Adenosine Receptors and Inflammation

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Abstract Extracellular adenosine is produced in a coordinated manner from cells following cellular challenge or tissue injury. Once produced, it serves as an autocrine- and paracrine-signaling molecule through its interactions with seven-membrane-spanning G-protein-coupled adenosine receptors. These signaling pathways have widespread physiological and pathophysiological functions. Immune

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cells express adenosine receptors and respond to adenosine or adenosine agonists in diverse manners. Extensive in vitro and in vivo studies have identified potent anti-inflammatory functions for all of the adenosine receptors on many different inflammatory cells and in various inflammatory disease processes. In addition, specific proinflammatory functions have also been ascribed to adenosine receptor activation. The potent effects of adenosine signaling on the regulation of inflammation suggest that targeting specific adenosine receptor activation or inactivation using selective agonists and antagonists could have important therapeutic implications in numerous diseases. This review is designed to summarize the current status of adenosine receptor signaling in various inflammatory cells and in models of inflammation, with an emphasis on the advancement of adenosine-based therapeutics to treat inflammatory disorders.

Keywords Adenosine · Adenosine Receptor · G-Protein Coupled Receptor · Inflammation

Abbreviations

A_1AR	Adenosine A_1 receptor
$A_{2A}AR$	Adenosine A_{2A} receptor
$A_{2B}AR$	Adenosine A_{2B} receptor
A_3AR	Adenosine A_3 receptor
AC	Adenylate cyclase
ADA	Adenosine deaminase
AR	Adenosine receptor
CD26	Dipeptidyl pepsidase
CD39	Ectonucleoside triphosphate diphosphohydrolase
CD73	Ecto-5′-nucleotidase
CLP	Cecal ligation and puncture
CNTs	Concentrative nucleoside transporters
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
ERK	Extracellular signal-related protein kinase
fMLP	Formyl methionyl-leucyl-phenylalanine
GRK	G-protein-coupled receptor kinase
HIF	Hypoxia-inducible factor
HMVECs	Human microvascular endothelial cells
HPAECs	Human pulmonary artery endothelial cells
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
IL	Interleukin

imDC	Immature dendritic cell
KO	Knockout
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NF-κB	Nuclear factor kappa B
NK	Natural killer
NR	Normothermic recirculation
PAF	Platelet-activating factor
PBMCs	Peripheral blood mononuclear cells
PDCs	Plasmacytoid dendritic cells
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
TCR	T-cell receptor
TNF-α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

1 Introduction

Adenosine is an endogenous signaling molecule that engages cell surface adenosine receptors to regulate numerous physiological and pathological processes (Fredholm et al. 2001). Extracellular adenosine is produced in excess in response to cellular stress, largely from the breakdown of released adenine nucleotides. Substantial evidence demonstrates that adenosine is an important signaling molecule and adenosine receptors are important molecular targets in the pathophysiology of inflammation. All inflammatory cells express adenosine receptors, and research into the consequences of adenosine receptor activation has identified numerous avenues for adenosine-based therapeutic intervention. Indeed, adenosine-based approaches are currently being developed for the treatment of various disorders where inflammatory modulation is a key component (reviewed in Jacobson and Gao 2006). This chapter was designed to review the contribution of adenosine and adenosine receptors to the regulation of key inflammatory and immune responses.

1.1 *Adenosine Production and Metabolism*

Regulation of extracellular adenosine levels is orchestrated by the actions of proteins that regulate adenosine production, metabolism and transport across the plasma

membrane. The release and catabolism of adenine nucleotides to adenosine is believed to be the major route of adenosine production following cellular stress or injury. Possible routes of ATP release include the constitutive release of ATP through vesicle fusion with the plasma membrane, and programmed release through membrane channels such as the ATP binding cassette family of membrane transporters, including the cystic fibrosis transmembrane conductance regulator (Reisin et al. 1994) and multiple drug resistance channels (Roman et al. 2001), connexin hemichannels (Cotrina et al. 1998), maxi-ion channels (Bell et al. 2003), stretch-activated channels (Braunstein et al. 2001) and voltage-dependent anion channels (Okada et al. 2004). A number of different cell types are sources of adenine nucleotides, including platelets, neurons, and endothelial cells. In addition, inflammatory cells such as mast cells (Marquardt et al. 1984), neutrophils (Madara et al. 1993) and eosinophils (Resnick et al. 1993) are able to release adenine nucleotides and adenosine into the local environment.

Extracellular ATP is rapidly dephosphorylated by ectonucleoside triphosphate diphosphohydrolases such as CD39 to form ADP and AMP (Kaczmarek et al. 1996), and extracellular AMP is dephosphorylated to adenosine by the 5′-nucleotidase CD73 (Resta et al. 1998). CD39 and CD73 are widely expressed on the surface of cells and are essential for the production of adenosine following cellular stress or injury (Thompson et al. 2004; Volmer et al. 2006). Recent findings demonstrate that CD73 and CD39 are novel markers on regulatory T cells (Tregs), where they serve to convert extracellular adenine nucleotides to adenosine, which in turn promotes immunosuppressive activities (Kobie et al. 2006; Deaglio et al. 2007). This process is an example of the concerted role of extracellular adenosine production and signaling in the regulation of inflammatory processes. Adenosine is also generated inside cells by either the dephosphorylation of AMP by cytosolic nucleotidases (Sala-Newby et al. 1999) or the hydrolysis of S-adenosylhomocysteine (Hermes et al. 2005). Alterations in cellular metabolic load or methylation reactions that utilize S-adenosylmethonine as a methyl donor can lead to increased intracellular adenosine levels and subsequent release.

Adenosine is transported across the plasma membrane by both facilitated and cotransport mechanisms. The facilitated nucleoside transporters, known as the equilibrative nucleoside transporters, are bidirectional transporters (Baldwin et al. 2004). They are widely distributed in mammalian tissues, and play a major role in transporting adenosine in and out of the cell. Adenosine transported across the cell membrane also occurs through concentrative nucleoside transporters (CNTs), which are Na^+-dependent concentrative transporters (Gray et al. 2004). The tissue distributions of the CNTs vary, with CNT1 localized primarily to epithelial cells, while CNT2 and CNT3 are more widely distributed.

Finally, adenosine is metabolized by one of two pathways. It can be phosphorylated to form AMP intracellularly by the enzyme adenosine kinase (Spychala et al. 1996), or it can be deaminated to inosine by adenosine deaminase (ADA) (Blackburn and Kellems 1996). ADA is a predominantly cytosolic enzyme. However, it is also found outside the cell as a component of plasma. In humans,

ADA can complex with the cell surface protein CD26 (Hashikawa et al. 2004). This interaction may play an important role in localizing adenosine metabolism to certain regions of the cell surface to impact adenosine signaling. These enzymes, together with rapid cellular uptake, serve to regulate the levels of intra- and extracellular adenosine. In homeostatic situations, adenosine levels range from 10 to 200 nM, whereas extracellular adenosine levels can be elevated to 10–100 μM in hypoxic or stressed tissue environments (Fredholm 2007). The concerted production and metabolism of adenosine is an important mechanism that contributes to the ability of this signaling molecule to regulate aspects of immunobiology and tissue homeostasis.

1.2 Adenosine Receptors

Adenosine exerts its effects by interacting with receptors located on the cell surface. Four adenosine receptor subtypes, A_1, A_{2A}, A_{2B} and A_3, have been defined by pharmacological and molecular biological approaches (Fredholm et al. 2001). These receptors belong to the superfamily of G-protein-coupled receptors and are characterized by seven-transmembrane-spanning α-helical domains with an extracellular amine terminus and a cytoplasmic carboxy terminus. Receptor subtypes are distinguished based on their affinity for adenosine, pharmacological profiles, G-protein coupling and signaling pathways, and genetic sequence. The physiological effects of adenosine are mediated by intracellular signaling processes that are specific to the receptor subtype and the type of cell. The adenosine A_1 receptor (A_1AR) is coupled to the pertussis toxin (PTX)-sensitive inhibitory G proteins (G_i) or G_o. Activation of the A_1AR can lead to the activation a number of effector systems, including adenylate cyclase (AC), phospholipase A_2, phospholipase C (PLC), potassium channels, calcium channels, and guanylate cyclase (Akbar et al. 1994; Olah and Stiles 2000; Fredholm et al. 2001). The primary changes in second messengers associated with A_1AR activation are decreased production of cAMP or increased Ca^{2+}, depending on the effector system. Like the A_1AR, the adenosine A_3 receptor (A_3AR) is coupled to the PTX-sensitive G_i protein and also to G_q (Fredholm et al. 2001). Activation of the A_3AR results in an inhibition of AC (leading to decreased cAMP) or stimulation of PLC and phospholipase D (Gessi et al. 2008). The adenosine A_{2A} receptor (A_{2A}AR) and adenosine A_{2B} receptor (A_{2B}AR) share a relatively high homology and are coupled to Gs (Fredholm et al. 2001), leading to increased levels of cAMP. In addition, the A_{2B}AR has been shown to couple to G_q (Feoktistov et al. 2002), thereby regulating intracellular Ca^{2+} levels. In general, the A_1AR, A_{2A}AR and A_3AR subtypes have high affinity for adenosine, while the A_{2B}AR has a lower affinity (Fredholm 2007).

2 Adenosine Receptors on Immune Cells

2.1 Neutrophils

Neutrophils are the most abundant leukocyte and represent the body's first line of defense in response to a pathogenic challenge; they are the predominant leukocyte involved in acute inflammation (Burg and Pillinger 2001; Edwards 1994; Witko-Sarsat et al. 2000). All four adenosine receptor subtypes are expressed on neutrophils (Bours et al. 2006; Marone et al. 1992; Fortin et al. 2006; Fredholm 2007). At submicromolar adenosine concentrations, A_1AR activation on human neutrophils produces a proinflammatory response by promoting chemotaxis and adherence to the endothelium (Bours et al. 2006; Cronstein et al. 1990, 1992; Forman et al. 2000; Rose et al. 1988). A_1AR-mediated chemotaxis in neutrophils is disrupted by PTX, an agent that inhibits the function of G_i-linked receptors, and requires an intact microtubule system (Cronstein et al. 1990, 1992).

Activation of $A_{2A}AR$ and $A_{2B}ARs$ on neutrophils is anti-inflammatory. High concentrations (micromolar) of adenosine inhibit neutrophil adhesion to endothelial cells by activating $A_{2A}AR$ and $A_{2B}ARs$ on neutrophils (Bours et al. 2006; Eltzschig et al. 2004; Sullivan et al. 2004; Thiel et al. 1996; Wakai et al. 2001). In human neutrophils, $A_{2A}AR$ activation inhibits the formation of reactive oxygen species (Cronstein et al. 1983, 1990; Salmon and Cronstein 1990). In addition, $A_{2A}AR$ activation inhibits the adherence of N-formyl methionyl-leucyl-phenylalanine (fMLP)-activated neutrophils to endothelium (Cronstein et al. 1992) and downregulates Mac-1 (Wollner et al. 1993), β_2-integrin (Thiel et al. 1996; Zalavary and Bengtsson 1998), and L-selectin (Thiel et al. 1996). Activation of the $A_{2A}AR$ also downregulates the activity of other endothelial cell surface proteins, including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) (McPherson et al. 2001), alpha 4/beta 1 integrin VLA4 (Sullivan et al. 2004), and platelet cell adhesion molecule (Cassada et al. 2002). Activation of $A_{2A}ARs$ on activated human neutrophils produces an anti-inflammatory effect by decreasing the formation of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) (Harada et al. 2000, Thiel and Chouker 1995), chemokines such as macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, MIP-2α/CXCL2, and MIP-3α/CCL20 (McColl et al. 2006), and leukotriene LTB4 (Flamand et al. 2000, 2002; Grenier et al. 2003; Krump et al. 1996, 1997; Krump and Borgeat 1999; Surette et al. 1999), and platelet activating factor (PAF) (Flamand et al. 2006). Other important immunoregulatory effects mediated by the $A_{2A}AR$ include the inhibition of Fc gamma (Fcγ) receptor-mediated neutrophil phagocytosis and inhibition of degranulation (Bours et al. 2006; Cronstein et al. 1983; Harada et al. 2000; Salmon and Cronstein 1990; Sullivan et al. 1999; Visser et al. 2000; Zalavary et al. 1994, Zalavary and Bengtsson 1998). Activation of the $A_{2B}AR$ inhibits neutrophil extravasation across human umbilical vein endothelial cell (HUVEC) monolayers and inhibits the release of vascular endothelial growth factor (VEGF) (Wakai et al. 2001).

Conflicting reports suggest that activation of A_3ARs on neutrophils may produce proinflammatory or anti-inflammatory effects. Studies with A_3AR knockout mice suggest that the A_3AR promotes recruitment of neutrophils to lungs during sepsis (Inoue et al. 2008). Moreover, A_3ARs play an important role in the migration of human neutrophils in response to chemoattractant molecules released by microbes (Chen et al. 2006). In isolated human neutrophils, extracellular adenosine (1–1,000 nM) induces a redistribution of A_3ARs to the neutrophil's leading edge, the portion of the membrane closest to the chemoattractant stimulus (Chen et al. 2006). In addition, selective A_3AR antagonists inhibit fMLP-mediated chemotaxis in human neutrophils (Chen et al. 2006). In other studies, activation of A_3ARs on human neutrophils has been shown to counteract inflammation by inhibiting degranulation and oxidative burst (Bouma et al. 1997; Fishman and Bar-Yehuda 2003; Gessi et al. 2002).

