Chapter 8
Animal Models of Airway Diseases

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Abstract Over the past 20 years, the growing awareness that purinergic signaling events literally shape the immune and inflammatory responses to infection and allergic reactions warranted the development of animal models to assess their importance *in vivo* in acute lung injury and chronic airway diseases. The pioneer work conducted with the adenosine deaminase (ADA)-deficient mouse provided irrefutable evidence that excess adenosine (ADO) accumulating in the lungs of asthmatic patients, constitutes a powerful mediator of disease severity. These original studies launched the development of murine strains for the two major ectonucleotidases responsible for the generation of airway ADO from ATP release: CD39 and CD73. The dramatic acute lung injury and chronic lung complications, manifested by these knockout mice in response to allergens and endotoxin, demonstrated the critical importance of regulating the availability of ATP and ADO for their receptors. Therapeutic targets are currently evaluated using knockout mice and agonists/antagonists for each ADO receptor (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) and the predominant ATP receptors ($P2Y_2R$ and $P2X_7R$). This chapter provides an in-depth description of each *in vivo* study, and a critical view of the therapeutic potentials for the treatment of airway diseases.

Keywords Adenosine deaminase · CD73 · CD39 · Fibrosis · Pulmonary edema

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8.1 Introduction: Pulmonary Edema

Pulmonary edema is a life-threatening complication of acute lung injury, acute respiratory distress syndrome and pneumonia, which affects both barriers separating the bloodstream from the airspace: hyper-permeability of the pulmonary endothelial and epithelial barriers to macromolecules and inflammatory cells, and reduction of alveolar fluid clearance capacity mediated by ion channels (review: [1]). This complication is routinely documented in murine models targeting the enzymes and receptors regulating purinergic signaling. Yet, considerable discrepancies are reported between the beneficial or detrimental effects of receptor agonists/antagonists. The following section summarizes the current knowledge on the purinergic regulation of pulmonary edema taking place at the endothelial and epithelial barriers, which reconciles all apparent disagreements. This information will allow the reader to appreciate the impact of excess airway ATP and ADO in respiratory complications *in vivo*, the usefulness of certain animal models for drug development, and the appropriate administration route for therapeutic compounds.

8.1.1 The Permeability of the Endothelial Barrier

Until recently, our understanding of endothelial barrier regulation was limited to ADO, which plays an essential role in resealing the gate after the transmigration of inflammatory cells (review: [2]). Then again, we have learned through the first seven chapters of this book that purinergic signaling generally initiates an ATP-mediated pro-inflammatory response, which is followed by an ADO-mediated anti-inflammatory response. Therefore, several groups tested the impact of P2 receptor agonists on the regulation of endothelial barrier permeability. While the use of stable analogues and P2 receptor antagonists clearly demonstrated the sensitivity of the barrier to ATP, these studies provided contradictory conclusions (review: [1]). In this section, we provide a thorough analysis of all data which reconciles the literature under a unifying concept: opposing regulations of barrier permeability by P2Y$_1$Rs (ADP) and P2Y$_2$Rs (ATP), depending on local ATP/ADP concentrations. While these studies were conducted on various endothelia, these receptors are co-localized on pulmonary endothelial cells [3].

   In human pulmonary artery and lung microvascular endothelial cultures, the low micromolar ATP concentrations induced a dose-dependent increase in transendothelial electrical resistance (TER) [4]. Likewise, 10 μM ATP reduced the thrombin-mediated barrier permeability of cultured human umbilical vein endothelial cells [5]. Similar data were obtained with non-hydrolysable ATP analogs known to interact with P2Y receptors, ruling out the possibility that these effects were mediated by ADO after ATP metabolism by the CD39-CD73 tandem [4]. This ATP concentration also inhibited the passage of macromolecules across endothelial monolayers, thus labeling this nucleotide as barrier protective [6]. Pharmacological
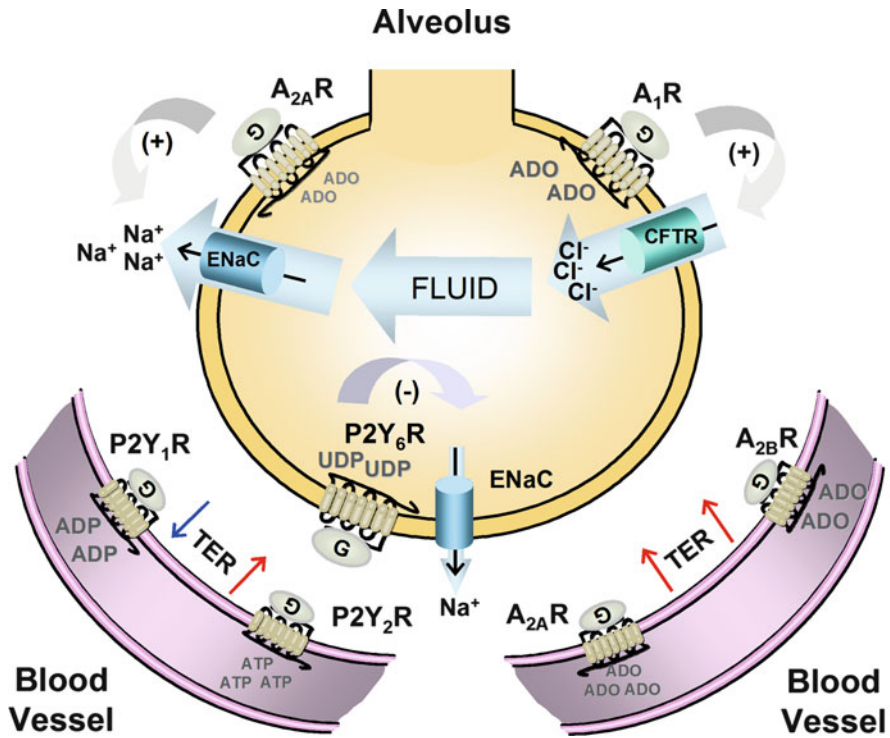
Fig. 8.1 Purinergic regulation of fluid fluxes and barrier permeability in the lung. In the circulation, nucleotides regulate endothelial permeability to fluid and leukocytes by the balancing activities of ATP, ADP and ADO receptors. Whereas $P2Y_1R$ activation reduces the transmembrane electrical resistance (*TER*), $P2Y_2Rs$, $A_{2A}Rs$ and $A_{2B}Rs$ all protect the lungs against vascular leakage. A second barrier protects the airways against excess fluid accumulation: the epithelial barrier. Alveolar cells express A_1Rs and $A_{2A}Rs$ which regulate fluid fluxes in and out of the airspace along ion gradients generated by the CFTR chloride (Cl^-) channel and the ENaC sodium (Na^+) channel. During viral infection, pulmonary edema is caused by the production, release and conversion of UTP into UDP, which activates $P2Y_6Rs$ to inhibit Na^+ and fluid absorption via ENaC

analysis ascribed this response to $P2Y_2Rs$ which initiates a signaling cascade involving the activation of phospholipase C (PLC), but not PKC or Ca^{2+} mobilization. Incidentally, Kolosova et al. demonstrated that the enhancement of barrier integrity by low micromolar ATP involves reorganization of the intercellular tight junctions, which occurs independently of Ca^{2+} mobilization or ERK1/2 [4] (Fig. 8.1). The signaling cascade is reminiscent of $P2Y_2R$-mediated induction of cell migration during epithelial repair (see Chap 6 for details). The $P2Y_2Rs$ located near tight junctions are coupled to G proteins G_q or G_{i2} (not G_{12} or G_{13}), which induce protein kinase A (PKA) activation, leading to the dephosphorylation of myosin light chain, and phosphorylation of vasodilator-stimulated phosphoprotein (VASP). When phosphorylated, VASP inhibits actin polymerization and the formation of stress fibers that would disrupt the barrier.

The barrier resistance of human umbilical vein endothelial cells is reduced by selective agonists of P2Y$_1$Rs [7]. Contrary to P2Y$_2$Rs, the signaling mechanism is Ca^{2+}-dependent [7] and requires myosin light chain phosphorylation [8]. Incidentally, the use of ionomycin to trigger Ca^{2+} entry into the cells facilitated the passage of albumin across aortic endothelial monolayers [6]. On the other hand, this receptor is only activated by ADP, released at high micromolar concentrations by platelets and red blood cells. While CD39 expressed on the endothelial surface generates ADP from ATP, the enzyme does not allow this product to accumulate significantly before further dephosphorylation into AMP. This is an important point, since the affinity of P2Y$_1$Rs for ADP (EC$_{50}$ = 8 μM) is considerably lower than P2Y$_2$Rs for ATP (EC$_{50}$ = 0.2 μM) (review: [9]). Under normal conditions, low micromolar ATP would prevent pulmonary edema primarily via P2Y$_2$Rs. On the other hand, massive local ADP release from platelets or red blood cells squeezing into the pulmonary vascular bed would transiently raise barrier permeability via P2Y$_1$Rs, before the nucleotide is dephosphorylated by CD39. In vascular beds, P2Y$_1$Rs have been shown to facilitate the passage of circulating cells through the narrow vessels by inducing nitric oxide-mediated vasodilatation [10]. However, conditions reducing CD39 activity, such as hypoxia/oxidative stress (see Chap. 4), may overwhelm the protective effects of P2Y$_2$Rs and cause pulmonary edema.

The highly efficient surface conversion of ATP and ADP into ADO generates a secondary wave of purinergic signaling for the regulation of endothelial barrier integrity. An *in vivo* study comparing the extent of hypoxia-induced vascular leakage among the knockout mice for each ADO receptor revealed that only the A$_{2B}$R$^{-/-}$ mice respond to hypoxia by significantly worse lung edema than wild-type mice, which was prevented by the A$_{2B}$R agonist, BAY60-6583 [11]. The capacity of A$_{2B}$R activation to raise endothelial TER was confirmed on human microvascular endothelial cultures using an antagonist (PSB 1115) and by RNA silencing technique [11]. In fact, Rounds et al. recently showed that A$_{2A}$Rs, A$_{2B}$Rs and ADO transporters work in concert to prevent pulmonary edema through endothelial barrier enhancement [12]. The adenosine deaminase inhibitor, pentostatin, raised surface ADO levels by tenfold, which enhanced the formation of endothelial adherens junction and focal adhesion through Rac1 GTPase activation. The fact that A$_1$Rs and A$_3$Rs were ineffective at regulating endothelial barrier permeability supports the well-established notion that barrier resistance is cyclic AMP (cAMP)-dependent (review: [2]). Under baseline conditions, ADO-mediated TER would be maintained by the high-affinity A$_{2A}$Rs (EC$_{50}$ = 0.56−0.95 μM) (review: [9]), whereas crisis situations raising circulating ADO levels (i.e. hypoxia or infection) would recruit the low-affinity A$_{2B}$Rs (EC$_{50}$ = 16.2−64.1 μM) (review: [9]) for additional protection against vascular leakage.

The therapeutic potential of A$_{2B}$R agonists for lung edema was demonstrated in a study conducted with endotoxemic pigs [13]. Endotoxin-induced acute lung injury and increase in lung extravascular water were, both, reduced by intravenously infused ADO. A similar response was obtained with the ADO receptor agonist, 2-chloroadenosine, in endotoxemic guinea-pigs [14]. Interestingly, the microvascular endothelial cells express predominantly ADO receptors causing an

increase in cyclic AMP (cAMP) production ($A_{2A}Rs$ and A_2BRs), which leads to PKA-dependent VASP phosphorylation, like for the abovementioned $P2Y_2Rs$ [15]. These data suggest that circulating nucleotides normally maintain TER by successive waves of ATP- and ADO-mediated signals, which by the same token, would regulate leukocyte migration induced by inflammatory mediators.

