Published in final edited form as:

Crit Care Med. 2016 April ; 44(4): e181–e189. doi:10.1097/CCM.0000000000001370.

Inhibition of adenosine kinase attenuates acute lung injury

David Köhler, PhD^{1,*}, Ariane Streißenberger, BSc^{2,*}, Julio C. Morote-García, PhD^{1,*}, Tiago F. Granja, PhD^{1,*}, Mariella Schneider, BSc^{2,*}, Andreas Straub, MD^{3,*}, Detlev Boison, MD PhD^{4,*}, and Peter Rosenberger, MD PhD^{4,*}

*Department of Anaesthesiology and Intensive Care Medicine, Tübingen University Hospital; Eberhard-Karls University Tübingen, Germany

+Department of Neurology, Neurobiology Laboratories, Legacy Research Institutes, Portland OR, USA

Abstract

Objective—Extracellular adenosine has tissue protective potential in several conditions.

Adenosine levels are regulated by a close interplay between nucleoside transporters and adenosine kinase (ADK). Based on evidence of the role of ADK in regulating adenosine levels during hypoxia, we evaluated the effect of ADK on lung injury. Furthermore, we tested the influence of a pharmacological approach to blocking ADK on the extent of lung injury.

Design—Prospective experimental animal study.

Setting—University based research laboratory.

Subjects—In vitro cell lines, wildtype (Wt) and *ADK*^{+/-} mice.

Methods—We tested the expression of ADK during inflammatory stimulation in vitro and in a model of lipopolysaccharide (LPS) inhalation in vivo. Studies using the ADK promoter were performed in vitro. Wt and *ADK*^{+/-} mice were subjected to LPS inhalation. Pharmacological

Address correspondence to: Peter Rosenberger, MD, PhD, Department of Anesthesiology and Intensive Care Medicine, Universitätsklinikum Tübingen, Hoppe-Seyler-Straße 3, 72076 Tübingen, Tel.: +49 7071 / 29-86622, peter.rosenberger@medizin.uni-tuebingen.de.

¹Research Associate

²Medical Student

³Staff Physician

⁴Chairman

Copyright form disclosures:

Dr. Boison received royalties from Springer (Book on Adenosine), is employed by Legacy Research Institute, and received support for article research from the National Institutes of Health (NIH). His institution received grant support from the NIH, US Department of Defense, and CURE Foundation. The remaining authors have disclosed that they do not have any potential conflicts of interest.

Conflict of interest: None.

Author Contribution Statement:

David Köhler: designed research, performed experiments, analysed data, and wrote the manuscript

Ariane Streißenberger: designed research, performed experiments, and analysed data

Julio C. Morote-García: designed research, performed experiments, and analysed data

Mariella Schneider: performed experiments and analysed data

Tiago Granja: performed experiments

Andreas Straub: wrote the manuscript

Detlef Boison: checked the manuscript and provided animals

Peter Rosenberger: designed research and wrote the manuscript

inhibition of ADK was performed in vitro, and its effect on adenosine uptake was evaluated. The pharmacological inhibition was also performed in vivo, and the effect on lung injury was assessed.

Measurements and Results—We observed the repression of ADK by pro-inflammatory cytokines and found a significant influence of NF- κ B on regulation of the ADK promoter. Mice with endogenous ADK repression ($ADK^{+/-}$) showed reduced infiltration of leukocytes into the alveolar space, decreased total protein and myeloperoxidase levels, and lower cytokine levels in the alveolar lavage fluid. The inhibition of ADK by 5-iodotubercidine increased the extracellular adenosine levels in vitro, diminished the transmigration of neutrophils and improved the epithelial barrier function. The inhibition of ADK in vivo showed protective properties, reducing the extent of pulmonary inflammation during lung injury.

Conclusions—Taken together, these data show that ADK is a valuable target for reducing the inflammatory changes associated with lung injury and should be pursued as a therapeutic option.

Keywords

ADK; acute lung injury; inflammation; ITU

Introduction

The acute respiratory distress syndrome (ARDS) and lung injury (ALI) are associated with high morbidity and mortality in the critically ill (1). Clinically, ALI is characterised by the acute onset of hypoxaemia (which requires oxygen support), pulmonary inflammation and changes in the pulmonary structure during radiological assessment. These changes are caused by the reduced function of the alveolar-capillary barrier, which leads to increased pulmonary vascular permeability, and by the infiltration of inflammatory cells into the alveolar space (2, 3). The severity of these pathophysiological changes determines the severity of the associated lung injury and the patient outcome (4).

