

# Adenosine in inflammatory joint diseases

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**Abstract** Inflammatory joint diseases are a group of heterogeneous disorders with a variety of different etiologies and disease manifestations. However, there are features that are common to all of them: first, the recruitment of various inflammatory cell types that are attracted to involved tissues over the course of the disease process. Second, the treatments used in many of these diseases are commonly medications that suppress or alter immune function. The demonstration that adenosine has endogenous anti-inflammatory functions and that some of the most commonly used anti-rheumatic medications exert their therapeutic effects through stimulation of adenosine release suggest an important role for purinergic signaling in inflammatory rheumatic disorders.

**Key words** adenosine · adhesion molecules · cytokines · methotrexate · P1 receptors · superoxide

Inflammatory joint diseases are a heterogenous group of disorders afflicting not only joint tissues but often multiple organs in the body. While known etiologies differ among the variety of diseases, treatment often involves medications with immuno-modulatory functions. It has been known for a long time that deficiency of adenosine

deaminase, the principal catabolizing enzyme for adenosine and deoxyadenosine, is associated with immunity dysfunction. Indeed, one-third of patients with autosomal recessive severe combined immunodeficiency exhibit adenosine deaminase deficiency. The disorder is characterized by abnormal development of lymphocytes in the thymus and may affect maturation of T cells as well as that of B cells [1]. This suggests that endogenous adenosine might have an important immunosuppressive role. The immune effects of adenosine deaminase deficiency have been well studied in animal models of this disease. It appears that the toxic effects on T and B lymphocytes are related to increased concentrations of 2'-deoxyadenosine, which, through various mechanisms, increase the rate of apoptosis in the developing thymocytes [2, 3]. With the discovery of the effects of adenosine in modulating the generation of free oxygen radicals in neutrophils, it was clear that the effects of adenosine on the immune system were widespread and not limited to lymphocytes alone [4]. As interest in the anti-inflammatory effects of adenosine grew, it became known that the release of adenosine is important in mediating the therapeutic effects of methotrexate, one of the most widely used disease-modifying medications for rheumatoid arthritis. We will explore some of the physiological mechanisms of adenosine and its receptors relevant to the perpetuation as well as treatment of the rheumatic diseases (Fig. 1).

