the extent of collateral tissue damage reaches some 'unacceptable level', changes in the local tissue environment will effect adaptive changes in cell metabolism. Consequently, these changes will result in the accumulation of intracellular metabolic intermediates that, after their release into the extracellular space, might signal downregulation of inflammation through inhibitory receptors on activated immune cells. Such metabolites would report excessive tissue damage and signal the need to terminate proinflammatory cytokine secretion by activated immune cells. In addition, this model predicts that enhanced inflammation and increased tissue damage would be a consequence of the absence of receptors for these physiological signals of excessive tissue damage [4]. It was further assumed that extracellularly accumulated immunosuppressive metabolic intermediates might trigger increased levels of immunosuppressive intracellular cAMP, which would lead to the inhibition of overactive immune cells [5]. Intracellular cAMP is an attractive candidate as a mediator

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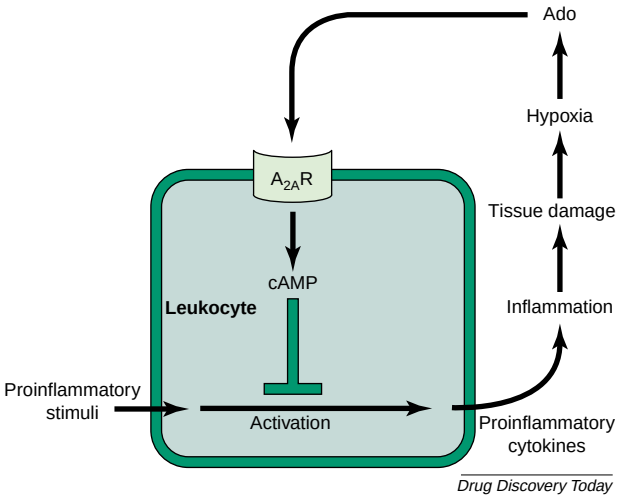

Figure 1. Negative-feedback mechanism of A_{2A} adenosine receptor-mediated downregulation of inflammation and protection from excessive tissue damage. Secretion of proinflammatory cytokines by leukocytes results in local tissue damage and diminished blood supply. Hypoxia and cell lysis lead to an accumulation of extracellular adenosine, which triggers accumulation of immunosuppressive cAMP in leukocytes through the $A_{2A}R$. Immunosuppressive cAMP is able to attenuate activation of the leukocyte. Abbreviations: $A_{2A}R$, A_{2A} adenosine receptor; Ado, adenosine.

for the prevention of excessive inflammatory tissue damage [6], but such a role has yet to be shown for this molecule *in vivo*.

Accordingly, the identification of receptors that are capable of elevating levels of cAMP and ligands that might function as endogenous inhibitors of inflammation has been an important unresolved issue in understanding the regulation of inflammation and in testing the role of cAMP. This issue has been complicated by the difficulties encountered in identifying ligands that are involved in the physiological elevation of cAMP levels from among a great number of molecules that trigger different G_s-protein-coupled receptors [7]. Indeed, several different cAMP-elevating ligands, including catecholamines, prostaglandins, dopamine, histamine, extracellular adenosine and other anti-inflammatory molecules, are known to have immunosuppressive properties and have been considered as potential anti-inflammatory stimuli *in vivo* [3,8,9].

In this review, the implications of studies intended to determine whether or not extracellular adenosine might serve as an endogenous, physiological regulator of immune response *in vivo* and whether or not hypoxia-inducible factor-1α (HIF-1α) might contribute to adenosine receptor-mediated inhibition are described [9–15]. The emergence of genetic tools and models of acute inflammation have helped to provide the first evidence for the crucial roles of extracellular adenosine as the 'signal' and A_{2A} adenosine receptor ($A_{2A}R$) as the 'sensor' of excessive tissue damage [4].

The combined action of various proinflammatory stimuli results in local tissue damage and changes in the local tissue environment, including diminished blood supply, local tissue hypoxia and adaptive changes in cell metabolism (Figure 1). These adaptive changes might eventually lead to the accumulation of extracellular adenosine, which could then trigger immunosuppressive signaling (because of elevated cAMP levels) via the adenosine receptor. This cascade of events culminates in a delayed, negative-feedback downregulation of acute inflammation as the result of cAMP inhibition of unidentified molecular intermediates of the proinflammatory signaling pathway (Figure 1). Recent findings of negative regulation of tissue damage by hypoxia and HIF-1α [16–18] point to the need for comprehensive studies of the mechanism through which HIF-1α and adenosine receptors might inhibit immune cell response in a coordinated manner. These findings could potentially have clinical implications.

