Chapter 4

Pharmacology of Adenosine Receptors and Their Signaling Role in Immunity and Inflammation

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1. Introduction

Since the late 1920s, the importance of the physiological role of adenosine triphosphate (ATP) and its metabolites, mainly adenosine, has been clear. In 1970, strong evidence now suggests ATP as a neurotransmitter in nonadrenergic, noncholinergic (NANC) nerves supplying the gut. Initially, the hypothesis of purinergic transmission encountered some resistance, however, this concept is now widely accepted and purines are considered powerful extracellular messengers in peripheral and central nervous system and to non-neuronal cells, including immune, and inflammatory cells. Implicit in the purinergic hypothesis was the presence of purinoceptors. The first evidence for the existence of adenosine receptors, responsible for the physiological effects of adenosine, was only published in the beginning of the 1970s. Throughout the 1990s and 2000s adenosine receptors were cloned, and the mechanisms of signal transduction mediated by these receptors were described. Currently, it is well known that adenosine activates four G protein-coupled receptors named A_1, A_{2A}, A_{2B} and A_3. The A_1 and A_3 receptors preferentially interact with members of the $G_{i/o}$ family of G proteins, lowering the intracellular levels of cyclic adenosine monophosphate (cAMP), whereas the A_{2A} and A_{2B} receptors interact with members of the Gs family of G proteins, elevating of intracellular cAMP.

Throughout the 1980s and 1990s, several studies demonstrated that ATP metabolites, especially adenosine, are important signaling molecules, and adenosine receptors are important molecular targets in inflammation and immunity. During inflammation, the generation of the appropriate immune response can itself cause considerable damage and thus requires effective regulation. It has been suggested that this regulation of the immune system requires the

sensing of specific signals called “danger signals”. Thus, in the context of purinergic signaling, it has been proposed that the regulation of the immune system requires at least two ‘danger’ signals, the first (ATP) indicating the presence of danger from pathogens or other injurious events and leading to the activation of immune cells and defensive effector function and the second (adenosine) indicating the danger from overactive immune cells and triggering the downregulation of the proinflammatory activities of the immune system. These discoveries increased the interest in the purinergic signaling pathways, and there was an increase in publications studying the effects of adenosine and inosine in inflammation and immunity (Figure 1 A and B).

Substantial evidence demonstrates that adenosine receptors are expressed in most inflammatory cells and may therefore modulate different steps involved in inflammatory and immune responses. Moreover, several recent studies have indicated that inosine, a metabolite of adenosine, once believed to be an inert metabolite, can exert many immunomodulatory actions through adenosine receptors, mainly the A_2 and A_3 receptors. In this regard, adenosine receptors have become potential therapeutic targets for the treatment of several pathologies in which inflammatory modulation is a key component. In this regard, adenosine and inosine can be considered molecules with valuable therapeutic potential, performing the desired effect with minimal side effects, such as those present in therapy with adenosine (Adenocard®) for the treatment of paroxysmal supraventricular tachycardia (facial flushing, chest pressure, hyperventilation, dizziness, numbness and tingling). Moreover, no studies clearly demonstrate the side effects of inosine. However, it is important to warn that the purines can play deleterious effects not observed clinically, depending on the target tissue and the receptor that is activated according to figure 3.

Thus, this chapter was designed to highlight the importance of ATP metabolites, especially adenosine and inosine, and their modulatory effect on inflammation through the activation of adenosine receptors. In addition, we aimed to provide updated information about the pharmacology of adenosine receptors, especially about its proinflammatory versus anti-inflammatory effects. Furthermore, we are able to clarify the overall effect of adenosine and inosine in different inflammatory diseases. Finally, we intend to present a short overview concerning the advances in drug development targeting adenosine receptors.

2. The ATP metabolic pathways

Carbohydrates, lipids and proteins, also called "metabolic fuels" are constantly being oxidized to provide energy. Glucose is generally the primary energy source for cellular metabolism. It is catabolized by the following three main processes: glycolysis, the tricarboxylic acid (TCA or Krebs) cycle and oxidative phosphorylation, which lead to the production of ATP, the final energy-rich product that is used in many different active processes in an organism. Macromolecule synthesis, muscle contraction, active transport of ions and thermogenesis are some of the key processes that require energy. Since the mid-1920s, when ATP was discovered as a substrate used in muscle contraction, knowledge about this high-energy molecule has

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constantly been expanded. The literature has shown that many aspects of cellular metabolism are directly linked to the production and consumption of ATP and has also emphasized its importance in purinergic signaling mechanisms. In this context, taking into account the importance of ATP in the maintenance of homeostasis and the evidence indicating the role of its metabolites in the control of immunity and inflammation, understanding ATP metabolism in the body has become more imperative [1].

2.1. The degradation of ATP and formation of ADP, adenosine and inosine

In situations of high energy demand such as inflammation and hypoxia, ATP may be converted into adenosine monophosphate (AMP) in the intracellular environment through a reaction dependent on ATPase and adenylate kinase. AMP can be converted into adenosine by the intracellular enzyme 5-nucleotidase and thereafter can be transported to the extracellular environment via bidirectional nucleoside transporters. ATP in the extracellular environment can activate P2 –type receptors in the surroundings or generate adenosine via ecto-5-nucleotidase (NT5'E), the primary enzyme responsible for ATP metabolism under physiological conditions [2]. In the extracellular space, ATP and ADP are converted to AMP through the ectonucleoside triphosphate diphosphohydrolase-1 (CD39). The second step for extracellular adenosine formation is the ecto-5'-nucleotidase (CD73) conversion of extracellular AMP into adenosine. During inflammation and hypoxia, an increase in the activity and expression of adenosine deaminase and in its binding partner, CD26, has also been demonstrated [3]. This increase promotes adenosine conversion into inosine within seconds, terminates adenosine signaling and can thus initiate inosine signaling. Inosine can be converted into hypoxanthines and uric acid (UA) by purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO), respectively [4, 5]. Current studies using mice lacking the CD39 and CD73 genes have revealed the importance of these enzymes in contributing to extracellular adenosine generation in different organs and situations [5]. In agreement with those studies, certain CD39 polymorphisms increase ATP and ADP, lowering extracellular adenosine levels, which can lead to increased susceptibility to inflammatory pathological conditions such as inflammatory bowel disease (IBD) and multiple sclerosis (MS) [6, 7]. Furthermore, the loss-of-function mutation of CD73 in humans is suggested to be the basis for the development of peripheral arterial calcifications, indicating that adenosine generation can be vasoprotective [8, 9]. Currently, it is known that after adenosine is released from cells or generated in extracellular space, it diffuses into the surroundings, where it binds to adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) on adjacent cells. Finally, after adenosine generation and receptor activation, adenosine diffuses away from the receptor and is rapidly transported into the intracellular space mainly through equilibrate nucleoside transporters (ENT-1 and ENT-2) [5, 10, 11].

3. ATP metabolites as danger signals: the role of adenosine and inosine

During inflammation, infection or hypoxia, the generation of the appropriate immune response can itself cause considerable damage and thus requires effective regulation [12]. It has been suggested that this regulation of the immune system requires the sensing of specific

signals called "danger signals" [13-15]. Although the immune response can be activated by recognizing the signatures of foreign pathogens, collectively called pathogen-associated molecular patterns (PAMPs), it is also able to respond to endogenous host molecules to trigger inflammatory responses. Most of these are produced as a result of cell death or injury or by tumor cells; they include degradation products of the extracellular matrix (ECM), heat-shock proteins and high-mobility group box 1 (HMGB1) proteins, UA crystals, amyloid-β and oxidized LDL (Ox-LDL), which act as stimulators for pattern recognition receptors (PRRs) and have been referred to as danger-associated molecular patterns (DAMPs) [15, 16].

Extracellular ATP and UA are well-characterized dangers signals, likely released from cells as a consequence of cell damage or nonapoptotic cell death. The exposure of local cells to elevated extracellular ATP and monosodium urate crystals has been described as proinflammatory because it activates P2X7 receptors, NALP3 (a member of NOD-like receptors, also called cryopyrin) and caspase-1 [17-19]. This activation leads to the processing and release of interleukin 1β (IL-1β) and results in inflammation [14, 20]. Furthermore, the elevation of extracellular ATP has been demonstrated to guide circulating neutrophils to the inflammatory microenvironment and can function as a "find-me signal" to attract inflammatory cells (particularly phagocytes) and direct the inflammatory response [12, 21].

Recent studies have shown that in situations of inflammation, trauma or hypoxia when extracellular ATP concentrations are elevated, there is an increased expression of ectonucleotidases that rapidly convert ATP/ ADP into adenosine, terminating the proinflammatory effects of ATP [12]. Thus, in the context of purinergic signaling, it has been proposed that the regulation of the immune system requires at least two 'danger' signals, the first (ATP) indicating the presence of danger from pathogens or other injurious events and leading to the activation of immune cells and defensive effector function and the second (adenosine) indicating the danger from overactive immune cells and triggering the downregulation of the proinflammatory activities of the immune system [13, 22].

The effects of adenosine in different tissues may depend upon the repertoire of adenosine receptors present on the cell surfaces [22]. In this context, the A_{2A} and A_{2B} receptors have been described as the receptors most involved in the control of immunity and inflammation [13, 22, 23]. By binding to A_{2A} and A_{2B} receptors, adenosine triggers cAMP elevation in T cells, which results in the activation of CREB/ATF (cAMP-responsive element (CRE)-binding protein/ activating transcription factor), an immunosuppressive mechanism [12, 23, 24]. This activation has been shown to trigger Treg cell activation, the production of anti-inflammatory cytokines such as TGF-β and IL-10 and inhibit the functional response of TCR- activated T effector cells, reducing the secretion of IL-2 and IFN-γ [24]. Moreover, similar to adenosine, the metabolite inosine is also known to exert wide raging anti-inflammatory effects, which include inhibition of proinflammatory cytokines, chemokine production and protection from septic shock, colitis and acute lung injury [25-27]. Some studies have shown that inosine can stimulate adenosine A_{2A} receptors and is protective in models of concanavalin A-induced liver damage, endotoxin-induced sepsis [28, 29] and TNBS-induced colitis [30]. In this context, although there is no description of inosine as a danger signal in the current literature, some studies have described inosine as tissue protective. Taking into account that it can activate the same sensors (receptors)

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as adenosine, we speculate that inosine might be an additional danger signal that could work with adenosine to dampen inflammation.