2.2 Monocytes and Macrophages

Monocytes and macrophages are a heterogenous group of mononuclear cells that present an early line of innate immune defense. They represent a primary source of inflammatory modulators and are highly adaptable with a phenotype that can change rapidly in response to the local environment of the inflamed tissue (Hasko et al. 2007; Rutherford et al. 1993). Macrophages also serve an important role in terminating the inflammatory process, which is critical for preventing excessive tissue injury (Duffield 2003; Gilroy et al. 2004; Hasko et al. 2007; Wells et al. 2005; Willoughby et al. 2000). All four adenosine receptors are expressed on monocytes and macrophages, although expression levels differ markedly throughout the maturation and differentiation process (Eppell et al. 1989; Thiele et al. 2004). In quiescent monocytes, adenosine receptor expression is low and is increased following activation by inflammatory stimuli. It is hypothesized that the temporal changes in the expression of adenosine receptor subtypes play an important role in the resolution of inflammation. In human monocytes, A_1AR activation produces a proinflammatory effect whereas A_{2A}AR activation produces an anti-inflammatory effect. A key function of the A_1AR is a rapid enhancement of the activity of the Fcγ receptor (Salmon et al. 1993). Activation of A_{2A}ARs limits inflammatory reactions by inhibiting phagocytosis in monocytes (Salmon et al. 1993) and macrophages (Eppell et al. 1989), decreasing the production of reactive oxygen species (Thiele et al. 2004), and altering cytokine release. In addition, A_3AR activation inhibits fMLP-triggered respiratory burst in human monocytes (Broussas et al. 1999).

Monocytes and macrophages are a primary source of TNF-α, a proinflammatory cytokine involved in the pathophysiology of a number of chronic inflammatory diseases. Early studies suggested that activation of the A_{2A}AR suppresses production of TNF-α in human monocytes activated by bacterial lipopolysaccharide (LPS) (Le Vraux et al. 1993). In primary cultures of human monocytes activated by LPS (Zhang et al. 2005) and LPS-stimulated mouse macrophages (Ezeamuzie and Khan

2007), activation of the $A_{2A}AR$ attenuated the release of TNF-α, whereas activation of the A_1AR and A_3AR subtypes had no effect on the formation of TNF-α (Zhang et al. 2005). Similar results were obtained in studies with primary cultures of mouse peritoneal macrophages, in which activation of the $A_{2A}AR$ inhibited LPS-induced TNF-α release, while activation of the A_3AR had no effect (Kreckler et al. 2006). In other studies, activation of the A_3AR was shown to inhibit LPS-induced TNF-α release in vitro in the RAW 264.7 murine leukemia macrophage line (Haskó et al. 1996; Martin et al. 2006), U937 human leukemic macrophage cell line (Sajjadi et al. 1996), murine J774.1 macrophages (Bowlin et al. 1997; McWhinney et al. 1996) and in vivo in endotoxemic mice (Hasko et al. 1996). In the RAW 264.7 macrophage line, the inhibitory effects of A_3ARs were mediated by a mechanism involving Ca^{2+}-dependent activation of nuclear factor-kappa B (NF-κB) (Martin et al. 2006).

Interleukin (IL)-12. IL-12 is a proinflammatory cytokine that is produced in response to certain bacterial and parasitic infections. IL-12 activates naïve T lymphocytes to mount a T helper 1 response. The production of IL-12 is modulated by adenosine and ARs (Hasko et al. 1998, 2000, 2007; Le Vraux et al. 1993; Link et al. 2000). Pharmacological studies (Hasko et al. 2000; Le Vraux et al. 1993; Link et al. 2000) and studies with $A_{2A}AR$ knockout (KO) mice (Hasko et al. 2000) have demonstrated that $A_{2A}AR$ activation downregulates IL-12 production, thereby producing an anti-inflammatory response. In human peripheral monocytes, $A_{2A}AR$ activation decreases IL-12 and IL-12p40 (Link et al. 2000). The effects of the $A_{2A}AR$ on IL-12 production are strongly influenced by the presence of proinflammatory cytokines (Khoa et al. 2001). In THP-1 monocytic cells, TNF-α and IL-1 enhanced $A_{2A}AR$-mediated inhibition of IL-12 production, whereas interferon (IFN)-γ attenuated $A_{2A}AR$-mediated inhibition of IL-12 production (Khoa et al. 2001). The effects of TNF-α and IL-1 were associated with an upregulation of $A_{2A}ARs$, while IFN-γ effects were associated with downregulation of $A_{2A}ARs$.

Activation of the A_3AR negatively regulates the synthesis of IL-12 in murine RAW 264.7 macrophages (Szabo et al. 1998), human monocytes (la Sala et al. 2005), and mice treated with LPS (Hasko et al. 1998). The A_3AR-mediated effects appear to be mediated through the phosphatidyl inositol 3-kinase signaling pathway (la Sala et al. 2005). Taken together, these studies suggest an anti-inflammatory role for the A_3AR via negative regulation of IL-12.

IL-10. IL-10 is an anti-inflammatory cytokine (Kotenko 2002; Moore et al. 1993, Mosmann 1994; Hasko et al. 2007) that functions by inhibiting the secretion of proinflammatory cytokines, including TNF-α and IL-12 (Moore et al. 2001). IL-10 is produced by T helper 2 cells, monocytes, and macrophages (Moore et al. 2001). Following the induction of proinflammatory cytokines, IL-10 regulates the termination of inflammatory processes. Both the $A_{2A}AR$ and $A_{2B}AR$ subtypes have been implicated in the stimulation of IL-10 production in monocytes and macrophages (Haskó et al. 1996, 2000, 2007; Khoa et al. 2001; Link et al. 2000; Nemeth et al. 2005).

Other cytokines, chemokines, and adhesion molecules. Treatment of peripheral blood mononuclear cells (PBMCs) with IL-18, a proinflammatory cytokine released by T cells and dendritic cells, results in increased TNF-α, IL-12, IFN-γ release,

and increased expression of ICAM-1 (Takahashi et al. 2003). In PBMCs, adenosine inhibited the IL-18-induced release of TNF-α, IL-12, and IFN-γ, and expression of ICAM-1. This inhibitory effect was mimicked by an $A_{2A}AR$ agonist and blocked by $A_{2A}AR$ antagonism (Takahashi et al. 2007a). Moreover, the $A_{2A}AR$-mediated anti-inflammatory effects on the IL-18-induced production of TNF-α, IL-12, IFN-γ, and ICAM-1 were reversed by an A_1AR agonist and an A_3AR agonist. The results of these studies suggest that the anti-inflammatory effect of adenosine on human PBMCs activated by IL-18 occurs by activation of the $A_{2A}AR$; however, an A_1AR proinflammatory effect predominates when the $A_{2A}AR$ is saturated with agonist. Thus, the net effect of adenosine on PBMCs activated by IL-18 is a function of the activation of multiple adenosine receptor subtypes, including an anti-inflammatory effect via $A_{2A}ARs$ and proinflammatory effects via A_1AR and A_3ARs (Takahashi et al. 2007a).

With respect to activation of $A_{2B}AR$ and A_3ARs on monocytes and macrophages, in both in vivo and in vitro studies, activation of the $A_{2B}AR$ induces the release of the proinflammatory cytokine IL-6 from macrophages (Ryzhov et al. 2008a), and activation of the A_3AR inhibits the production of MIP-α in LPS-stimulated RAW 264.7 macrophages (Szabo et al. 1998) and inhibits tissue factor expression in LPS-stimulated human macrophages (Broussas et al. 2002). In human monocytes, A_1AR activation induces the release of VEGF (Clark et al. 2007).

2.3 Dendritic Cells

Dendritic cells (DCs) are highly specialized antigen-presenting cells that play an important role in the initiation and regulation of immune responses by migrating to sites of injury and infection, processing antigens, and activating naive T cells (Banchereau and Steinman 1998; Macagno et al. 2007). Immature DCs (imDCs) undergo a maturation process following exposure to proinflammatory signals, including pathogens, LPS, TNF-α, IL-1, and IL-6 (Banchereau and Steinman 1998). The maturation process results in decreased phagocytic activity and increased expression of membrane major histocompatibility complex (MHC), CD54, CD80, CD83, and CD86. Mature DCs release a number of cytokines, including TNF-α, IL-12 and IL-10. IL-12 is a major contributor to the differentiation of Th1 cells. In human blood, DCs are classified as the CD1c^{+} DCs and the CD123^{+} DCs (Shortman and Liu 2002). CD123^{+} DCs, also known as plasmacytoid DCs (PDCs), are located in blood and secrete IFN-γ (Siegal et al. 1999). In addition, PDCs are powerful regulators of T-cell responses (Gilliet and Liu 2002; Kadowaki et al. 2000).

Adenosine receptors are differentially expressed on human DCs (Fossetta et al. 2003; Hofer et al. 2003; Panther et al. 2001, 2003; Schnurr et al. 2004). Immature, undifferentiated human DCs express mRNAs for the A_1AR, $A_{2A}AR$ and A_3AR but not for the $A_{2B}AR$ (Fossetta et al. 2003; Hofer et al. 2003; Panther et al. 2001; Schnurr et al. 2004). Activation of the A_1AR and A_3AR subtypes in undifferentiated DCs induces chemotaxis and mobilization of intracellular Ca^{2+}, while activation of

the $A_{2A}AR$ subtype has no effect (Panther et al. 2001; Fossetta et al. 2003). Activation of the $A_{2A}AR$, but not A_1AR and A_3ARs, in imDCs is linked to increased cell surface expression of CD80, CD86, human leukocyte antigen-DR, and MHC-I (Panther et al. 2003). Activation of A_1ARs in resting DCs suppresses vesicular MHC class I cross-presentation by a G_i-mediated pathway (Chen et al. 2008).

Following treatment with LPS to induce differentiation and maturation, human DCs primarily express the $A_{2A}AR$ (Fossetta et al. 2003; Panther et al. 2001). Activation of the $A_{2A}AR$ increases AC activity and inhibits production of the proinflammatory cytokine IL-12, thereby reducing the ability of the DC to promote the differentiation of T cells to the Th-1 phenotype, and stimulates the production of the anti-inflammatory cytokine IL-10 (Banchereau and Steinman 1998; Panther et al. 2001, 2003).

In immature PDCs, adenosine acting via the A_1AR promotes the migration of PDCs to the site of infection. As PDCs differentiate and mature, the expression of the A_1AR is downregulated, corresponding to a decrease in migratory capability. In mature PDCs, the $A_{2A}AR$ is the predominant subtype and $A_{2A}AR$ activation decreases the production of IL-6, IL-12 and IFN-α (Schnurr et al. 2004). Moreover, IL-3-induced maturation of human PDCs results in a downregulation of A_1ARs and an upregulation of $A_{2A}ARs$ (Schnurr et al. 2004). The mouse DC line XS-106 expresses functional adenosine $A_{2A}AR$ and A_3ARs (Dickenson et al. 2003). $A_{2A}AR$ activation increases cAMP levels and p42/p44 mitogen-activated protein kinase (MAPK) phosphorylation, whereas activation of the A_3AR inhibits cAMP accumulation and increases in p42/p44 MAPK phosphorylation. Functionally, the activation of both subtypes produces a partial inhibition of LPS-induced release of TNF-α.

2.4 Lymphocytes

Lymphocytes are critically involved in adaptive immunity (Alam and Gorska 2003; Larosa and Orange 2008). Adenosine regulates multiple physiologic processes and inflammatory actions on lymphocytes (Bours et al. 2006; Marone et al. 1986, 1992; Priebe et al. 1988, 1990a, b, c). In early studies, it was demonstrated in mixed human lymphocytes that R-PIA (N^6-R-phenylisopropyladenosine) and low concentrations of adenosine (1–100 nM) inhibit cAMP accumulation in human lymphocytes via an A_1AR mechanism, while high concentrations of adenosine (100 nM–100 μM) stimulate cAMP via an $A_{2A}AR$ mechanism (Marone et al. 1986, 1992).

$CD4^+$ and $CD8^+$ T lymphocytes express $A_{2A}AR$, $A_{2B}AR$, and A_3ARs (Gessi et al. 2004, 2005; Huang et al. 1997; Hoskin et al. 2008; Koshiba et al. 1997, 1999; Mirabet et al. 1997). In activated human $CD4^+$ and $CD8^+$ T lymphocytes, $A_{2B}AR$ expression is increased and $A_{2B}AR$ activation is linked to decreased IL-2 production (Mirabet et al. 1999). Activation of human $CD4^+$ T lymphocytes with phytohemagglutinin results in increases in A_3AR mRNA and protein levels that are accompanied by increased agonist potency (Gessi et al. 2004).

A number of studies suggest that $A_{2A}AR$ engagement on CD4$^+$ T lymphocytes results in anti-inflammatory effects. In mouse CD4$^+$ T lymphocytes, $A_{2A}AR$ engagement inhibits T-cell receptor (TCR)-mediated production of IFN-γ (Lappas et al. 2005). TCR activation results in $A_{2A}AR$ mRNA upregulation, which functions as an anti-inflammatory mechanism for limiting T-cell activation and subsequent macrophage activation in inflamed tissues (Lappas et al. 2005). In vitro and in vivo studies suggest that the $A_{2A}ARs$ selectively inhibit TCR-activated T cells, thereby inhibiting lymphocyte inflammatory activity (Apasov et al. 1995, 2000; Erdmann et al. 2005; Huang et al. 1997; Lappas et al. 2005). Activation of the $A_{2A}AR$ on CD4$^+$ T lymphocytes prevents myocardial ischemia-reperfusion injury by inhibiting the accumulation and activation of CD4$^+$ T cells in the reperfused heart (Yang et al. 2006b). Moreover, an anti-inflammatory role in chronic inflammation was demonstrated for the $A_{2A}AR$ in an in vivo murine model of inflammatory bowel disease, where activation of the $A_{2A}AR$ attenuated the production of IFN-γ, TNF-α, and IL-4 in mesenteric T lymphocytes in a rabbit model of colitis (Odashima et al. 2005).