## The cellular components of inflammatory joint diseases

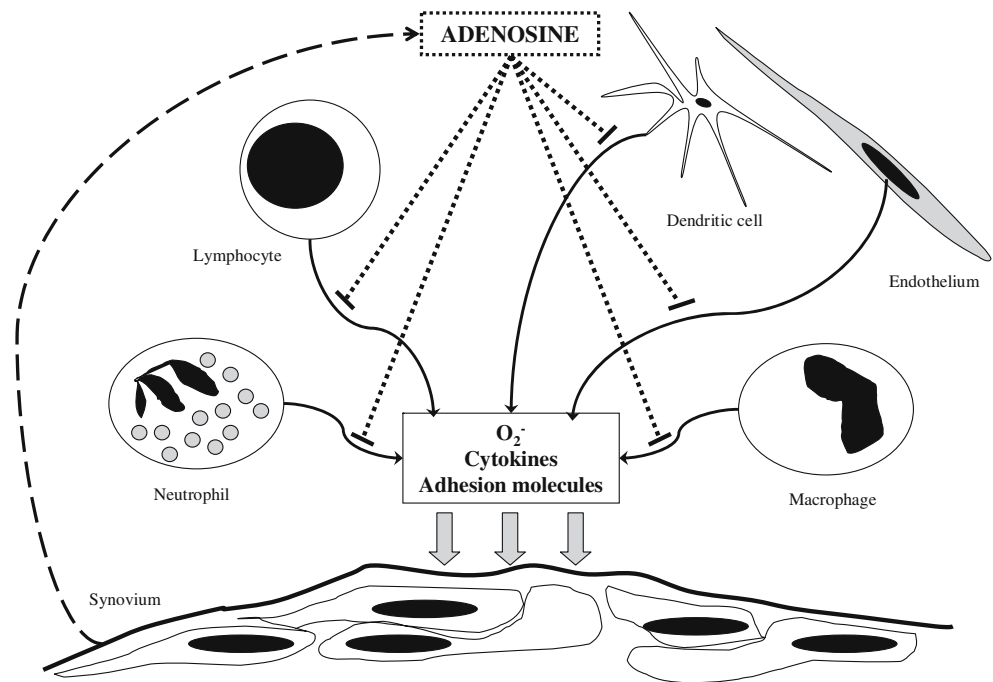
### Neutrophils

A hallmark of the inflammatory response is the recruitment of inflammatory cells into the site of inflammation. Histological observation of the cell types involved has

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**Fig. 1** The effects of adenosine in inflammatory tissue injury



been carried out for as long as the microscope has existed. Early observers have identified the polymorphonuclear neutrophil as an important player in the acute inflammatory response. These cells, when activated, can generate inflammatory damage via various mechanisms. One of the most important of these mechanisms is the generation of free oxygen radicals. In the initiation phase of oxygen radical formation, an NADPH oxidase is assembled within the membrane and the superoxide anion is generated from molecular oxygen in a chain of chemical reactions. There are many known physiological stimulators for the superoxide anion, including the chemo-attractant *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), the complement component C5a, and artificial stimulants such as the calcium ionophore A23187. In 1983 it was shown for the first time that adenosine can dramatically suppress the generation of superoxide anions in neutrophils via interaction with cell surface receptors, which was thought to be the A<sub>2</sub> receptor at the time [4–7]. The inhibition of superoxide anion generation was reversed by the addition of adenosine deaminase to the FMLP-stimulated neutrophils in culture. Furthermore, G<sub>αs</sub> receptor stimulation led to a rise in intracellular 3', 5' cyclic adenosine monophosphate (cAMP), and inhibition of protein kinase A downstream of this signaling pathway by the inhibitor KT5720 reversed the effects of cAMP analogs but not adenosine receptor agonists on superoxide anion generation in the stimulated neutrophils. These observations are somewhat controversial, as Sullivan et al. have reported that cAMP and protein kinase A mediate the effects of adenosine receptor occupancy in tumor necrosis factor (TNF)-treated neutro-

phils [8]. Recent observations suggest that it is possible that cAMP–protein kinase A-mediated effects are more important in regulating function in TNF-treated cells because of the dramatic increase in cAMP generated in TNF-treated cells [9–11]. It was later reported that the receptor in question responsible for these functions was, indeed, the adenosine A<sub>2A</sub> receptor [12, 13]. These findings led to interest in the immuno-modulatory functions of adenosine.

Not only is adenosine important in modulating neutrophilic function, but, much earlier than that in the cascade of inflammatory events, it also influences the recruitment of cells into the inflammatory site by regulating the expression of adhesion molecules [14–16]. Adhesion of the stimulated neutrophil to the endothelium and its chemotaxis are promoted by stimulation of the adenosine A<sub>1</sub> receptor, whereas stimulation of the A<sub>2</sub> receptor suppresses neutrophil adhesion and the generation of oxygen radicals [17]. The effects on neutrophil adhesion are relevant to clinical practice, since the expression of adhesion molecules has been shown to be upregulated in circulating blood neutrophils in real-life patients with diseases associated with widespread inflammatory activation such as sepsis [18]. Furthermore, adenosine A<sub>2A</sub> receptor activation counteracts the enhancement of adhesion molecule expression in the stimulated neutrophils from these patients. Those findings highlight the opposing roles of the A<sub>1</sub> and A<sub>2</sub> receptors in inflammatory modulation, i.e., proinflammatory and anti-inflammatory, respectively. Transmigration of the neutrophil through the endothelium, as well as the release of vascular endothelial growth factor by neutrophils, are both modulated by the A<sub>2B</sub> receptor [19].