In summary, the immunosuppressive effects of adenosine have been broadly described in the literature as a "retaliatory metabolite" [31] or an "engineer" of inflammation [22], indicating that adenosine can manipulate the intensity and the time course of inflammatory process in vivo and suggesting biochemical control of immunity. These events appear to be biologically coordinated and may constitute a homeostatic mechanism of tissue integrity. Therefore, the failure of this protective mechanism may contribute to beginning and perpetuating chronic inflammation response.

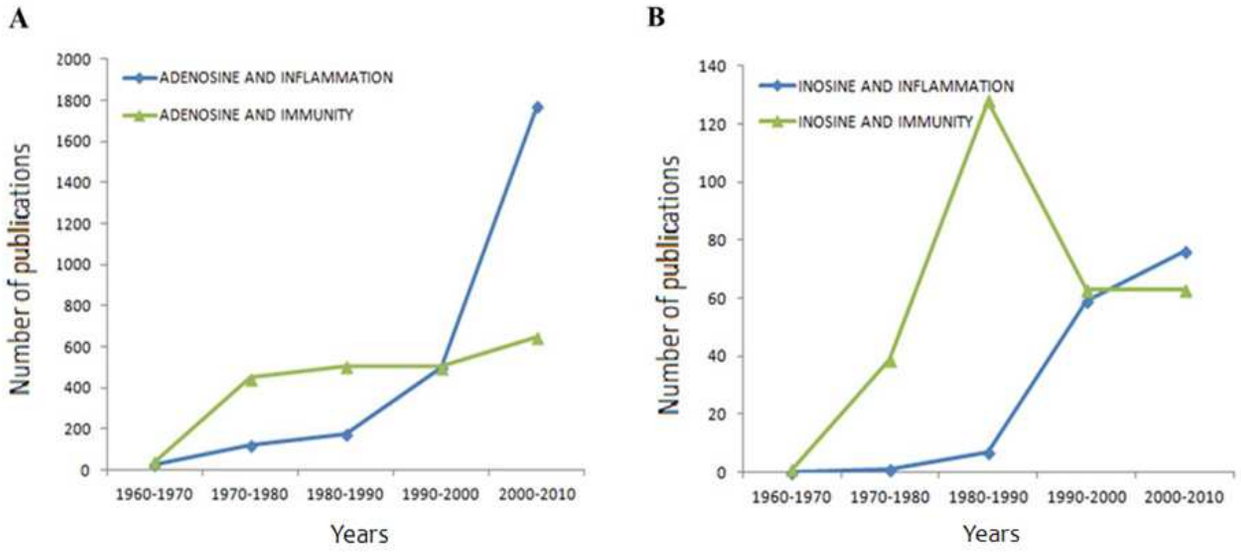

Figure 1. The number of publications regarding the investigation of adenosine and inosine effects in inflammation and immunity since the 1960s.

4. Adenosine receptors and inflammation: proinflammatory versus anti-inflammatory effects

4.1. A_1 adenosine receptors

The adenosine A_1 receptor is coupled to the $G_{i/0}$ family of G proteins, lowering the intracellular levels of cAMP [32, 33]. Activation of A_1 receptors leads to increased intracellular Ca^{+2} levels due to the stimulation of phospholipase C, which in turn promotes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Figure 2). Moreover, the enhancement of intracellular calcium can activate certain enzymes, such as protein kinase C (PKC), phospholipase D (PLD), phospholipase A_2 (PLA_2) and others [10, 33].

The recently reported crystal structure confirms that adenosine receptors display the typical topology of GPCRs, a common central core domain consisting of seven transmembrane (TM) helices numbered from 1 to 7 that are composed of 20-27 amino acids and that are largely α-helical. The TM domains are also slightly bent and linked by three intracellular (IL-1, IL-2 and IL-3) and three extracellular (EL1, EL2, and EL3) loops [34]. The A_1 receptor amino acid sequence varies from 324 to 328 residues [35]. In 1992, Ijzerman and colleagues highlighted the role of two histidine residues in ligand binding, one located in TM6 and one in TM7 [36]. This result was in agreement with the first mutagenesis data on adenosine receptors, published in the same year, performed on bovine A_1 receptors, which demonstrated that residues His251 (6.52) and His278 (7.43) were important for ligand binding. The same study also revealed that the mutation of histidine residues to leucine led to a decrease in ligand affinity, especially His278 [37]. In 1994, two mutagenesis studies suggested a role of residue 270 (7.35, isoleucine or methionine in the bovine/canine receptor) in the binding of *N*6-adenine substituted compounds (A_1 receptor agonists) and the importance of residue 277 (7.42, threonine in human/bovine receptor) in the recognition and interaction of the adenosine ribose moiety [38, 39]. It has also been demonstrated that the Pro25Leu (1.48) mutation and substitution of Leu88 (3.33), Thr91 (3.36), and Gln92 (3.37) by alanine reduces the affinity for *N*6-unsubstituted adenosine derivatives [39]. In the same study, it was also demonstrated that the Gly14Thr (1.37) mutation increases the receptor's affinity for agonists, suggesting the constitutively active form of this mutant receptor [40]. Finally, analysis of the Thr277Ala (7.42) mutation by an allosteric enhancer suggested an allosteric role for this residue [41].

There is no consensus regarding the effects of A_1 receptors in the inflammatory response; some studies suggest proinflammatory effects, while others suggest anti-inflammatory effects. The A_1 receptors are expressed in leukocytes. At submicromolar adenosine concentrations, the activation of these receptors in human neutrophils produces a proinflammatory response by promoting chemotaxis and adherence to the endothelium [42, 43]. In lymphocytes, A_1 receptor antagonism contributes to adenosine's anti-inflammatory effects by reducing the expression of intracellular adhesion molecule-1 (ICAM-1), production of IL-12 and IFN-γ and lymphocyte proliferation [44]. In addition to these data, various studies with selective agonists and antagonists demonstrated the proinflammatory effects of A_1 receptors in different inflammatory models, some of which are summarized here. During acute pancreatitis induced with cerulein or taurocholate in rats, the selective A_1 receptors agonist CCPA (2-chloro-*N*6-cyclopentyladenosine) produced an increase in leukocyte infiltration and interstitial edema in pancreatic tissue, which was attenuated by FK-838 (6-oxo-3-(2-phenylpyrazolo[1,5-α]pyridin-3-yl)-1*(6H)*-pyridazinebutanoic acid), a selective A_1 receptor antagonist [45]. A_1 receptor antagonism was also protective in the lungs; treatment with DPCPX (8-cyclopentyl-1,3-dipropylxanthine), an A_1 receptor antagonist, prevented endothelial damage, neutrophil migration and alveolar injury in a model of ischemia reperfusion in the lungs [46]. In another interesting study, the A_1 receptor antagonist DPSPX (1,3-dipropyl-8-*p*-sulfophenylxanthine) decreased the area of cardiac necrosis and improved ventricular function in a canine model of myocardial ischemia reperfusion, most likely due to inhibition of neutrophil chemoattraction [43]. Pretreatment with KW3902, a selective A_1 receptor antagonist, preserved hepatic architecture, decreasing the infiltration of neutrophils into hepatic tissue in a hepatic ischemia

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reperfusion injury model in dogs [47]. Collectively, these findings demonstrate that the A_1 receptor is an important target in inflammation and that antagonists may be efficacious as anti-inflammatory drugs.

In opposition to such data, studies using pharmacological (selective A_1 receptor agonist or antagonists) and genetic tools (knockout animals) have shown that activation of the A_1 receptor can promote anti-inflammatory effects. CCPA, an A_1 receptor agonist, presented a protective effect in a mouse model of renal ischemia reperfusion, an effect reverted by DPCPX, a selective A_1 receptor [48]. Studies performed with knockout mice confirmed the renal protective effects of the A_1 receptor [49] and also revealed the protective effects of this receptor in other tissues and inflammatory conditions because the absence of the A_1 receptor promotes proinflammatory effects in the lungs, enhancing leukocyte migration and levels of cytokines, including IL-4 and IL-13 [50]. In the central nervous system (CNS), A_1 receptor knockout animals exhibited severe demyelination and axonal injury, involving the activation of macrophages and microglial cells [51]. In sepsis induced in mice, the A_1 receptor knockout animals had a higher degree of renal dysfunction induced by higher release of pro-inflammatory cytokines [52]. A previous study described the mechanism by which activation of A_1 receptor leads to anti-inflammatory effects, which involves phosphorylation of ERK, MAPK and Akt (Figure 2), all of which are involved in the upregulation of cytoprotective genes and also increase the phosphorylation of heat shock protein (HSP) 27, a molecular chaperone that prevents the denaturation and aggregation of cellular proteins, a cytoprotective effect [53]. In summary, the lack of consensus regarding the existence of the proinflammatory and anti-inflammatory effects of the adenosine A_1 receptor could be explained assuming that the A_1 receptor can activate intracellular signaling pathways that result in tissue injury or protection, through proinflammatory or anti-inflammatory effects, respectively, because the activated pathways depend on the following:

- the species/tissue/organ and the stage/progression of injury;
- Predominant inflammatory cell type as a function of species;
- Intracellular signaling and desensitization mechanisms as a function of species or cell/tissue/organ.

4.2. A_{2A} adenosine receptors

Most A_{2A} receptors are coupled to the Gs protein family. A subset, preferentially located in the striatum, is coupled to the G_{olf} protein family. It is well established that the biological effects triggered by A_{2A} receptors are due to enhancement of cAMP production followed by adenylate cyclase activation. The increase of the cAMP level stimulates cAMP-dependent kinase (PKA) (Figure 2), which, in turn, activates several pathways through calcium channels, potassium channels, cAMP responsive element-binding (CREB), mitógeno-activated protein kinase (MAPK) and phospholipase C (PLC) activation [23, 54].