8.1.2   Fluid Clearance on Alveolar Epithelia

The respiratory tract is frequently subjected to mechanical stress and infections, which stimulate the release of nucleotides into the airways. Decades of research clearly established that extracellular nucleotides are the major regulators of airway hydration (review: [16]). More importantly, they showed that the epithelial barrier regulates fluid fluxes by purinergic regulation of ion channels. The vectorial movements of Na^+ and Cl^- ions across the barrier create an osmotic gradient for water to follow. The absorption of Na^+ by the epithelial sodium channel (ENaC) mediates fluid clearance, and Cl^- secretion by the Ca^{2+}-dependent Cl^- channels (CaCC) and cystic fibrosis transmembrane regulator (CFTR) hydrates airway surfaces. This subject is covered in details in Chap. 5 for the large airways, where nucleotides are identified as stimulators of fluid accumulation into the airspace. This concept seems to apply to the regulation of alveolar fluid clearance (AFC), as intra-tracheal instillation of ATP caused lung edema in mice [17]. This initial observation launched various initiatives to clarify the involvement of purinergic signaling in the impaired epithelial fluid clearance observed during pulmonary edema.

   In mice, the respiratory syncytial virus (RSV) causes a transient reduction in AFC, likely to promote clearance by the mucociliary escalator [18]. Using *in vivo* and *in vitro* studies, Matalon et al. showed that RSV impairs Na^+-driven AFC through ENaC in distal lung and upper airway epithelia [19–21]. In the H441 Clara cells, RSV-mediated ENaC inhibition was partially prevented by blockers of nitric oxide production (1,400 W), or *de novo* UTP synthesis (A77-1726). During RSV infection, the accumulation of both purines (ATP, ADP) and pyrimidines (UTP, UDP) in the alveolar space [20], and the prevention of fluid accumulation by P2Y receptor antagonists [19], support a role for UTP synthesis and release in the purinergic regulation of AFC.

   The predominant P2Y receptor capable of mediating pyrimidinergic signals in the respiratory tract is the $P2Y_2R$, which is activated by ATP and UTP, not by ADP or UDP (Chap. 7, Table 7.1). On the other hand, only soluble enzymes degrading pyrimidines (UDP-glucose pyrophosphorylase or apyrase) prevented RSV-induced edema [19]. In addition, intratracheal UTP and UDP, both, reproduced RSV-mediated AFC reduction [18]. And, UTP is readily dephosphorylated into UDP by ectonucleotidases on airway surfaces [22, 23]. Contrary to the endothelial surface, the ectonucleotidase population of the epithelial cells allows for significant accumulation of UDP before it is dephosphorylated further into UMP and uridine (see Chap. 2 for details). These results raised the possibility that UTP and UDP,

both, mediated their effects through $P2Y_6R$ activation. This receptor is functionally expressed on the apical surface of airway and alveolar epithelial cells (review: [24]). In A549 human alveolar Type 2 cells, $P2Y_6Rs$ activation accounts for the majority of Ca^{2+}-dependent ATP secretion [25]. The identity of this receptor was later confirmed using mice infected with the influenza A virus [26]. Like RSV, this virus induced hypoxemia and inhibited AFC, and was associated with a rapid increase in ATP and UTP concentrations in the BAL fluid. The use of various ion channel blockers and P2Y receptor antagonists confirmed that viral infections induce *de novo* UTP synthesis and secretion, followed by UTP dephosphorylation into UDP and $P2Y_6R$ activation. This signaling cascade inhibits Na^+ absorption by ENaC, allowing fluid to accumulate into the alveolar space.

This purinergic receptor does not support the ATP-mediated pulmonary edema observed in mice during mechanical ventilation [17]. On the other hand, most purinergic networks accommodate a dual regulation of cellular functions mediated by the balancing effects of nucleotide (P2) receptors and ADO (P1) receptors. Davis et al. demonstrated that CFTR inhibitors, or agents preventing the formation of ADO from ATP metabolism, improved AFC during viral infection [26]. The use of selective P1 receptor antagonists identified the A_1R as the culprit responsible for the CFTR-mediated reduction of AFC in infected mice. These findings were quite surprising considering the well established role of $A_{2B}Rs$ in the stimulation of fluid secretion by CFTR in human airways (see Chap. 5). As will be described below, the purinergic network of the alveolar wall differs from the airways in terms of ectonucleotidases (see Chap. 2), as well as purinoceptors.

We are grateful for Factor et al., who meticulously determined the identity and polarity of the ADO receptors on alveolar epithelial cells [27]. Based on laser capture microdissection and quantitative PCR, they showed that all four receptors are detected on the murine epithelial barrier. Contrary to the upper airways, alveolar cells predominantly express the $A_{2A}R$, with an mRNA level at least fivefold higher than for the other receptors. Membrane fractionation indicated that A_1Rs and $A_{2A}Rs$ are concentrated on the apical surface, whereas $A_{2B}Rs$ and A_3Rs were undetected by Western blot. However, one must keep in mind that *in vivo* measurements of AFC will include the $A_{2B}R$-mediated CFTR activation and fluid secretion taking place in the airways. Incidentally, the lung edema induced, in mice, by mechanical ventilation was significantly reduced by intratracheal instillation of an $A_{2B}R$ antagonist (PSB1115) [28]. To specifically study the purinergic regulation of alveolar ion channels, Factor et al. used two approaches: the alveolar cell monolayers and the isolated perfused lungs [27]. Both models revealed that low ADO concentrations ($0.01-10$ nM) and $A_{2A}R$ agonists (CGS 21680) stimulate AFC via ENaC activation. In contrast, low micromolar ADO (≥ 1 μM) and A_1R agonists (CCPA) reduce AFC via CFTR activation [27], as reported during viral infection [26]. Since both ADO receptors enhance opposing fluid fluxes across the epithelial barrier, the net directionality will depend on the local fluctuations in alveolar ADO concentrations.

Collectively, these studies support a complex purinergic regulation of AFC which involves $P2Y_6Rs$ (UDP), $A_{2A}Rs$ (ADO) and A_1Rs (ADO) (Fig. 8.1). In healthy

lungs, the mechanical stress induced by normal rhythmic breathing induces the release of purines and pyrimidines into the airspace. Under these conditions, only the ADO concentration maintained by ATP release/metabolism reaches the activation thresholds of purinergic receptors, which mediates a balanced ENaC/CFTR activation to optimize AFC. During mechanical ventilation, the excessive ATP release raises ADO levels, tilting the balance toward the CFTR-mediated fluid accumulation into the lung. In addition, mechanical ventilation activates lung phosphoinosides 3-kinase gamma, which degrades cAMP [29], thereby weakening the protective effects of the ADO-A_{2A}R-cAMP pathway against lung edema. Consequently, patients with chronic illnesses associated with high airway ADO levels would be particularly at risk of developing pulmonary edema during mechanical ventilation, combining a dominant A_1R-mediated flooding and a weak A_{2A}R-mediated AFC. Likewise, bacterial infection causes ATP release from alveolar epithelial cells [30, 31], and thus initiates the same A_1R/A_{2A}R imbalance toward flooding. In the case of viral infection, the *de novo* synthesis and secretion of UTP provides additional UDP for $P2Y_6$R activation, which causes alveolar flooding via ENaC inhibition.

In terms of therapeutic application, simultaneous inhibition of CFTR (CFTR$_{inh-172}$), and prevention of ENaC inhibition using a blocker of UTP *de novo* synthesis (A77-1726), restored normal AFC in infected mice [26]. While this approach seem attractive for the treatment of pulmonary edema, clinical studies reported considerable side-effects during treatments involving A77-1726, or its prodrug leflunomide (review: [32]). Inhibition of UTP synthesis within the cells is expected to impair critical functions, including DNA synthesis. Instead, the development of drugs targeting A_1Rs, A_{2A}Rs or $P2Y_6$Rs would allow more flexibility in dose optimization with minimal side-effects. Since CFTR and ENaC are primarily regulated by $P2Y_2$Rs and A_{2B}Rs in the large airways, targeting these receptors would not compromise the clearance of pathogens by the mucociliary escalator.

This section emphasizes the distinct purinergic regulations of barrier permeability on the endothelial and epithelial barriers separating the bloodstream from the alveolar space. Armed with this information, we will begin our description of the murine models which were developed to appreciate the contribution of impaired ATP and ADO regulation in the development of acute and chronic disorders, with a critical eye on the therapeutic strategies proposed to restore lung homeostasis.

8.2 Murine Models of Aberrant Purine Regulation

Murine models were developed to determine the contribution of aberrant ATP and/or ADO concentrations to the development and progression of complex disorders, including respiratory diseases. Most mammalian cells regulate surface ATP and ADO by the sequential activities of CD39, CD73 and ADA. First, CD39 dephosphorylates ATP and ADP into AMP, and then CD73 converts AMP into ADO.

Finally, cell surface ADA converts a fraction of the extracellular ADO into inosine, whereas the remaining portion is transported back into the cells through concentrative transporters (see Chap. 2 for details). Consequently, animal models were designed to target each of these ectonucleotidases. In the following section, we compare the phenotype of these mice to chronic respiratory diseases bearing the same metabolic disorders, to gain an appreciation of their role in the lung complications, and determine the potential of purinergic-based therapeutic approaches.

8.2.1   Too Much Adenosine in ADA-Deficient Mice

Humans with mutations in the *ada* gene resulting in a lack of enzyme activity suffer from severe combined immunodeficiency (ADA-SCID) [33]. Because ADA-deficient patients are extremely rare, considerable effort was expended in the generation of *ada* gene-targeted mice to better understand this genetic disease. Unfortunately, mice in which the *ada* gene was deleted in all tissues suffered from perinatal lethality [34, 35]. Surprisingly, the cause of death was not immunodeficiency, as they survive quite well in a clean animal facility, but rather hepatotoxicity. In an attempt to create viable $ada^{-/-}$ mice, Blackburn et al. engineered an $ada^{-/-}$ mouse strain that expressed an *ada* mini-gene in the placenta using trophoblast-specific controlling elements in the *ada* promoter [36]. These mice were viable and had normal liver function. However, they died at about 3 weeks of age from respiratory distress, characterized by severe inflammation, rapid and labored breathing. Their lifespan was extended by treatment with polyethylene glycol-conjugated bovine ADA (PEG-ADA) [37], the same material used for enzyme replacement therapy in ADA-deficient patients [38]. These mice received PEG-ADA intramuscularly every 4 days after birth, which partially suppressed the ADO-mediated lung inflammation. Contrary to intranasal instillation, intramuscular injection of PEG-ADA suppresses early events in the development of lung inflammation, like leukocyte and dendritic cell migration (see Chap. 7 for details). For the purpose of this review, these mice will be referred to as "ADA-deficient mice". A second strain with partial ADA activity was generated by ectopic expression of a minigene in the gastrointestinal tract of ADA-deficient mice [39]. They developed a milder pulmonary disease and had a lifespan of about 5 months. They will be referred to as "partially ADA-deficient" mice.

  The ADA-deficient mice appeared normal at birth, but their lungs exhibited larger alveolar spaces by day 5 [36, 37]. There was no inflammatory cell in the lungs at that time, so the defect in alveogenesis preceded the onset of inflammation, which occurred around day 10. By day 18, abnormal alveogenesis was obvious, and was characterized by thickened vascular smooth muscles and marked enlargement of the alveolar spaces. There was also hypertrophy of bronchial epithelia with increased mucus production, and cell debris accumulated in the airways. Most inflammatory cells in bronchoalveolar (BAL) fluid were macrophages, in the form of enlarged and foamy multinucleated giant cells, clustered around bronchioles and

pulmonary vessels. Few eosinophils accumulated in the interstitium and the luminal space. It was suspected that these structural changes and inflammatory responses were caused by the excess ADO measured in the total lung tissue of ADA-deficient mice. Whereas lung ADO levels were barely detectable in wild-type mice, they rose about 20-fold in ADA-deficient mice, reaching a concentration of 4 $nmol \cdot mg^{-1}$ protein. The concentration of the other ADA substrate, deoxyADO was also elevated, but to a lesser extent (<0.05 $nmol \cdot mg^{-1}$ protein), suggesting that the toxicity of this molecule for DNA synthesis played a minor role in lung pathology. The critical role of ADO was clearly demonstrated by the fact that ADA-deficient mice were essentially "cured" by PEG-ADA treatments. The improvements in lung physiology and histology correlated with the decrease in lung ADO concentrations.