## Macrophages and dendritic cells

Cells of the monocyte–macrophage lineage are significant contributors to tissue damage in the rheumatoid synovium. Their capacity to do so is exemplified by their ability to phagocytose opsonized particles, generate oxidant-induced damage, and produce a whole host of proinflammatory cytokines that augment the inflammatory response. These cytokines can modulate the function and expression of adenosine  $A_{2A}$  as well as  $A_{2B}$  receptors [9–11, 20]. Adenosine and its analogs can, in turn, regulate production of these cytokines, as well as nitric oxide synthase expression in macrophages [20, 21].

Lipopolysaccharide-induced interleukin (IL)-12 production by monocytes and macrophages is an effect mediated by toll-like receptor-4 (TLR4), and IL-12 elicits a strong inflammatory response. Adenosine exerts its anti-inflammatory effects in part by suppressing the production of IL-12 [9, 22–24]. Much like the case with neutrophils, FMLP induces the generation of superoxide anions in monocytes. Adenosine, in micromolar concentrations, suppresses this respiratory burst response to FMLP but not to the induction of phorbol myristate acetate [25]. The Fc gamma receptor serves an important function in mediating phagocytosis in monocytes. Stimulation of the adenosine  $A_1$  receptor promotes Fc gamma receptor-mediated phagocytosis by monocytes, whereas  $A_2$  receptor agonists suppress phagocytosis. Another example of the opposing effects of  $A_1$  and  $A_2$  receptors can be seen with multi-nucleated giant cell formation. Multi-nucleated giant cells, formed by the fusion of multiple macrophages, are a distinctive feature of the subcutaneous nodules. These are seen both as a manifestation of rheumatoid arthritis itself and as a consequence of one of its most common drug treatments, methotrexate. Multi-nucleated giant cell formation from stimulated human monocytes is promoted by  $A_1$  receptor stimulation, while activation of the  $A_2$  receptor has the opposing effect [26].

Dendritic cells are also important players in antigen presentation, and they too express adenosine receptors. Lipopolysaccharide induces maturation of dendritic cells, and, as they mature, the  $A_1$  and  $A_3$  receptor message is downregulated, while no change is observed with  $A_{2A}$  receptor mRNA [27]. Following activation of the dendritic cell,  $A_{2A}$  receptor ligation inhibits its production of IL-12, much as occurs in macrophages [24]. Other cytokine-modulating effects exist, to include suppression of tumor necrosis factor- $\alpha$  as well as enhancement of secretion of the anti-inflammatory cytokine, interleukin-10 [28]. These effects are also attributable to the  $A_{2A}$  receptor. Furthermore, dendritic cells that matured in the presence of adenosine were less able to promote the differentiation of CD4-positive naïve T lymphocytes towards a Th1-pheno-

type, and this has great implications in limiting a T cell-initiated proinflammatory response [28].

## Lymphocytes

The T lymphocyte remains one of the key cell types responsible for the inflammatory state in the rheumatoid synovium. At low concentrations of extracellular adenosine, inhibition of T cell receptor-triggered proliferation and induction of interleukin-2 $\alpha$  chain expression has been observed, and these may be important contributing mechanisms for causing peripheral T cell depletion [29]. These effects are mediated through the adenosine  $A_{2A}$  receptor. Indeed, adenosine acts as a strong suppressant of many known T cell receptor-triggered effector functions of T lymphocytes, such as induced FasL expression on cytotoxic T lymphocytes. While exposure to ligands may be brief, effector functions and cellular changes in cAMP may be more prolonged and contribute to T cell memory [30]. Much as the response to ligand activation varies among T lymphocytes, adenosine receptor expression is also cell type-dependent, and lymphokine-producing T helper cells have been cited to be much more likely to express  $A_{2A}$  receptors [31]. T cell receptor activation is associated with a rapid induction of adenosine  $A_{2A}$  receptor message expression, and  $A_{2A}$  receptor activation profoundly suppresses T cell receptor-mediated interferon- $\gamma$  production [32]. Thus, adenosine  $A_{2A}$  receptor activation is generally beneficial for the impairment of T lymphocyte-triggered events in the pathogenesis of rheumatic disease.