In a mixed lymphocyte reaction of human PBMCs and lymphocytes, adenosine-induced inhibition of IL-18-induced increases in IL-12, IFN-γ, ICAM-1, and lymphocyte proliferation was blocked by an $A_{2A}AR$ antagonist, ZM-241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol), was enhanced by an A_1AR antagonist, DPCPX (8-cyclopentyl-1,3-dipropylxanthine), and an A_3AR antagonist, MRS1220 (N-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo [1,5-c]quinazolin-5-yl]benzene acetamide), and was not affected by an $A_{2B}AR$ antagonist (Takahashi et al. 2007b). Moreover, the anti-inflammatory effect of an $A_{2A}AR$ agonist, CGS 21680 (2-(p-(2-carnonylethyl) phenylethylamino)-5-N-ethylcarboxamido adenosine) on IL-18-induced increases in IL-12, IFN-γ, ICAM-1, and lymphocyte proliferation were reversed by A_1AR and A_3AR agonists. These results suggest that the anti-inflammatory effects of adenosine in a mixed lymphocyte reaction are mediated by $A_{2A}ARs$; however, an A_1AR proinflammatory effect predominates when the $A_{2A}AR$ is saturated with agonist. As such, the net effect of adenosine on a mixed lymphocyte reaction activated by IL-18 is a function of activation of multiple adenosine receptor subtypes, including an anti-inflammatory effect via $A_{2A}ARs$ and proinflammatory effects via A_1AR and A_3ARs (Takahashi et al. 2007b).

In primary cultures of B lymphocytes, activation of B-cell antigen receptors results in the activation of NF-κB pathways (Minguet et al. 2005). Adenosine inhibits the NF-κB pathway by a mechanism related to increased cAMP levels and activation of protein kinase A. This study suggests that adenosine-mediated signals represent an important step in mediating the activation of B lymphocytes.

In activated human and mouse natural killer (NK) cells, adenosine inhibited the production of cytokines and chemokines (Raskovalova et al. 2005, 2006). In in vitro studies with lymphocytes derived from mouse spleen, A_1AR activation increased NK cell activity while A_2AR activation decreased NK cell activity (Priebe et al. 1990a). In mouse LAK cells, the adenosine agonist CADO (2-chloroadenosine) inhibited the cytotoxic activity and attenuated the production of IFN-γ, granulocyte

macrophage colony-stimulating factor, TNF-α, and MIP-1α (Lokshin et al. 2006). Taken together, these results suggest that elevated adenosine levels in tumors may inhibit the tumoricidal effects of activated NK cells (Raskovalova et al. 2005; Lokshin et al. 2006). In addition, recent studies have shown that adenosine exhibits anti-inflammatory activities by engaging A_{2A}ARs on regulatory cells (Deaglio et al. 2007).

2.5 Mast Cells

Mast cells are important effector cells of allergic diseases such as asthma (Shimizu and Schwartz 1997). They can be stimulated to release mediators that have both immediate and chronic effects on airway constriction and inflammation. Adenosine can impact both the degranulation of mast cells and the production of inflammatory mediators. Rodent and human mast cells express the A_{2A}AR, A_{2B}AR and A_3AR (Feoktistov and Biaggioni 1995; Salvatore et al. 2000; Zhong et al. 2003b; Ryzhov et al. 2008b). Engagement of the A_3AR on rodent mast cells mediates degranulation in a manner that involves phosphoinositide 3-kinase (PI3K) activation and increase in intracellular Ca^{2+} (Salvatore et al. 2000; Zhong et al. 2003b). With regards to humans, it is not clear which adenosine receptor mediates the degranulation of mast cells, particularly in the airways. However, emerging evidence suggests that the A_{2B}AR mediates the production and release of proinflammatory mediators such as IL-8, IL-4 and IL-13 from both mouse and human mast cells (Feoktistov and Biaggioni 1995; Ryzhov et al. 2004, 2008b). The role of A_3AR and A_{2B}AR contributions to mast cell degranulation and the production of mediators are areas of active research that will aid in the development of adenosine-based therapeutics for diseases such as asthma, where mast cells play an important role.

2.6 Eosinophils

Eosinophils are involved in the pathophysiology of allergic diseases, including asthma (Frigas and Gleich 1986; Frigas et al. 1991; Gleich et al. 1983). During airway inflammation, eosinophils infiltrate tissues and release inflammatory mediators, including leukotrienes, reactive oxygen species, and granular proteins such as major basic protein. Activation of the A_1AR on human eosinophils enhances O_2^- release (Ezeamuzie and Philips 1999), whereas activation of A_3ARs on human eosinophils elevates intracellular Ca^{2+} (Kohno et al. 1996), inhibits PAF-induced chemotaxis (Knight et al. 1997; Walker et al. 1997), inhibits C5a-induced degranulation (Ezeamuzie and Philips 1999, 2001), and inhibits C5a-induced O_2^- release (Ezeamuzie et al. 1999). Eosinophils isolated from the lungs of patients with airway inflammation have higher levels of A_3AR mRNA compared to controls (Walker et al. 1997). In contrast to these findings, adenosine and A_3AR engagement has been shown to have proinflammatory effects on mouse (Young et al. 2004) and

guinea pig eosinophils (Walker 1996). Together, these studies have led to the suggestion that selective A_3AR ligands may be useful therapies for the treatment of eosinophil-dependent inflammatory disorders such as asthma.

2.7 Endothelial Cells

Under normal physiological conditions, the endothelium provides several important regulatory and protective functions by serving as a physical barrier with both anti-coagulant and anti-inflammatory properties (Hordijk 2006; Mehta and Malik 2006; Sands and Palmer 2005). An initiating event in inflammation is the recruitment and adhesion of leukocytes to the vascular endothelium and changes in endothelial permeability that permit the passage of leukocytes out of the vasculature and into the site of infection or tissue damage.

Adenosine receptors are expressed heterogeneously on endothelial cells, with the predominant subtypes generally being $A_{2A}AR$ and $A_{2B}AR$ (Deguchi et al. 1998; Feoktistov et al. 2002, 2004; Iwamoto et al. 1994; Khoa et al. 2003; Lennon et al. 1998; Olanrewaju et al. 2000; Sexl et al. 1997). In cell culture, endothelial cells derived from different sources have unique expression patterns of adenosine receptor subtypes (Feoktistov et al. 2002, 2004; Khoa et al. 2003). For example, mRNA levels of the $A_{2A}AR$ are approximately tenfold greater than mRNA levels for the $A_{2B}AR$ in HUVECs, whereas mRNA expression of the $A_{2B}AR$ is approximately fourfold greater than $A_{2A}AR$ mRNA expression levels in human microvascular endothelial cells (HMVECs) (Feoktistov et al. 2002). In endothelial cells, activation of the $A_{2A}AR$ inhibits the expression of VCAM-1 (Zernecke et al. 2006), E-selectin (Bouma et al. 1996; Hasko and Cronstein 2004), and tissue factor (Deguchi et al. 1998). Furthermore, activation of the $A_{2A}AR$ (Sullivan et al. 1999) and $A_{2B}AR$ (Eltzschig et al. 2003; Lennon et al. 1998; Yang et al. 2006a) is associated with decreased permeability of the vascular endothelium. These studies suggest that the $A_{2A}AR$ and $A_{2B}AR$ on endothelial cells play an important role in the prevention and mitigation of the inflammatory process. As opposed to these anti-inflammatory effects, activation of A_1ARs on human pulmonary artery endothelial cells (HPAECs) induces the release of thromboxane A_2 and IL-6, substances that are cytotoxic to endothelial cells and increase endothelial permeability (Wilson and Batra 2002). A_1AR antagonists prevented endothelial adhesion and digestion of the endothelial plasmalemma of alveolar capillaries by granulocytes, as well as the diapedesis of neutrophils toward the alveolar lumen in endotoxin-induced acute lung injury (Neely et al. 1997). In addition, activation of A_1ARs and A_3ARs on stimulated HUVECs results in an upregulation and downregulation of tissue factor expression, respectively, representing a potential mechanism for regulating the procoagulant activity of vascular endothelial cells in vivo by adenosine receptors (Deguchi et al. 1998).

3 Regulation of Adenosine Receptor Expression in Inflammatory Environments

Adenosine receptor expression is under dynamic regulation during various forms of physiological and pathophysiological stress, including hypoxia/ischemia and inflammation. For example, a distinct time-dependent alteration in adenosine receptor levels was observed in primary HMVECs subjected to hypoxic culture conditions (Eltzschig et al. 2003). After 12 h, hypoxia induced a selective upregulation of the $A_{2B}AR$, while at later time points (18 and 24 h), expression levels of the A_1AR and $A_{2A}AR$ were downregulated and expression levels of the A_3AR were unchanged. This example demonstrates how a single stimulus can lead to complex alterations in adenosine receptor subtype expression.

Expression of the A_1AR is upregulated under conditions of stress. Numerous studies have demonstrated that stress-induced upregulation of the A_1AR involves increased transcriptional regulation by NF-κB, including in vitro oxidative stress (Nie et al. 1998), in vivo oxidative stress (Ford et al. 1997), in vivo cerebral ischemia (Lai et al. 2005), in vitro hyperosmotic stress (Pingle et al. 2004), in vivo exposure to LPS (Jhaveri et al. 2007), cardiac dysfunction induced by TNF-α overexpression (Funakoshi et al. 2007), and sleep deprivation stress (Basheer et al. 2007). Studies with genetically modified mice lacking the p50 subunit of NF-κB underscore the role of NF-κB in regulating A_1AR expression under basal conditions and pathogenic conditions (Jhaveri et al. 2007). LPS, an activator of NF-κB, increases A_1AR expression levels in the cortices of wild-type but not NF-κB p50 KO mice. In addition, expression of the A_1AR is upregulated in the bronchial epithelium and bronchial smooth muscle of asthmatics (Brown et al. 2008).

In a number of different cell types, the expression of the $A_{2A}AR$ increases following exposure to proinflammatory conditions (Thiel et al. 2003). Following exposure to proinflammatory cytokines, including TNF-α and IL-1β, the expression and functional activity of the $A_{2A}AR$ increases in cultured human monocytic THP-1 cells (Khoa et al. 2001), HMVECs (Nguyen et al. 2003), isolated human neutrophils (Fortin et al. 2006), and A549 human lung epithelial cells (Morello et al. 2006). In A549 cells, the upregulation of $A_{2A}AR$ expression is regulated by NF-κB (Morello et al. 2006). Conversely, IFN-γ downregulates $A_{2A}AR$ expression in THP-1 cells (Khoa et al. 2001) and HMVECs (Nguyen et al. 2003). Following exposure to LPS, mRNA levels for the $A_{2B}AR$ and A_3AR were slightly upregulated in primary mouse intraperitoneal macrophage and WEHI-3 cells (Murphree et al. 2005); however, $A_{2A}AR$ mRNA levels increased dramatically. The increased transcription of $A_{2A}AR$ mRNA in the mouse intraperitoneal macrophages occurred via an NF-κB pathway. In WEHI-3 cells, the LPS-induced upregulation of $A_{2A}AR$ mRNA was accompanied by an increase in cell surface $A_{2A}AR$ expression and increased $A_{2A}AR$ agonist-mediated cAMP production. Functionally, $A_{2A}AR$ agonists inhibited TNF-α production with greater potency in the LPS-treated mouse intraperitoneal macrophages as compared to untreated control cells. These findings demonstrate the role of inflammatory stimuli in the upregulation of $A_{2A}AR$ signaling.

In models of inflammatory bowel disease, characterized by altered levels of proinflammatory cytokines and local tissue hypoxia (Taylor and Colgan 2007), the expression of A_1AR, A_3AR (Sundaram et al. 2003) and $A_{2B}ARs$ (Kolachala et al. 2005a) are altered. In a rabbit model of chronic ileitis, transcription of the A_1AR and A_3AR is upregulated in the ileum (Sundaram et al. 2003). The $A_{2B}AR$ is upregulated in intestinal epithelia of a mouse model of colitis and in human intestinal epithelial mucosa during active colitis (Kolachala et al. 2005a). In T84 human colonic mucosal epithelial cells, TNF-α increases $A_{2B}AR$ mRNA and protein levels (Kolachala et al. 2005a). IFN-γ inhibits $A_{2B}AR$-mediated effects without changing protein expression or $A_{2B}R$ membrane recruitment (Kolachala et al. 2005b). Thus, in inflammatory conditions of the bowel, the regulation of the low-affinity $A_{2B}AR$ occurs via direct effects on receptor expression and indirect effects on signal transduction pathways (Kolachala et al. 2005a, b).

Expression of the $A_{2B}AR$ is upregulated during hypoxic and ischemic conditions (Linden 2001; Eltzschig et al. 2003; Zhong et al. 2005). In primary cultures of human lung fibroblasts, hypoxia induces an increase in $A_{2B}AR$ expression levels (Zhong et al. 2005). Moreover, activation of the $A_{2B}AR$ acts synergistically with hypoxia to increase the release of IL-6 from fibroblasts and promotes differentiation to myofibroblasts, suggesting that the upregulation of the $A_{2B}AR$ may be relevant to chronic lung inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) (for more information on the role of the $A_{2B}AR$ in asthma, see Chap. 11 of this volume, "Adenosine Receptors and Asthma," by Wilson et al.).

Hypoxia also selectively increases the expression of the $A_{2B}R$ in HMVECs and T84 cells (Kong et al. 2006). The $A_{2B}AR$ promoter contains a functional binding site for hypoxia-inducible factor (HIF)-1α, a transcriptional regulator that is important for adaptive responses to hypoxia. Disruption of this element blocks hypoxia-induced $A_{2B}AR$ upregulation, and hypoxia-induced $A_{2B}AR$ expression is directly proportional to HIF-1α activity (Kong et al. 2006). In an in vivo mouse model of colitis, a disorder characterized by increased HIF-1α, $A_{2B}AR$ expression in colon endothelial tissue was increased (Kong et al. 2006). Moreover, HIF-1α KO mice have decreased $A_{2B}AR$ levels in intestinal epithelia. Thus, HIF-1α directly regulates the expression of the $A_{2B}AR$ by modulating gene transcription.