Extensive gene profiling was conducted to gain insight into the lung pathology of the ADA-deficient mice. In the initial report [40], data were generated with Atlas mouse cDNA expression arrays containing 1,176 known genes and RNA isolated at day 18 of life, when the mice were near death. The two genes, whose expression levels were most altered in ADA-deficient mice, were vascular endothelial growth factor (VEGF) which was down-regulated by about 50%, and monocyte chemoattractant protein-3 (MCP-3) which was up-regulated about tenfold. These data are consistent with the inhibitory effect of ADO on the secretion of VEGF from neutrophils, a potent inducer of endothelial barrier permeability [41]. Therefore, these mice are not expected to develop vascular leakage or excessive leukocyte infiltration, as indicated by the vast majority of resident macrophages in the BAL fluid [36, 37]. Other genes related to eosinophil trafficking, cell adhesion, inflammation and fibrosis were also significantly up-regulated in the lungs of the ADA-deficient mice. Importantly, the expression of all these genes was largely normalized by treating the mice with PEG-ADA under dosing conditions that prevented lung pathology. The second study was more extensive and used arrays containing 7,056 oligonucleotides (70-mers) that were produced at the UCSF Sandler Center of Basic Research in Asthma [42]. The aim of this study was to identify the pathways causing abnormal alveogenesis in ADA-deficient mice. Therefore, RNA samples were collected at earlier time points: at birth and at 5 and 10 days of life. This study showed that ADA deficiency up-regulates genes involved in apoptosis, but down-regulates genes coding growth factors and their receptors, as well as surfactant proteins and angiogenic factors. As for the first study, these changes in gene expression were largely reversed by treatment with PEG-ADA. These data suggest that excess ADO, reported in the airways of patients with asthma and COPD [43, 44] impacts the developing lung via multiple pathways including apoptosis, proliferation and migration, which would promote the development of airway remodeling and sub-epithelial fibrosis. Yet, the short lifespan of the ADA-deficient mice did not allow for adequate documentation of these manifestations.

The long-term consequences of high lung ADO concentrations were investigated using two different approaches: untreated partially-deficient ADA mice [39] and ADA-deficient mice treated with low doses of PEG-ADA over the course of 16 weeks [45]. Under these conditions, both cohorts eventually maintained comparable lung ADO levels (≤ 1.0 $nmol \cdot mg^{-1}$ protein), which were at least

fourfold lower than untreated ADA-deficient mice. As a consequence, both cohorts developed severe lung pathology and died from respiratory distress at about 5 months of age. These models allowed the authors to analyze the long-term impact of high ADO concentrations on the respiratory system to better understand the implications for chronic airway diseases. After completion of the 16 weeks of treatment, the ADA-deficient mice exhibited lung complications comparable to untreated partially ADA-deficient mice [45]. While the inflammatory response was still dominated by multinucleated macrophages, BAL fluid also accumulated lymphocytes, neutrophils and eosinophils. The histology revealed extensive remodeling, characterized by epithelial cell hyperplasia and hypertrophy, pulmonary fibrosis, matrix deposition and smooth muscle cell thickening. These observations were confirmed by real-time PCR analysis of the lung tissue, which showed that chronically-elevated ADO up-regulates several pro-fibrotic factors (TGFβ1, osteopontin, plasminogen activator inhibitor-1 and matrix metalloproteinase-2) and pro-inflammatory Th2 cytokines (IL-1β and IL-13) [45]. Functional experiments conducted on dendritic cells showed that chronic exposure to excess ADO reduces the capacity to initiate and amplify a Th1 immune response [46]. These complications were largely reversed by 5 weeks of high-dose PEG-ADA in ADA-deficient mice initially treated 13 weeks with low-dose PEG-ADA [45].

The partially ADA-deficient mice also adopted Th2-skewed lung inflammatory responses typical of pulmonary fibrosis and asthma [47–49]. The transcript of IL-13 was undetected in the lung tissue of wild-type animals, but easily measured in partially ADA-deficient mice [50]. Other Th2 cytokines were up-regulated (IL-4 and IL-5), whereas the Th1 cytokine, IFNγ, was down-regulated, compared to wild-type mice [50]. Interestingly, IL-13 transgenic mice share many lung complications with partially ADA-deficient mice [51, 52]. They develop inflammatory infiltrates enriched in macrophages and eosinophils, airway epithelial cell hypertrophy and fibrosis, mucus cell metaplasia and hypersecretion. Furthermore, IL-13 transgenic mice exhibit high lung ADO levels and a reduced ADA expression. The fact that most lung complications of IL-13 transgenic mice and partially-deficient ADA mice were resolved by PEG-ADA treatment supports the existence of a positive amplification pathway for IL-13 and ADO accumulation in the lungs. Whereas high ADO levels up-regulate IL-13, the overexpression of this cytokine promotes ADO accumulation by a reduction in ADA activity. These findings are consistent with an *in vitro* study conducted on cultures of human bronchial epithelia cells, whereby chronic exposure to excess ADO raised IL-13 mRNA levels by sixfold [53]. This Th2 cytokine, which promotes eosinophilia, remodeling and fibrosis (review: [54]), likely plays a major role in the development of lung complications associated with excess airway ADO.

Neovascularization is a major complication of chronic inflammatory disorders, including asthma (review: [55]). Inflammation stimulates the growth of new blood vessels, which enhances inflammatory cell recruitment, airway obstruction and hyper-responsiveness. The ADA-deficient mice exhibited significantly enhanced vascularity in the trachea than wild-type mice as early as 18 days after birth, which was quantified by immunolocalization of the endothelial cell marker CD31 [56]. This complication was prevented by PEG-ADA treatment, and was attributed to the

up-regulation of the pro-angiogenic chemokine, CXCL1, and its receptor CXCR2. These findings are consistent with the corneal micropocket assay of angiogenesis using lung extracts from wild-type and ADA-deficient mice. The angiogenic activity of lungs extract from ADA-deficient mice was inhibited by pre-treatment with PEG-ADA, or neutralizing antibodies to either CXCL1 or CXCR2. The source of CXCL1 appears to be macrophages, which accumulate in high numbers in the lung of ADA-deficient mice. While the gene expression surveys support an increased resistance against leukocyte transmigration into the airspace of the ADA-deficient mice, the longer lifespan of partially-deficient ADA mice and PEG-ADA treated ADA-deficient mice allows sufficient time to develop intense neovascularization as zones of vulnerability for leukocyte infiltration. Interestingly, genetic inactivation of A_{2A}Rs attenuated the pathological, but not developmental, angiogenesis in mouse retina [57]. Understanding the mechanisms by which excess ADO enhances neovascularization may lead to preventive therapies for airway remodeling and chronic inflammation.

8.2.2 Too Little Adenosine in CD73$^{-/-}$ Mice

Most chronic respiratory diseases are associated with aberrances in extracellular nucleotide metabolism which maintain excess ADO in airway secretions (see Chap. 4 for details). Given the devastating consequences predicted from the ADA-deficient mice, therapeutic strategies are being formulated to restore normal airway ADO levels in these patients. The importance of careful dosing the pharmacological agents is emphasized by an animal model which restricts the production of this receptor agonist. Studies were conducted with $Cd73^{-/-}$ mice to determine the impact of too little ADO on the overall health of the subject, as well as airway responses to acute insults and chronic conditions.

Pulmonary hypoxia/ischemia initiates the translocation of inflammatory cells from the bloodstream into the interstitium as a pathological healing response (review: [58]). Hypoxic tissue releases ATP, which constitutes a powerful neutrophil chemoattractant to eliminate apoptotic cells as part of the repair process (see Chap. 7 for details). Without the existence of an efficient feedback mechanism, the excess recruitment of inflammatory cells would cause extensive tissue injury. Purinergic signaling provides a critical defense mechanism by terminating neutrophil transmigration across the endothelial barrier. The sequential activities of CD39 and CD73 on endothelial cells convert circulating ATP into ADO, which raises barrier resistance via A_{2A}Rs/A_{2B}Rs (Fig. 8.1). Although ADO induces the production of VEGF, a potent inducer of endothelial barrier permeability, in many circumstances, it can also block its secretion from neutrophils [41]. Incidentally, adherent neutrophils were shown to facilitate their transmigration by inhibiting CD73 activity on endothelial cells [59], which may explain the lack of CD73 on these inflammatory cells [60]. For all these reasons, ADO formation by the CD39-CD73 tandem is essential for the prevention of excessive inflammation and acute lung injury during hypoxia.

Hypoxia triggers the up-regulation of CD39, CD73 and $A_{2B}Rs$ [11, 61, 62]. While CD39 up-regulation is mediated by the Sp1 transcription factor [61], CD73 and $A_{2B}R$ up-regulation require binding of the hypoxia inducible factor-1α (HIF-1α) to their promoter site [11, 62]. The higher CD39-CD73 efficiency is expected to raise the circulating ADO concentration above the activation threshold of the $A_{2B}Rs$ to enhance barrier resistance against vascular leakage and leukocyte transmigration (review: [2]). The importance of this purinergic regulation is demonstrated in mice denied CD73. The $Cd73^{-/-}$ mice exhibit significant vascular leakage in multiple organs, including the lung [63]. Acute hypoxia (8% O_2; 4 h) causes more severe vascular leakage in $Cd73^{-/-}$ mice than in wild-type mice, characterized by perivascular edema and inflammatory infiltrates. These complications are partially reversed by the intraperitoneal injection of P1 receptor agonists, or soluble 5′-nucleotidase to replace CD73 [63, 64]. These results are consistent with the relative affinities of $A_{2A}Rs$ and $A_{2B}Rs$, both involved in barrier protection (Fig. 8.1). While high-affinity $A_{2A}Rs$ maintain baseline barrier resistance, the dramatic rise in circulating ADO level caused by hypoxia recruits the activities of low-affinity $A_{2B}Rs$. This study suggests that therapies aiming to suppress ADO production in chronic respiratory diseases could leave the patients vulnerable to hypoxia-induced pulmonary edema, and even aggravate lung inflammation, if the suppression of CD73 activity is not carefully optimized and restricted to the airways.

Animal models are currently available to assess the potential of new therapies for acute lung injury (review: [65]). The intratracheal instillation of bleomycin ranks among the five most popular models, as it recapitulates most features: neutrophilic alveolitis, damage to alveolar epithelia and endothelia, hyaline membrane formation, and thrombus accumulation in the microvasculature. This antibiotic is isolated from *Streptomyces verticillus* and complexes with oxygen and metals, leading to the production of oxygen radicals, DNA breaks and ultimately cell death. Within 24 h, mice develop inflammation, depicted by the accumulation of macrophages, lymphocytes and neutrophils in the BAL fluid, which lasts about 11 days [66]. Lung tissue contains higher mRNA levels of pro-inflammatory cytokines (IL-1β and TNFα) and many pro-fibrotic factors (osteopontin, plasminogen activator inhibitor-1, TGFβ1 and tissue inhibitor of metalloproteinase-1), as well as excess collagen. Fibrosis appears around day 11, and is reversed within 10 days.

Acute lung injury reproduces the high airway ADO concentrations reported in asthmatic and COPD patients [43, 44]. The bleomycin-challenged mice maintain threefold higher total lung ADO levels, and a proportional increase in CD73 activity, during 14 days [66]. To determine whether this metabolic imbalance promotes or suppresses the pulmonary complications, $Cd73^{-/-}$ mice were subjected to the bleomycin challenge [66]. These mice developed more severe complications to bleomycin, and died within 18 days. However, their lung ADO levels remained normal, identifying AMP dephosphorylation as the source of the agonist. These data suggest that a transient increase in airway ADO production is a defense mechanism to suppress acute lung injury. Incidentally, enzyme replacement therapy by intranasal instillation of 5′-nucleotidase restored the ability of $Cd73^{-/-}$ mice to accumulate

lung ADO, which reduced all symptoms of inflammation and fibrosis. The fact that 5′-nucleotidase, given intranasally, was able to reduce the number of inflammatory cells in BAL fluid suggests that airway ADO can reach the interstitium to suppress leukocyte migration, or regulates epithelial permeability. Our current knowledge does not support a role for ADO in the regulation of neutrophil epithelial transmigration (Fig. 8.1). However, Hirsh et al. demonstrated that polarized bronchial epithelia support the vectorial transport of airway ADO into the interstitial tissue via apical concentrative, and basolateral equilibrative, nucleoside transporters [53]. Hence, the benefits of aerosolized drugs, given to acutely raise ADO levels during acute lung injury, may extend beyond the airways.