Studies of adenosine receptors on immune cells

Investigation of the immunosuppressive properties of cAMP-elevating, adenosine receptor-mediated signaling can be divided into three major stages. The original findings (from 1964 to 1982) dealt with studies into the immunosuppressive properties of extracellular adenosine on different immune cells, the role of the cAMP-elevating pathway and of adenosine (purinergic) receptors, in addition to descriptions of multiple stimuli and conditions that induce adenosine release by various cells [6,19–27].

Over the two decades that followed, advances in molecular cloning techniques enabled the characterization of four subtypes of adenosine receptors [28–30] which, together with the realization that some important anti-inflammatory drugs might exert their pharmacological effects through endogenous adenosine-mediated signaling, were important findings in the study of adenosine receptor-mediated immunosuppression [31]. Important clues to the possible roles for extracellular adenosine *in vivo* were provided by detailed studies of its effects on various immune cells [12–15,31–34]. Such studies were facilitated by the availability and continued development of selective agonists and antagonists that act on the various adenosine receptors [30,35].

The investigators of today are poised to understand fully the physiological and pathophysiological roles of adenosine receptor-mediated signaling *in vivo* and to use such knowledge in the modulation of inflammation. Studies of adenosine receptors have been greatly facilitated by the development of $A_{2A}R$ gene-deficient mice [36,37]; the use of

these mice provided the first conclusive *in vivo* evidence that the $A_{2A}R$ plays a crucial role in the downregulation of acute inflammation [4]. Optimistic expectations for the development of adenosine receptor-based therapies include promising strategies to enhance tissue protection and treat diseases using new generations of agonists, antagonists [38,39] and 'neoceptors–neoligands' [40]. The use of these drugs could potentially be maximized by the development of synergizing partners for adenosine receptor agonists for the targeting of inflammatory cells [41].

Observations from studies of gene dose effect on $A_{2A}R$ expression are of great importance for future drug development [42]. These experiments were performed in thymocytes and T cells from heterozygous (*Adora2a*$^{+/-}$) mice, and demonstrated that the reduction observed in the number of $A_{2A}R$ was proportionately reflected in a decrease in the functional cAMP response of the cells to adenosine, thus identifying the absence of a 'receptor reserve' (i.e. there are no spare $A_{2A}R$) in T cells [42]. Furthermore, the detection of attenuated adenosine-induced cAMP levels in thymocytes from $A_{2A}R$-deficient mice suggests that the loss of $A_{2A}R$ was not compensated by the A_{2B} receptors on thymocytes. The level of apoptosis of thymocytes from *Adora2a*$^{+/-}$ heterozygous mice induced by the selective $A_{2A}R$ agonist CGS21680 was reduced compared with thymocytes from wild-type mice [42].

Examination of $A_{2A}R$-deficient mice using real-time RT–PCR revealed no significant compensatory increases in the expression of A_{2B}, A_1 or A_3 adenosine receptor mRNA in lymphoid organs [43]. In acute models of inflammation, it appears that the $A_{2A}R$ has a non-redundant role in protecting against excessive inflammatory tissue damage. This could be in part a consequence of the insufficient upregulation of other immunosuppressive adenosine receptors in lymphoid organs of $A_{2A}R$-deficient mice to significantly compensate for the lack of $A_{2A}R$. However, chronic inflammation could present a different scenario.

cAMP-elevating immunosuppressive extracellular adenosine receptors

The search for the physiological 'off' signal was based on the assumption that the physiological immunosuppressive mechanism might employ the intracellular cAMP-dependent anti-inflammatory pathway [6,44–46]. This was prompted by pharmacological demonstrations of the ability of cAMP to inhibit immune cells [17]. Indeed, in 1974 it was proposed that '…cAMP serves to protect the host from the dangerous consequences of an unregulated immune response' [47]. In the past three decades, many investigations have demonstrated that cAMP can function through a high-fidelity immunosuppressive pathway that inhibits early and late events in signal transduction pathways in activated immune cells [6,44]. However, it has not been proven directly that cAMP-dependent signaling in immune cells functions as the physiological, endogenous regulator of immune response *in vivo*.