## Endothelial cells

The vascular endothelium limits the flow of traffic between the vascular lumen and surrounding tissues, and as such, it serves an important function in regulating the influx of inflammatory cells into the site of inflammation. Activation of the endothelium may result from the influence of a vast number of inflammatory mediators, including cytokines such as tumor necrosis factor- $\alpha$  and interleukin-1 as well as immune components such as immune complexes and complement components. Endothelial permeability is increased by oxidant injury, and the resultant extravasation of fluid normally contained within the vessel lumen can be seen as inflammatory edema. The neutrophil, once activated, is induced to release AMP and adenosine, which augments this barrier function in an *in vitro* endothelial paracellular permeability model [33]. The endothelium has the intrinsic ability to generate adenosine by dephosphorylation of AMP through the action of 5'-ectonucleotidase. Passage of Evan's blue dye-labeled albumin across confluent monolayers of human umbilical vein endothelial cells (HUVECs) is hampered in the presence of adenosine, a

function thought to be mediated by adenosine  $A_1$  receptors [34]. Others have argued that the function of the endothelial barrier is more closely related to adenosine  $A_{2B}$  receptor activation, since  $A_{2B}$  receptor antagonists effectively reverse ATP-induced changes in endothelial permeability following hypoxic insult [35]. These data demonstrate that adenosine exerts its overall anti-inflammatory function in part by limiting extravasation at the endothelial level. In contrast, adenosine has also been reported to induce apoptosis in endothelial cells, a physiological activity that requires protein tyrosine phosphatase [36]. The outflow of inflammatory cells is similarly suppressed in the presence of adenosine. This occurs as a result of alteration in adhesion molecule expression such as E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in the presence of adenosine [37]. In addition, dose-dependent suppression of the expression of inflammatory cytokines IL-6 and IL-8 by stimulated HUVECs has also been observed [37]. Proliferation of endothelial cells is stimulated by the presence of adenosine, and this signals a role for adenosine in the promotion of angiogenesis, since neo-vascularization in the retina is suppressed by ribozymes that cleave  $A_{2B}$  receptor mRNA [38, 39]. The proliferative effect is, in part, mediated through induction of the formation of vascular endothelial growth factor [40]. Response to adenosine receptor occupancy varies between cell types, since agonist-induced upregulation of interleukin-8, vascular endothelial growth factor and basic fibroblast growth factor occurs in human microvascular endothelial cells (HMEC-1) but not HUVECs [41]. This effect, attributable to  $A_{2B}$  receptor activation, occurs via Gq and possibly  $G_{12/13}$  coupling. Tissue hypoxia has a modulatory effect on endothelial adenosine receptor expression in favor of an  $A_{2B}$  pro-angiogenic phenotype [42]. The adenosine  $A_{2A}$  receptor also has an important role to play in regulating angiogenesis.  $A_{2A}$  receptor stimulation in primary human endothelial cells is known to activate the mitogen-activated protein kinase signaling pathway [43]. Using a murine air pouch model, Montesinos et al. first demonstrated that angiogenesis is inhibited in adenosine  $A_{2A}$  receptor-deficient mice compared with wild-type littermates [44, 45]. It is now known that this occurs at least in part by a reduction in the expression of the anti-angiogenic matrix protein thrombospondin-1 and increased macrophage production of vascular endothelial growth factor [46, 47]. Moreover, topical adenosine  $A_{2A}$  receptor agonists stimulate recruitment of endothelial precursor cells from the bone marrow to injured sites [45].