The chronic model of allergic asthma supports the detrimental effects of chronic excess ADO in the airways. Mice are generally sensitized with ovalbumin (OVA) by intraperitoneal injections taking place over 14 days, followed by four challenges with nebulized OVA over an additional week [67]. Within 24 h after the last challenge, their lungs develop features of allergic asthma, including eosinophilic inflammation, goblet cell metaplasia and hyperresponsiveness to methacholine [68]. Furthermore, the BAL fluid of OVA-challenged mice contains eightfold higher ATP concentrations than in saline-challenged control mice, as reported for asthmatic patients following an allergen challenge [68]. Schreiber et al. tested the impact of preventing ADO production, from this excess nucleotide, in this model of allergic asthma [69]. The $Cd73^{-/-}$ mice exhibited no baseline allergen-induced inflammation, and failed to develop hyperresponsiveness to methacholine following OVA treatment. These data demonstrate that the airway ADO produced by CD73, and not excess ATP, is responsible for the hyperresponsiveness of OVA-challenged mice. Aerosolized PEG-ADA may constitute a potential therapeutic intervention for airway hyperresponsiveness in asthmatic patients.

8.2.3 How Informative Are CD39$^{-/-}$ Mice?

The impact of chronically-high airway ATP concentrations has been investigated by genetic deletion of the ATP/ADP-hydrolyzing ectonucleotidase, CD39. This choice was originally based on the predominant role of endothelial CD39 in the prevention of ADP-mediated thrombus formation (review: [70]). Consequently, it was quite surprising to find that $Cd39^{-/-}$ mice exhibit no symptom of pulmonary complication, unless they are subjected to an insult. For instance, mechanical ventilation has been used as a model of acute lung injury in over 500 publications (review: [65]). Patients kept on a ventilator for less than 3 days usually recover from an acute inflammatory response, but prolonged intubation initiates tissue fibrosis and loss of lung function. For this reason, mechanical ventilation remains the focus of most animal studies on acute lung injury.

Eckle et al. demonstrated that mice subjected 90 min to high-pressure mechanical ventilation develop typical manifestations of acute lung injury, characterized by vascular leakage, pulmonary edema, neutrophil infiltration and hemorrhage [71].

Interestingly, as in the case of hypoxia, this insult up-regulated both CD39 and CD73 by threefold within 2 h, resulting in a fourfold increase in total lung ADO concentration [71]. To test whether these metabolic disruptions constitute a defense mechanism, or promote the lung damage, $Cd39^{-/-}$ and $Cd73^{-/-}$ mice were sub-jected to the same insult. These knockout mice developed the same symptoms, but they were dramatically more severe than in the wild-type mice [71]. Surprisingly, similar BAL albumin level and neutrophil myeloperoxidase activity, as well as lung water content, were detected in the ventilated $Cd39^{-/-}$ and $Cd73^{-/-}$ mice. During acute insults, it is widely accepted that ATP mediates predominantly pro-inflammatory effects, whereas ADO generally provides a feedback anti-inflammatory response (review: [72]). Since $Cd39^{-/-}$ mice allow ATP to accumulate and prevent ADO formation, we would have expected a more severe lung inflammation than in the $Cd73^{-/-}$ mice, which simply deny ADO formation. This study highlights the critical protective role of excess ADO production during an acute insult, which outweighs any short-term detrimental effect of ATP accumulation. The significance of this purinergic protection was confirmed by the beneficial effects of enzyme replacement therapy on the ventilated wild-type mice. The intraperitoneal adminis-tration of apyrase (commercial form of CD39) or 5′-nucleotidase (commercial form of CD73), significantly reduced the accumulation of BAL albumin during mechan-ical ventilation. The excess circulating ADO, likely, provided additional endothe-lial barrier protection against vascular leakage via the low-affinity A_{2B}Rs (Fig. 8.1). Incidentally, this study also showed that intraperitoneal injection of an antagonist of A_{2B}Rs, not A_{2A}Rs, before the onset of mechanical ventilation reproduced the severity of the lung edema observed in the knockout mice. Consequently, this study confirms the protective role of excessive ADO against pulmonary edema during acute lung injury. Endothelial A_{2B}Rs should be targeted at the first manifestations of acute lung injury.

Most animal models of acute lung injury are based on clinical disorders, including mechanical ventilation, gastric aspiration and sepsis (review: [65]). The intratracheal administration of bacterial lipopolysaccharide (LPS) is particularly suited to investigate the impact of purinergic signaling on neutrophil migration into the airways. Aside from the usual BAL fluid cytokines, vascular leakage and disruption of the lung architecture, the mice accumulate neutrophils in all compart-ments of the respiratory system. This LPS challenge also induced a coordinated up-regulation of CD39 and CD73 [73], as described above for hypoxia [74] and mechanical ventilation [71]. The exposure of $Cd39^{-/-}$ mice and $Cd73^{-/-}$ mice to intratracheal LPS generated more severe accumulation of neutrophils in the pulmo-nary tissue than in LPS-treated wild-type mice. To determine which step of neutrophil migration was affected in the knockout mice, Reutershan et al. labeled their neutrophils by intravenous injection of Alexa 633-labelled Gr-1 [73]. They showed that the absence of CD39 or CD73 raised neutrophil numbers in the interstitium and the airspace, but not in the bloodstream. Since both knockout mice respond similarly to the LPS challenge, this study suggests that the prevention of ADO formation in the $Cd39^{-/-}$ mice and $Cd73^{-/-}$ mice was responsible for the exaggerated lung neutrophilia. The fact that ADO deficiency allowed neutrophils to

accumulate to greater extent, both, in the interstitium and BAL fluid, suggest that this P1 receptor agonist normally restrict their transmigration across the endothelial and alveolar epithelial barriers. As in the other models of acute lung injury described above, the low circulating ADO levels of $Cd39^{-/-}$ mice and $Cd73^{-/-}$ mice were associated with a loss of endothelial barrier resistance, as determined by extravasation of Evans blue dye. On the other hand, this study provides the first evidence of a purinergic regulation of leukocyte migration across airway epithelia.

A common pattern emerges from studies conducted with mouse models of aberrant purine regulation. The fact that $Cd39^{-/-}$ mice and $Cd73^{-/-}$ mice respond similarly to acute insults indicates that circulating/airway ADO, not ATP, significantly influences the short term and reversible inflammatory responses. In these situations, a robust and transient increase in circulating ADO appears essential to raise endothelial barrier integrity, which restricts pulmonary edema and the infiltration of inflammatory cells. In contrast, models of chronic airway diseases clearly show that the sustained elevation of airway ADO is associated with a wide array of detrimental effects, ranging from severe inflammation to airway remodeling and hyperresponsiveness. Therefore, any therapeutic strategy that will be envisioned to reduce purine-mediated lung complications must be carefully tailored with the most appropriate murine model.

8.3 Murine Models Targeting Adenosine Receptors

8.3.1 Can A_1Rs Go Either Way?

Ischemia-reperfusion injury causes significant morbidity and mortality, which remains a major obstacle after lung transplant. Neely et al. provided evidence that A_1R antagonists attenuate inflammation and injury in a feline model of ischemic lung injury [75]. In these animals, ischemia-reperfusion induced lung edema and the infiltration of inflammatory cells (neutrophils, macrophages and red blood cells) in the alveolar space. Intralobar arterial infusion of an A_1R antagonist, xanthine amine congener (XAC) or 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), before the onset of ischemia fully prevented alveolar injury. This study suggests that a proactive treatment with A_1R antagonists may reduce the ischemia-reperfusion injury developing after lung transplant surgery.

Bacterial LPS initiates a number of pro-inflammatory responses, such as priming of neutrophils and macrophages, and the EGFR-mediated signaling cascades of cytokine and growth factor secretion from airway epithelial cells (see Chaps. 6 and 7 for details). Yet, the specific mechanism by which LPS initiates these cascades of pathophysiological events in the lung has not been described. Neely et al. used an intralobar arterial injection of LPS as a model of acute lung injury in spontaneously breathing cats [76]. These animals developed a dose-dependent lung injury characterized by the presence of macrophages, neutrophils and red blood cells in the alveoli, alveolar edema and necrosis. Pre-treatment by intravenous bolus injection

of DPCPX prevented all aspects of the acute lung injury. This study suggests that A_1R antagonists may decrease the symptoms of adult respiratory distress syndrome induced by sepsis. Based on the human model of fluid flux regulation, this approach may prevent edema by suppressing A_1R-mediated CFTR activity and fluid secretion in the alveolar space (Fig. 8.1). On the other hand, since few studies documented purinergic signaling in cats, it would be reassuring to repeat these experiments in mice, especially in the light of the following study.

In mice, the administration of aerosolized LPS triggers classic manifestations of acute lung injury, summarized by microvascular leakage and neutrophil accumulation in the vascular, interstitial and alveolar compartments. Interestingly, a study showed that LPS also causes a gradual increase in the total lung mRNA and protein level of A_1Rs. This response was considerably weaker in mice depleted in neutrophils by injection of anti-granulocyte antibodies [77]. Therefore, the authors tested whether this receptor regulates neutrophil recruitment by exposing knockout mice to aerosolized LPS. These $A_1R^{-/-}$ mice developed exaggerated neutrophil migration and microvascular permeability to LPS, supporting an anti-inflammatory role for this ADO receptor. Incidentally, the pre-treatment of wild-type mice by intraperitoneal injection of the new A_1R agonist, named 2'Me-2-chloro-N6-cyclopentyladenosine (2′Me-CCPA), significantly reduced vascular leakage and neutrophil counts in the interstitium and alveolar space. This agonist also reduced the accumulation of pro-inflammatory cytokines (TNF-α, IL-6 and CXCL2/3) in the BAL fluid. A combination of *in vivo* and *in vitro* experiments revealed that A_1Rs protect against the loss of endothelial and epithelial barrier permeability induced by LPS through inhibition of cytoskeletal rearrangement. Also, pre-treatment of neutrophils with 2′Me-CCPA reduced their transmigration across endothelial cells, supporting a role for A_1Rs on the inflammatory cells. Since the receptor specificity of 2′Me-CCPA has been fully assessed [78], this study supports the therapeutic potential of the agonist for the treatment of acute lung injury regarding airway bacterial infection.

Chronic airway diseases are associated with higher mRNA levels of A_1Rs in the animal models [39, 79, 80], as well as bronchial epithelial and airway smooth muscle cells from asthmatic patients [81]. An up-regulation of this receptor in asthma supports a role in the pathophysiology of this disease, and the potential of selective antagonists for the treatment of asthmatics. In the dust mite-conditioned allergic model of asthma, rabbits receiving aerosolized antisense oligodeoxynucleotides targeting A_1Rs were desensitized to subsequent challenges with ADO or dust mite [82]. The intratracheal ingestion of the water soluble A_1R antagonist, L-97-1, also attenuated bronchoconstriction in this model [83]. In addition, allergic animals pre-treated with L-97-1 accumulated significantly less BAL eosinophils and neutrophils in response to a dust mite challenge than the untreated allergic animals [83]. Whether ADO-induced bronchoconstriction is mediated via A_1Rs expressed on airway smooth muscle, inflammatory cells or nerve endings is not clear. A recent study conducted with $A_1R^{-/-}$ mice supports a neuronal component [84]. But the fact that aerosolized and gastric deliveries of the A_1R antagonist reduce hyperresponsiveness supports a multi-cellular mechanism (see Chap. 9 for details). Detrimental roles for the A_1Rs in chronic diseases also include the capacity to raise the

expression of MUC2 and MUC5AC [85], which contributes to mucus overproduction, a major feature of asthma.