The A_{2A} receptor structure is very similar to other adenosine receptors. However, it differs in the four disulfide links observed at the extracellular level, which are critical for the packing and stabilization of the restricted conformation of the seven transmembrane helices. Another difference concerns the A_{2A} receptor length of the C-terminal region, which consists of

approximately 120 residues [55]. In 1995, Kim et al. published the results of site directed mutagenesis experiments on A_{2A} receptors [56], revealing the essential role of some residues for ligand interaction, particularly Phe182 (5.43), Asn253 (6.55), Ile274 (7.39), and Ser281 (7.46). The key role of His250 (6.52), Ser277 (7.42) and His278 (7.43) was also confirmed. After 1995, several mutagenesis studies revealed some of the amino acids residues involved in direct or indirect interaction with ligands or in allosteric regulation. It was observed that the conserved Glu 1.39 is critical for agonist but not antagonist binding [57, 58]. Glu13 (1.39) and His278 (7.43) were found to be critical for the allosteric regulation of A_{2A} receptors [59], and Gln89 (3.37) was suggested to play an indirect role in ligand binding, while Ser281 (7.46) mutation to asparagine improved agonist affinity [60]. Additional studies were carried out to analyze the role of loop residues of A_{2A} receptors, revealing the importance of Glu151 and Glu169 (EL2) for ligand binding [61]. Unlike A_1 receptors, there is a considerable consensus regarding the effects of A_{2A} receptors on the inflammatory response. A broad range of investigations using *in vivo* and/or *in vitro* approaches have provided evidence that A_{2A} receptor activation limits inflammation and tissue damage, therefore playing an anti-inflammatory role [13, 31]. The A_{2A} receptor signaling in suppressing inflammation is related to cAMP-increased levels, which also have a well-known immunosuppressive effect on immune cells. Corroborating these data, the fact that A_{2A} receptors are expressed on most cells of the immune system is crucial to its anti-inflammatory properties [62]. Thus, the anti-inflammatory properties of these receptors are due, at least in part, to the prevention of the activation of effector cells of the immune system. For example, they may prevent neutrophil migration and interfere in the activity of proteins on the surface of neutrophils and endothelial cells that are crucial to the adhesion and migration of the former into the inflammatory site. In these sense, A_{2A} receptor activation inhibits the adherence of *N*-formyl methionyl-leucyl-phenylalanine (fMLP)-activated neutrophils to the endothelium [63] and downregulates Mac-1 [64], β2-integrin [65] and L-selectin [66]. Furthermore, activation of the A_{2A} receptors also downregulates the activity of other endothelial cell surface proteins, including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) [67], alpha 4/beta 1 integrin (VLA4) [68] and platelet cell adhesion molecule [69]. On the other hand, the anti-inflammatory properties resulting from A_{2A} receptor activation are due to the reduction of the release of inflammatory mediators such as IL-12, INFγ, TNF-α, and IL-4 from important immunomodulatory cells such as neutrophils, monocytes, dendritic cells and T lymphocytes [70, 71].

Many of the anti-inflammatory effects of A_{2A} receptors are mediated by adenosine itself. It is known that inflammatory tissue damage is accompanied by the accumulation of extracellular adenosine in inflamed sites due to its release from non-immune and immune cells. Because endogenous adenosine levels are elevated during an inflammatory process and endogenous adenosine can activate A_{2A} receptors to attenuate inflammation and tissue damage, strategies that aim to foment adenosine production and increase its availability to activate A_{2A} receptors present extraordinary anti-inflammatory potential. In this regard, an interesting study demonstrated that the immunosuppressive effects of activated Treg lymphocytes could be in part related to adenosine production in the extracellular environment through both CD39 (an ectonucleoside triphosphate diphosphohydrolase that converts ATP and ADP to AMP) and CD73 (an ectonucleoside that converts AMP to adenosine) expressed on the surface of these

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cells. Moreover, the Treg immunosuppressive effects have been shown to be modulated by A_{2A} receptors [72]. In a model of ischemia reperfusion liver injury, treatment with ATL146e (4-(3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9*H*-purin-2-yl]-prop-2-ynyl)- cyclohexanecarboxylic acid methyl ester), an agonist of A_{2A} receptors, was associated with decreased inflammation and protection from liver damage. When A_{2A} receptor knockout mice were subjected to the same insult, the effectiveness of ATL146e was lost [73]. Later, similar approaches showed the protective effect of the A_{2A} receptor in models of myocardial infarction, acute lung injury and spinal cord compression injury [74] [75, 76].

In another study, the same A_{2A} receptor agonist, ATL146e, was associated with decreased leukocyte infiltration, inflammatory mediator production and necrosis in a model of inflammatory bowel disease [77]. In a model of LPS-induced lung injury, treatment with the A_{2A} receptor agonist ATL202 was associated with decreased recruitment of neutrophils to the lung together with reduced cytokine levels and pulmonary edema, whereas A_{2A} receptor knockout mice treated with LPS showed an increase in neutrophil recruitment [75]. In a mouse model of allergic lung inflammation, treatment with an A_{2A} receptor (CGS-21680) agonist resulted in diminished pulmonary inflammation [78]. On the other hand, A_{2A} receptor knockout mice have been shown to have higher lung inflammation compared to wild-type mice [79].

4.3. A_{2B} adenosine receptors

The A_{2B} receptor couples to Gi-type G proteins, leading to the inhibition of adenylate cyclase upon receptor activation [80]. In some cell systems, such as HEK-293 and HMC-1 mast cells, A_{2B} receptors are also coupled to phospholipase C via the action of Gq proteins in increasing intracellular Ca^{2+} levels [81-83]. The A_{2B} receptor has also been described to be involved in the ERK1/2 [80, 83, 84] and p38 MAPK pathways in mast cells (Figure 2) [85]. Furthermore, a link between A_{2B} receptor signaling and the arachidonic acid signal transduction pathway leading to vasoconstriction has been described [83, 86]. Among the four adenosine receptors, A_{2B} is the least well characterized receptor, mainly due to the lack of suitably specific ligands [87].

The A_{2B} receptor has low affinity for most agonists, so only some agonists are useful, including the non-selective and related adenosine derivative NECA [88] and the highly selective A_{2B} agonist BAY60-6583 [89]. Conversely, highly selective A_{2B} antagonists have been developed, such CVT-6883 and an A_{2B}-specific antagonist radioligand, [3H]PSB-603, which has high potency and specificity across species, including rodents and humans [90]. The A_{2B} receptors have a closely related structure to A_{2A} receptors, and sequence analysis of the human A_{2A} and A_{2B} receptors show an overall identity of 58% and a similarity of 73%. The most conserved residues are found within the seven transmembrane domains. [83]. In contrast to the A_{2A} receptor, the A_{2B} receptor possesses the longest extracellular loop 2 (ECL2) of all four adenosine receptor subtypes, with four cysteine residues – the highest number found in any GPCR – of which three (C154, C167, C171) are homologous to the three (C146, C159, C166) found in the A_{2A} receptor [83]. These cysteine residues are involved in disulfide bonds formed between the ECL and transmembrane domains (TM) of GPCRs and have been reported to play an important role in ligand binding affinity and receptor stability and function.

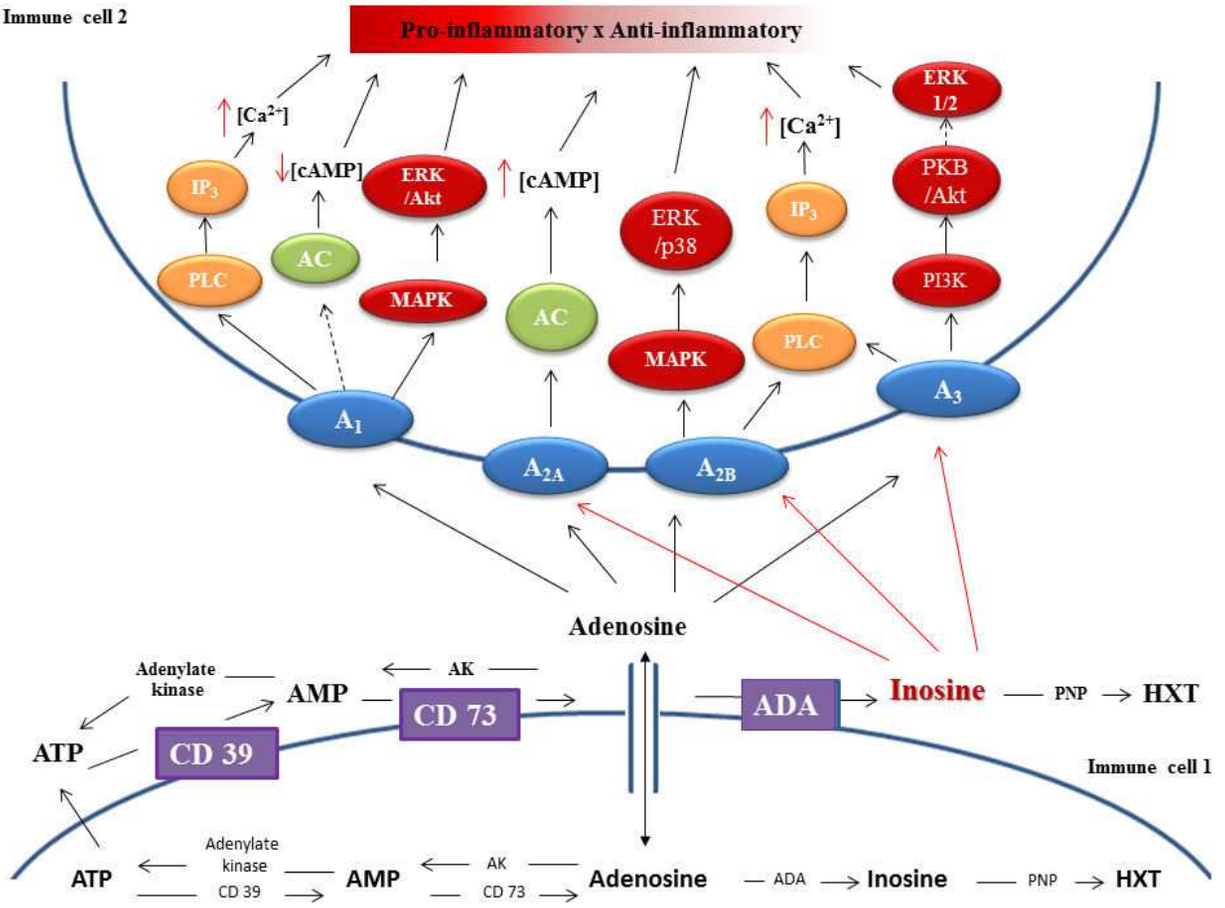

Figure 2. A brief summary of the role of the purinergic system in inflammation. A_1, adenosine A_1 receptor; A_{2A}, adenosine A_{2A} receptor; A_{2B}, adenosine A_{2B} receptor; A_3, adenosine A_3 receptor; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; ERK1/2, extracellular signal-regulated kinases 1 and 2; IP3, inositol triphosphate; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; PLC, phospholipase C; PI3K, Phosphatidylinositide 3-kinase; ATP, adenosine triphosphate; AMP, adenosine monophosphate; HXT, hypoxanthine; AK, adenosine kinase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; ENTs, equilibrate nucleoside transporters; CD 39, ectonucleoside triphosphate diphosphohydrolase; Inhibition → ; Activation - - →