In an in vivo mouse model of LPS-induced peritonitis, a distinct time course for the differential expression of adenosine receptor subtypes is observed (Rogachev et al. 2006). In mouse mesothelial cells, the early stages of peritonitis are characterized by an induction of the A_1AR, with a peak in receptor protein at 12 h and a return to baseline by 24 h. During this phase of peritonitis, activation of the A_1AR is proinflammatory and results in the recruitment and extravasation of leukocytes, with the peak in A_1AR expression correlating with peak leukocyte counts. The $A_{2A}AR$ protein reached a plateau between 12 and 24 h, and the expression of the $A_{2B}AR$ reached a peak after 48 h in mesothelial cells. Functionally, the $A_{2A}AR$ reduced TNF-α and IL-6 levels and decreased leukocyte accumulation. A similar adenosine receptor upregulation profile and time course was observed for the $A_{2A}AR$ and $A_{2B}ARs$ in mouse peritoneal neutrophils (Rogachev et al. 2006). In addition, the effect of proinflammatory cytokines on adenosine receptors was evaluated in human

primary peritoneal mesothelial cells (Rogachev et al. 2006). Following exposure to IL-1 and TNF-α, early proinflammatory cytokines, mRNA and protein expression levels of $A_{2A}AR$ and $A_{2B}ARs$ were upregulated. IFN-γ, secreted later during the course of peritonitis, decreased $A_{2A}AR$ levels but increased $A_{2B}AR$ expression levels in human primary peritoneal mesothelial cells. These results suggest that the acute phase of the peritoneal infection involves a proinflammatory A_1AR response and increased release of proinflammatory cytokines, which upregulate the expression of the anti-inflammatory $A_{2A}AR$ and $A_{2B}ARs$ (Rogachev et al. 2006). These findings further demonstrate the intricate regulation of adenosine receptor expression in specific inflammatory environments (Rogachev et al. 2006).

3.1 Adenosine Receptor Desensitization

Desensitization, a mechanism by which a cell attenuates its response to prolonged agonist stimulation, has been studied for all four adenosine receptor subtypes and is driven by a number of factors, including receptor subtype, compartmentalization and scaffolding, and the complement of intracellular proteins involved in the desensitization and signaling process (Klaasse et al. 2008). While both the A_1AR and A_3AR are G_i-coupled receptors, their desensitization responses to agonist stimulation are very different. The cloned rat A_3AR desensitizes rapidly via the phosphorylation of serine and threonine residues on the intracellular carboxy terminus (Palmer et al. 1995a, 1996; Palmer and Stiles 2000). In contrast, the cloned human A_1AR is not phosphorylated in response to agonist and only becomes desensitized after prolonged agonist exposure (Ferguson et al. 2000, 2002).

A number of in vitro studies with cultured cells have demonstrated agonist-mediated $A_{2A}AR$ desensitization, including Chinese hamster ovary cells (Palmer et al. 1994), rat pheochromocytoma PC-12 cells (Chang et al. 1997), NG108-15 mouse neuroblastoma $\times$ rat glioma hybrid cells (Mundell and Kelly 1998; Mundell et al. 1998), rat aortic vascular smooth muscle cells (Anand-Srivastava et al. 1989), and bovine aortic endothelial cells (Luty et al. 1989). In addition, $A_{2A}AR$ desensitization has been demonstrated in native tissue, including rat brain (Barraco et al. 1996) and porcine coronary artery (Makujina and Mustafa 1993). Desensitization of both the $A_{2A}AR$ and $A_{2B}AR$ is mediated by the G-protein-coupled receptor kinase (GRK) 2 isozyme (Mundell et al. 1998). In human astroglial cells, chronic treatment with TNF-α increases the functional responsiveness of $A_{2B}ARs$ (Trincavelli et al. 2004); however, short-term treatment with TNF-α causes $A_{2B}AR$ phosphorylation, impaired $A_{2B}AR$–G protein coupling, and reduced cAMP production (Trincavelli et al. 2008).

In addition to direct effects on expression level, adenosine receptor signaling can be modified by indirect changes in intracellular signal transduction components. In HMVECs treated with TNF-α and IL-1β, there is an increase in $A_{2A}AR$ activity related to receptor upregulation and increased levels of the G protein $\beta 4$ isoform (Nguyen et al. 2003). Moreover, TNF-α prevents $A_{2A}AR$ desensitization in human

monocytoid THP-1 cells by blocking the translocation of GRK2 and β-arrestin to the cell membrane, which together with TNF-α stimulation results in upregulation of the $A_{2A}AR$ (Khoa et al. 2001) and enhanced $A_{2A}AR$ activity (Khoa et al. 2006). In T84 human colonic mucosal epithelial cells treated with IFN-γ, a reduction in $A_{2B}AR$ signaling occurs in response to a downregulation of AC isoforms 5 and 7 without affecting $A_{2B}AR$ expression levels or membrane recruitment (Kolachala et al. 2005b). In human astrocytoma ADF cells, TNF-α increased $A_{2B}AR$ functional responses and receptor G-protein coupling without altering expression levels. This increased functional response was mediated by attenuating agonist-mediated phosphorylation and desensitization of the $A_{2B}AR$ (Trincavelli et al. 2004).

Thus, desensitization is an important phenomenon that contributes to the net effect of adenosine signaling on specific cell types involved in inflammation and to the development of agonists as therapeutic agents, since the potential for tolerance/tachyphylaxis as an unwanted effect could limit their efficacy with chronic use.

4 Adenosine Receptor Contributions to the Regulation of Inflammation

4.1 A_1AR and Inflammatory Responses

4.1.1 Historical Perspective

A seminal study published in 1983 by Cronstein and colleagues demonstrated that the A_1AR mediates proinflammatory events and the A_2AR mediates antiinflammatory effects in isolated human neutrophils (Cronstein et al. 1983). In recent years, the role of the A_1AR in inflammation has been extensively studied using a number of approaches, including selective agonists and antagonists, monoclonal antibodies, selective antisense molecules, and genetically modified animals (Bours et al. 2006; Hasko and Cronstein 2004; Salmon et al. 1993; Sun et al. 2005). These studies have contributed to the delineation of the role of A_1AR in inflammation.

4.1.2 Proinflammatory Effects

Activation of the A_1ARs produces proinflammatory effects in a number of different tissues and cell types. On human neutrophils, A_1AR activation induces neutrophil chemotaxis, adherence to endothelial cells, and Fcγ receptor-mediated phagocytosis and O_2^- generation (Cronstein et al. 1990, 1992; Forman et al. 2000; Salmon and Cronstein 1990). In cultured human monocytes, the A_1AR enhances Fcγ receptor-mediated phagocytosis (Salmon et al. 1993; Salmon and Cronstein 1990), and promotes multinucleated giant cell formation on synovial fluid mononuclear phagocytes of patients with rheumatoid arthritis (Salmon et al. 1993; Merrill et al.

1997). Furthermore, A_1AR activation induces VEGF release from human mono-cytes (Clark et al. 2007). In human PBMCs, A_1AR antagonist enhanced and A_1AR agonist reversed the anti-inflammatory effects of adenosine mediated by $A_{2A}ARs$ on expression of ICAM-1 and production of IFN-γ, IL-12 and TNF-α in the presence of IL-18 (Takahashi et al. 2007a). In addition, A_1AR antagonism enhanced and A_1AR agonism reversed the anti-inflammatory effects of adenosine mediated by $A_{2A}ARs$ on expression of ICAM-1 and production of IL-12 and IFN-γ and lymphocyte proliferation during a human mixed lymphocyte reaction (Takahashi et al. 2007b). These findings suggest that activation of the A_1AR on a number of different inflammatory cells results in proinflammatory effects.

A proinflammatory role for the A_1AR has also been demonstrated in in vivo studies in a number of different species and disease states, including a rat model of pancreatitis (Satoh et al. 2000), ischemia-reperfusion injury of the lung (Neely and Keith 1995), ischemia-reperfusion injury of the heart in cats (Neely et al. 1996), dogs (Auchampach et al. 2004; Forman et al. 2000), and rats (Katori et al. 1999), ischemia-reperfusion injury of the liver in dogs (Magata et al. 2007) and pigs (Net et al. 2005), and endotoxin-induced lung injury in cats (Neely et al. 1997). Moreover, in an allergic model of asthma, L-97-1 (3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl-1-propyl-3,7-dihydro-purine-2,6-dione), a selective A_1AR antagonist reduced airway inflammation following allergen challenge, specifically reducing the number of eosinophils, neutrophils and lymphocytes in the airways (Nadeem et al. 2006). Recently, Ponnoth et al. have shown a proinflammatory role for A_1AR in vascular inflammation using a mouse model of allergic asthma (Ponnoth et al. 2008).

In rat models of acute pancreatitis induced with cerulein or taurocholate, the pancreas showed morphological changes that included interstitial edema and leukocyte infiltration (Satoh et al. 2000). Intraperitoneal administration of CCPA (2-chloro-N^6-cyclopentyladenosine), a selective A_1AR agonist, produced similar dose- and time-dependent effects on leukocyte infiltration and interstitial edema in pancreatic tissue, while $A_{2A}AR$ and A_3AR agonists had no effect (Satoh et al. 2000). The proinflammatory histopathological effects produced by CCPA in this model were attenuated by FK-838 (6-oxo-3-(2-phenylpyrazolo[1,5-α]pyridin-3-yl)-1(6H)-pyridazinebutanoic acid), an A_1AR-selective antagonist. These results suggest that activation of the A_1AR may play an important role in the tissue damage observed in acute pancreatitis.

Whole animal studies in models of ischemia-reperfusion in the lungs (Neely and Keith 1995), heart (Auchampach et al. 2004; Forman et al. 2000; Neely et al. 1996), and liver (Magata et al. 2007; Net et al. 2005) demonstrated that the A_1AR plays a proinflammatory role in these systems. In a feline model of ischemia-reperfusion injury of the lung, infusion of the A_1AR antagonists XAC (xanthine amine congener) or DPCPX reduced the percentage of injured alveoli (Neely and Keith 1995). In addition, DPCPX prevented endothelial damage, as well as margination and adhesion of neutrophils to pulmonary endothelial cells. Moreover, in a feline regional cardiac infarct model, pretreatment with the A_1AR antagonists DPCPX, bamiphylline, and XAC prevented ischemia-reperfusion injury; in other

words, reduced infarct size (Neely et al. 1996). Similarly, in the canine model of myocardial ischemia-reperfusion, pre- and posttreatment with the A_1AR antagonist DPSPX (1,3-dipropyl-8-p-sulfophenylxanthine) decreased the area of cardiac necrosis and improved regional ventricular function (Forman et al. 2000). Based on studies with isolated human neutrophils demonstrating that DPSPX and DPCPX blocked fMLP-induced chemoattraction, it was hypothesized that the cardioprotective effect of the A_1AR antagonist DPSPX in the canine model was due to inhibition of neutrophil chemoattraction (Forman et al. 2000). For more information on A_1ARs and ischemia-reperfusion injury of the heart, please refer to Chap. 7 of this volume, "Adenosine Receptors and Reperfusion Injury of the Heart," by Headrick and Lasley.

The role of the A_1AR in hepatic ischemia-reperfusion injury was studied in a model using total hepatic vascular exclusion in beagles (Magata et al. 2007) and in a normothermic recirculation (NR) model of liver transplantation in pigs (Net et al. 2005). In the canine model, pretreatment with KW3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine), an A_1AR antagonist, significantly increased survival following hepatic ischemia-reperfusion (Magata et al. 2007). Moreover, histopathological examination of liver tissue revealed that pretreatment with KW3902 preserved hepatic architecture and decreased the infiltration of neutrophils into hepatic tissue. In the porcine model, NR following warm ischemia reversed the injury associated with liver transplantation and increased five-day survival. This protective effect of NR was simulated by the preadministration of adenine (Net et al. 2005). Blockade of the A_1AR with DPCPX during NR further protected the liver. Taken together, these studies suggest that the A_1AR plays a proinflammatory role in hepatic ischemia-reperfusion injury.

In an in vivo feline model of LPS-induced lung injury, blockade of the A_1AR prevents acute lung injury (Neely et al. 1997). In this model, an intralobar arterial infusion of LPS produced dose-dependent lung injury characterized by perivascular and peribronchial edema and hemorrhage, margination of neutrophils along the venular endothelium, thickened alveolar septae, alveolar infiltration of neutrophils and macrophages, alveolar edema, and alveolar hemorrhagic necrosis. In this study, lungs from animals treated with the A_1AR antagonists DPCPX or bamiphylline could not be distinguished from controls, suggesting that LPS-induced pulmonary injury involves activation of the A_1AR (Neely et al. 1997). To further evaluate A_1AR function in LPS-induced lung injury, LPS from various Gram-negative bacterial sources were evaluated using cultured cells derived from HPAECs (Wilson and Batra 2002). LPSs from *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* bind directly to the A_1AR. Additional studies with HPAECs demonstrated that the CCPA- and LPS-induced release of IL-6 and thromboxane A_2, cytotoxic substances that increase permeability of the endothelium, was blocked by DPCPX. Together, these studies suggest that activation of the A_1AR on pulmonary artery endothelial cells by LPS during sepsis directly contributes to the pathology of acute lung injury (Neely et al. 1997; Wilson and Batra 2002).

To demonstrate the efficacy of an A_1AR antagonist as an antiendotoxin, antisepsis adjunctive therapy in combination with antibiotics in sepsis, the A_1AR antagonist

L-97-1 has been tested in a model of polymicrobial sepsis and endotoxemia (rat cecal ligation and puncture, CLP). Administration of L-97-1 as an intravenous therapy postCLP improved the seven-day survival in a dose-dependent manner (30–40% survival) as compared to untreated CLP controls (17% survival) or antibiotics alone (23% survival) (Wilson et al. 2006). In combination with antibiotics, L-97-1 increased survival to 50–70% in a dose-dependent manner. Improvement in seven-day survival was statistically significant for L-97-1 versus CLP, as well as for L-97-1 versus antibiotics. Moreover, L-97-1 plus antibiotics had a significant trend towards increased survival time based on the dose of L-97-1. Furthermore, efficacy for the A_1AR antagonist L-97-1 has been demonstrated in a bioterrorism animal model of pneumonic plague (Wilson, Endacea, Inc., unpublished data). In this model, rats are infected via intratracheal administration with *Yersinia pestis*, a Gram-negative bacterium that releases endotoxin, a major virulence factor for *Y. pestis*. In these studies, L-97-1 plus antibiotics (ciprofloxacin) improves six-day survival and lung injury scores versus antibiotics alone in 72 h delay treatment groups. During sepsis, the expression of A_1ARs is upregulated (Rogachev et al. 2006), and furthermore, LPS upregulates A_1AR expression (Jhaveri et al. 2007). These studies, taken together with the findings that A_1AR antagonists block LPS-induced acute lung injury and improve survival in both a CLP and a Gram-negative sepsis model induced by *Y. pestis*, suggest that the A_1AR is an important target in sepsis, and that A_1AR antagonists may represent an attractive class of compounds for development as antisepsis drugs.