Murine models of chronically-high ADO concentrations in the lung are associated with enhanced or induced A_1Rs expression, depending on the cell type [86]. In the ADA-deficient mice, the transcript levels of A_1Rs were threefold higher in lung tissue, and 50-fold higher in BAL cell pellet, compared to wild-type mice. Immunolocalization showed enhanced protein expression on macrophages, and the receptor appeared on endothelial and epithelial cells. For these reasons, ADA/A_1R double knockout mice were developed to determine the impact of A_1R up-regulation on airway defenses. The genetic removal of A_1Rs from the ADA-deficient mice resulted in an additional twofold increase in the total lung ADO level. Compared to the ADA-deficient mice, they had more severe pulmonary inflammation, mucus metaplasia and alveolar destruction, exaggerated expression of Th2 cytokines (IL-4 and IL-13) and matrix metalloproteinases. This study suggests that, in the presence of chronically-high ADO, A_1Rs are anti-inflammatory in the respiratory system. On the other hand, it is important to note that these double knockout mice also exhibit a twofold decrease in $A_{2A}R$ transcript, which mediates potent anti-inflammatory responses in the lung, as described in the section below. A weaker protection by $A_{2A}R$ may account, at least partially, for the severity of lung complications in ADA/A_1R knockout, compared to ADA-deficient mice.

These animal models provide important information on the contribution of A_1Rs to acute and chronic lung disorders. However, work is needed to determine the role of this receptor, which is expected to appear on alveolar and airway epithelial cells of patients with high lung ADO content, as in asthma and COPD. The murine study, which used the new selective A_1R agonist, 2′Me-CCPA [77], supports the benefits of this compound in chronic diseases. And yet, the delivery route is a key determinant in treatment design, as vascular administration reduces lung inflammation, whereas aerosolized agonists may induce bronchoconstriction. The A_1R provides an excellent example for the importance of understanding the physiological background to develop a safe and efficient treatment.

8.3.2 The All Mighty $A_{2A}Rs$

The potent anti-inflammatory activities mediated by $A_{2A}Rs$ on various cell types motivated the development of animal models to evaluate the potential of agonists for the treatment of inflammation and injury in pulmonary disorders (review: [87, 88]). Sharma et al. conducted a series of studies to investigate the impact of $A_{2A}Rs$ on the acute lung injury caused by ischemia-reperfusion [89]. Perfused isolated lungs developed significant dysfunction (higher airway resistance, pulmonary artery pressure and lower compliance), tissue injury (vascular leakage and edema), and the accumulation of cytokines (TNFα, KC, MIP-2 and RANTES) in the BAL fluid. The addition of the selective $A_{2A}R$ agonist, ATL313, to the perfusate after ischemia reduced all reperfusion-related complications by at least 50%. This

study shows that $A_{2A}Rs$ expressed by resident cells, such as alveolar macrophages, endothelial and epithelial cells, play a major role in the anti-inflammatory properties of these receptors during acute lung injury.

The number of circulating neutrophils is regulated by IL-17A, a pro-inflammatory cytokine released by CD4+ T lymphocytes (review: [90]). Sharma et al. used an *in vivo* murine model of ischemia-reperfusion to test whether the anti-inflammatory roles of the $A_{2A}R$ include the suppression of CD4+ T cell activities [91]. As in the case of isolated lungs [89], perfusion of the agonist, ATL313, significantly prevented lung injury and the loss of compliance in mice [91]. The significant reduction of neutrophil infiltration was demonstrated by immunohistochemistry and BAL fluid myeloperoxidase activity. The mice subjected to ischemia-reperfusion after depletion of the CD4+ T cells or neutrophils also had significantly reduced lung injury and BAL fluid levels of inflammatory mediators, including IL-17A. However, ATL313 further reduced the symptoms only in neutrophil-depleted mice. This study shows that $A_{2A}R$ activation attenuates ischemia-reperfusion injury by inhibiting CD4+ T cell activation, and the subsequent neutrophil infiltration in the lung. Systemic administration of $A_{2A}R$ agonists, before a transplant procedure, could offer two waves of protection against excessive inflammation and injury.

The airway defenses supported by the $A_{2A}R$ are also sensitive to acute lung injury initiated within the airspace. Aerosolized LPS was associated with an up-regulation of the ADO receptor [92]. Therefore, the consequences of this deregulation were determined by exposing $A_{2A}R^{-/-}$ mice to aerosolized LPS. Consistent with the anti-inflammatory role of this receptor, knockout mice accumulated more neutrophils into the lungs in response to LPS than the wild-type mice. Accordingly, intranasal instillation of the selective agonist, ATL 202, only reduced neutrophil transmigration in wild-type mice [92]. Together, bone marrow transplant between wild-type and $A_{2A}R^{-/-}$ mice, and conditional knockout mice lacking $A_{2A}Rs$ on macrophages, demonstrated that $A_{2A}R$ overexpression on macrophages plays a major role in the anti-inflammatory properties of airway ADO [92]. Since LPS was added to the airways, it is not expected to affect endothelial barrier permeability. Instead, LPS stimulates the secretion of pro-inflammatory cytokines (i.e. IL-1β, TNFα) from alveolar macrophages, which bind specific receptors on the epithelia to stimulate the release of neutrophil chemoattractants. On macrophages, $A_{2A}R$ activation suppresses the production and secretion of these cytokines [93–95]. During an acute airborne insult, the overexpression of $A_{2A}Rs$ on resident macrophages would prevent the development of excessive inflammatory responses.

The intense preclinical research on $A_{2A}R$ agonists currently conducted in murine models of chronic airway diseases is not surprising, given the multiple roles of this P1 receptor in the prevention of excessive and damaging airway inflammation. Interestingly, contrary to the up-regulating effect of acute insults, chronic exposures to ragweed or Th2 cytokines (IL-4 or IL-13) are all associated with a down-regulation of $A_{2A}Rs$ [79, 80, 96]. Similar findings were observed in the ADA-deficient model of chronically-elevated lung ADO [39]. The ragweed sensitization/challenge caused more severe airway inflammation and hyperresponsiveness in the $A_{2A}R^{-/-}$ mice than in the wild-type mice [96]. Altogether, these studies suggest that the

down-regulation of A_{2A}Rs contributes to disease severity in Th2 driven chronic lung disorders. Therefore, preclinical studies support a substantial benefit for A_{2A}R-based aerosolized therapies in chronic diseases. In mice subjected to the OVA sensitization/ challenge model of allergic asthma, the intratracheal instillation of CGS 21680 before the last challenge significantly reduced lung inflammation, quantified in BAL fluid in terms of leukocyte numbers, neutrophil myeloperoxidase and eosinophil peroxidase activities [97]. A similar protective effect of intratracheal CGS 21680 was reported in Norway rats subjected to this OVA exposure protocol [97]. On the other hand, airway hyperresponsiveness and mucus hypersecretion are not treated by A_{2A}R agonists, in accordance with the lack of regulation of these functions by this receptor [84, 98]. Aerosolized A_{2A}R agonists are expected to target specifically the activities of immune and inflammatory cells, and their recruitment (see Chap. 7 for details).

Lung transplant is currently the preferred option for various end-stage pulmonary diseases. While remarkable progress in the outcome has been made, chronic allograft rejection still leads to the development of bronchiolitis obliterans, a chronic inflammatory disorder. Given the well established anti-inflammatory profile of A_{2A}R signaling, a study examined the therapeutic potential of A_{2A}R agonists in a murine model of transplant rejection [99]. Non-revascularized tracheal transplants exhibited precocious signs of rejection in A_{2A}R knockout mice, compared to wild-type mice, manifested by enhanced inflammation, epithelial loss and fibrosis. Pre-treatment of the wild-type mice with an A_{2A}R agonist (ATL 313) before receiving the transplant significantly protected against the infiltration of inflammatory cells (lymphocytes, macrophages and neutrophils) and luminal occlusion by collagen deposition, measured over 21 days. This approach shows potential for the prevention of bronchiolitis obliterans in lung transplant.

Collectively, these studies support an anti-inflammatory role for A_{2A}Rs in acute and chronic lung diseases. The expression of this receptor on key regulatory and effector cells, such as lymphocytes, neutrophils and macrophages, suppresses their migration and the secretion of cytokines and chemokines to dampen immune responses. Hence, there is substantial preclinical evidence that A_{2A}R agonists may be useful to treat inflammatory features in acute lung injuries and chronic lung diseases.

8.3.3 A_{2B}Rs: Balancing Clearance and Inflammation

Over the past 5 years, models of acute lung injury applied to $A_{2B}R^{-/-}$ mice have assigned a protective role to the receptor (review: [100]). The first model considered was the hypoxia-induced vascular leakage and recruitment of inflammatory mediators to the lungs. Initial profiling of each ADO receptor was obtained by silencing RNA techniques in human microvascular endothelial cells. The results showed that only A_{2B}R elimination resulted in a significant increase in endothelial leakage in response to hypoxia [11]. The same selective response was reported in

knockout mice exposed to normobaric hypoxia [11]. Only the $A_{2B}R^{-/-}$ mice exhibited enhanced pulmonary edema and vascular leakage, compared to the hypoxic wild-type mice. Interestingly, hypoxia selectively up-regulated $A_{2B}Rs$, likely as a defense mechanism against vascular leakage [11]. Therefore, agonists and antagonists were evaluated in wild-type mice for their capacity to prevent acute lung injury. Pretreatment of the mice by intraperitoneal injection of an agonist (BAY60-6583) almost completely prevented hypoxia-induced injury, whereas an antagonist (PSB1115) aggravated these lung complications. To determine the relative contribution of $A_{2B}Rs$ expressed on pulmonary or hematopoietic cells to the vascular leakage during hypoxia, the authors generated bone marrow chimeric mice. Mice expressing $A_{2B}Rs$ on the tissues, but not on the hematopoietic cells, showed reduced Evan's blue extravasation in response to BAY 60-6583, whereas the opposite chimera offered no protection. Taken together, these studies provide pharmacological and genetic evidence for vascular $A_{2B}R$ signaling as a central control point of hypoxia-associated vascular leak. These data are consistent with the purinergic regulation of vascular permeability in human lungs (Fig. 8.1), thereby supporting a role for $A_{2B}R$ agonists in the treatment of hypoxia-related acute lung injury.

Bacterial infection triggers an acute increase in $A_{2B}R$ expression throughout the respiratory system, by a mechanism involving cytokine release and autocrine responses. In cultured human microvascular endothelial cells and A549 alveolar epithelial cells, IL-1β, IL-4, IL-6 and prostaglandin E2 induce a progressive increase in $A_{2B}R$ mRNA levels, reaching more than sixfold higher values after 12 h, then returning to baseline within 24 h [101]. These findings were reproduced *in vivo*, as mice responded to LPS inhalation by a nearly fivefold increase in total lung $A_{2B}R$ transcript and protein levels. When the mice were pre-treated by inhalation of an $A_{2B}R$ antagonist (MRS 1754), they reacted to LPS by dramatically more severe lung edema, higher BAL fluid cytokine concentrations (IL-1β and TNFα) and neutrophil myeloperoxidase activity. Similar findings were obtained with $A_{2B}R^{-/-}$ mice. As in the case of hypoxia [11], the bone marrow chimeric mice support a predominant role for $A_{2B}Rs$ expressed on pulmonary cells, not hematopoietic cells, in the protection against acute lung injury. Accordingly, the $A_{2B}R$ agonist BAY 60-6583 clearly prevented LPS-mediated lung inflammation and edema. Collectively, these studies show that hypoxia and bacterial infection acutely up-regulate $A_{2B}Rs$ as a defense mechanism, which offers a platform for the effective treatment of acute lung injury.