In 1970, Sattin and Rall [48] provided one of the first demonstrations of the link between adenosine and cAMP levels. Their research indicated that adenosine is one of the most potent stimulators of cAMP accumulation in brain slices of guinea pigs. The observation that adenosine is able to suppress the processes that are involved in inflammation has been described independently by Born *et al.* [19], Wolberg *et al.* [21] and by scientists who have studied the mechanisms of immunosuppression in adenosine deaminase (ADA)-deficient patients that are also suffering from severe combined immune deficiency syndrome (SCID) [49]. These observations were followed by multiple demonstrations of the immunosuppressive effects of adenosine on various types of immune cell, including mast cells, neutrophils and more detailed studies of T cells [12–15,31–34]. It has been shown that adenosine effects strong inhibition of T-cell receptor (TCR)-triggered CD25 upregulation and T-cell proliferation, [13] and also suppresses virtually all tested T-cell functions, including cytotoxicity and FasL production [14]. In addition, adenosine induces apoptosis in double-positive ($CD4^+CD8^+$) thymocytes [12].

One reason that studies of adenosine effects on immune cells elicited so much interest was the need to understand the pathogenesis of human SCID, which represents a group of inherited disorders that are characterized by the lack of immune response to infectious disease [49]. About a third of SCID patients suffer from a deficiency of the enzyme ADA, which participates in the catabolism of adenosine to inosine. In the absence of ADA, adenosine is not degraded, and accumulates intracellularly and extracellularly [50]. ADA immunodeficiency is accompanied by a severe depletion of T and B cells, and defects in lymphocyte development [51].

Further evidence for a possible physiological role for extracellular adenosine in the downregulation of inflammation has been supplied by studies on the release of adenosine from cells. It was observed that cells that are exposed to different stimuli, including those frequently found in inflamed areas and in anoxia or hypoxia, release adenosine in concentrations that are sufficient to affect other cells and physiological processes [24–27,32,33]. Taken together, the findings from these studies were consistent with the possibility that adenosine plays a regulatory role in immune response, which helps to maintain interest in the *in vivo* role of extracellular adenosine and in adenosine receptors that mediate the effects of adenosine on immune cells.

For more than two decades, adenosine receptors have been considered pharmacological triggers of anti-inflammatory signals to immune cells [22]. It is now understood that the effects of extracellular adenosine are mediated by G_s- and G_i-protein-coupled receptors, which are subdivided into A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. A_1 and A_3 are coupled to G_i proteins, whereas A_{2A} and A_{2B} interact with G_s proteins. Activation of G_s proteins leads to the accumulation of intracellular cAMP, whereas stimulation of G_i proteins leads to decreases in cAMP levels [29].

Depending on the repertoire of adenosine receptors present on the cell surface, adenosine could have opposite effects on particular physiological processes. Cronstein *et al.* [52] demonstrated that neutrophil adherence to endothelium could be inhibited by A_2 receptors and enhanced by A_1 receptors. A general observation is that the activation of A_{2A}R in lymphoid cells generates an anti-inflammatory response [13,14,31,32,34,53]. T cells were shown to express immunosuppressive, cAMP-elevating A_{2A}R that were capable of blocking TCR-triggered proliferation, granule exocytosis, FasL expression and secretion of cytokines such as IFN-γ [13,14,22]. Similarly, adenosine receptors on human basophils that inhibit immune function upon activation have been reported [54]. The observations that A_1 adenosine receptors are predominantly expressed on macrophages in brain and peripheral blood, and that their expression is decreased in patients with multiple sclerosis [55] suggests a role for these receptors in the pathogenesis of the disease through the increase in secretion of proinflammatory cytokines, which could then cause damage to the central nervous system. The effects of A_3 receptors on inflammatory processes are more complex and variable, for example, inhibition of eosinophil migration and triggering the degranulation of mast cells [30,56]

In vivo evidence for the downregulation of inflammation by the A_{2A} adenosine receptor

In wild-type mice, agonists of A_{2A}R blocked inflammatory tissue damage *in vivo*, thereby confirming that this receptor is capable of inducing an anti-inflammatory response [31,33,35]. The treatment of a variety of diseases, including sepsis [4], wound healing [57,58] and rheumatoid arthritis [59], might benefit from the targeted stimulation of A_{2A}R. For instance, methotrexate, which is used as a treatment for rheumatoid arthritis, has been shown to engage this pathway via endogenous adenosine [60]. However, the genetic *in vivo* models of acute inflammation with multiple controls were required to provide a conclusive answer [4].

According to the proposed model of adenosine receptor-mediated regulation of inflammation (Figure 1), the absence of A_{2A}R was expected to result in an increased and prolonged secretion of proinflammatory cytokines and exacerbated tissue damage and, indeed, this was the case in various *in vivo* inflammation models.

The role of the A_{2A}R as a 'stop' signal was confirmed in *in vivo* models of T-cell-dependent autoimmune and viral hepatitis, *Pseudomonas aeruginosa* exotoxin A-induced fulminant hepatitis and carbon tetrachloride-mediated hepatotoxicity. A similar anti-inflammatory role of the A_{2A}R was highlighted by the demonstration of enhanced tissue damage and elevated levels of proinflammatory cytokines during endotoxin-induced sepsis and in 'infected wound' models [4].