Previous research has identified extracellular adenosine as a protective molecule in hypoxia (5), during ischaemia reperfusion (6) and in inflammatory conditions, including acute and chronic lung diseases (7, 8). Extracellular adenosine is generated by the conversion of extracellular ATP to ADP and AMP as a result of dephosphorylation by the enzymes ectonucleotidase triphosphate diphosphohydrolase (ENTPDase; CD39) and ecto-5'-nucleotidase (CD73), which are primarily located on the vascular endothelium (9). Signal transduction by extracellular adenosine then functions through the four purinergic G-protein-coupled adenosine receptors. These adenosine receptors are widely distributed throughout the organism, and all of these receptors are proposed to mediate tissue protection (10, 11). Following its generation, adenosine is mainly cleared from the extracellular space by the equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs), resulting in the movement of adenosine into cells. In the intracellular space, adenosine is then either degraded to inosine via adenosine deaminase (12) or converted to AMP and ADP by adenosine kinase (ADK) (13). Therefore, the intracellular enzyme ADK also regulates the extracellular adenosine levels. The inhibition of ADK results in elevated extracellular adenosine levels; therefore, this inhibition has a high therapeutic potential for protecting tissue in several pathological conditions (13, 14).

Because changes in the vascular barrier function and infiltration of leukocytes into the alveolar space are key processes during the initial stages of lung injury, we sought to describe the role of ADK in the development of this condition. In addition, we evaluated whether ITU can inhibit ADK, as a possible pharmacological intervention that acts on ADK to ameliorate the changes in lung injury.

Materials and Methods

Ethic Statement

All animal protocols were in accordance with the German guidelines for the use of living animals and were approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. For experiments using materials from human blood samples, ethics committee approval was obtained, and each participant provided written informed consent.

Mice

ADK^{+/-} mice (*Adk*^{tm1Bois}) were generated, validated and characterised as described previously (15). For further details, please see the supplement material.

Transcriptional analysis

To assess the relative expression levels of ADK, we tested various tissues from WT and *ADK*^{+/-} mice. For details, please see the online supplement.

In a separate experiment, human lung epithelial A549 cells were grown to confluence and stimulated with TNF- α (100 ng/ml), IL-6 (20 ng/ml), or IL-1 β (20 ng/ml) for 2, 4, 8 and 24 hours. For further details, please see the supplement material.

Protein analysis

Cell culture and mouse tissue samples were normalised for the protein levels before being applied to SDS containing poly-acrylamide gels and were processed according to standard protocols.

Immunofluorescence staining

Lung epithelial A549 cells and murine tissues for immunofluorescence staining of the lungs were harvested and processed according to standard protocols. For further details, please see the supplement material.

Immunohistochemistry

Immunohistochemistry was performed as previously described (7, 16).

Isolation of human neutrophils and the transepithelial migration assay

Peripheral blood was taken from healthy donors into Sarstedt monovettes containing sodium citrate (Sarstedt) and neutrophils were isolated as previously described (17). For transendothelial migration studies, A549 cells were grown on the basolateral aspect of

permeable transwell inserts (0.4 µm pore size, 6.5 mm diameter) until they were confluent. Migration assays were performed as described elsewhere (17). In a subset of experiments, ADK A549 cells were inhibited by ITU for 30 min before the transepithelial migration assay was started.

Macromolecule paracellular permeability assay (Flux)

Human A549 cells were seeded and grown to a monolayer on the permeable membrane as described above. Flux assays were performed as previously described (18).

Measurement of the intracellular adenosine and adenosine uptake

Human lung epithelial A-549 cells were cultured for 2 days in 6-well plates to confluence. At the start of the experiment, the cell media were replaced with HBSS+ containing 10 or 25 µm/ml 5-iodotubercine (ITU). As a control, we used cells without ITU treatment, and to control adenosine reuptake, we administered 25 µm/ml ITU plus 10 µm/ml dipyridamole, which is a cellular adenosine uptake inhibitor. For details, please see the online supplement.

ADK Reporter Assays

Promoter analysis and identification of both the transcription start site and the potential NF-κB-binding sites were evaluated using MatInspector by Genomatix. The pGL4.17-expressing sequence vector, which corresponds to the full-length ADK promoter region, was purchased from GeneArt. For further details, please see the supplement material.

NF-κB Chromatin immunoprecipitation (ChIP)

The ChIP assay (CHP1; Sigma-Aldrich) was performed according to the manufacturer's instructions. As the ChIP-qualified antibody of interest, we used the NF-κB antibody Anti-NF-κB p105/p50 – ChIP Grade Abcam (ab7971). For the PCR detection of ADK, we used the following primers: ttt cct agg ctg agg ctt ccc (forward) and tca gct ccc tgt aac agc act (reverse).