Collectively, in vitro studies in inflammatory cells and in vivo studies in animal models suggest that the A_1AR is an important target in inflammation, and that A_1AR antagonists may be efficacious as anti-inflammatory drugs. Several biotechnology, biopharmaceutical, and pharmaceutical companies have engaged in developing A_1AR antagonists for different medical conditions. Phase I/II/III clinical trials demonstrate that A_1AR antagonists as a class of drugs appear to be safe in humans. For example, Aderis Pharmaceuticals (formerly Discovery Therapeutics) developed an A_1AR antagonist, N-0861 (N^6-endonorboran-2-yl-9-methyladenine), for the treatment of bradyarrhythmias (Bertolet et al. 1996). This compound was in Phase I/IIa clinical trials until it was put on clinical hold due to solubility problems. The high volume of diluent required to administer the drug intravenously, not the safety of N-0861, prevented further clinical development of this molecule. CV Therapeutics (Palo Alto, CA, USA) licensed CVT 124 to Biogen as BG-9719 (ENX) (1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)]xanthine) for the treatment of congestive heart failure with renal impairment (Gottlieb et al. 2002). In clinical trials, both N-0861 and BG-9719 were well tolerated. However, problems with solubility, bioavailability, and formulation prevented the further clinical development of these A_1AR antagonists (Bertolet et al. 1996; Gottlieb et al. 2002; Doggrell 2005). Biogen (Biogen Idec, Cambridge, MA, USA) is developing another A_1AR antagonist, Adentri (BG 9928; 1,3-dipropyl-8-[1-(4-propionate)-bicyclo-[2,2,2]octyl]xanthine) for chronic congestive heart failure and renal impairment. This molecule is safe and is in Phase III clinical trials (Doggrell 2005; Greenberg et al. 2007; Press release Biogen, August 21, 2008). Two other A_1AR antagonists, SLV320 (Solvay)

and KW-3902 (rolofylline) (previously NovaCardia, now Merck) are in Phase II and Phase III clinical trials for congestive heart failure with renal impairment, respectively, and are well tolerated (Givertz et al. 2007; Dittrich et al. 2007; http://www.clinicaltrials.gov).

4.1.3 Anti-inflammatory Effects

Studies with genetically modified mice suggest that the A_1AR also has context-dependent anti-inflammatory functions (Sun et al. 2005; Joo et al. 2007; Lee and Emala 2000; Lee et al. 2004a, b, 2007). A_1AR function was evaluated in adenosine deaminase (ADA) knockout mice by generating ADA/A_1AR double-knockout mice. ADA knockout mice exhibit increased levels of adenosine and increased levels of the A_1AR transcript, which was most predominant in activated alveolar macrophages (Sun et al. 2005). These animals developed pulmonary inflammation, characterized by an increase in macrophages, eosinophils, fibrosis, and airway hyperreactivity. Pulmonary inflammation was exacerbated in mice lacking both ADA and the A_1AR (Sun et al. 2005). The lungs of ADA/A_1AR double-knockout mice were characterized by higher levels of cytokines, including IL-4 and IL-13, and chemokines such as eotaxin 2 and thymus- and activation-regulated chemokine. Interestingly, lung adenosine levels in ADA/A_1AR double-knockout mice were approximately 200% higher than those found in ADA-deficient mice (Sun et al. 2005). Furthermore, an anti-inflammatory role of the A_1AR was demonstrated in A_1AR knockout mice with experimental allergic encephalomyelitis, an in vivo model of multiple sclerosis (Tsutsui et al. 2004). A_1AR knockout mice exhibited severe demyelination and axonal injury, enhanced activation of macrophages and microglial cells, increased transcription of proinflammatory cytokines, and decreased transcription of anti-inflammatory cytokines.

The anti-inflammatory role associated with the A_1AR has been extensively studied in models of renal ischemia-reperfusion in mice and rats and in cultured renal tubule cells (Joo et al. 2007; Lee and Emala 2000; Lee et al. 2004a, 2007). Initially, the anti-inflammatory role for the A_1AR was described in a rat model of renal ischemia-reperfusion, where preconditioning, adenosine, and an A_1AR agonist, R PIA, produced a protective effect improving renal function and morphology (Lee and Emala 2000). Interestingly, an A_3AR agonist, IB-MECA (1-deoxy-1-(6-((3-iodophenyl)methyl)amino-9H-purin-9-yl)-N-methyl-D-ribofuranuronamide), worsened and an A_3AR antagonist, MRS 1191 (3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-dihydropyridine-3,5-dicarboxylate), improved renal function in this model. The protective effect of the A_3AR antagonist was greater than that of adenosine. In these studies, DPCPX blocked the protective effect of adenosine but not that of preconditioning (Lee and Emala 2000). Subsequently, a protective effect for CCPA, an A_1AR agonist, was demonstrated in a mouse model of renal ischemia-reperfusion (Lee et al. 2004b). In these studies, DPCPX worsened renal function and increased expression of inflammatory markers, necrosis and apoptosis, and blocked the protective effect of CCPA. Next, the protective effect of

the A_1AR in renal ischemia-reperfusion was studied in A_1AR knockout mice (Lee et al. 2004a). In these studies, A_1AR knockout mice showed worsened renal function and histology compared to the wild-type controls. Moreover, DPCPX increased markers of renal inflammation while CCPA reduced markers of renal inflammation. Interestingly, the A_3AR antagonist MRS 1191 improved renal function in A_1AR knockout mice with an efficacy similar to that produced by CCPA in wild-type mice (Lee et al. 2004a). Collectively, these studies suggest that both A_1AR and A_3ARs play an important role in ischemia-reperfusion injury in the kidney of rats and mice.

In mice, the mechanism of renal protection was found to consist of an acute and a delayed phase. Renal protection involved A_1AR-mediated phosphorylation of ERK MAPK and Akt, which are involved in the upregulation of cytoprotective genes (Joo et al. 2007). Activation of the A_1AR also resulted in increased phosphorylation of heat shock protein (HSP) 27 (Joo et al. 2007; Lee et al. 2007), a molecular chaperone involved in the cytoprotection of cellular proteins through the prevention of denaturation and aggregation under conditions of oxidative stress (Joo et al. 2007). In contrast, A_1AR knockout mice had decreased levels of basal HSP27 (Lee et al. 2007). Specific inhibitors of HSP synthesis blocked the A_1AR-mediated renal protection in A_1AR wild-type mice. Inhibition of G_i proteins with PTX blocked both the early phase and the late phase protective effects mediated by the A_1AR. The early phase of the A_1AR-mediated antiinflammatory effect was blocked with chelerythrine, a protein kinase C (PKC) inhibitor. The early and delayed phases of renal protection were blocked by deletion of PI3K gamma and inhibition of Akt, but not inhibition of ERK.

The role of A_1AR activation has also been studied in an immortalized porcine renal tubule cell line (LLC–PK1 cells) overexpressing the human A_1AR, and in primary cultures of renal proximal tubule cells from A_1AR knockout mice (Lee et al. 2007). In the LLC–PK1 cells, overexpression of the A_1AR was associated with increased basal expressions of total and phosphorylated HSP27, reportedly due to A_1AR-mediated stimulation of p38 and MAPK. Renal epithelial cells overexpressing the A_1AR showed decreased peroxide-induced necrosis and TNF-α-induced apoptosis, which was blocked by selective blockade of the A_1AR. In contrast, primary cultures of proximal tubule cells from A_1AR knockout mice showed increased levels of necrosis and apoptosis. Taken together, these studies suggest that A_1AR activation exerts a protective preconditioning effect in renal ischemia-reperfusion by modulating the inflammatory response and tissue necrosis, and that this process may involve HSP27.

Furthermore, studies with A_1AR knockout and wild-type mice suggest that activation of the A_1AR protects against sepsis (Gallos et al. 2005). Following CLP, mortality was increased in both the A_1AR knockout mice and in wild-type mice treated with DPCPX to antagonize A_1ARs. In addition, A_1AR knockout mice had increased levels of TNF-α, suggesting that the A_1AR modulates TNF-α production during sepsis. Finally, renal tissue in the A_1AR knockout mice exhibited increased levels of neutrophils, ICAM-1, and proinflammatory cytokines, indicating a higher degree of renal dysfunction induced by sepsis. These results suggest that the A_1AR attenuates the inflammatory response and diminishes the hyperacute inflammatory response characteristic of sepsis.

The differences in the studies suggesting both a proinflammatory role for the A_1AR in the pancreas, lung, heart, and liver as well as an anti-inflammatory role in the lung and kidney may be due to differences in the models (i.e., genetically modified mice and cell lines overexpressing adenosine receptors versus other models). For example, in the A_1AR knockout models and cell lines, other proteins such as the $A_{2B}AR$ and A_3ARs may be responsible for the protective effects of adenosine described. There is substantial evidence suggesting that the protective effect of preconditioning is mediated by PTX-sensitive G_i-coupled proteins, including the A_1AR and A_3ARs. The studies that described a protective effect of both A_1AR and A_3ARs in the kidney are consistent with what is reported in the literature for other species. Therefore, the protective effects of adenosine and the selective A_1AR agonists R-PIA and CCPA in studies of renal ischemia-reperfusion are not surprising. The protective effect of overexpression of the A_1AR in LLC–PKC1 cells is also not surprising, for the same reasons.

The deleterious effects of DPCPX on the renal function and histology of the kidney in the rat and mouse is surprising in light of the protective effect of a number of A_1AR antagonists in different models of inflammation. DPCPX is at best tenfold selective for A_1ARs versus $A_{2B}ARs$ (Fredholm et al. 2001). In the in vivo renal ischemia-reperfusion studies and sepsis studies in mice, it is possible that DPCPX may be blocking the anti-inflammatory effects of the G_s-coupled $A_{2B}AR$ (Yang et al. 2006a). Although another highly selective A_1AR antagonist, FSCPX 8-cyclopentyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]propyl]-1-propylxanthine), reversed the resistance to cell death in LLC–PK1 cells produced by overexpressing the A_1AR, FSCPX was used in a high concentration (20 µM), and a dose–response relationship for the blocking effects of FSCPX was not demonstrated. In other models of inflammation, anti-inflammatory effects for highly selective A_1AR antagonists, including BG-9928 in ischemia-reperfusion injury of the heart in dogs (Auchampach et al. 2004), FK-838 in pancreatitis in the rat (Satoh et al. 2000), FK-352 ([R]-1-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl) acryloyl]piperidin-2-yl acetic acid) in ischemia-reperfusion injury of the heart in rats (Katori et al. 1999), and L-97-1 in allergic asthma in the rabbit (Nadeem et al. 2006), were demonstrated.

Differences in the studies described above suggesting both a proinflammatory and an anti-inflammatory role for the A_1AR may be due to differences in (i) the A_1AR-activated signaling pathway that results in tissue injury (i.e., proinflammatory pathway) versus that for protection (i.e., anti-inflammatory pathway), and which pathway predominates as a function of species and the stage/progression of injury; (ii) predominant inflammatory cell type as a function of species; in other words, the role of the macrophage where the transcript for the A_1AR was high in mice (Sun et al. 2005) versus the predominant role of the neutrophil in acute inflammation in other species; (iii) expression of A_1ARs on endothelial cells and different inflammatory cells, possibly a function of species differences; (iv) intracellular signaling and desensitization mechanisms as a function of species or cell/tissue/organ; and (v) density of homo- and heterodimers of adenosine receptors and their functional properties as a function of the cell type, organ, and species. For example, there is evidence from in vitro studies with native neural tissue as well as in vivo

studies to suggest that adenosine receptors, including the A_1AR, can form homo-
and heterodimers that have unique pharmacological profiles and functional effects,
including altered ligand affinity, G-protein coupling, and desensitization character-
istics (Ciruela et al. 2006; Ferre et al. 2008; Franco et al. 2006; Fuxe et al. 1998;
Nakata et al. 2004). To date, adenosine receptor dimerization has not been studied
in cells from the immune system, but it is likely that this phenomenon is relevant
in inflammatory processes. These considerations are of particular significance given
that inflammatory cells undergo significant phenotype changes (e.g., adenosine re-
ceptor expression levels, altered cytokine profiles, altered cell surface protein levels)
that are unique to various physiological and pathophysiological challenges. Finally,
differences in the phenotypes of genetically modified animals and cells are very
complex and are not yet completely understood. It is possible that genetically ma-
nipulated animals and cells exhibit compensatory expression or functions of other
proteins that alter the phenotype of cells and organs in a manner that is not fully
appreciated at this time.

4.2 $A_{2A}AR$ and Inflammatory Responses

4.2.1 Historical Perspective

Numerous investigations in cellular and animal model systems have provided ev-
idence that $A_{2A}AR$ signaling pathways are active in limiting inflammation and
tissue injury (Hasko and Cronstein 2004; Linden 2005; Sitkovsky and Ohta 2005;
Hasko and Pacher 2008). Some of the earliest observations that $A_{2A}AR$ signaling
is anti-inflammatory came from Cronstein and colleagues, who demonstrated that
engagement of the $A_{2A}AR$ could inhibit elicited superoxide formation from neu-
trophils (Cronstein et al. 1983). Expression of the $A_{2A}AR$ has subsequently been
found on most inflammatory cells (Sitkovsky et al. 2004), where it has numerous
anti-inflammatory properties, including inhibiting T-cell activation (Huang et al.
1997; Erdmann et al. 2005) and limiting the production of inflammatory media-
tors such as IL-12, TNF-α and INFγ (Hasko et al. 2000; Pinhal-Enfield et al. 2003;
Lappas et al. 2005). Ohta and colleagues performed a series of studies in vivo us-
ing $A_{2A}AR$ knockout mice to demonstrate that this receptor plays an important
role in limiting the degree of inflammatory mediator production and tissue injury
in response to challenges with concanavalin A or endotoxin (Ohta and Sitkovsky
2001). Subthreshold doses of these agents that caused minimal responses in wild-
type mice led to extensive inflammatory mediator production, tissue damage and
death in $A_{2A}AR$ knockout mice. Thus, adenosine signaling through the $A_{2A}AR$ ap-
pears to serve as a critical endogenous regulator of tissue inflammation and damage.
Given that hypoxia and subsequent adenosine generation is likely an acute response
to numerous injuries, this pathway is likely to have important and widespread im-
plications in dictating the balance of tissue injury and repair.