Studies of mutagenesis, X-ray receptor analysis and radioligand binding have shown that C78 in transmembrane domain 3 (TMD3) and C171 in ECL2 form an important disulfide bond related to ligand-binding affinity and receptor expression levels [83, 91, 92]. In agreement with these data, in a mutagenesis model in which the complete ECL2 of human A_{2B} was exchanged for the ECL2 of the A_{2A} receptor, the mutant A_{2B} (ECL2-A_{2A}) receptor mice had increased affinity and selectivity for A_{2B} agonists and antagonists as well as receptor-mediated cAMP accumulation, indicating that ECL2 is a ligand binding site [93]. ECL2 also seems to contribute to the low affinity of ligands to A_{2B} receptors because it has been shown to have between 4 and 10 more amino acids than A_{2A} receptors. The longer ECL2 may, in some cases, partially block the entrance of ligands into the binding pocket and explain why A_{2B} receptors typically show lower affinity for adenosine and adenosine derivatives (agonists) than A_{2A} receptors, which have a shorter ECL2, which may facilitate the entrance of ligands to the binding pocket [83]. In addition, residues Thr42, Val54 (2.51) and Phe84 (3.31) are also involved in the binding site;

mutation to alanine, leucine or serine, respectively, decreased agonist binding [94]. As mentioned above, in many tissues, A_{2B} receptors are considered low-affinity receptors with mostly low expression levels, so adenosine needs to reach higher concentrations to activate them [83, 95].

During hypoxia, a critical role of HIF-1 in the transcriptional induction of the adenosine A_{2B} receptor has been suggested. In such conditions, as in $A_{2B}^{-/-}$ receptor mice, the presence of A_{2B} receptors attenuates hypoxia-induced increases in vascular leakage, mainly in the lungs, a protective effect [95]. Accordingly, Yang and colleagues reported that A_{2B}-null mice present augmentation of proinflammatory cytokine levels, such as TNF-α, upregulation of vascular adhesion proteins and leukocyte migration in response to LPS-induced acute inflammation. Conversely, in adenosine-deficient mice and bleomycin-induced lung inflammation, the antagonist CVT-6883 attenuated pulmonary inflammation [96]. Moreover, A_{2B} activation resulted in an increase in IL-8 [85], IL-1beta, IL-3, IL-4, IL-13 and IgE leading to mast cell and Th2 and B lymphocyte activation [97]. Mice treated with CVT-6883 and MRS-1754 or A_{2B}-null mice developed less severe experimental autoimmune encephalomyelitis (EAE) with reduced IL-6 release and Th17 cell differentiation [98].

In spite of the structural similarity, adenosine has been recognized as the natural ligand of A_{2B} receptors, but inosine was not [99, 100]. However, a recent study conducted by our group has shown evidence that inosine can reduce both acute pleural inflammation and allergic lung inflammation through a mechanism that involves both A_{2A} and A_{2B} receptors, suggesting a regulatory role of inosine and A_2 in these process [11, 101]. Taking into account the lack of information about the A_{2B} receptor and its involvement in several inflammatory diseases, it may be a candidate target for future therapeutic intervention

4.4. A_3 adenosine receptors

Studies have demonstrated that both adenosine and inosine can activate A_3 receptors in vitro and therefore can directly modulate its activation and biological effects [99, 100]. Similar to other adenosine receptors, the A_3 receptors is a GPCR with seven transmembrane domains (TM). It is coupled to classical second-messenger pathways such as inhibition of adenylyl cyclase, stimulation of PLC and calcium mobilization [33, 102-104].

In the heart, A_3 mediates cardioprotective effects through the activation of K_{ATP} channels that are coupled to RhoA–phospholipase D signaling, mediating the protection of cardiac myocytes from ischemia [104]. With regard to cardiac protection, signaling through cAMP response element-binding protein (CREB)-Bcl2 pathways after A_3 receptor activation has also been described [104]. In addition, like other adenosine receptors, A_3 receptors are coupled to MAPK and lead to stimulation of extracellular signal-regulated kinases (ERK1/2), which relates A_3 receptor activation to cell growth, survival, death and differentiation [33, 80].

A_3 receptor activation in melanoma cells stimulates PI3K-dependent phosphorylation of protein kinase B (PKB/Akt), leading to the reduction of basal levels of ERK1/2 phosphorylation, which in turn inhibits cell proliferation (Figure 2) [105]. Consistent with these data, treatment with IB-MECA induced inhibition of tumor growth [33, 104], although in the hypoxic condi-

tions that occur in solid tumors, A_3 activation mediates angiogenesis and cell survival through increased HIF-1 α and VEGF production [106]. Moreover, the A_3 receptor is involved in adenosine and inosine induced-mast cell degranulation [99, 107]; inhibition of endotoxin-induced neutrophil granulation and TNF-α production [108, 109]; reduction of T cell tumoricidal activity and enhancement of natural killer cell cytotoxicity [104]; reduction of neuropathic pain [110]; and augmentation of bone marrow cells proliferation favoring myeloprotection [111]. One of the characteristics of the A_3 receptor is the rapid (within a few minutes) desensitization through phosphorylation by G-protein-coupled receptor kinase 2 (GRK2) at the intracellular threonine residues within the C-terminal domain after exposure to an agonist, which can limit the agonist's effect [112-114].

A_3 receptors exhibit the lowest degree of identity among species compared with other adenosine receptor subtypes [115]. In a study that used a combination of mutagenesis, radioligand binding, functional activity and molecular modeling approaches, a mutation of TMD3 His95 (3.37), which is conserved in A_3 receptors in various species including humans, sheep and rats, resulted in a decreased affinity of agonists and antagonists and is therefore considered critical to ligand binding. The same was observed when His272 (7.43) and Asn250 (6.55) were mutated. Moreover, residues Tyr243 (6.48) and Lys152 (ECL2) were needed only for antagonist binding [115], and Trp243 (TM6) is involved in the functional activation of the A_3 receptor based on the impairment of the coupling of the receptor to the G protein in the Trp243 mutant [115, 116]. Furthermore, molecular modeling suggested that Trp243 is in the binding pocket and might occupy a strategic position as a switch in the TM6-mediated structural transition from the resting to the active state [115]. Given the involvement of A_3 receptors in important pathological process, as described above, several studies evaluating the structure-activity relationships of agonists and antagonists with A_3 receptors have been conducted to identify new potential drugs for the treatment of deleterious diseases.

5. Adenosine and inosine regulation on leukocyte function

The action of adenosine and inosine on the immune system is determined by their bioavailability and adenosine receptor expression in immune cells. The rapid release of adenosine in response to tissue-disturbing stimuli such as hypoxia, ischemia, inflammation or trauma and the rapid conversion of adenosine into inosine have been reported to modulate the function of leukocytes, a basic constituent of immune system [117]. In this section, we discuss adenosine and inosine modulation of immune cells and the corresponding involvement of adenosine receptors.

Neutrophils are the first immune cells recruited to inflamed sites by a combination of chemoattractant cytokines and adhesive interactions between leukocytes and the vascular endothelium [118, 119]. Adenosine mainly acts on A_{2A} receptors, signaling through cAMP-PKA-dependent pathways. It decreases neutrophil activation, neutrophil-mediated injury to endothelial cells, production of reactive oxygen species, PAF, and leukotriene B_4, secretion of cytokines such as TNF-α and chemokines such as MIP-1alpha/CCL3, MIP-1beta/CCL4,

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MIP-2alpha/CXCL2 and MIP-3alpha/CCL20 and expression of adhesion molecules such as selectins and integrins [31, 120-128]. Similar to adenosine, inosine also interferes with neutrophil activation by blocking formyl-Met-Leu-Phe-induced superoxide generation [31, 100], neutrophil migration and release of the proinflammatory cytokines TNF-α and IL-1β during acute inflammation, acting through A_{2A} and A_{2B} receptors [11]. A_3 and A_{2A} receptors are involved in the reduction of superoxide anion generation [127, 129]. A_3 receptors can also direct neutrophil migration [130]. Interestingly, the adenosine interaction with A_1 and A_3 receptors induces G-CSF production, which leads to a stimulatory effect on bone marrow cells, suggesting that adenosine is a chemoprotective agent that could restore the number of leukocytes and neutrophils to normal levels after chemotherapy [131].

Macrophages and dendritic cells are phagocytes that are widely dispersed throughout the body at portals of microorganism entry [31]. They initiate an effective innate immune response against microbes by recognizing pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) [119, 132]. This response involves pathogen processing and is regulated by the secretion of several cytokines and activation of lymphocytes and other immune cells.