Contrary to acute lung injury, abundant literature supports pro-fibrotic and tissue destructive roles for $A_{2B}Rs$ in chronic lung diseases, such as asthma, COPD and fibrotic pulmonary diseases [102, 103]. Numerous studies demonstrated the ability of $A_{2B}R$ activation to induce the expression and secretion of pro-inflammatory mediators from various cell types, including IL-4, IL-8 and IL-13 from mast cells [104–106], IL-6 and IL-19 from airway epithelial cells [107, 108] and monocyte chemoattractant protein-1 from bronchial smooth muscle cells [109]. Consequently, it is not surprising that $A_{2B}R^{-/-}$ mice, subjected to the

OVA sensitization/challenge model of allergic asthma, develop milder features of pulmonary inflammation and airway remodeling than wild-type mice [110]. Major inflammatory findings included a reduced production of IL-4 and lower airway eosinophil numbers in $A_{2B}R^{-/-}$ mice chronically exposed to the allergen. With respect to airway remodeling, these mice presented lower levels of TGFβ, mucus cell metaplasia and thickening of the smooth muscle layer surrounding bronchial airways. Similar data were reported for mice subjected to the ragweed sensitization/NECA challenge model of allergic asthma [111]. In the sensitized mice, pretreatment by intraperitoneal injection of an $A_{2B}R$ antagonist (CVT-6883) significantly attenuated the airway inflammation and hyperresponsiveness induced by nebulized NECA. These studies provide evidence for the potential of $A_{2B}R$ antagonists in the treatment of chronic diseases, like asthma.

The contribution of $A_{2B}Rs$ to chronic lung disease is further supported by studies conducted in the ADA-deficient model of ADO-mediated lung injury [103]. These mice develop progressive increases in total lung ADO level, in association with inflammation and tissue remodeling [37, 45]. This model is characterized by the accumulation of activated alveolar macrophages producing various inflammatory mediators, alveolar airspace destruction, mucus cell metaplasia and fibrosis. The expression of the $A_{2B}R$ is elevated in the lungs of ADA-deficient mice. Once lung complications are established, the selective $A_{2B}R$ antagonist CVT-6883 significantly reduced the production of pro-inflammatory and pro-fibrotic mediators, alveolar airspace enlargement and fibrosis [112]. Similar findings were observed in the bleomycin model of pulmonary fibrosis [112]. Interestingly, $A_{2B}Rs$ are also up-regulated in the lungs of IL-4 [80] and IL-13 [79] overexpressing mice which develop features of asthma, COPD and pulmonary fibrosis. Moreover, high $A_{2B}R$ expression was recently described as a feature of patients with accelerated pulmonary fibrosis [113]. Thus, the exaggerated activation of $A_{2B}R$ signaling pathways appears to constitute a widespread feature of chronic airway diseases with fibrosis. In support of this, $A_{2B}R$ activation has been shown to promote the differentiation of human pulmonary fibroblast into collagen producing myofibroblasts [114], and to stimulate the production of pro-fibrotic fibronectin in alveolar epithelial cells [115]. These findings suggest that the sustained up-regulation and engagement of $A_{2B}Rs$ might collectively serve to enhance chronic remodeling features of chronic lung diseases.

As in chronic lung diseases, the post-transplant bronchiolitis obliterans syndrome presents many histological characteristics of allograft injury and inflammation consistent with the pro-inflammatory and pro-fibrotic effects of $A_{2B}R$ activation (review: [116]). Therefore, a murine model of tracheal transplant rejection was used to determine the role of $A_{2B}Rs$ in the development of bronchiolitis obliterans [117]. When the tracheas were transplanted into $A_{2B}R^{-/-}$ mice, they exhibited less luminal obliteration and inflammatory cell infiltration (macrophages, neutrophils, CD3+ T cells) after 21 days, than in wild-type recipients. In contrast, the allografts of the $A_{2B}R^{-/-}$ mice contained higher numbers of the immunosuppressive CD4/CD25/Foxp3 T regulatory (Treg) cells [117]. Therefore, apart from

the well-known pro-inflammatory and pro-fibrotic effects of $A_{2B}R$ activation, this receptor may also stimulate the development of bronchiolitis obliterans by inhibiting the recruitment of the immunosuppressive Treg cells into the allograft.

8.3.4   The A₃R Models the Infiltrates

As with the other ADO receptors, A_3Rs play complex roles in inflammation, with both pro- and anti-inflammatory functions being described in different models [118]. Pharmacological studies have demonstrated that treatment with A_3R agonists is beneficial in the feline model of reperfusion injury [119], as documented above for A_1Rs [75]. In spontaneously breathing cats subjected to ischemia-reperfusion in the left lower lobe, the perfusion of IB-MECA or MRS 3558 before ischemia reduced acute lung injury, which was quantified by the number of apoptotic cells, lung edema, and BAL fluid neutrophil myeloperoxidase assay. Western blots conducted on tissue samples collected over 10 h indicated that A_3R engagement activates the secondary messengers extracellular signal-regulated kinases 1 and 2 (ERK1/2), but not c-Jun amino-terminal protein kinase (JNK) and p38, which essentially shifts the signaling balance from cell death to cell survival. Therefore, A_3R agonists offer a therapeutic benefit for the treatment of acute lung injury related to ischemia-reperfusion.

The role of A_3Rs in acute lung injury was also recently documented in a murine model of airway infection [120]. In mice, LPS inhalation triggered general characteristics of acute lung injury, including neutrophil accumulation in all lung compartments, as well as an up-regulation of the A_3R. Therefore, pharmacological experiments were initiated to determine the directionality of A_3R-mediated responses, with respect to the neutrophilic inflammation. Mice pretreated by intralobar arterial infusion of the specific A_3R agonist, Cl-IB-MECA, responded to aerosolized LPS by significantly lower neutrophil counts into the interstitium and alveolar airspace, but not the vasculature. Incidentally, measurement of Evans blue extravasation showed that Cl-IB-MECA inhibits the LPS-induced increases in endothelial barrier permeability. The mechanism was addressed *in vitro* using cultures of pulmonary microvascular endothelial cells. The A_3R agonist interfered with the LPS-induced cytoskeletal rearrangement and cell retraction known to initiate vascular leakage. On the other hand, experiments conducted with bone marrow chimeric mice revealed that Cl-IB-MECA requires A_3Rs expressed, both, on hematopoietic cells and pulmonary cells to suppress the transmigration of neutrophils *in vivo*. Possible mechanisms include cell adhesion or the secretion of mediators (see Chap. 7 for details). Regardless, this study proposes a new role for A_3Rs in the recruitment of neutrophils to the airways. Whereas A_3Rs expressed on neutrophils were shown to accelerate chemotaxis (review: [121]), the present study demonstrates that A_3Rs also restricts neutrophil infiltration into the lung tissue and airspace. The administration of Cl-IB-MECA into the

bloodstream may be useful to suppress the damaging neutrophilic inflammation associated with sepsis.

Intratracheal instillation of bleomycin ranks among the most popular models of acute lung injury. The mice transiently develop damage to the alveolar epithelial and endothelial barriers, extensive fibrosis, thrombus formation in the microvasculature and the accumulation of inflammatory cells in the interstitial space (review: [65]). Genetic deletion of the A_3R yielded no significant phenotype, but the mice reacted more strongly to the bleomycin challenge [122]. After 14 days, the lungs of $A_3R^{-/-}$ mice accumulated three times more inflammatory cells than the wild-type mice, including eosinophils. Interestingly, whereas bleomycin caused an increase in eosinophil peroxidase activity in the BAL fluid of wild-type and $A_3R^{-/-}$ mice, the secreted enzyme activity was only higher in the wild-type mice. These data show that eosinophils require the A_3R for degranulation. On the other hand, the loss of this receptor did not influence the formation of pulmonary fibrosis in response to bleomycin, as shown by measurement of collagen and α1procollagen in lung tissue. Altogether, these data suggest that A_3Rs generally serve anti-inflammatory functions in the bleomycin model of acute lung injury, without affecting pulmonary fibrosis. On the other hand, lung complications associated with a predominant eosinophilic inflammation would ascribe a pro-inflammatory role to this receptor, such as asthma. This study highlights the disease-specificity of purinergic regulation which arises from the cell type specificity of receptor expression.

Chronic lung diseases are generally associated with an up-regulation of the A_3R, reported in the lung biopsies of asthmatic and COPD patients [123]. The importance of this disturbance in purinergic regulation is highlighted by three murine models of Th2-mediated airway inflammation and remodeling, which are also associated with enhanced A_3R expression [67, 79, 124]. This receptor affects several aspects of the lung disease. First, human and murine eosinophils abundantly express A_3Rs, to inhibit chemokine-induced migration [123–125], whereas it accelerates neutrophil chemotaxis [121]. The airway epithelial cells of wild-type mice do not normally express A_3Rs. However, the receptor appears selectively on the hyperplastic mucin-secreting cells of the OVA sensitized/challenge mice, IL-13 transgenic mice and ADA-deficient mice [67, 79, 124]. Young et al. determined that the receptor does not regulate the mRNA expression or packaging of mucins into granules, as wild-type and $A_3R^{-/-}$ mice exhibit similar storage sizes following OVA sensitization/challenge [67]. In contrast, the selective A_3R agonist, IB-MECA, stimulated mucin secretion in the OVA challenged, but not the untreated, animals. This study supports a role for A_3R induction in the mucin hypersecretion and airway obstruction reported in many chronic airway diseases, including asthma, COPD and CF (review: [126]). On the other hand, the induction of A_3R expression during mucus cell metaplasia and hyperplasia remains to be confirmed in human airways, where mucin secretion is normally mediated by ATP ($P2Y_2$) receptors (reviews: [98, 127]) (see Chap. 5 for details).

8.3.5   General Patterns in Acute and Chronic Disorders

This area of purinergic research is evolving so rapidly that the notions formulated when this book was initially designed had to be revised along the way. Thankfully, we can finally discern general patterns of behavior for each ADO receptor in murine models of acute and chronic lung complications. First, the literature shows that acute and chronic disorders up-regulate all ADO receptors, except for a down-regulation of the $A_{2A}R$ in chronic lung diseases. Such general mobilization of the purinergic network is a testimony to the critical role of ADO in airway defenses against insults and intruders. Second, knockout mice do not present any intrinsic phenotype, but respond dramatically to insults or exposures, supporting the primary role of this network in alarm situations. This inductive behavior would prioritize the receptors responding to abnormally high ADO concentrations generated in circulation and airway secretions by ATP release and metabolism: $A_{2A}Rs$ and $A_{2B}Rs$ (see Chap. 7, Table 7.1).

A summary of the effects of agonists/antagonists in the murine studies identify aspects of acute and chronic lung complications regulated by each ADO receptor (Fig. 8.2). Regardless of the exposure protocol, the engagement of all receptors initiated anti-inflammatory and protective responses against acute lung injury. This came as a surprise, considering their widespread biphasic effects on immune and inflammatory cells (see Chap. 7 for details). Then again, the initial inflammatory response to an insult is the recruitment of hematopoietic cells to the lung tissue. Since they must all cross the endothelial barrier, this site constitutes a bottle neck for airway defenses to efficiently regulate the extent of airway inflammatory responses. The fact that all ADO receptors participate in the maintenance of barrier integrity identifies the endothelium as primary site for therapeutic applications regarding hypoxia, acute lung injury and sepsis. The $A_{2A}R$ agonists represent the safest choice because the receptor only supports protective or anti-inflammatory responses.