Bronchoalveolar lavage

After animals were sacrificed, bronchoalveolar lavage (BAL) was collected by performing a tracheotomy and flushing the lungs three times with 0.6 ml of PBS. For further details, please see the supplement material.

Data analysis

We performed statistical analysis using one-way analysis of variance (ANOVA) to determine the differences between the groups using Dunnetts post-hoc analysis. Unpaired Student *t* test was used where appropriate. A value of P-value < 0.05 was considered to be statistically significant.

Results

Adenosine kinase is repressed during pulmonary inflammation in mice

In an initial experiment, we sought to evaluate whether the ADK levels change during lung injury in mice. For this, we exposed mice to a lung injury model involving LPS inhalation. Following this, we found significant repression of ADK at the transcriptional level in pulmonary tissue (Figure 1A). The protein levels of ADK also reflected this finding (Figure 1B). To illustrate the repression of ADK, we performed immunofluorescence staining of the pulmonary tissue. The ADK staining in pulmonary tissue sections showed reduced ADK-specific immunofluorescence following LPS exposure. An epithelial marker, cytokeratin, was used for co-immunostaining (Figure 1C, Supplemental Figure E1).

Expression of epithelial ADK is diminished by inflammatory cytokines in vitro via NF- κ B

To determine whether ADK expression is regulated at the transcriptional level in response to pro-inflammatory stimuli, we exposed confluent pulmonary epithelial cell monolayers (A549) to TNF- α (100 ng/ml), IL-6 (20 ng/ml) or IL-1 β (20 ng/ml) for 0, 2, 4, 8 and 24 hours. In addition, we measured ADK transcriptional expression following exposure to different concentrations of these cytokines. We found significant repression of ADK at the transcriptional level, and we detected an early onset of repression, within 2 hours of the start of exposure (Figure 2A and B, Supplemental Figure E2A and B). To verify our results, we examined ADK expression at the protein level in A549 cells using different cytokine concentrations. The protein analysis confirmed the results that were observed at the transcriptional level, showing repression of ADK protein expression after pro-inflammatory stimulation with TNF- α , IL-6 or IL-1 β (Figure 2C, Supplemental Figure E2C). To visualise the repression of ADK, we used immunofluorescence staining of alveolar A549 cells. Following 24 hours of stimulation with TNF- α , there was reduced ADK immunofluorescence (Figure 2D, Supplemental Figure E1). This finding could be confirmed by measurement of ADK through ELISA (Supplemental Figure E3).

To gain further insight into the regulatory mechanisms controlling ADK at the transcriptional level, we performed a transcription factor binding site analysis of the ADK promoter region using the available public databases. We identified one NF- κ B binding site at -309 bp (Figure 3A). To identify the functional role of this NF- κ B responsive element, we first tested the binding activity of NF- κ B to the ADK promoter in A549 cells by using chromatin immunoprecipitation. There was a strong binding response of NF- κ B after stimulation with TNF- α , which indicated an NF- κ B-driven mechanism that regulated ADK production at the transcriptional level (Figure 3B). To assess the functional role of the NF- κ B binding site, we designed luciferase reporter constructs that expressed the putative full-length ADK (ADK-FL) promoter (Figure 3C). In addition, we manufactured site directed mutations in the NF- κ B response element regions to prevent NF- κ B binding. We transfected A549 cells with the promoter constructs and stimulated the cells with 100 ng/ml TNF α for 24 h. The luciferase activity of the unmodified ADK-FL promoter was strongly reduced in response to the inflammatory stimulus. Following mutation of the binding site for NF- κ B (-309), this repression of luciferase activity was reversed.

Extracellular adenosine levels are influenced by ADK, reducing transepithelial migration and flux

Because high extracellular adenosine levels are known to be involved in tissue protection during ischaemic or inflammatory events, we first tested a potential increase in intracellular adenosine by blocking ADK through the addition of ITU to pulmonary epithelial (A549) cells. Eight hours after incubation with either 25 μ M or 10 μ M ITU, the intracellular adenosine levels increased approximately 3-fold compared with the starting adenosine level (Figure 4A). Compared with the controls, the intracellular adenosine concentration was approximately 2-fold higher 8 hours after blocking ADK with ITU. To determine the impact of adenosine uptake after adenosine enhancement as a result of the ITU-mediated inhibition of intracellular conversion of adenosine, we added adenosine to the extracellular space and detected the changes in extracellular adenosine concentration over time. We used untreated cells as a negative control and treatment with the adenosine uptake inhibitor dipyridamole as a positive control. We found that the loss of extracellular adenosine from the supernatant was significantly attenuated after ADK inhibition by ITU. The extracellular adenosine levels in all ITU treated samples were elevated in a concentration dependent manner, but these levels were influenced by ENT-dependent adenosine uptake inhibition with dipyridamole (Figure 4B).