In this context, studies have demonstrated that adenosine inhibited TNF-α, IL-6 and IL-8 release from macrophages stimulated with thioglycollate or LPS via A_{2A} receptors, although the A_{2B} receptors seems to play an underlying inhibitory role that may contribute to anti-inflammatory action [133, 134]. Some studies have demonstrated that adenosine can increase IL-10 production and release through a mechanism involving adenosine A_{2A} receptor-CEBPβ axis activation [135]. The augmentation of the production of IL-10 and the decrease in systemic endotoxin-induced levels of TNF-α, IL-12, MIP-1α and IFN-γ have also been ascribed to inosine [136]. Current data have shown that activation of both A_{2A} and A_3 receptors inhibited IFN-γ and IL-12 release after TLR-4 stimulation by LPS [137-139]. Adenosine and inosine were described to decrease M1 activation and the release of mediators, reducing Th1 response, by interfering with TLR-4 activation. Moreover, there is growing evidence that A_{2A} receptor activation also reduces the TLR-2, 3, 7, and 9 responses in M1 macrophages, upregulating VEGF and IL-10 expression and therefore polarizing macrophages into an M2-like phenotype, called M2d, which favors an angiogenic switch and plays a protective role in ischemia [70, 140].

Adenosine also interferes with mature dendritic cell stimulation by producing a dose-dependent inhibition of TNF-α and IL-12 release, whereas it enhanced the secretion of IL-10, preventing tissue injury mediated by innate immune mediators during overwhelming immune response [141]. Furthermore, dendritic cells matured in the presence of adenosine had a reduced capacity to induce T helper 1 (Th1) polarization of naive $CD4^+$ T lymphocytes, evidence that adenosine diminishes the capacity of dendritic cells (DCs) to initiate and amplify Th1 immune responses [141].

Mast cells are resident in all normal tissues, where they are believed to play an important role in tissue homeostasis, wound healing and host defense, particularly in terms of bacterial infection. When activated, they secrete the autacoid mediators histamine, prostaglandin (PG)D2 and leukotriene (LT)C4, which contribute to the pathophysiology of many diverse diseases including rhinitis and asthma [142-144].

Rodent and human mast cells express the A_{2A}, A_{2B} and A_3 receptors [145-149]. It has been reported that adenosine and inosine binding to A_3 receptors expressed in mast cell membranes induces degranulation and release of vasoactive mediators [99, 107]. Accordingly, the release of reactive mediators following A_3 activation in mast cells is directly related to the bronchoconstrictor effects observed after topical administration of adenosine in the airways of patients with asthma and chronic obstructive pulmonary disease [109, 150]. In contrast, inosine has no effect on airway caliber, indicating that bronchoconstriction is a specific response to adenosine [151]. Engagement of the A_3 receptors on rodent mast cells mediates degranulation and cell migration through a mechanism that involves phosphoinositide 3-kinase (PI3K) or protein C kinase (PKC) activation and an increase in intracellular Ca^{2+} [146, 147]. Moreover, A_1 and A_{2B} receptor activation has been related to the release of histamine, IL-8, IL-4 and IL-13 by mouse and human mast cells [97, 152-154]. In contrast to the A_1 and A_{2B} receptors, A_{2A} activation results in the suppression of histamine and tryptase release from human mast cells [155]. A_{2A} and A_{2B} receptors provide a balanced control mechanism for mast cell activation. It is possible that at low concentrations of adenosine, only the 'off' signal provided by the engagement of the higher affinity A_{2A} receptors prevails, thus downregulating mast-cell mediator release. Conversely, in situations in which high concentrations of adenosine are reached, such as in asthma and COPD [156], the low-affinity A_{2B} receptor becomes activated, resulting in significant mast cell degranulation [108, 109, 157]. Although the effects of adenosine regarding mast cell activation are well described, there is a lack of information regarding the effects of inosine on the activation of the A_3 receptor. Recently, a study from our group suggested that inosine can activate A_{2A}, A_{2B} and A_3 receptors and decrease mast cell migration during allergic pulmonary inflammation, suggesting that inosine can modulate allergic inflammation [101].

Lymphocytes cells play a vital role in the induction of adaptive immune responses and in steering them toward particular effector phenotypes [119]. Several pieces of evidence suggest that adenosine and inosine generated in the site of inflammation can modulate lymphocyte function [23, 24, 28]. Experimental data demonstrated that in ConA-induced liver injury (an in vivo model mediated by T cells), inosine inhibited hepatocyte apoptosis and reduced the accumulation of proinflammatory cytokines (e.g., TNF-α) and alanine transaminase in $A_3^{+/+}$ but not $A_3^{-/-}$ or $A_2^{-/-}$ mice, suggesting the endogenous inosine can influence inflammatory responses and indicating the importance of A_3 receptors in controlling liver injury [28].

The extracellular adenosine generated in inflammatory or hypoxic environments affects regulatory T cell lymphocytes (Treg) through the activation of A_{2A} receptors. Treg cells are a specialized population of $CD4^+$ T cells implicated in the regulation of immune responses, maintenance of immunological self-tolerance and protection from excessive inflammatory damage [24, 158]. A_{2A} receptor stimulation expanded the Treg population [159] coordinated by coexpressed CD39 ecto-ATPase/ADPase and CD73 ecto-5'-nucleotidase and generating adenosine pericellularly [72, 160]. The CD39- and CD73-mediated generation of extracellular adenosine might provide Treg cells with the capacity to directly inhibit DC and T effector cells by activating their respective cAMP-elevating A_{2A} receptors [160]. Consistent with these data, the A_{2A} receptor has been described to suppress the development of T-cell receptor (TCR) - stimulated naive T cells into both Th1 and Th2 cells [135], interfering with early development

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as well as the late effector stages of Th1- and Th2-cell responses [135]. The activation of A_{2B} receptors seems to indirectly inhibit Th17 activation. A recent study using EAE in mice indicated that blocking A_{2B} receptors with specific antagonists, such as CVT-6883 and MRS-1754, alleviated the clinical symptoms of EAE and protected the CNS from immune system-mediated damage. Confirming this hypothesis, the deletion or blockade of A_{2B} receptors inhibited Th17 cell differentiation by blocking IL-6 production from APCs such as dendritic cells. The activation of phospholipase Cβ-protein kinase C and p38 MAPK pathways was found to be involved in A_{2B}-mediated IL-6 production, suggesting A_{2B} as a target for the development of anti-multiple sclerosis drugs and indicating that adenosine might participate in regulation of this pathology [98].

6. Overall effect of adenosine and inosine on inflammatory diseases

6.1. Asthma and COPD

A great deal of evidence suggests that adenosine plays a detrimental role in asthma and COPD and perhaps other chronic airway disorders [161]. The potential role of adenosine triphosphate and adenosine in the pathogenesis of asthma and COPD has been supported by the broncho-constrictor effects observed after their topical administration in the airways of patients with these diseases and the lack of reaction in healthy subjects [150, 162-164]. However, there is some controversy about the role of adenosine during allergic reactions in the airways, such as asthma. When administrated topically, as mentioned above, it seems to trigger airway hyperactivity, but the administration of non-selective and selective agonists of different adenosine receptors can suppress (such as adenosine A_{2A} receptors) [165-167] or contribute with airway allergic inflammation, as described below.

Treatment with CGS21860, a selective A_{2A} receptor agonist, reduced the number of leukocytes in bronchoalveolar lavage fluid, protein content and eosinophil peroxidase activity in Brown Norway rats immunized and challenged with OVA, a compound similar to the glucocorticos-teroid budesonide [165]. Moreover, treatment with NECA, a non-selective adenosine A_2 receptor agonist, reduced the total leukocyte infiltration and eosinophilia in a model of allergic airway inflammation [166]. Interestingly, inosine but not adenosine was described to reduce leukocyte infiltration into the lungs, Th2 pro-inflammatory cytokine levels and improve pulmonary mechanics in OVA-induced airway inflammation through a mechanism involving the A_{2A} and A_3 receptors [101]. Consistent with these data, inosine and its stable analogue INO-2002 were described to reduce LPS-induced airway inflammation [26, 168]. Several studies have hypothesized that the bronchial response to adenosine observed in asthma and COPD in humans can be attributed to an indirect mechanism involving mast cell activation, likely via A_{2B} or A_3 receptors and the release of mediators such as histamine and leukotriene (LT)C4 [109, 150, 169]. These are low-affinity receptors that can modulate the deleterious effect of high concentrations of adenosine in chronic airways diseases [108, 109, 157]. Likely, adenosine signaling through the A_{2B} receptor also plays a role in asthma development, promoting the upregulation of pro-inflammatory cytokines, leukocyte migration and airway

remodeling (Figure 3) [170, 171]. Other studies have described a pro-fibrotic role for A_{2B} receptor signaling, which results in the differentiation of human pulmonary fibroblasts into collagen-producing myofibroblasts, increasing the production of the pro-fibrotic molecule fibronectin in alveolar epithelial cells [147, 161, 172]. These data demonstrate the potential involvement of A_{2B} receptors in the remodeling and fibrosis observed in asthma and COPD. Although the A_3 receptors appear to reduce inflammation and eosinophil activation in humans, in mice, A_3 activation induces mast cell degranulation and increases inflammation, activating eosinophils and mucus production [161, 173].

The literature data describes differences in mast cell expression between rodents and humans and maybe it can explain the different effects of adenosine in modulating these cells during asthma and allergy. The adenosine A_3 receptor that was expressed in mast cells in rodents [174], have recently been described to be expressed in human lung mast cells [175]. While the expression of adenosine A_{2A} and A_{2B} receptors in rats was not described; in mice, both were expressed in bone marrow derived mast cells. The A_{2A} receptors were also described in cardiac mast cells, in mice [174]. In humans, both receptors have been described in lung mast cells and in human mast cells line HMC-1 [174, 176]. The adenosine A_1 receptor were not described to be expressed in mice and humans but recent data have shown that agonists of adenosine A_1 receptor can potenciate human cultured mast cell activation, suggesting a modulatory effect [152, 177, 178].