The ADA-deficient mouse model conceptualizes the consequences of maintaining lung ADO concentrations elevated in chronic respiratory diseases, including asthma, CF and COPD. The severity of the purine-related complications results from the combined effects of $A_{2A}R$ down-regulation and $A_{2B}R$ up-regulation, which dramatically shifts the balance in favor of pro-inflammatory and remodeling responses to ADO concentrations capable of significantly activating low-affinity receptors. The murine models of chronic lung diseases revealed that ADO receptors inhibiting cAMP production (A_1Rs and A_3Rs) promote mucin hypersecretion and bronchoconstriction. On the other hand, $A_{2B}R$ activation stimulates most aspects of the inflammatory responses, remodeling and fibrosis. The ideal aerosolized therapy would combine A_1R and $A_{2B}R$ antagonists to address all aspects of inflammatory diseases associated with airway hyperresponsiveness. On the other hand, careful dosing will be required for $A_{2B}R$ antagonists to minimize side-effects regarding the mucociliary clearance of allergen and infectious particles, as this receptor plays
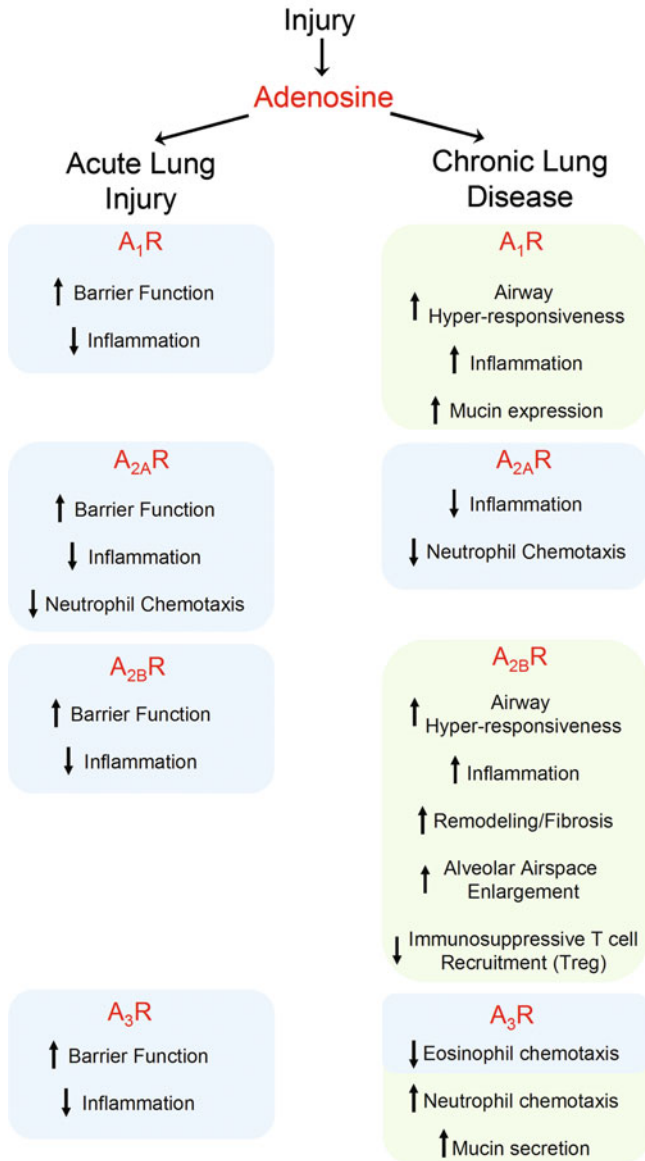
Fig. 8.2 Summary of the adenosine-mediated responses in murine models of acute lung injury and chronic lung disease. The *blue boxes* indicate anti-inflammatory and protective effects, the *green boxes* indicate pro-inflammatory and damaging responses

critical roles in fluid secretion and cilia beating (see Chap. 5 for details). The importance of findings in pre-clinical and cellular models will have to be validated in carefully designed clinical trials that take into account the potential impact of ADO receptors signaling in acute and chronic lung disorders.

8.4   Murine Models Targeting ATP Receptors

The field of purinergic inflammation formulated the unifying concept that ATP is released as a "danger signal" to induce inflammatory responses upon binding cell surface purinergic receptors (see Chap. 7 for details). Our current understanding of purinergic signaling in the respiratory system suggests that this concept can be extended to airway defenses against infection and obstruction (see Chap. 5 for details). The mouse models currently available for P2X and P2Y receptors, and for ectonucleotidases, highlight the airway functions particularly targeted by ATP and altered under pathological conditions. In general, these mice present no apparent phenotype or lung complication, unless they are subjected to an insult. As such, this section exposes the critical role of extracellular ATP in acute lung injury and chronic respiratory diseases.

8.4.1   The P2Y$_2$R in Acute and Chronic Disorders

The most convincing evidence that the P2Y$_2$R is engaged primarily during alarm situations is derived from studies conducted on the knockout mice [128, 129]. They are fertile, undistinguishable from their wild-type littermates, and show no abnormality in their organs, including the heart, lung, pancreas, intestines, kidneys and trachea. The fact that their airways are not obstructed is consistent with the distinct roles of P2Y$_2$R-CaCC and A$_{2B}$R-CFTR signaling pathways in mucociliary clearance (MCC), established using cultures of human airway epithelial cells (review: [24]). Under the steady-state (baseline) conditions, the surface ADO concentrations partially activate A$_{2B}$Rs, which maintains sufficient CFTR-mediated fluid secretion for MCC in a sterile environment. In contrast, the surface ATP concentrations periodically reach the activation threshold of P2Y$_2$Rs during normal tidal breathing, as a result of mechanical stress-induced nucleotide release. This transient and weak activation stimulates fluid and mucin secretion to maintain a thin shield above the ciliated epithelium. Together, these ADO- and ATP-mediated signaling events continuously produce and evacuate this protective film along the ciliary escalator. On the other hand, an alarm situation, sensed by the interaction of an irritant or pathogen with the epithelium, initiates a robust and transient MCC response through ATP release and P2Y$_2$R activation. The most powerful trigger for this flushing mechanism is a cough. The rapid metabolism of ATP into ADO by ectonucleotidases triggers a second wave of fluid secretion through enhanced A$_{2B}$R-CFTR activity and ciliary beating, both gradually returning to baseline with ADO concentration. Hence, P2Y$_2$R-mediated MCC only predominates during alarm situations.

The possibility that different P2 receptors may predominate in humans and rodents was addressed by comparing the capacity of airway epithelia from wild-type and P2Y$_2$R$^{-/-}$ mice to regulate ion and fluid secretion. Measurements of ion channel activity in Ussing chambers indicated that the loss of P2Y$_2$R decreases ATP-induced

Cl$^-$ secretion by 75% in the nose and 90% in the trachea [129]. Also, mucin secretion induced by the selective P2Y$_2$R agonist, ATPγS, was reduced by >80% in P2Y$_2$R$^{-/-}$ mice, compared to their wild-type littermates [130]. These studies demonstrate that the "danger signal", provided by stress-induced ATP release, triggers airway clearance primarily by P2Y$_2$R activation in murine airway epithelia.

The beneficial effects of P2Y$_2$R signaling is extended to the mobilization of cells engaged in the destruction of inhaled pathogens, including monocytes/macrophages and leukocytes. In most instances, these actions are essential to resolve airway infection. On the other hand, chronic or exaggerated activation of these functions lead to irreversible damage to the respiratory system. Whether P2Y$_2$Rs are beneficial or detrimental in these two scenarios was examined using models of acute and chronic lung complications.

The overall impact of P2Y receptors on the susceptibility to airway infection was investigated using P2Y$_1$R/P2Y$_2$R double knockout mice [131]. When subjected to intra-tracheal instillation of the common airway pathogen, *Pseudomonas aeruginosa*, all the double knockout mice succumbed within 30 h, whereas 85% of the wild-type mice lived a normal lifespan. After 24 h, the BAL fluid of P2Y$_1$R/P2Y$_2$R$^{-/-}$ mice contained more proteins as a measure of edema, but less pro-inflammatory mediators (IL-1β, IL-6, GM-GSF and MIP-2). In contrast, the two mouse groups presented similar total cell counts and cell composition, which were primarily neutrophils (>80%). These data suggest that P2Y receptors exert a protective role during lung infection by suppressing pulmonary edema, while promoting the inflammatory responses required to eradicate the infection. The severe pulmonary edema developed by the P2Y$_1$R/P2Y$_2$R$^{-/-}$ mice is consistent with our model of fluid regulation (Fig. 8.1), whereby the loss of endothelial P2Y$_2$Rs would prevent ATP from protecting barrier functions. On the other hand, it is intriguing that these mice accumulate BAL neutrophils to the same extent as the wild-type mice, given the critical role of P2Y$_2$Rs for their chemotaxis (see Chap. 7 for details). Unfortunately, double knockout mice are of limited value to discriminate the roles of each receptor.

An acute model of airway infection was used to determine the impact of P2Y$_2$Rs localized in the vasculature, on circulating cells or the endothelial surface [132]. Wild-type mice received intravenous saline, or a selective P2Y$_2$R agonist (ATPγS), before intranasal instillation of LPS. The mice which received the P2Y$_2$R agonist exhibited about 50% less inflammatory cells, cytokines and proteins in the BAL fluid, than the saline-treated LPS-exposed mice. The protective effect of ATPγS against vascular leakage was confirmed by Evans blue albumin extravasation into the lung tissue, pointing toward a primary function at the endothelial barrier. This was confirmed *in vitro* in human lung microvascular endothelial cultures, where ATPγS significantly inhibited the LPS-mediated reduction of transmembrane electrical resistance. These data suggest that intravenous P2Y$_2$R agonists may reduce the airway inflammatory responses and lung injury caused by airborne pathogens. Using this administration route, ATPγS would not promote neutrophil chemotaxis through tissue along a chemotactic gradient [121], which is a major role postulated for this P2Y receptor.

On inflammatory cells, $P2Y_2R$ activation initiates the pro-inflammatory responses required to eradicate microbial pathogens through a series of highly coordinated events culminating in phagocytosis and destruction. In pathological circumstances, like sepsis, they release antimicrobial compounds which cause injury and death of bacteria and host cells (see Chap. 7 for details). The lung damage associated with excessive neutrophilia contributes to a wide array of respiratory complications, including ARDS, COPD and CF (review: [133]). Incidentally, genetic deletion of the $P2Y_2R$ improves the survival of mice subjected to sepsis by cecal ligation and puncture [134]. These mice exhibited fewer lung neutrophils and less pulmonary damage than wild-type mice. Since $P2Y_2R$ activation provides directionality during neutrophil chemotaxis [121], the leukocytes of $P2Y_2R^{-/-}$ mice lack the ability to follow an ATP gradient toward the lung. This study suggests that intraperitoneal $P2Y_2R$ antagonists may improve the survival of sepsis patients.

Chronic respiratory diseases are commonly associated with elevated airway ATP concentrations, as reported in CF, IPF and COPD patients [135–138], and they are raised by allergens in asthmatic patients [68]. The expression of $P2Y_2Rs$ is also expected to be elevated in these diseases, as chronic exposure to pathogens or allergens up-regulate the receptor in animal models. For instance, house dust mites caused an increase in $P2Y_2R$ expression in eosinophils and dendritic cells from asthmatic patients, which significantly enhanced chemotaxis [139]. It is important to point out that cells from healthy subjects did not up-regulate $P2Y_2Rs$ in response to house dust mites, indicating that the purinergic regulation of inflammatory responses is not normally modified by allergens in the general population. On the other hand, rat alveolar Type 2 cells exposed overnight to LPS reacted by raising $P2Y_2R$ transcripts by threefold, which resulted in a significant increase in the ATP-mediated surfactant secretion [30]. Whereas $P2Y_2R$ overexpression may improve MCC, the pro-inflammatory reactions of immune and inflammatory cells may aggravate airway inflammation. Therefore, it is particularly important to understand the costs and benefits of $P2Y_2Rs$ in chronic diseases before we can envision therapeutic applications.