#### Mast cells

Adenosine is known to be a stimulus for mast cell degranulation. It has been demonstrated that this may occur

through  $A_3$  receptor ligation, though it is interesting to note that inosine can also bind to the  $A_3$  receptor and cause degranulation of the mast cell [48, 49]. Since mast cell degranulation is generally thought of as a potent pro-inflammatory signal, it is difficult to reconcile this seemingly untoward physiological function with the overall anti-inflammatory nature of adenosine release. One possible explanation is that the release of histamines contained within the granules is a stimulus for suppression of the production of tumor necrosis factor- $\alpha$  and interleukin-12 while enhancing the release of the anti-inflammatory interleukin-10, at least in a murine model of endotoxemia [50]. Furthermore, upregulation of Th2 cytokines in mast cells may affect the immune function of other cell types, since adenosine-activated mast cells induce the production of IgE by B lymphocytes, an effect mediated by the adenosine  $A_{2B}$  receptor [51].

#### Methotrexate

Treatment of rheumatoid arthritis took a great leap half a century ago with the discovery of the beneficial effects of corticosteroids on this disease, a discovery that was, indeed, crowned by the award of a Nobel prize. It was around this time that methotrexate came into clinical use. While long in use for the treatment of malignant diseases, the use of methotrexate in rheumatoid arthritis has only become widespread in the past 20 years [52–54]. Since then, it has been a mainstay of rheumatoid therapy, and its popularity, as well as its efficacy, in this disease has remained undisputed, even with the appearance of newer biological agents.

Methotrexate was originally developed as an anti-folate agent. As such, it has antiproliferative properties against immune cells active in the flaring rheumatoid synovium, such as the lymphocyte. This was indeed thought to be the principal mechanism of action of methotrexate in rheumatoid arthritis. Methotrexate induces clonal deletion of activated T lymphocytes in mixed lymphocyte reactions, and this effect holds true for peripheral blood lymphocytes taken from patients with rheumatoid arthritis [55]. However, the effect is short-lived, making it difficult to explain why administration of methotrexate in a dose frequency of only once a week, as is normally the case in rheumatoid arthritis treatment, could result in clinical immunosuppression from its lymphocyte antiproliferative properties alone.

Other theories have existed to explain the anti-inflammatory actions of methotrexate. Since methotrexate is an inhibitor of dihydrofolate reductase, it suppresses tetrahydrofolate synthesis. Tetrahydrofolate is a methyl group donor that enables the formation of methionine from homocysteine. A metabolite of methionine, *S*-adenosyl-

methionine, is also a methyl donor in the formation of the polyamines spermine and spermidine. These polyamines are processed by immune cells, including monocytes, resulting in toxic metabolites. Thus, methotrexate can inhibit the formation of detrimental polyamines. It has been shown that methotrexate can inhibit the generation of spermine and spermidine in stimulated rheumatoid lymphocytes [56]. Inhibitors of polyamine production increased interleukin-2 production by peripheral blood mononuclear cells from rheumatoid arthritis patients, suggesting that polyamines may be associated with diminution of T cell effector function [57]. Urinary polyamine levels are increased in patients with rheumatoid arthritis and may be associated with disease activity [58]. These effects are, however, reversed by folic acid, and are therefore insufficient to explain the anti-inflammatory effects of low-dose methotrexate that are unaffected by folic acid administration.

In the molecular pathways leading to the de novo synthesis of purines, accumulation of various intermediates can lead to the release of adenosine [59]. Methotrexate can be thought of as a prodrug. Once ingested, it is taken up by cells via the reduced folate carrier and, in turn, polyglutamated [60]. These polyglutamates, which remain in cells for time periods measured in weeks, are potent inhibitors of one of the key enzymes involved in the de novo biosynthesis of purines, aminoimidazole-carboxy-amido-adenosine ribonucleotide (AICAR) transformylase [61–63]. This enzyme inhibition effect occurs at pharmacologically relevant concentrations of methotrexate and can readily explain the clinically persistent anti-inflammatory effect of the infrequent once-a-week dosing interval. Thus, it is likely that the principal mechanism of action of low-dose methotrexate as used in inflammatory joint diseases such as rheumatoid arthritis is different from its cellular effects as a chemotherapeutic agent. Urinary excretion of aminoimidazole carboxamide was increased following methotrexate administration in a rat adjuvant arthritis model [64]. Inhibition of AICAR transformylase leads to an increase in its substrate, AICAR. The accumulation of AICAR, in turn, has important effects on key enzymes that influence adenine nucleotide metabolism. AICAR itself is an inhibitor of AMP deaminase, which converts AMP to inosine monophosphate. A metabolite of AICAR, aminoimidazole-carboxamido-ribonucleoside (AICAside), inhibits adenosine deaminase. The net effect of AICAR accumulation, therefore, is a rise in intracellular AMP and adenosine levels, an effect known to occur with methotrexate in pharmacological concentrations in animal models [14, 65].