4.2.2 Anti-inflammatory Effects

Substantial lines of evidence suggest that the $A_{2A}AR$ is the major adenosine receptor mediating the anti-inflammatory properties of adenosine (Hasko and Pacher 2008). The ability of $A_{2A}AR$ activation to suppress Th1 cytokine and chemokine expression by immune cells is likely the dominant mechanism involved. For example, $A_{2A}AR$ activation can attenuate IL-12, INFγ and TNF-α production from important immunomodulatory cells such as monocytes (Hasko et al. 2000), dendritic cells (Panther et al. 2003) and T cells (Lappas et al. 2005). The ability to diminish the production of such cardinal inflammatory molecules likely contributes to the decreased inflammation and tissue damage due to effector cell activation that is often seen with $A_{2A}AR$ activation. However, there is also evidence that $A_{2A}AR$ activation can prevent effector cell activities such as neutrophil migration (Cronstein et al. 1992) and oxidative burst (Cronstein et al. 1983). Collectively, these anti-inflammatory properties of the $A_{2A}AR$ represent a sensitive and widespread mechanism for the immunoregulation of tissue injury and repair.

Findings in disease-relevant animal models suggest that $A_{2A}AR$ activation on immune cells is beneficial in environments associated with acute inflammation and hypoxia. $A_{2A}AR$ agonists have remarkable anti-inflammatory and tissue-protective effects in models of ischemic liver damage (Day et al. 2004,) myocardial injury (Lasley et al. 2001; Glover et al. 2005), spinal cord injury (Reece et al. 2004), renal injury (Day et al. 2003), inflammatory bowel disease (Naganuma et al. 2006), and lung transplantation (Ross et al. 1999). Many of these models involve postischemic environments and suggest that $A_{2A}AR$ activation (on various immune cells) limits or inhibits the degree of inflammation and subsequent tissue damage. Activation of the $A_{2A}AR$ has also been shown to play an important role in the promotion of wound healing and angiogenesis (Montesinos et al. 2002), and the $A_{2A}AR$ and A_3AR are responsible for the anti-inflammatory actions of methotrexate in the treatment of inflammatory arthritis (Montesinos et al. 2003). Collectively, these studies suggest that activation of the $A_{2A}AR$ has a significant impact on stemming inflammation and tissue damage in a number of disease-relevant models, suggesting that there may be numerous clinical benefits from the use of $A_{2A}AR$-activating compounds.

Recent studies have utilized bone marrow transplantation approaches together with gene knockout and selective $A_{2A}AR$ agonist treatments to identify populations of immune cells that contribute to the anti-inflammatory properties of this receptor in disease models. In a model of ischemia-reperfusion liver injury, activation of the $A_{2A}AR$ with the selective agonist ATL146e (4-(3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl)- cyclohexanecarboxylic acid methyl ester) was associated with decreased inflammation, and the liver was protected from damage brought about by reperfusion following ischemia (Day et al. 2004). When $A_{2A}AR$ knockout mice were subjected to the same insult, the effectiveness of ATL146e was lost. Moreover, $A_{2A}AR$ knockout mice exhibited increased liver damage, suggesting that endogenous adenosine is involved in the tissue protection seen. Subsequent studies using

bone marrow transplantation approaches and $A_{2A}AR$ knockout mice suggested that it was $A_{2A}AR$ on bone marrow-derived cells that conferred $A_{2A}AR$ agonist protection. Subsequent studies identified CD1d-activated NK T cells as being the critical cells mediating the protective effects of $A_{2A}AR$ agonist treatment in this model, where $A_{2A}AR$ engagement reduced the production of IFN-γ from NK T-cells in association with blocking liver reperfusion injury (Lappas et al. 2006). Bone marrow transplantation studies using $A_{2A}AR$ knockout mice were also used to demonstrate that the protective effect of $A_{2A}AR$ agonist in a model of renal ischemia-reperfusion injury was due to $A_{2A}AR$ activation on marrow-derived cells (Day et al. 2003). Although the exact cell type has not been identified, there is evidence to suggest that it is a cell type other than macrophages, which have been shown to be important in mediating the protective effects of $A_{2A}AR$ agonism in a model of diabetic nephropathy (Awad et al. 2006). Similar approaches demonstrate that $A_{2A}AR$ expression on bone marrow-derived cells is responsible for $A_{2A}AR$ agonist anti-inflammatory and tissue-protective effects in models of myocardial infarction (Yang et al. 2006b), acute lung injury (Reutershan et al. 2007), and spinal cord compression injury (Li et al. 2006).

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are associated with severe tissue inflammation and damage. $A_{2A}AR$ activation has anti-inflammatory and tissue protective properties in several studies investigating inflammation in the gastrointestinal tract (Odashima et al. 2005; Cavalcante et al. 2006; Naganuma et al. 2006). $A_{2A}AR$ knockout mice are more sensitive to experimental colitis, and treatment with the $A_{2A}AR$ agonist ATL 146e is associated with decreased leukocyte infiltration, inflammatory mediator production and necrosis in a model of inflammatory bowel disease (Odashima et al. 2005). CD25+ CD4+ Tregs play an important role in regulating inflammatory responses, including those associated with inflammatory bowel disease (Izcue et al. 2006). Recent studies have identified $A_{2A}ARs$ on Tregs as playing an important role in regulating inflammation in inflammatory bowel disease (Naganuma et al. 2006). Tregs isolated from wild-type mice and transferred to immunodeficient mice together with colitis-inducing CD4+ T cells were able to confer protection from the development of colitis, whereas Tregs isolated from $A_{2A}AR$ knockout mice were not. These studies highlight the importance of $A_{2A}AR$ signaling as an anti-inflammatory pathway in inflammatory bowel disease.

Given that endogenous adenosine acting through the $A_{2A}AR$ appears to be a potent regulator of inflammation and tissue injury, it stands to reason that mechanisms must exist to tightly regulate adenosine's actions during the natural course of the inflammatory response. This could occur at multiple levels, including the regulation of adenosine production and the availability of effective receptor signaling pathways. A recent study by Deaglio and colleagues provided new and interesting information on the mechanisms of adenosine generation and immunoregulation by Tregs (Deaglio et al. 2007). Extracellular adenosine is generated from the dephosphorylation of extracellular nucleotides (Zimmermann 2000). ATP and ADP are converted to AMP by the ectonucleoside triphosphate diphosphohydrolase CD39. AMP is dephosphorylated to adenosine by the ectonucleotidase CD73. Both of

these enzymes play critical roles in producing extracellular adenosine (Deaglio et al. 2007). A newly recognized feature of Tregs is that they express a unique combination of both CD39 and CD73 together with the forkhead transcription factor Foxp-3 (Deaglio et al. 2007). These findings provide an important new signature for defining Tregs, but more importantly they demonstrate that the production of adenosine through this cascade on the surface of Tregs is important to the $A_{2A}AR$-mediated immunosuppressive effects of these cells (Deaglio et al. 2007). These findings provide an elegant example of how the coordinate regulation of adenosine production and signaling can impact the immune response.

Anti-inflammatory properties of $A_{2A}AR$ signaling have also been noted in animal models of inflammatory lung disease. In a model of LPS-induced lung injury, treatment with the $A_{2A}AR$ agonist ATL202 was associated with decreased recruitment of neutrophils to the lung, together with reduced cytokine levels and pulmonary edema (Reutershan et al. 2007). There was enhanced neutrophil recruitment in $A_{2A}AR$ knockout mice treated with LPS and bone marrow transplantation, as well as tissue-specific $A_{2A}AR$ deletion studies suggested that $A_{2A}AR$ expression on leukocytes was important in the anti-inflammatory effects seen. In addition to models of acute lung injury, anti-inflammatory effects of $A_{2A}AR$ signaling have been noted in allergic lung inflammation. Treatment of allergic rats with the $A_{2A}AR$ agonist resulted in diminished pulmonary inflammation (Fozard et al. 2002). Similar findings were seen in a mouse model of allergic lung inflammation (Bonneau et al. 2006). $A_{2A}AR$ knockout allergic mice have also been shown to have higher lung inflammation as compared to $A_{2A}AR$ wild-type mice upon allergen challenge (Nadeem et al. 2007). Finally, in a recent study on the ADA knockout model of adenosine-dependent lung inflammation and damage, genetic removal of the $A_{2A}AR$ led to enhanced pulmonary inflammation, mucus production and alveolar airway destruction (Mohsenin et al. 2007), further implicating $A_{2A}AR$ signaling pathways as important anti-inflammatory networks in the lung. These findings suggest that $A_{2A}AR$ agonism may be beneficial in the treatment of diseases such as asthma; however, recent investigations into this in humans have been inconclusive (Luijk et al. 2008).

4.2.3 Detrimental Aspects of $A_{2A}AR$ Engagement

It is becoming increasingly clear that the suppression of various T-cell functions is a major mechanism by which $A_{2A}AR$ signaling limits tissue inflammation and damage in response to acute injury. Whereas this has obvious benefits in protecting tissues and promoting repair, there are examples where this paradigm is detrimental. A recent study by Ohta and colleagues demonstrated that $A_{2A}AR$-mediated immunosuppressive activities serve to protect cancer cells from the activities of antitumor T cells, and thus promote the survival and growth of tumors (Ohta et al. 2006). It was shown that the hypoxic environment of certain tumors promotes adenosine formation, and that treatment of wild-type mice with $A_{2A}AR$ antagonists can decrease tumor size. Moreover, injection of $A_{2A}AR$ knockout mice with cancer cells was associated with remarkable decreases in tumor size and animal survival

relative to what was seen in wild-type mice injected with the same cancer cells. This effect appears to be mediated largely by CD8+ T cells and the production of INFγ and TNF-α. These findings suggest that the well-characterized anti-inflammatory properties of $A_{2A}AR$ signaling may actually serve to protect certain tumors from the body's attempt to eliminate them. This raises several attractive avenues for novel cancer therapies. The use of $A_{2A}AR$ antagonists or strategies to lower adenosine levels in tumors may prove beneficial in allowing the immune system to attack cancer cells. In addition, dampening the anti-inflammatory effects of $A_{2A}AR$ signaling in certain tumors with the development of targeted antitumor T cells with $A_{2A}AR$ gene deletion may improve strategies for cancer immunotherapy.

Another area where $A_{2A}AR$ receptor signaling has received substantial attention as a potential target for therapeutic intervention is neurodegenerative disorders. Adenosine levels markedly increase in the brain in response to hypoxic, traumatic, and inflammatory insults (Pedata et al. 2001). Interestingly, engagement of the $A_{2A}AR$ in brain injury models appears to have both protective and detrimental effects. Akin to what is seen in other organ systems (see above), activation of the $A_{2A}AR$ has been shown to reduce brain damage in kainate-induced hippocampal injury and hemorrhagic brain injury (Jones et al. 1998; Mayne et al. 2001). In contrast, $A_{2A}AR$ antagonists have been shown to attenuate ischemic brain injury (Monopoli et al. 1998) and neurotoxicity induced by kainate and quinolinate (Jones et al. 1998; Popoli et al. 2002). In addition, $A_{2A}AR$ antagonists are protective in neurotoxic models of Parkinson's disease (Ikeda et al. 2002). The contribution of $A_{2A}AR$ signaling to the promotion of neuronal injury has been validated genetically. $A_{2A}AR$ knockout mice were found to have smaller infarct volumes and neural behavioral deficit scores following ischemic brain injury than wild-type mice (Yu et al. 2004). The $A_{2A}AR$ is expressed on many cell types in the brain, including neuronal components such as striatal neurons and glial cells (Svenningsson et al. 1999), endothelial cells and various bone marrow-derived cells such as neutrophils, macrophages and dendritic cells (Hasko and Cronstein 2004). Engagement of $A_{2A}ARs$ on different cells during different types or even stages of injury may be responsible for the destructive or protective effects of this receptor in the injured brain. A series of experiments using bone marrow transplantation of cells from $A_{2A}AR$ knockout mice and tissue-specific knockout of the $A_{2A}AR$ in neuronal cells has recently provided evidence that it is the expression of the $A_{2A}AR$ on bone marrow-derived cells that is responsible for the detrimental effects of $A_{2A}AR$ signaling in ischemic brain injury and 3-nitropropionic acid-induced striatal damage (Yu et al. 2004; Huang et al. 2006). The mechanisms underlying potential protective effects of $A_{2A}AR$ are not clear. However, the observation that both protective and detrimental effects have been noted in different types of brain injury suggests that multiple sites of action for injury-induced adenosine production and $A_{2A}AR$ signaling must be understood before $A_{2A}AR$ agonists and antagonists can be effectively utilized in the treatment of neurodegenerative disorders. For more information on $A_{2A}ARs$ in neuroprotection and neurodegenerative diseases, please refer to other chapters in this volume, including Chap. 16, "Adenosine Receptors and the Central Nervous System" (by Sebastião and Ribeiro), Chap. 17, "Adenosine Receptors and Neurological

Disease: Neuroprotection and Neurodegeneration" (by Stone et al.), and Chap. 18, "Adenosine A_{2A} Receptors and Parkinson's Disease" (by Morelli et al.).