The A_1 receptor is also described to have pro or anti-inflammatory effects on airway inflammation. Treatment with an A_1 receptor antagonist [179] or with antisense oligodeoxynucleotides targeting this receptor reduced the bronchoconstrictor responses in an allergic rabbit model [180]. Conversely, in knockout $A_1^{-/-}$ mice, an increase in transmigration of polymorphonuclear cells and microvascular permeability in comparison to wild type mice was observed in a model of LPS-induced lung injury, suggesting a possible protective effect of the A_1 receptor in airways [181]. The engagement of adenosine receptors on inflammatory and pulmonary cells appears to play an important role in regulating chronic lung disorders such as asthma and COPD, so the complete and full characterization of adenosine receptor subtype distribution in the airways and their specific role in the response to adenosine and inosine in health and disease is important for the development of new therapies to treat asthma and COPD [161].

6.2. Skin inflammation and wound healing

The skin is a highly specific immune defense organ. Physical, chemical or immune-specific insults rapidly evoke cellular responses, characterized by the increased expression of a wide range of pro-inflammatory mediators [182]. Controlling the extent of an immune response is thus a major challenge for maintaining skin integrity, which is of paramount importance for host survival [183]. In this context, several lines of evidence have shown that adenosine and adenosine receptors contribute to the regulation of skin inflammation.

A recent study showed that activated Treg cells can produce adenosine in a CD39-dependent manner and abrogated the ear-swelling reaction induced by 2,4,6-trinitro-1-chlorobenzene (TNCB), indicating a role of adenosine in the Treg cell–induced suppression of contact

hypersensitivity responses. Moreover, the same study demonstrated that adenosine's effects involve the impairment of effector T cell adhesion to inflamed endothelium and downregulation of E- and P- selectin in the vascular endothelium [184]. A complementary study of IL-10-deficient (IL-$10^{-/-}$) Tregs showed impaired adenosine production, which contributes with their inability to suppress contact hypersensitivity responses, indicating that the reduced suppressive effects observed may not be exclusively attributable to the lack of IL-10 production [185]. Several lines of evidence indicated that adenosine's effects on skin are mediated by the activation of adenosine receptors.

The activation of A_1 receptors has been demonstrated to decrease the numbers of circulating neutrophil granulocytes and ear swelling in a model of stress-induced contact hypersensitivity response [186]. In addition to receptor A_1 activation, activation of receptor A_{2A} was described to reduce leukocyte activation and to prevent ischemia reperfusion wound formation in a rat model of a pressure ulcer [187]. Evidence from experiments performed on A_{2A} receptor knockout mice and with CGS21680 demonstrated that the A_{2A} receptor is the main adenosine receptor subtype involved in wound healing [149, 188]. In addition, histological analysis of mice treated with the same agonist showed faster re-epithelialization and increased matrix deposition, fibroblast density and vascularity in the granulation tissue of the agonist treated wounds as soon as 3 days after injury [188]. A study from the same group revealed that treatment of human microvascular endothelial cells (HMVEC) with the selective A_{2A} receptor agonists CGS21680 and MRE0094 (Sonedenoson) favors vascular tube formation by cultured HMVEC and downregulated the antiangiogenic matrix protein thrombospondin 1 (TSP1) secretion by these cells, indicating that A_{2A} activation induces angiogenesis [189]. Furthermore, treatment with MRE0094 increased the rate of wound closure in comparison to recombinant human platelet-derived growth factor (Becaplermin gel), an agent currently used to promote the healing of diabetic ulcers, indicating the importance of A_{2A} receptors in wound healing [190]. Moreover, the A_{2A} and A_{2B} receptors were described to contribute to tissue formation because their activation leads to enhanced fibroblast and endothelial cell migration [191].

Adenosine seems to have a fibrogenic role in the skin. A study using ADA-deficient mice reported a direct fibrogenic effect of adenosine on the skin, and pharmacological treatment with the A_{2A} receptor antagonist ZM-241385 prevented the development of dermal fibrosis by reducing dermal collagen content and the expression of profibrotic cytokines and growth factors (Figure 3) [192]. The data mentioned above are interesting and strongly suggest that adenosine and A_{2A} receptors have important modulatory effects in skin homeostasis. Although several studies have shown a role for adenosine in the skin, nothing has been found regarding a role for inosine. Additional studies addressing the real role of the purinergic system in the skin would be extremely useful to improving wound management and care, as well as controlling chronic inflammatory diseases in skin.

6.3. Arthritis

Arthritis is the term used to designate a particular pathological condition that encompasses a constellation of more than 100 diseases, among which osteoarthritis (OA) and rheumatoid arthritis (RA) stand out. OA is the most common adult joint disease and is increasing in

frequency and severity, with an estimated US prevalence of more than 25 million affected adults [193]. It is characterized by gradual loss of articular cartilage and is therefore being considered a slowly progressing degenerative disease. The etiology of arthritis involves biochemical and genetic factors as well as repetitive mechanical injury, which has been proposed as the critical mechanisms contributing to alterations in the normal functional activities of chondrocytes, the main cellular component of hyaline cartilage, disrupting chondrocyte–matrix associations and culminating in the initiation and progression of OA [194].

In early OA, a transient proliferative chondrocytes response (clonal growth) occurs along with increased synthesis of the cartilage matrix as an early repair attempt and increased synthesis of catabolic cytokines (such as IL-1, TNF-α and IL-18) and matrix-degrading enzymes (such as metalloproteinases, especially collagenases, and aggrecanases). Fibroblast- and macrophage-like cells in the synovia also generated catabolic cytokines in response to breakdown products from the damaged cartilage. All these events contribute greatly to the local loss of proteoglycans and cleavage of type II collagen, which initially occurs at the cartilage surface, contributing to water content increases and loss of tensile strength in the cartilage matrix as the lesion progresses [195, 196]. It is characterized by several inflammatory cascades, which all lead towards a final common pathway in which persistent synovial inflammation and associated damage to articular cartilage and underlying bone are present [197].

One inflammatory cascade that deserves attention in the pathogenesis of RA is the overproduction and overexpression of TNF-α. Interactions between T and B lymphocytes, synovial-like fibroblasts and macrophages are likely to be involved in TNF-α and IL-6 overproduction, contributing to both synovial inflammation and joint destruction [198]. In addition to inflammatory cytokines, rheumatoid factors are key pathogenic markers of classic RA. In this case, the immunoglobulins IgM and IgA are directed against the Fc fragment of immunoglobulin IgG, resulting in the formation of immune complexes, which are able to activate the complement system, initiating an immune response [199]. Adenosine has a known therapeutic potential against inflammatory joint diseases; studies have demonstrated its ability to limit synoviocyte [200] and chondrocyte [201] inflammatory responses and to minimize articular damage in adjuvant-induced models of arthritis in rats [202].

In an interesting study, Tesch and colleagues demonstrated that endogenously produced adenosine regulates articular cartilage matrix homeostasis in vitro. In this study, authors showed that the depletion of endogenous adenosine through exposure to adenosine deaminase (ADA) in cartilage explants resulted in cartilage matrix degradation, involving matrix metalloproteinases-3 and -13 (MMP-3, MMP-13), prostaglandin E_2 (PGE_2), and nitric oxide (NO) release. In addition to these data, this study suggested that endogenously released adenosine can regulate chondrocyte production of matrix-degrading enzymes and matrix loss, an effect believed to be in part mediated via A_{2A} receptors, because N^6-[2-(3,5-dimethoxyphenyl)-ethyl]adenosine (DPMA, an A_{2A} receptor selective agonist) was able to prevent the release of PGE_2, NO and glycosaminoglycan (GAG) (Figure 3) [203]. Another in vitro study demonstrated that adenosine, N^6-methyladenosine (a substituted adenosine derivative that is resistant to breakdown by adenosine deaminase), DPMA and 5′-N-ethylcarboxamidoadeno-

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sine (NECA, a non-selective adenosine receptor agonist) suppressed NO production by LPS-stimulated equine chondrocytes [201]. The addition of exogenous adenosine and erythro-9-(2-Hydroxy-3-nonyl) adenine hydrochloride (EHNA, an adenosine deaminase inhibitor) further suppressed NO production by LPS-stimulated chondrocytes [201]. These two studies clearly demonstrate the protective effect of adenosine against degradation of articular cartilage in vitro, suggesting its potential to prevent joint damage and hence its effectiveness in the prevention of joint diseases such as osteoarthritis.

In the second half of the 1980s, remarkable attention was focused on investigating the role played by ADA in RA pathophysiology, which provided the first evidence of the role of adenosine in RA. These studies showed increased levels of ADA activity in the synovial fluid from patients with seropositive rheumatoid arthritis, suggesting a local release of this catabolic enzyme by cells within joints [204]. Subsequent investigations were aimed at characterizing the activities of different ADA isoforms in tissues, cell homogenates and serum samples obtained from patients with rheumatic disorders. The highest level of enzyme activity was found in lymphocytes and monocytes from patients with rheumatoid arthritis, and ADA-2 was the isoform specifically expressed in monocytes [205]. Iwaki-Egawa and colleagues observed a significant positive correlation between high activity of ADA isoforms in the synovial fluid of rheumatic patients and metalloproteinase-9 (MMP-9), an enzyme critical to the regulation of the cell matrix composition [206]. These results suggest that the high activity of ADA in the synovial fluid of patients with RA and consequently the reduction in local levels of adenosine are directly related to the development and maintenance of RA. In support of this hypothesis, Forrest and colleagues demonstrated that adenosine is able to suppress the elevated levels of proinflammatory cytokines such as TNF-α and IL-1β in RA patients and that this effect appears to be mediated by adenosine receptors [207].

Recent studies have shown adenosine A_3 receptor upregulation in RA patients, suggesting the potential of this receptor as a therapeutic target. In agreement with these results, after oral treatment with CF101, a selective A_3 receptor agonist, a marked decrease in RA clinical manifestations including inflammation, pannus formation, cartilage destruction and bone reabsorption and lysis was observed [208].

6.4. Ischemia

Ischemia is defined as a lack of blood supply to an organ or tissue, resulting in cellular oxygen deprivation. Although ischemia itself is a serious condition, the phenomenon that seems to be the definitive treatment for ischemia, reperfusion of the ischemic tissue, can promote further tissue injury, especially after prolonged ischemia [209]. Thus, the tissue can be injured by both ischemic and reperfusion processes and can be defined as ischemia reperfusion (IR) injury. IR injury involves a complex cascade of events including oxidative stress, inflammation and interactions among many cell types [209]. Furthermore, it has widespread clinical relevance and is encountered in a variety of surgical settings (e.g., transplantation, cardiopulmonary bypass and aneurysm repair) as well as non-surgical settings (e.g., myocardial infarction, stroke, hemorrhage, trauma and shock).