Asthmatic patients maintain normal airway ATP levels, but these concentrations rise tenfold in response to an allergen [68]. The consequences of this excess ATP were examined in the OVA sensitization/challenge model of allergic asthma [68]. The mice are sensitized by intra-peritoneal injection, and then challenged by nebulization 10 days later. After 24 h, their BAL fluid had accumulated eightfold higher ATP concentrations than in the saline-challenged control animals, as reported in the allergen-challenged asthmatic patients. They also developed all major features of allergic asthma, including eosinophil-dominant lung infiltrates, goblet cell metaplasia, hyperresponsiveness to methacholine and Th2 inflammation in the lymph nodes (IL-4, IL-5 and IL-13). These complications were significantly reduced when the OVA challenges were conducted in the presence of an ATP-metabolizing enzyme (apyrase) or P2Y receptor antagonist (PPADS or suramin). Through a series of elegant *in vivo* and *in vitro* protocols, Idzko et al. demonstrated that the excess ATP generated after an allergen challenge triggers airway inflammation by the activation of resident dendritic cells, and their mobilization to the

lymph nodes to trigger a Th2 type of inflammation. It is interesting that excess ATP in the BAL fluid was not detected 10 min, but rather 24 h, after the challenge in asthmatic patients and in the mice. The mechanisms behind the delayed accumulation of ATP into the airways have not been identified. However, oxidative stress building gradually with airway inflammation has been shown to inhibit CD39 [140], and ATP-hydrolyzing ectonucleotidases expressed on airway epithelial [141], immune and inflammatory [142] cells. Consequently, this study supports the therapeutic potential of P2Y receptor antagonists for the treatment of airway inflammation in asthmatic patients.

More recently, $P2Y_2R^{-/-}$ mice were subjected to this model of allergic asthma to identify the mechanism by which $P2Y_2Rs$ promote eosinophilia [139, 143]. The $P2Y_2R^{-/-}$ mice exhibited a weaker capacity to recruit eosinophils in response to the OVA exposure protocol than the wild-type mice [143]. *In vitro* assays showed that circulating dendritic cells and bone marrow eosinophils collected from $P2Y_2R^{-/-}$ mice fail to migrate within an ATP gradient [139]. Three findings support a role for the $P2Y_2R$-mediated up-regulation of VCAM-1 in the purinergic stimulation of lung eosinophilia. This molecule is a potent chemoattractant and adhesion molecule for eosinophils (review: [144]). First, the OVA challenge caused the accumulation of soluble VCAM-1 in the BAL fluid, but to a lower level in $P2Y_2R^{-/-}$ mice [143]. Second, OVA also induced an up-regulation of membrane-bound VCAM-1 at the surface of lung endothelial cells, but to a lower extent in $P2Y_2R^{-/-}$ mice. Finally, adhesion assays confirmed that ATP promotes eosinophil adhesion to the endothelial surface through a $P2Y_2R$-mediated increase in VCAM-1 surface expression. Collectively, these studies suggest that an amplification of the ATP-$P2Y_2R$ signaling in asthmatic patients during an allergic reaction, contributes to the development of lung eosinophilia. Accordingly, asthmatic patients may benefit from the intravenous injection of $P2Y_2R$ antagonists to suppress eosinophil recruitment to the airways.

Chronic obstructive pulmonary disease is another interesting example of $P2Y_2R$-dominant lung pathology. The discovery that the murine model of smoke-induced lung inflammation and emphysema reproduces the high airway ATP concentrations of COPD patients provided the necessary tools to investigate the role of purinergic deregulation in the pathogenesis of this disease [145]. First, the role of ATP in the early events of airway inflammation was investigated using an acute cigarette smoke exposure protocol, which consisted of a daily 20 min session for 4 days. This acute insult caused an eightfold increase in BAL fluid ATP level, the typical macrophage and neutrophil infiltrates, and the accumulation of their cytokines (IL-1β, IL-6, IFNγ, MIP-2 and KC). Intratracheal instillation of the ATP-metabolizing apyrase, or a P2Y receptor antagonist (PPADS or suramin), before each smoke inhalation reduced all inflammatory parameters by more than 50%. This acute protocol shows that the excess ATP, accumulating after the first cigarettes, is critical to the early development of smoke-induced airway inflammation.

The contribution of ATP-induced inflammatory responses to the development of emphysema was studied using a chronic smoke exposure protocol lasting 4 months [145]. These mice developed foci of emphysema throughout the parenchyma,

which was essentially prevented by oral administration of a P2Y receptor antagonists, suramin or PPADS. Real-time PCR analysis of P2 receptor expression in the inflammatory cells and lung parenchyma revealed a dramatic (eightfold) and selective up-regulation of $P2Y_2Rs$ in the neutrophils of smoke-exposed, compared to air-exposed, animals. The importance of the amplified ATP-$P2Y_2R$ signaling axis in the development of smoke-induced airway inflammation and emphysema was clearly demonstrated using $P2Y_2R^{-/-}$ mice and chimera [137]. While the source of BAL fluid ATP has not been addressed, *in vitro* experiments showed that human neutrophils release ATP in response to cigarette smoke in a dose-dependent manner, and as efficiently as fMLP [137]. Also, cigarette smoke induced an ATP-dependent secretion of neutrophil elastase, which is an important mediator of lung destruction in emphysema (review: [146]). These studies suggest that the initial increase in BAL fluid ATP concentration detected after the first cigarette exposures initiates the development of airway inflammation, which is amplified over time by an up-regulation of $P2Y_2Rs$ on immune and inflammatory cells (see Chap. 7 for details). Therefore, the oral prescription of apyrase, or a P2 receptor antagonist, could prevent and reduce the irreversible lung damage and loss of lung function in COPD patients.

8.4.2   The $P2X_7R$ in Chronic Lung Diseases

For years, the importance of $P2X_7Rs$ for ATP-mediated inflammatory responses has been largely underestimated on the premise that the EC_{50} of this receptor is orders of magnitude above that of $P2Y_2Rs$ (review: [9]). However, the elegant work of Di Virgilio et al. clearly demonstrated that sites of inflammation or tissue injury accumulate ATP in the high micromolar range *in vivo*, above the activation threshold of $P2X_7Rs$ (Chap. 7, Figs. 7.1 and 7.2). The recent finding that major pro-inflammatory cytokines involved in the initiation of airway inflammatory responses (i.e. IL-1β) depend on the $P2X_7R$ for their production, maturation and secretion (see Chap. 7 for details) is only one example of the many critical roles emerging for this receptor. Therefore, it is not surprising that the inhibition or deletion of $P2X_7Rs$ has such profound implications for airway defenses.

The mouse model of COPD, described above by Idzko et al., revealed a remarkable up-regulating effect (10- to 20-fold) of cigarette smoke on the $P2X_7Rs$ of neutrophils and macrophages within 4 days [145]. The use of selective antagonists demonstrated that $P2X_7R$ activation on these cells promotes all aspects of airway inflammation and fibrosis leading to the development of COPD and emphysema. In wild-type mice subjected to the acute smoke protocol, intratracheal injection of KN62 or A438079, before each exposure, markedly reduced the accumulation of macrophages, neutrophils and their cytokines (IL-1β, IL-6, IFNγ, MIP-2 and KC) in the BAL fluid [147]. In mice subjected to the chronic exposure protocol, these agents prevented the development of fibrosis and emphysema. The widespread

effects of P2X$_7$Rs on airway inflammation are explained by their pivotal role in the secretion of IL-1β from the resident macrophages, which initiate the events leading to leukocyte recruitment. Furthermore, P2X$_7$R-overexpressing neutrophils and macrophages would cause considerable lung destruction by massive discharge of potent cytotoxic molecules which do not discriminate between the bacteria and host cells [146] (see Chap. 7 for details). Since smoke triggers such dramatic increases in BAL fluid ATP level [145] and P2X7R expression, an early treatment with aerosolized antagonists may reduce disease progression and prevent emphysema.

Idiopathic pulmonary fibrosis (IPF) is a fatal respiratory disorder characterized by inflammation, tissue fibrosis and destruction of the alveolar architecture. Since the IPF patients maintain high airway ATP concentrations [138], the bleomycin-induced murine model of lung fibrosis was used to test the contribution of purinergic deregulation to the pathogenesis of this disease [138]. In mice, the intratracheal administration of bleomycin initiates neutrophil recruitment within 24 h, which normalizes around day 11. First signs of fibrosis appear around day 11, and are reversed within 10 days (review: [65]). Time-course analysis indicated that bleomycin induces a transient increase in BAL fluid ATP level over the first 6 h, which is followed by the initiation of neutrophil recruitment. This time line supports a role for an ATP gradient in neutrophil recruitment to the lungs. Disruption of this ATP gradient by apyrase treatment reduced by >60% the BAL content in neutrophils, myeloperoxidase and metalloproteinases. Similar findings were obtained when comparing wild-type and P2X$_7$R$^{-/-}$ mice after bleomycin exposure [138]. This study suggests that the higher availability of ATP for P2X$_7$R activation contributes to the inflammatory processes launching tissue fibrosis and lung destruction.

In asthmatic patients, airway ATP concentrations only rise following an allergen challenge [68]. On the other hand, the purinergic network is permanently altered in these patients, as P2X$_7$R expression is up-regulated in BAL fluid macrophages and circulating eosinophils, compared to healthy subjects [148]. A murine model of allergic asthma was used to determine the consequences for airway defenses. Mice sensitized and challenged with OVA summarize the inflammatory responses of asthmatic patients during an allergic reaction [148]. The lung infiltrates contain mainly eosinophils, followed by mast cells, neutrophils and lymphocytes, whereas BAL fluid exhibits the typical Th2 inflammation (IL-4, IL-5 and IL-13). As reported in asthmatic patients, the P2X$_7$R was up-regulated 15-fold in the inflammatory cells and lung tissue of OVA-treated mice. The use of a selective P2X$_7$R antagonist (KN62) on OVA-treated wild-type mice, or OVA-treated P2X$_7$R$^{-/-}$ mice, was associated with milder lung inflammation compared to OVA-treated wild-type mice. The mechanism behind the pro-inflammatory role of P2X$_7$Rs in allergic asthma was investigated using bone marrow-derived dendritic cells from wild-type and P2X$_7$R$^{-/-}$ mice. The loss of P2X$_7$Rs reduced the capacity of OVA-treated dendritic cells to induce Th2 immunity *in vivo*. This study highlights another critical role of P2X$_7$Rs in the early events initiating an inflammatory response to an airborne insult, and the potential of selective antagonists for the suppression of Th2 inflammation in asthma.

8.5   Conclusion

All animal models of acute lung injury and chronic lung diseases are associated with an accumulation of extracellular purines, and an up-regulation of their receptors, except for the anti-inflammatory $A_{2A}R$. Such massive amplification of purinergic signals in response to hypoxia, ischemia-reperfusion, mechanical ventilation, bacterial products and allergens supports critical and widespread functions in airway defenses. During an acute lung injury, all ADO receptors provide protection against excessive lung inflammation, damage and pulmonary edema. The intravenous administration of agonists yielded the most effective resolution of all lung complications, which identified the microvascular endothelial barrier as primary target. Treatments should focus on agonists of the high-capacity $A_{2A}Rs$ and $A_{2B}Rs$ to minimize receptor desensitization (Chap. 7, Table 7.1). Whereas $P2Y_2R$ agonists may also be considered, possible side-effects may arise from their role in neutrophil and eosinophil recruitment. All models of chronic lung diseases are associated with a down-regulation of the anti-inflammatory $A_{2A}Rs$ in inflammatory cells and lung tissue, which tilts the balance toward pro-inflammatory responses to ADO. Whereas $A_{2A}R$ agonists were shown to suppress airway inflammation, they would not address airway hyper-responsiveness, remodeling and fibrosis. Alternatively, the models of asthma, COPD and emphysema suggest that aerosolized antagonists of $A_{2B}Rs$, $P2Y_2Rs$ and $P2X_7Rs$ resolve most lung complications. However, the $A_{2B}R$ or $P2Y_2R$ antagonists must be carefully dosed to avoid any significant impairment of the mucociliary clearance mechanisms. Of particular interest are the $P2X_7R$ antagonists, which show tremendous benefits in reducing the development of COPD and emphysema in smokers through a reduction of macrophage activities responsible for the initiation of leukocyte recruitment to the airways. In asthma, aerosolized $P2X_7R$ antagonists may prevent resident dendritic cells from initiating the Th2 immunity. One important point to remember is that delivery route will dictate the cost/benefits of a purine drug depending on the local effects of the target receptor, and whether a treatment is corrective or preventive.

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