Thus, studies in cellular models and preclinical investigations in animal models suggest that $A_{2A}AR$ agonists will be useful in the treatment of many diseases where inflammation is a detrimental component, while $A_{2A}AR$ antagonists may be beneficial in the treatment of neurological disorders such as Parkinson's disease. As clinical trials advance, it will become evident whether these preclinical observations translate into beneficial effects in humans (Schwarzschild et al. 2006; Gao and Jacobson 2007).

4.3 $A_{2B}AR$ and Inflammatory Responses

4.3.1 Historical Perspective

Initial identification of the A_2ARs was based on the ability of this class of receptor to activate AC (Londos et al. 1980). Both high-affinity and low-affinity A_2AR subtypes were described (Bruns et al. 1986), and it was not until the successful molecular cloning of these receptors in the early 1990s that it became clear that the high-affinity A_2AR was the $A_{2A}AR$ and the low-affinity A_2AR was the $A_{2B}AR$ (Pierce et al. 1992; Rivkees and Reppert 1992). Subsequently, studies in HMC-1 cells have shown that the $A_{2B}AR$ can not only couple AC through G_s (Feoktistov and Biaggioni 1997), but that it also interacts with G_q to activate PLC (Feoktistov et al. 1999). However, the majority of in vitro studies in normal human cell lines, including human airway epithelial and bronchial smooth muscle cells as well as lung fibroblasts, and in vivo studies in mice suggest that the primary signaling pathway for the $A_{2B}AR$ is via AC, to produce an increase in intracellular cAMP (see below). The $A_{2B}AR$ is expressed on most inflammatory cells, and its expression is induced in hypoxic and inflammatory environments (Xaus et al. 1999a; Eltzschig et al. 2003). Furthermore, as with the other adenosine receptors, both anti- and proinflammatory activities have been associated with $A_{2B}AR$ activation. However, the observation that the $A_{2B}AR$ has a relatively low affinity for adenosine has led to the notion that $A_{2B}AR$ activation may serve important functions in pathological situations where adenosine production is increased (Fredholm 2007). Deciphering the contributions of $A_{2B}AR$ signaling in models of tissue inflammation and tissue injury is an active area of research with exciting possibilities for novel adenosine-based therapeutics.

4.3.2 Anti-inflammatory Effects

Perhaps the best-characterized anti-inflammatory actions associated with the $A_{2B}AR$ are its ability to inhibit monocyte and macrophage functions. Along these

lines, IFN-γ can upregulate the $A_{2B}AR$ expression on macrophages as part of a proposed mechanism for macrophage deactivation (Xaus et al. 1999a). Consistent with this, $A_{2B}AR$ activation can inhibit the production or release of proinflammatory cytokines such as TNF-α and IL-1β from macrophages or monocytes, and it can inhibit macrophage proliferation following inflammatory stimulation (Xaus et al. 1999b; Sipka et al. 2005; Kreckler et al. 2006). In addition, $A_{2B}AR$ activation can increase the production of IL-10 from macrophages (Nemeth et al. 2005), a process that can be considered anti-inflammatory. As with the $A_{2A}AR$, these anti-inflammatory effects on macrophages likely stem from the ability of the $A_{2B}AR$ to couple to adenylate cyclase to increase cAMP levels.

Recent observations in $A_{2B}AR$ knockout mice provide compelling evidence that this receptor is associated with anti-inflammatory events in vivo. Yang and colleagues published on the initial characterization of $A_{2B}AR$ knockout mice and demonstrated that these mice show evidence of increased inflammation at baseline, in that levels of cytokines such as TNF-α and IL-6 were elevated in naïve $A_{2B}AR$ knockout mice, while IL-10 levels were elevated (Yang et al. 2006a). Exposure of $A_{2B}AR$ knockout mice to LPS resulted in the exaggerated production of TNF-α and IL-6, further suggesting an anti-inflammatory role for the $A_{2B}AR$ in vivo. These findings were surprising given data suggesting that $A_{2B}AR$ signaling is proinflammatory in vitro and in vivo (see below). Furthermore, these studies suggest a role for physiological $A_{2B}AR$ activation, which is paradoxical considering the notion that $A_{2B}AR$ signaling is only activated during pathological situations when adenosine levels are high, and that it is a low-affinity receptor. Interestingly, in $A_{2B}AR$ knockout mice there was evidence of increased expression of vascular adhesion molecules that mediate inflammation (Yang et al. 2006a), a finding that was associated with the $A_{2B}AR$-dependent production of cytokines from macrophages. These findings are consistent with the anti-inflammatory properties previously attributed to this receptor on macrophages.

A second manuscript by Hua and colleagues using independently generated $A_{2B}AR$ knockout mice demonstrated enhanced mast-cell activation in the absence of the $A_{2B}AR$ (Hua et al. 2007). These studies showed a reduction in basal levels of cAMP in mast cells isolated from $A_{2B}AR$ knockout mice, suggesting that $A_{2B}AR$ engagement may play a role in regulating mast-cell activation at baseline. Furthermore, experiments in this study demonstrated that mice lacking the $A_{2B}AR$ exhibit increased sensitivity to IgE-mediated anaphylaxis, suggesting that this receptor may limit the magnitude of antigen-driven responses on mast cells. These findings are somewhat paridoxical given the touted proinflammatory functions of the $A_{2B}AR$ receptor on HMC-1 cells (Feoktistov et al. 1998). A recent manuscript by Ryzhov and colleagues probed this further by examining adenosine-dependent effects on mast cells isolated from $A_{2B}AR$ knockout mice (Ryzhov et al. 2008b). These studies confirmed the anti-inflammatory effects of $A_{2B}AR$ signaling on mast cells and demonstrated that the $A_{2B}AR$ is necessary for antigen-induced proinflammatory cytokine production from these cells. Thus, the $A_{2B}AR$ seems to play both anti- and proinflammatory functions on mast cells.

A recent study by Eckle and colleagues demonstrated increased vascular leakage in $A_{2B}AR$ knockout mice exposed to hypoxia (Eckle et al. 2008). In addition, there was an increase in neutrophil influx into tissues of $A_{2B}AR$ knockout mice exposed to hypoxia. Bone marrow transplantation studies suggested that this enhanced neutrophilia was, in part, due to $A_{2B}AR$ expression on bone marrow-derived cells. The ability of the $A_{2B}AR$ to regulate vascular leak represents a potentially major anti-inflammatory role for this receptor. Moreover, the anti-inflammatory effects of $A_{2B}ARs$ are not surprising for a G_s-coupled receptor that increases intracellular cAMP, and are similar to those produced by other agents that increase cAMP, in other words $A_{2A}AR$ agonists and phosphodiesterase IV inhibitors.

4.3.3 Proinflammatory Effects

Numerous studies have demonstrated a proinflammatory function for the $A_{2B}AR$, largely through its regulation of proinflammatory cytokine and chemokine production. A prominent example of this is the promotion of IL-6 release from a number of cells, including intestinal (Sitaraman et al. 2001) and airway epithelial cells (Sun et al. 2008), macrophages (Ritchie et al. 1997; Ryzhov et al. 2008a), pulmonary fibroblasts (Zhong et al. 2005), bronchial smooth muscle cells (Zhong et al. 2003a), astrocytes (Schwaninger et al. 1997) and cardiomyocytes (Wagner et al. 1999). In addition, $A_{2B}AR$ activation can promote the release of IL-8 from HMC-1 cells (Feoktistov and Biaggioni 1995), IL-4 and IL-13 (Ryzhov et al. 2004, 2008b) from HMC-1 cells and murine bone marrow-derived mast cells, and the release of IL-19 from airway epithelial cells (Zhong et al. 2006) and MCP-1 from bronchial smooth muscle cells (Zhong et al. 2003a). In addition, $A_{2B}AR$ activation can stimulate the production of VEGF (Feoktistov et al. 2003; Ryzhov et al. 2008b), which can be considered proinflammatory in certain disease states. The $A_{2B}AR$ driven production of these proinflammatory molecules has been attributed to both G_s and G_q pathways (Feoktistov et al. 1999; Sitaraman et al. 2001).

$A_{2B}AR$ expression has been shown to be increased in the gastrointestinal track during inflammatory bowel disease and colitis (Hosokawa et al. 1999). Evidence in animal models of inflammatory bowel disease demonstrates that $A_{2B}AR$ activation can stimulate the release of IL-6, an important proinflammatory cytokine in inflammatory bowel disease, from the apical surface of the colonic epithelium (Sitaraman et al. 2001). This increased IL-6 secretion is proposed to promote the degranulation of neutrophils and contribute to disease progression. In support of this model, emerging work from the Sitaraman laboratory suggests that treatment of animal models of inflammatory bowel disease with the $A_{2B}AR$ antagonist ATL 801 inhibits IL-6 production and is associated with an improvement in clinical and histological signs in these models (Kolachala et al. 2008a, b). Collectively, these findings suggest that $A_{2B}AR$ signaling may play a role in the progression of inflammatory bowel disease, and so an $A_{2B}AR$ antagonists may have therapeutic benefit in related conditions.

As mentioned earlier, engagement of the $A_{2B}AR$ on HMC-1 cells appears to have predominantly proinflammatory functions (Feoktistov et al. 1998); however, recent studies in $A_{2B}AR$ knockout mice have revealed that this receptor plays an anti-inflammatory function in bone marrow-derived mast cells (Hua et al. 2007). Understanding the complexity of $A_{2B}AR$ signaling in this cell type may relate to the distinct inflammatory functions of this cell type. Following antigen priming and stimulation, mast cells undergo a degranulation process where preformed mediators are released (Shimizu and Schwartz 1997). This process is part of an acute response to antigen that mediates important processes such as bronchoconstriction in airways. In addition to this acute response, mast cells are stimulated to produce and release cytokines and chemokines. This inflammatory response is part of a more chronic or late-stage response that can promote additional tissue inflammation and injury. The recent work from Ryzhov and colleagues demonstrates that engagement of the $A_{2B}AR$ on mouse mast cells does not contribute to adenosine's ability to promote mast cell degranulation; however, the $A_{2B}AR$ does contribute to the production of IL-13 and VEGF (Ryzhov et al. 2008b). Thus, adenosine-mediated degranulation is likely mediated by the A_3AR in rodents, while the $A_{2B}AR$ regulates mediator production. It is important to note that significant species differences have been noted in relation to adenosine's effects on mast cells, and relatively little is known about the contribution of the $A_{2B}AR$ in human mast cells. Continued efforts to define the functions of adenosine receptors on mast cells in specific disease environments such as asthma will be critical to the development of adenosine-based therapeutics targeting mast-cell effector activities.

The apparent low affinity of adenosine for the $A_{2B}AR$ suggests that this receptor may have important roles in pathological environments where adenosine levels are elevated (Fredholm 2007). Consistent with this, work in the ADA-deficient model of adenosine-dependent lung inflammation and injury has demonstrated proinflammatory features of $A_{2B}AR$ signaling (Sun et al. 2006). In this model, mice that lack ADA exhibit progressive increases in lung adenosine concentrations in association with progressive pulmonary inflammation and tissue remodeling (Blackburn et al. 2000; Chunn et al. 2005). Noted features include the accumulation of activated alveolar macrophages that produce numerous inflammatory mediators, including IL-6, CXCL1, TGF-β1 and osteopontin (Sun et al. 2006). Production of these mediators is associated with alveolar airway destruction, mucus cell metaplasia and pulmonary fibrosis. $A_{2B}AR$ expression is elevated in the lungs of ADA-deficient mice, and treatment of these mice with the selective $A_{2B}AR$ antagonist CVT-6883 resulted in decreased production of proinflammatory mediators from macrophages, which was associated with decreased alveolar airway enlargement and pulmonary fibrosis (Sun et al. 2006). Similarly, a recent study using a mouse model of ragweed sensitization and challenge in the lung revealed that $A_{2B}AR$ antagonism with CVT-6883 (3-ethyl-1-propyl-8-[1-(3-trifluoromethylbenzyl)-1H-pyrazol-4-yl]-3,7-dihydropurine-2,6-dione) was associated with decreased airway inflammation and airway hyperreactivity (Mustafa et al. 2007). These studies demonstrate that $A_{2B}AR$ signaling plays a proinflammatory role in the lung, and suggest that $A_{2B}AR$ antagonists may prove beneficial in the treatment of lung disorders such as asthma, COPD, and pulmonary fibrosis.

4.4 A_3AR and Inflammatory Responses

4.4.1 Historical Perspective

The A_3AR was first identified through molecular cloning from a rat testis cDNA library based on 40% sequence homology with the canine A_1AR and $A_{2A}AR$ (Meyerhof et al. 1991). A_1AR, $A_{2A}AR$ and $A_{2B}AR$ are antagonized by methylxanthines, such as caffeine, theophylline and enprofylline, while the A_3AR is relatively xanthine insensitive, which may have been a reason for its relatively late discovery. In 1992, Zhou et al. cloned the A_3AR from rat striatum, expressed the protein in Chinese hamster ovary cells, and showed that A_3AR engagement leads to inhibition of AC (Zhou et al. 1992). Later it was shown that inhibition of AC is achieved through activation of the pertussis toxin-sensitive Gia2,3 protein (Palmer et al. 1995b). Ligand binding can also result in activation of PLC through $G_{q/11}$ or the βγ subunits, leading to increased release of Ca^{2+} (Abbracchio et al. 1995). More recent studies revealed several additional intracellular pathways that can be accessed by the A_3AR in different cell types to promote tissue-specific functions (for a review, see Gessi et al. 2008). Homologs of the A_3AR gene have been cloned from several species, and only 74% sequence homology was found between the genes from rat and human or sheep, while there is 85% homology between human and sheep. The recently cloned and characterized equine A_3AR gene shows a high degree of sequence homology to the human and sheep genes, but has a different pharmacological profile (Brandon et al. 2006). These species differences make it possible to design highly selective ligands for the human A_3AR, but the disadvantage is that these ligands cannot be adequately tested in rodent models. In an effort to circumvent this problem, Yamano and his colleagues created an A_3AR-humanized mouse by replacing the mouse A_3AR gene with the human gene (Yamano et al. 2006). When bone marrow-derived mast cells from the A_3AR-humanized mice were treated with an A_3AR agonist, an elevation of intracellular Ca^{2+} concentration was observed, and this increase could be completely antagonized by a human-selective A_3AR antagonist. However, the A_3AR agonist did not potentiate antigen-dependent degranulation, probably because the agonist-stimulated human A_3AR could not activate the phosphorylation of either ERK 1/2 or protein kinase B due to uncoupling of the receptor from G proteins (Yamano et al. 2006). To overcome the uncoupling, the group generated A_3AR functionally humanized mice by replacing the mouse A_3AR gene with a chimeric human/mouse sequence in which the intracellular regions of the human receptor were substituted for the corresponding regions of the mouse A_3AR. Activation of the chimeric A_3AR led to intracellular Ca^{2+} elevation and activation of the PI3Kγ-signaling pathway, which are equivalent to the actions induced by the A_3AR in wild-type mice. The human A_3AR antagonist had the same binding affinities for this chimeric receptor as for the human A_3AR, and completely antagonized the potentiation of antigen-dependent mast-cell degranulation. These studies provided the first direct evidence that the uncoupling of mouse G protein(s) to the human A_3AR is due to a sequence difference in the intracellular regions of

the receptor protein critical for G-protein recognition/coupling. It is expected that the A$_3$AR functionally humanized mice can be employed for pharmacological evaluations of the human A$_3$AR antagonists (Yamano et al. 2006).