One of the potentially most striking features of IR injury involves the generation of reactive oxygen species (ROS) (e.g., superoxide, peroxynitrite, hydrogen peroxide, and hydroxyl radical) [210, 211], especially during reperfusion of ischemic tissue, which, in turn, initiates an inflammatory cascade resulting in direct oxidative injury to cells and stimulation of pro-inflammatory mediators such as cytokines, chemokines and cell-adhesion molecules. Briefly, circulating and resident leukocytes, such as neutrophils, lymphocytes and macrophages as well as tissue resident cells, such as dendritic cells, contribute to the immune response to IR injury, particularly infiltrating neutrophils, which can impose significant tissue injury through ROS generation and further release of cytokines and proteases [212].

Strong evidence has indicated that cellular responses to hypoxia include robust increases in extracellular adenosine and signaling events through adenosine receptors. The hypoxic adenosine response in acute injury settings is able to promote tissue adaptation during hypoxia, including restoration of normal oxygen levels, enhancing metabolic ischemia tolerance and dampening inflammation [213]. Preclinical studies have shown that adenosine signaling is beneficial in ischemic acute injury in the lung [214, 215], kidney [216], heart [217], gastrointestinal track [218] and liver [73]. Some studies have demonstrated that chronic elevations of adenosine can contribute to tissue fibrosis in different organs including the lungs [170, 219], liver [220], skin [221], kidney [222] and following transplants [223]. Studies using CD39 and CD73 knockout mice, which lack both the enzyme that converts ATP to ADP/AMP and the enzyme that converts AMP to adenosine, and inhibitors of these enzymes demonstrated enhanced inflammation and tissue injury in models of hypoxia and ischemic injury [6, 224] concurrent with reduced production of adenosine. These results suggest that elevated extracellular levels of adenosine may display a protective effect in models of hypoxia and ischemia injury. An interesting strategy used to enhance extracellular levels of adenosine is the prevention of adenosine uptake by equilibrate nucleoside transporters (ENTs) [225].

Recently, Grenz and colleagues demonstrated that treatment with dipyridamole, an inhibitor of ENTs, led to increased adenosine levels in association with tissue protection in a mouse model of ischemic acute kidney injury [226]. Furthermore, genetic deletion of ENTs resulted in selective protection in $ENT1^{-/-}$ mice. A more detailed analysis using adenosine receptor-knockout mice exposed to acute kidney injury showed that renal protection promoted by ENT inhibitors involves the A_{2B} adenosine receptor. In addition, Eltzschig and colleagues demonstrated that the A_{2B} receptor serves anti-inflammatory and tissue-protective roles in various acute injury tissue models associated with hypoxic or ischemic injury including the heart [217], lung [227], intestine [218] and kidney [226] using genetic (A_{2B} receptor knockout mice) and pharmacological (A_{2B} receptor agonists) approaches. In summary, adenosine receptor signaling in hypoxic or ischemia-reperfusion injury varies depending on the receptor activation and cell/tissue type. In general, A_1 and A_{2A} receptor activation has been shown to be protective in the lung, kidney, heart and liver, whereas the role of the A_{2B} and A_3 receptors remains obscure. A considerable number of studies both in vitro and in vivo have demonstrated the potential of inosine in preventing injury in different models of hypoxia or ischemia.

In the early 1980s, it was demonstrated that infusion of 4 mM of inosine presented a protective effect in fetal mouse heart organ cultures deprived of oxygen, a model of ischemic-like injury

[228]. At the CNS level, purine catabolite concentrations were monitored for up to 15 h in the auditory and somatosensory cortices of cats using microdialysis/HPLC and hydrogen clearance following middle cerebral artery occlusion (MCAo). MCAo led to the release of inosine and its metabolite hypoxanthine from the ischemic cortex in stroke animals, which reached maximum levels 1-2 h after the onset of ischemia [229]. Cerebral infarct (stroke) often causes devastating and irreversible losses of function, in part because of the brain's limited capacity for anatomical reorganization. In an animal cerebral ischemia model, which results from infarction in the right dorsolateral cerebral cortex and underlying striatum, continuous infusion of inosine 50 mM into the cisterna magna using osmotic minipumps (0.25 μl/h) stimulated neurons on the undamaged side of the brain to extend new projections to denervated areas of the midbrain and spinal cord and consequently improved performance on several behavioral measures in adult rats [230].

Taken together, these data suggest that inosine promotes neuroregeneration after stroke. An interesting study recently published Shen and colleagues demonstrated that intracerebroventricular administration of inosine (25 nmol/L in 25 μL) before middle cerebral artery occlusion in rats resulted in a higher level of locomotor activity (lasting up to 2 weeks after stroke) and less cerebral infarction [231]. In addition, they indicated that coadministration of a selective A_3 receptor antagonist, MRS1191, significantly attenuated inosine-mediated protection. Moreover, in the electrophysiological study, inosine antagonized glutamate-induced excitation in cerebral cortical neurons. In summary, the authors proposed that inosine may inhibit glutamate postsynaptic responses and reduce cerebral infarction via the activation of the A_3 receptor, presenting a neuroprotective action (Figure 3) [231].

6.5. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a common and lifelong disabling gastrointestinal disease that includes Crohn's disease (CD) and ulcerative colitis (UC) [232]. Worldwide UC incidence varies greatly, ranging from 0.5-24.5 /100,000 habitants, and CD ranges from 0.1-16/100.000 habitants, with the highest incidence in developed countries [233]. Genetic, environmental, and immunological factors interplay in a complex manner to contribute to the genesis of IBD. Generally, the presence of one or more genetic factors triggers an over-reaction of the host mucosal immune system to normal constituents of the mucosal microflora. This over-reaction involves either a Th1-type T cell-mediated inflammation in the case of Crohn's disease or a Th2-type T cell-mediated inflammation in ulcerative colitis. This inflammatory process leads to the release of multiple cytokines, including interferon (INF)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1,, IL-6, IL-8, IL-12, IL-13,IL-17, and monocyte chemotactic protein (MCP)-1 [234]. These cytokines are responsible for the attraction and activation of neutrophils, eosinophils, mast cells and macrophages, which, in turn, produce large amounts of unstable chemical species such as reactive oxygen species (ROS) or oxyradicals (i.e., superoxide anions, hydrogen peroxide, hydroxyl radicals, and peroxynitrite), mediators that contribute greatly to the tissue injury seen in IBDs [235, 236].

In early stages of immune or inflammatory response, significantly elevated extracellular concentrations of adenosine are anti-inflammatory and tissue protective. Thus, one of the most

widely used strategies to study the effects of adenosine on different inflammatory processes involves inhibition of adenosine catabolism, mainly through inhibition of adenosine deaminase (increasing adenosine levels) or direct activation of adenosine receptors. Antonioli and colleagues have demonstrated, using a model of 2,4-dinitrobenzenesulfonic acid (DNBS)-induced colitis, that treatment with 4-amino-2-(2-hydroxy-1-decyl) pyrazole[3,4-d]pyrimidine (APP; novel adenosine deaminase inhibitor) or erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA; standard adenosine deaminase inhibitor) for up to 7 days was able to increase food intake and weight gain and ameliorate macroscopic and microscopic inflammatory colonic alterations with a concomitant reduction of mucosal and plasmatic pro-inflammatory mediators such as TNF-α and interleukin-6 [237]. In the same vein, Siegmund and colleagues showed that treatment with {4-amino-1-(5-amino-5-deoxy-1-β-d-ribofuranosyl)-3-bromo-pyrazol[3,4-*d*] pyrimidine} (GP515), an inhibitor of adenosine kinase, the enzyme responsible for the conversion of adenosine to AMP, resulted in a significant improvement of mucosal morphology and clinical score (weight loss, stool consistency, and bleeding) as well as decreased IFN-γ concentration in the colonic tissue in dextran sulfate sodium (DSS)-induced colitis [238]. These results clearly demonstrate that increased levels of adenosine can attenuate mucosal inflammation in experimental colitis. The activation of adenosine A_{2A} receptors seems to be one of the main mechanisms involved in the effects of adenosine (Figure 3).

Odashima and colleagues studied the anti-inflammatory effects of ATL-146e in acute and chronic rabbit formalin-immune complex models of colitis and the SAMP1/YitFc mouse model of spontaneous ileitis. ATL-146e (20 and 40 μg/kg, i.p.) significantly reduced the acute and chronic inflammatory index and tissue necrosis and prevented mortality. Furthermore, TNF-α, IFN-γ and IL-4 concentrations were significantly suppressed with ATL-146e treatment in supernatants from cultures of mesenteric lymph node cells of SAMP1/YitFc mice. Thus, these results support important anti-inflammatory actions of ATL-146e in the intestine, including the suppression of lymphocyte-derived cytokine-mediated pro-inflammatory responses, suggesting that the activation of A_{2A} receptor-mediated signaling through selective agonists may be a novel therapeutic approach for patients with IBD [77]. To this end, Cavalcante and colleagues evaluated the effects of a new selective A_{2A} receptor agonist (ATL 313) on *Clostridium difficile* toxin A-induced injury in murine ileal loops. ATL 313 treatment directly into ileal loops significantly reduced toxin A-induced secretion and edema, prevented mucosal disruption, neutrophil infiltration, TNF-α production, adenosine deaminase activity and prevented toxin A-induced cell death. Based on these findings, the adenosine system may represent a promising target for therapies for inflammatory intestinal disorders, either by manipulating its metabolism or through direct activation of its receptors, especially A_{2A} receptors [239].

6.6. Multiple sclerosis

MS is an autoimmune disease mediated by T cells that is characterized by CNS demyelination and neurodegeneration [98]. There are many other diseases that are associated with inflammation of the CNS, including meningitis, encephalitis, among others. However, one of the most common is MS. It affects more than 2.5 million people around the world and is characterized by the loss of neurological function, which occurs due to axonal demyelination and

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represents the main symptom of the disease. Recurrences, commonly associated with increased lymphocyte infiltration of the CNS, make the patient increasingly weak over time [240-242].