Studies of T-cell-, macrophage- and cytokine-dependent tissue injury in A_{2A}R-deficient mice revealed dramatically increased local tissue damage and the prolonged presence of proinflammatory cytokines such as TNF-α, IFN-γ and interleukin-12 (IL-12), which resulted in the death of some A_{2A}R-deficient mice [4]. By contrast, no deaths were observed among wild-type mice. Notably, sub-threshold doses of inflammatory stimuli, which were incapable of producing liver damage in wild-type mice, induced extensive liver damage and sustained levels of cytokines in mice lacking A_{2A}R expression. These data suggest that the A_{2A}R plays a crucial and non-redundant role in the downregulation of inflammation *in vivo*. Such a conclusion is justified by the failure of all other anti-inflammatory mechanisms to compensate for the absence of A_{2A}R in preventing dramatic tissue damage. However, it is likely that in less extreme conditions, with lower levels of inflammatory stimuli, there could be several different anti-inflammatory mechanisms acting in a coordinated manner, which might involve other adenosine receptors and other important anti-inflammatory molecules [3].

In a series of control experiments, it was shown that although A_{2A}R-deficient mice do not express functional A_{2A}R, they do express other cAMP-elevating candidate receptors (e.g. β-adrenergic and prostaglandin receptors) [4].

Studies in wild-type mice in which selective antagonists of the A_2 receptors were used to block the effects of endogenous extracellular adenosine *in vivo* addressed possible caveats for interpreting experiments using genetically engineered mice [4]. These experiments confirmed the conclusions derived from the studies of A_{2A}R gene-deficient mice – injection of A_{2A}R antagonists also exacerbates tissue damage by sub-threshold doses of inflammatory stimuli. In addition, control experiments confirmed that although extracellular adenosine-triggered cAMP accumulation was severely inhibited in A_{2A}R-deficient mice, ligands to other G_s-protein-coupled receptors were still capable of inducing similar degrees of elevation of cAMP in wild-type and A_{2A}R-deficient mice [43]. Other studies confirmed that A_{2A}R-deficient mice

did not have unanticipated spontaneous mutations in the cAMP-signaling, inflammation-inhibiting pathway downstream of the $A_{2A}R$ [4]. Although selective $A_{2A}R$ agonists were not able to inhibit acute inflammation in $A_{2A}R$-deficient mice, agonists to other G_s-protein-coupled receptors were strong inhibitors of inflammation in wild-type and $A_{2A}R$-deficient mice.