4.4.2 Anti-inflammatory Effects

The A$_3$AR has been shown to suppress LPS-induced TNF-α production in vitro from various macrophage cell lines (Le Vraux et al. 1993; McWhinney et al. 1996; Sajjadi et al. 1996; Martin et al. 2006) and microglial cells (Lee et al. 2006b), where, depending on the cell type, different signal transduction pathways are responsible for the inhibition. This inhibitory effect was also assessed in vivo by treating wild-type and A$_3$AR knockout mice with the A$_3$AR agonist 2-Cl—IB—MECA (2-chloro-N^6-(3-iodobenzyl)-adenosine-5$'$-N-methyluronamide) following LPS challenge, resulting in decreased TNF-α production that was more pronounced in wild-type mice (Salvatore et al. 2000). In peritoneal macrophages isolated from A$_3$AR knockout or wild-type mice, treatment with IB–MECA reduced TNF-α release to the same extent (Kreckler et al. 2006). Both of these in vivo studies demonstrate that A$_{2A}$AR activation inhibits the production of TNF-α regardless of the presence of A$_3$AR. Recent studies with human monocytes implicate both the A$_{2A}$AR and A$_{2B}$AR in the regulation of LPS-induced TNF-α production (Zhang et al. 2005; Hasko et al. 2007). In human neutrophils, both the A$_3$AR and the A$_{2A}$AR are involved in the reduction of O$_2^-$ generation (Bouma et al. 1997; Gessi et al. 2002), and the A$_3$AR also promotes neutrophil migration (Chen et al. 2006), thus performing both anti- and proinflammatory actions, respectively.

4.4.3 A$_3$AR in Disease Progression and Potential Agonist Therapies

A$_3$AR agonists can exert significant protective effects in animal models of arthritis. In a collagen-induced arthritis model, A$_3$AR activation inhibited CCL3 (MIP-1α) production (Szabo et al. 1998), while in autoimmune arthritis models, suppression of TNF-α production was found (Baharav et al. 2005). Moreover, inhibition of proinflammatory cytokine production was achieved by inhibiting the PI3K NF-$\kappa\beta$ signaling pathway in adjuvant-induced arthritis (Madi et al. 2007). Methotrexate, a therapeutic agent that is widely used to treat arthritis, exerts its anti-inflammatory effect through adenosine, and was shown to upregulate the expression of A$_3$AR on peripheral blood mononuclear cells both in rats with adjuvant-induced arthritis and in patients with rheumatoid arthritis (Ochaion et al. 2006). Concomitant treatment with IB—MECA and methotrexate resulted in additive anti-inflammatory effects in the adjuvant-induced arthritis animal model. IB—MECA (CF-101) has been tested in Phase I and Phase IIa clinical trials, where it was found to be safe, well tolerated, and shows evidence of an anti-inflammatory effects in patients with rheumatoid arthritis (Silverman et al. 2008). IB—MECA was also found to be protective in other mouse models of inflammatory diseases. In endotoxemic mice, pretreatment

with IB–MECA decreased mortality by reducing IL-12 and IFN-γ production independently of IL-10 production (Hasko et al. 1998). A similar effect was observed in different mouse models of colitis (Mabley et al. 2003) and septic peritonitis (Lee et al. 2006a), where IB–MECA treatment decreased the expression of proinflammatory cytokines, mainly TNF-α, while in A_3AR knockout mice inflammation was heightened. The A_3AR has been reported to have a protective role in vivo in lung injury following ischemia and reperfusion (Matot et al. 2006). Recently, in the bleomycin-induced lung injury model, significantly enhanced inflammatory cell recruitment was observed in the lungs of A_3AR knockout mice due to elevated expression of the chemokines CXCL-1, CCL11 (eotaxin-1) and GM-CSF (Morschl et al. 2008). These observations suggest that A_3AR agonists may represent a new family of orally bioavailable drugs in the treatment of inflammatory diseases (Bar-Yehuda et al. 2007). For more information on A_3ARs and inflammation, please refer to Chap. 10 of this volume, "A_3 Adenosine Receptor: Pharmacology and Role in Disease," by Borea et al.

4.4.4 Proinflammatory Effects

Early studies in mast cells indicated that A_3AR activation leads to increased inflammation by inducing the release of mediators and the potentiation of antigen-dependent degranulation (Ramkumar et al. 1993; Fozard et al. 1996; Reeves et al. 1997). A_3AR activation can enhance the degranulation of mast cells isolated from mouse lung through elevations of intracellular Ca^{2+} mediated by the coupling of G_i to PI3K (Zhong et al. 2003b). In contrast, in canine mast cells (Auchampach et al. 1997), degranulation was mediated by the $A_{2B}AR$ instead of the A_3AR. The specific adenosine receptors involved in the degranulation of human mast cells are not known.

A$_3$AR activation in sensitized guinea pigs resulted in increased inflammatory cell recruitment to the lung (Spruntulis and Broadley 2001). In rat mast cells, activation of the A_3AR inhibited apoptosis through protein kinase B phosphorylation (Gao et al. 2001), and this enhanced survival may contribute to inflammatory cell expansion in inflamed tissues.

A$_3$AR mRNA expression is elevated in transbronchial biopsy samples from asthma and COPD patients, where expression is localized to infiltrating eosinophils rather than mast cells (Walker et al. 1997). In ADA-deficient mice, which exhibit adenosine-mediated lung disease, genetic removal of the A_3AR or treatment with an A_3AR antagonist, MRS-1523 (3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate), prevented airway eosinophilia and decreased mucus production, suggesting that A_3AR signaling contributes to the regulation of features of chronic lung disease (Young et al. 2004). Additional ex vivo studies with mouse eosinophils confirmed the results of in vitro observations with human eosinophils, where A_3AR activation suppressed eosinophil chemotaxis (Ezeamuzie and Philips 1999). These observations suggest that the diminished eosinophilia in the ADA/A_3AR double-knockout mice or in the antagonist-treated ADA-deficient

mice in Young's study is not a direct effect on the eosinophils. Indirect regulators may be cytokines and chemokines that are known to be involved in eosinophil recruitment, but these were not affected by removal or inhibition of the A_3AR in ADA-deficient mice, suggesting that other mediators, such as proteases, extracellular matrix proteins and cell adhesion molecules, may be responsible (Young et al. 2004).

In addition to regulating chemotaxis, the A_3AR is also important for eosinophil activation and degranulation, although results are contradictory. For example, A_3AR activation decreased degranulation and O_2^- production in human eosinophils isolated from blood (Ezeamuzie and Philips 1999; Ezeamuzie 2001), while there was no degranulation in the absence of the A_3AR assessed by eosinophil peroxidase release in the bronchoalveolar lavage fluid in bleomycin-challenged mice (Morschl et al. 2008). Moreover, the treatment of human eosinophils with Cl$-$IB$-$MECA (a selective A_3AR agonist) elevated intracellular Ca^{2+} levels, suggesting the presence of PLC-coupled A_3AR and supporting the role of A_3AR in eosinophil degranulation and chemotaxis, which are both Ca^{2+}-driven events (Kohno et al. 1996).

4.4.5 Potential Use of Antagonist in the Treatment of Inflammation

Early observations showing an A_3AR-mediated enhancement of antigen-dependent degranulation of mast cells in mice and bone marrow-derived cell lines (Reeves et al. 1997; Salvatore et al. 2000) suggested that selective A_3AR antagonists may have therapeutic potential as antiasthmatic agents. A compound with dual antagonist properties for both the $A_{2B}AR$ and the A_3AR, QAF 805 (Novartis), is under development as an antiasthma drug (Press et al. 2005). However, this mixed A_{2B}/A_3 AR antagonist has now entered human clinical trials and has failed to increase the PC_{20} for AMP versus placebo in 24 AMP-sensitive asthmatics in a placebo-controlled, double-blind, randomized, two-way crossover Phase Ib clinical trial (Pascoe et al. 2007). Moreover, researchers at GlaxoSmithKline developed a compound with dual $A_{2A}AR$ agonist and A_3AR antagonist effects that was able to inhibit both the production of reactive oxygen species and degranulation from human eosinophils and neutrophils in vitro, but provided very little clinical benefit when used in a clinical study for the treatment of allergic rhinitis (Bevan et al. 2007; Rimmer et al. 2007).

Although several potent and selective antagonists of the human A_3AR have been identified, they show extremely low binding affinity for the rodent A_3AR (typically 1,000 times lower), and since rodent models are used for the pharmacological evaluation of new therapeutic agents, this poses a serious drawback. The humanized A_3AR chimera mice (Yamano et al. 2006) may prove useful in overcoming this problem; however, further in vivo studies are needed to confirm that these mice can be utilized to test human A_3AR-selective compounds.

The A_3AR exerts both pro- and anti-inflammatory effects on different cell types and cell functions, but how these cells interact and influence each other in their microenvironment is still not known. Methods to examine A_3AR protein expression

in situ are not sensitive, and it is possible that cells with low surface expression of A_3AR may be important in the regulation of inflammation in a manner that has not yet been appreciated.

Most cells express multiple types of adenosine receptors, and their actions can be overlapping or opposing, which may be an important mechanism to keep cell function in balance. It might prove beneficial to design analogs with dual or multiple affinities towards different types of adenosine receptors in order to influence various inflammatory actions at the same time. There are some reports of ligands that have $A_{2B}AR$ and A_3AR antagonist or $A_{2A}AR$ agonist and A_3AR antagonist properties that were designed to treat allergic airway diseases and that show promise in cell culture experiments, but further studies need to be performed with animal models of inflammation and in humans.

5 Conclusions

Orchestrated responses of cells to injury are essential for survival. As part of the body's ability to respond and recover from infection and injury, inflammatory processes help to limit infection and promote pathways for wound healing and the establishment of homeostasis. During various injurious situations, cells are placed under stress and must adapt to survive during the resolution of injury. Adenosine production and signaling has emerged as a major mechanism whereby cells respond to injury and regulate inflammation. There are precise mechanisms for regulating the production of extracellular adenosine at the cell surface, and there are now numerous studies demonstrating that this process helps to set in place pathways that can limit detrimental inflammatory processes while promoting beneficial inflammatory processes and promoting wound healing. The extensive research into the contributions of individual adenosine receptors on various immune cells, which has been extensively reviewed here, suggests that selective adenosine receptor agonists and antagonists may prove useful in regulating the immune response and hence the treatment of various injuries or diseases states. However, work from genetically modified mice and the use of selective adenosine receptor ligands in vivo have shown us that the path forward for the use of adenosine-based therapeutics will present many challenges. The engagement of all of the adenosine receptors has potent anti-inflammatory and tissue protective features in many situations. However, demonstrated proinflammatory and tissue destructive properties can also be ascribed to each of the adenosine receptors. Though this seems paradoxical, it may, in general terms, highlight the importance of adenosine signaling in regulating the balance between tissue injury and repair. For example, stimulation of anti-inflammatory adenosine receptor pathways will likely serve to stem inflammatory processes associated with numerous infections and challenges, as well as to promote wound-healing features such as angiogenesis and matrix deposition; however, overstimulation of such wound healing processes may actually promote disease. In addition, activation of anti-inflammatory pathways, such as the

downregulation IL-12 and upregulation of IL-10, may bias the tissue environment toward Th2-like inflammation, which may present exacerbations of inflammation in certain environments. Thus, therapeutic approaches must take into account numerous factors, including the stage of the disease, the immunological and pathological processes involved, and the duration of treatment. Finally, the numerous observations that adenosine receptor expression increases in inflammatory environments suggest that we must learn more about receptor number or availability on the cell surface during specific inflammatory insults. Despite these challenges, it is clear that selective adenosine receptor engagement can regulate many of the features of inflammation, and with time and continued research, adequate approaches will be developed for the treatment of human disease with adenosine-based approaches.

In this regard, it is clear that adenosine receptors are important molecular targets for adenosine-based therapeutics for the entire spectrum from inflammation to immune suppression. Approaches utilizing adenosine receptor-based therapeutics will be dependent on the role of the adenosine receptors in mechanisms of disease in humans, the timing of treatment with respect to the therapeutic window and the stage/progression of injury, and the duration and monitoring of treatment for both beneficial effects and adverse events. A number of adenosine receptor-based ligands with good safety profiles and high selectivity are available for testing in humans. Preclinical efficacy in animal models does not always translate into human efficacy. The development of preclinical model systems with relevance to the human condition of inflammation is essential for successful drug discovery. Only by testing adenosine receptor-based ligands that are safe and selective for the adenosine receptor subtypes in humans will we understand the role of these receptors in human conditions of inflammation, which will allow for the successful development of human therapeutics towards these important molecular targets.

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