Studies in mice using an EAE model demonstrated that adenosine signaling modulates the development of EAE. A_{2B} receptor blockade with CVT-6883 and MRS-1754 (both specific antagonists of A_{2B}) promoted a reduction in the symptoms of EAE, thus protecting against CNS immune response damage. Moreover, both deletion and A_{2B} receptor blockade promoted inhibition of the differentiation of Th17 cells due to the reduction of IL-6 production by APCs (dendritic cells), suggesting that the A_{2B} receptor is a potential new target for "anti-multiple sclerosis" drug action [98]. Post-mortem analysis of brain tissue from patients diagnosed with MS showed the presence of cells with high expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine in characteristic lesions of the disease [243, 244].

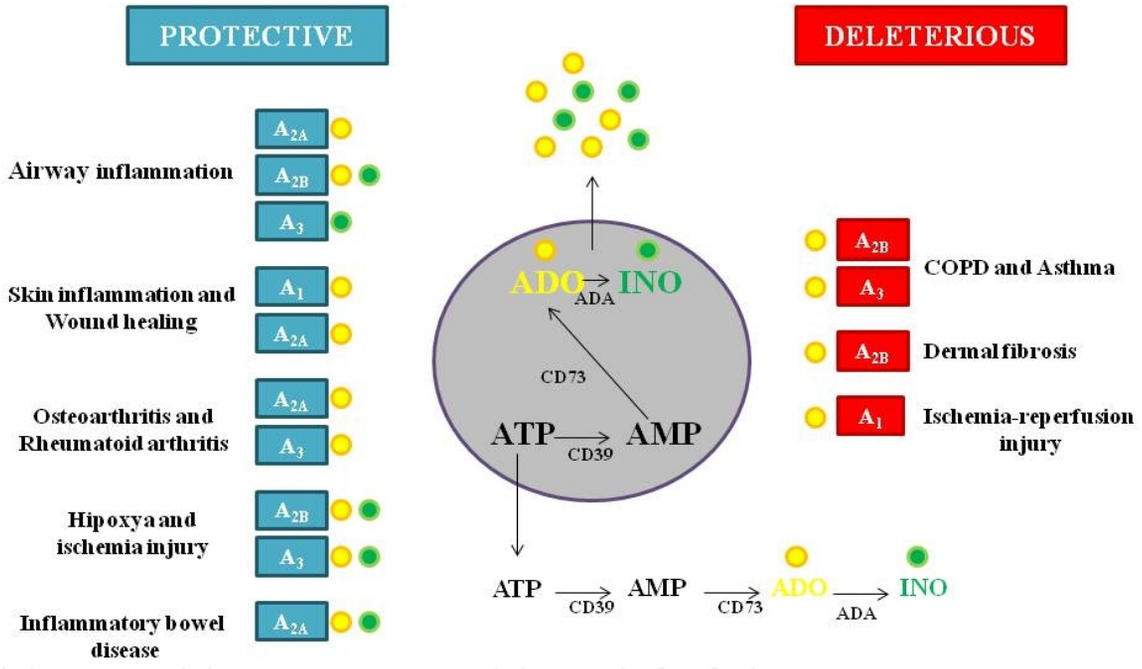

Figure 3. Adenosine deleterious and protective effects in inflammation and immunity. A_1, adenosine A_1 receptor; A_{2A}, adenosine A_{2A} receptor; A_{2B}, adenosine A_{2B} receptor; A_3, adenosine A_3 receptor; ATP, adenosine triphosphate; AMP, adenosine monophosphate; ADO, adenosine; CD 39, ectonucleoside triphosphate diphosphohydrolase.

Peroxynitrite, the end product of iNOS activity, which leads to the formation of NO and then to superoxide reaction, is highly reactive, causing a variety of toxic chemical changes in nerve tissue, including the nitration of tyrosine residues [245]. It has been previously described that UA, which is the final product of purine metabolism in humans, is a peroxynitrite scavenger [246], and for this reason UA therapy has become a potential method to alleviate the neuronal damage induced by peroxynitrite in MS treatment. Furthermore, inosine, an endogenous precursor of UA, seems to be an attractive candidate for the treatment of MS, given that patients who received inosine showed some evidence of clinical improvement and no sign of disease progression [247]. In addition, recent studies using adenosine receptor antagonists as well as

mice that were not capable of hydrolyzing adenosine from extracellular AMP ($CD73^{-/}$), have suggested that blockade of adenosine receptors or CD73 deletion protected mice from EAE, decreasing lymphocyte infiltration in the CNS [248]. One very interesting note about the involvement of the adenosinergic system during EAE is that both CD73 and A_{2A} receptor presented increased expression in the choroid plexus [248, 249] in comparison to other regions of the CNS. This result shows that choroid plexus is higher permeable to lymphocytes than other regions, therefore, it is speculated that ATP, released as result of damage, and its conversion to adenosine represent a signal that regulates the entry of lymphocytes into the CNS [250, 251].

7. Adenosine receptors as drug targets: future directions for new drug development

There is increasing interest in the therapeutic potential of adenosinergic compounds (including receptor agonists and antagonists, enzyme inhibitors and others), and many adenosine compounds have been evaluated for therapeutic use. For a long time, adenosine itself was the only adenosine agonist used in humans. It is widely used in the treatment of paroxysmal supraventricular tachycardia (Adenocard®) due to the activation of A_1 receptor and is also used as a diagnostic tool for myocardial perfusion imaging (Adenoscan®) as a consequence of its A_{2A} receptor-activating effects, resulting in vasodilation [87].

Spinal administration of adenosine and adenosine analogs in humans also exhibited an analgesic effect. A phase I clinical safety study in healthy volunteers demonstrated that 1000 μg of adenosine given intrathecally led to a significant decrease in mustard oil-induced inflammatory pain and tourniquet-induced ischemic pain and decreased areas of secondary allodynia after skin inflammation with low side effects [252]. Recently, some studies have demonstrated new approaches regarding the development of allosteric modulators that enhance the potency of endogenous agonists and aim to minimize the side effects [253]. Allosteric enhancers of the adenosine A_1 receptor have been linked to anti-arrhythmic and anti-lipolytic activity and also have therapeutic potential as analgesics. Oral administration of the A_1 receptor-selective allosteric enhancer T-62 was shown to reduce hypersensitivity in carrageenan-inflamed rats and was approved for phase I clinical trials for neuropathic pain treatment [254]. As was previously discussed, various studies of selective agonists and antagonists demonstrated pro-inflammatory effects of A_1 receptors in different inflammatory models [47]. One interesting study showed a decrease in leukocyte infiltration and reduced lung edema in rats treated with the A_1 receptor antagonist L-97-1 [255]. Furthermore, the selective A_{2A} receptor agonists, apadenoson, binodenoson and sonedenoson have been considered candidates for clinical use in cardiovascular disorders [189, 256, 257]. These agonists are of interest as vasodilator agents in cardiac imaging [258] and as inflammation suppressors. Moreover, as reported by Press and Fozard (2010), two clinical trial applications from Santen Pharmaceuticals claim that use of agonist of adenosine receptors such as regadenoson and sonedenoson [259] is useful in the treatment of glaucoma.

The A_{2A} receptor agonist BVT.115959 from Biovitrum completed clinical trials for diabetic neuropathic pain, and it was well tolerated but did not significantly improve pain symptoms [260].The primary indication claimed for A_{2A} receptor antagonists is in Parkinson's disease [261] because animal studies indicated that adenosine A_{2A} receptors are localized with dopamine D_2 receptors in the striatum and provide an antagonistic interaction between adenosine and dopamine [262]. Vernalis plc and Biogen Idec are currently profiling BIIB014 (V2006) in Phase II clinical trials for Parkinson's disease [263]. Patents for A_{2B} receptor antagonists generally claim asthma and allergic diseases as the primary indications, in line with current views on the receptor's role in vivo [163]. CVT-6883, an A_{2B}-adenosine receptor antagonist, is in clinical development for the treatment of asthma, and this antagonist significantly inhibits in vivo growth of B-16 tumors compared to taxol, as described in a recent review [259].

A_3 receptor selective agonists are also currently in clinical trials and exhibit nanomolar affinity to the receptor. In this context, CF101 (Can-Fite Biopharma) and Cl-IB-MECA (CF102) are in trials for autoimmune inflammatory disorders and liver cancer, respectively. Two other A_3 receptor selective agonists, CP-608,039 and its N6-(2,5-dichlorobenzyl) analog, CP-532,903, were previously under development for cardioprotection [264, 265]. Allosteric modulators of the A_3 receptor have also been developed, such as imidazoquinolines (LUF6000), which have been shown to inhibit adjuvant-induced paw joint swelling in an arthritis rat model [265].

8. Conclusion

This chapter presented a general and updated review of the therapeutic potential of adenosinergic system (including receptor agonists and antagonists, enzyme inhibitors and others) in the control of inflammatory and immune responses. Recently, is becoming clear that both adenosine and inosine play primordial roles in regulating the inflammatory process, working together for example as danger signals, in order to constitute a homeostatic mechanism of tissue integrity. Furthermore, adenosine as well inosine effects are mediated by adenosine receptors and depending on the tissue or cells where they are expressed, pro-inflammatory or anti-inflammatory effects are observed. In this regard, several preclinical studies are conducted to clarify the role of these purines during the inflammatory response and to better understand the adenosine receptors activation which has becoming interesting target to control inflammation. Currently, adenosine is used in the clinical setting, especially in emergency units, to convert sinus rhythm of paroxysmal supraventricular tachycardia (PSVT) to normal sinus rhythm, with excellent cost/effectiveness. Besides the differences in receptor expression between rodents and humans, the use of experimental animal models that can mimic the main features of inflammatory and immune diseases, the improvement of biochemical, genetic and molecular techniques have help us to better establish a translation between preclinical and clinical effects of adenosine and inosine, and to develop and test selective agonists and antagonist of adenosine receptors that can be used in the future treatment of chronic diseases with inflammatory and immune features.

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