These AMP and adenosine elevating effects of methotrexate are supported by various lines of evidence in vivo. The development of adjuvant arthritis in the rat was

suppressed by direct infusion of adenosine into the knee [66]. 8-Phenyl theophylline, a non-selective adenosine receptor antagonist, exacerbated leukotriene B<sub>4</sub>-mediated inflammatory responses in a hamster cheek pouch model of inflammation, suggesting that adenosine itself plays an anti-inflammatory role [67]. Szabo et al. reported that an adenosine A<sub>3</sub> receptor agonist, N<sup>6</sup>-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (IB-MECA), reduced the severity of joint inflammation in a collagen-induced model of arthritis. This was accompanied by a reduction in neutrophil infiltration and suppression of macrophage inflammatory protein (MIP)-1 $\alpha$  production in the paws of the animals [68].

We first reported that weekly treatment with methotrexate increased splenocyte AICAR concentrations and also increased adenosine levels in inflammatory exudates [14]. Blockade of adenosine receptors reversed the anti-inflammatory effects of methotrexate. Similarly, Montesinos et al. showed that weekly doses of methotrexate increased adenosine levels in the exudates of inflamed murine air pouches, which was associated with a decrease in leukocyte accumulation and tumor necrosis factor- $\alpha$  production, effects not seen in adenosine A<sub>2A</sub> or A<sub>3</sub> receptor-deficient mice [69]. The non-selective adenosine receptor antagonists, theophylline and caffeine, both reversed the beneficial effects of methotrexate in a rat adjuvant arthritis model of rheumatoid arthritis, as evidenced by disease severity index, hindpaw swelling, and hindpaw ankylosis [70].

Interestingly, the observation that caffeine reverses the anti-inflammatory effects of methotrexate has also been noted in humans. Silke et al. first reported that coffee ingestion was associated with poor clinical response to methotrexate, and patients with high caffeine intake were more likely to discontinue methotrexate treatment, presumably because of lack of response [71]. Coffee itself has been reported to have been directly correlated with rheumatoid factor positivity, and consumption of more than four cups of coffee a day was associated with a greater risk of developing frank rheumatoid factor-positive rheumatoid arthritis [72]. Neshet et al. have confirmed the reduction in efficacy of methotrexate treatment by caffeine consumption in patients with rheumatoid arthritis [73]. Recently, Ralph and colleagues reported that reduction in synovial expression of the orphan nuclear receptor NURR1 is the first change observed in patients treated with methotrexate and that the effects of methotrexate on NURR1 are mediated by adenosine acting at A<sub>2A</sub> receptors [74].

Although we have focused our discussion on methotrexate, other anti-rheumatic and immuno-modulatory agents have also been shown to exert their anti-inflammatory effects in an adenosine-dependent manner. Most important among these are sulfasalazine [75, 76], FK506 [77] and aspirin [78, 79].

## Conclusion

The role of adenosine in the modulation of inflammation has been appreciated only in recent years, yet this Cinderella of the inflammatory response has a clear influence on many cell types that govern the pathogenesis of inflammatory rheumatic diseases. The role of cytokines in the mediation of rheumatic joint damage has been exploited in recent anti-rheumatic drug development. Adenosine also has a part to play in modulating the generation of cytokines relevant to the development of rheumatic diseases. The overall effects of adenosine, often beneficial in rheumatic disease, are exemplified by its involvement in commonly used anti-rheumatic drug treatments. Adenosine analogs may one day find their way into the clinic.

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