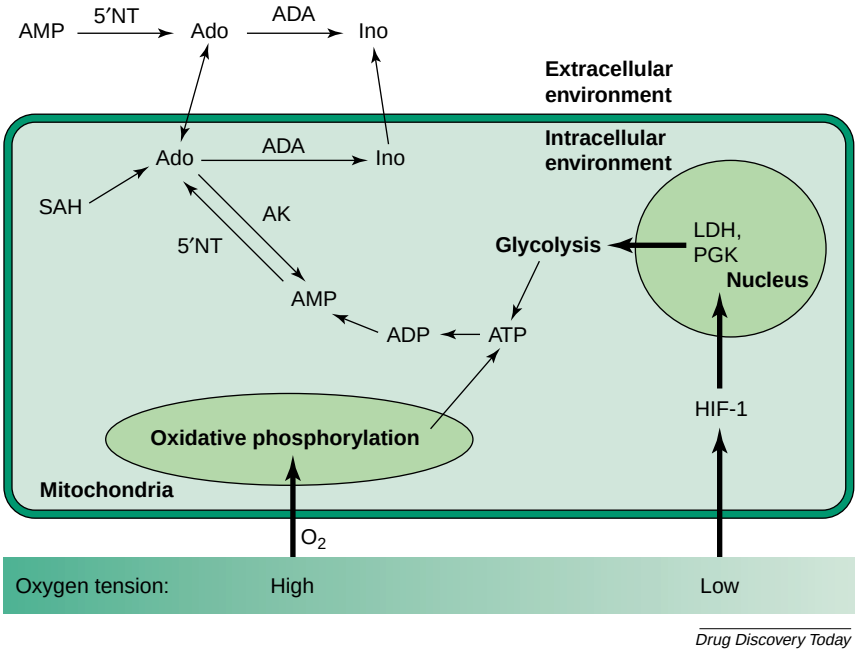

Figure 2. Cellular metabolism of adenosine at normoxia and hypoxia. At normal oxygen tension (normoxia) ATP is generated from the oxidative phosphorylation of ADP, whereas low oxygen tension (hypoxia) triggers the switch to hypoxia-induced glycolysis, which is regulated by the activity of HIF-1. HIF-1 is responsible for the transcription of several glycolytic enzymes (e.g. LDH, PGK, Glut-1 and ALDA). Ado is formed from SAH and from the hydrolysis of AMP by 5′-NT. Some Ado is rephosphorylated to AMP by AK and some is converted to Ino by ADA. Abbreviations: ADA, adenosine deaminase; Ado, adenosine; AK, adenosine kinase; ALDA, aldolase A; Glut-1, glucose transporter-1; HIF-1, hypoxia-inducible factor-1; Ino, inosine; LDH, lactate dehydrogenase; 5′NT, 5′-nucleotidase; PGK, phosphoglycerate kinase; SAH, S-adenosyl-homocysteine.

Mechanisms of accumulation of extracellular adenosine in the local tissue environment

Extracellular adenosine can be derived from pharmacological treatments and be produced during physiological and pathophysiological conditions. Adenosine is predominantly formed from AMP during the catabolism of cytosolic ATP. Studies of 5′-nucleotidase-I have provided additional insights into the mechanisms of adenosine metabolism [61,62] (Figure 2). The cellular availability of adenosine is under strict control through its catabolism by ADA and through phosphorylation by adenosine kinase to produce AMP. The relative contribution of the different pathways regulating adenosine availability in relation to different disease states and different local tissue environments has yet to be established. Such studies are complicated by the need for a better understanding of the important role of hypoxia in regulating adenosine metabolism and the difficulties encountered in mimicking *in vivo* redox conditions in *in vitro* experiments.

The accumulation of adenosine in ADA deficiency is a dramatic example of adenosine-based pathology; increased levels of adenosine and deoxyadenosine are detected *in vivo* using the ADA SCID mouse model [50]. In addition, the action of commonly used drugs such as the anti-inflammatory drug methotrexate leads to the accumulation of adenosine [63]. Pharmacologically induced extracellular adenosine has been implicated in the action of the anti-inflammatory drug nimesulide [64] and the neuroprotective drug propentofylline [65]. As a result of the release of adenosine from immune cells and other cell types, inflammatory tissue damage could be accompanied by accumulation of extracellular adenosine in inflamed areas [25,26]. Tissue damage-associated hypoxia represents another important source of adenosine [26]. Inhibition of adenosine kinase [66] and enhancement of 5′-nucleotidase activity [67] under hypoxic conditions might represent important mechanisms of adenosine accumulation in sites of local tissue injury. Thus, the understanding of extracellular adenosine-based mechanisms points to the need for extensive studies of local tissue hypoxia, and the role of hypoxia-inducible factors and their possible role in regulation of the immune system *in vivo* [16–18]. These questions are most often discussed in the context of normal cell metabolism and cancer tumor biology [68], but the tools and approaches developed by scientists working in these fields could be employed for the studies of inflammatory tissue damage. Indeed, the role of HIF-1α in the regulation of lymphocyte-mediated tissue damage [18] and myeloid cell-mediated inflammation [69] was shown using tissue-specific gene-knockout mice.

The activity of HIF-1α probably adds to or synergizes with the action of immunosuppressive extracellular adenosine via the $A_{2A}R$ (Figure 1). Local tissue hypoxia (developed as the result of local tissue damage caused by the action of proinflammatory cytokines) represents one of the first

events in the initiation of inflammation termination; this process creates conditions that are conducive to the accumulation of extracellular adenosine and stabilizes hypoxia-inducible factors such as HIF-1α and HIF-2α.

Conclusions

The development of drugs that are capable of minimizing tissue damage during inflammation might benefit from targeting the 'natural' pathways of hypoxia and adenosine receptor-mediated signaling. The crucial role of extracellular adenosine receptors in downregulating inflammation *in vivo* [4] represents an important example of physiological, metabolic changes in the inflamed local tissue environment that serve as 'reporters' of excessive tissue damage. In turn, these changes can provide a signal that can terminate the inflammatory process. Because adenosine can be produced by virtually all cells during the course of their metabolic activity, adenosine receptor-mediated signaling might form a negative-feedback loop through which extracellular adenosine can initiate immunosuppressive signaling that can inhibit immune cells involved in the initiation and maintenance of inflammation. Recent advances in the design, synthesis and analysis of adenosine-based ligands [38–40,69–71] have provided promising examples of potential applications of $A_{2A}R$ ligands in different clinical situations.

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