A hallmark of inflammation is the recruitment of PMNs to injured tissue, where they cross the endothelial and epithelial barrier in a transmigration process (19). Therefore, we sought to evaluate the influence of ADK inhibition on this process. For this, we used a transepithelial migration assay that was previously described (18). We found a significant reduction in the transepithelial migration of PMNs when they were treated with 25 μ M ITU (Figure 5A). We were also able to detect a reduction in paracellular permeability when testing FITC-Dextran flux following preincubation of the epithelium with ITU (Figure 5B).

Reduced pulmonary inflammation in $ADK^{+/-}$ mice following LPS inhalation

As homozygous ADK deficient mice are not viable, we exposed heterozygous $ADK^{+/-}$ mice to a model of lung injury induced through LPS inhalation. Before the start of the experiment, we validated the diminished ADK expression in several organs of these mice (Supplemental Figure E43 A and B) (15). Following exposure to LPS, $ADK^{+/-}$ mice had significantly reduced cell infiltration, total protein content and MPO amount in bronchoalveolar lavage fluid (BALF) compared with WT controls (Figure 6A–C). Diminished TNF- α , IL-1 β and IL-6 cytokine levels corroborated our findings of an attenuated inflammatory response in $ADK^{+/-}$ mice (Figure 6D–F). The decreased inflammation in $ADK^{+/-}$ mice was also associated with the decreased infiltration of PMNs into pulmonary tissue compared with WT controls (Figure 6G, Supplemental Figure E5). We found an increased expression of the adenosine receptor 2B in these animals (Supplemental Figure E6).

Pharmacological inhibition of ADK attenuates acute lung injury

Encouraged by the detected lung tissue protection in $ADK^{+/-}$ mice, we evaluated the pharmacological inhibition of ADK in WT animals as a potential therapeutic approach to ALI. For this, we administered the specific ADK inhibitor ITU via inhalation over a period of 30 minutes at concentrations of 0.1 mg/kg ($\triangle$ 3.33 μ g/kg-min) and 0.01 mg/kg ($\triangle$ 0.33

µg/kg·min) immediately after NaCl or LPS inhalation. As shown in the in vitro experiments using lung epithelial cells, ITU blocks ADK; as a result, the extracellular adenosine levels increase. Following the inhalation of ITU, we found significantly attenuated pulmonary inflammatory changes (Figure 7A–C). Low cytokine levels of TNF-α, IL-1β and IL-6 in the BALF confirmed that ITU was protective during pulmonary inflammation (Figure 7D–F). Reduced numbers of neutrophils infiltrating into the lung tissue were confirmed by immunohistochemistry (Figure 7G). Taken together, our findings provide evidence that the pharmacological inhibition of ADK decreases the pulmonary damage in ALI.

Discussion

To date, lung injury significantly contributes to in-hospital mortality (20). A crucial component of lung injury is the loss of alveolar-capillary function, which results in the trafficking of neutrophils from the vascular space into the alveolar space and in the development of tissue oedema (21). Based on previous observations that adenosine kinase has the potential to protect barrier function during hypoxia, we evaluated the role of ADK during ALI (22). We report here that ADK is repressed during inflammation both in vitro and in vivo. This repression translates into a protective response during pulmonary inflammation, as observed in animal experiments involving genetic repression of ADK. Moreover, pharmacological studies using ITU have demonstrated that the in vivo inhibition of ADK attenuates tissue injury during pulmonary inflammation (for mechanistic insight see Figure 8).

In the initial experiments, we observed both the transcriptional and translational repression of ADK during an acute inflammatory process in vitro. This observation is in line with our previous finding that ADK repression was induced in endothelial cells and intestinal epithelial cells during tissue hypoxia (22). To gain more insight into the regulation of ADK, we focused on NF-κB because it is one of the transcription factors that initiates inflammation (23). For this approach, we first analysed the promoter region of ADK and found one putative response element region for NF-κB binding in the promoter region. As proof of concept, we first checked for NF-κB binding during in vitro inflammatory conditions in lung epithelial cells, by using chromatin immunoprecipitation. Our results indicated strong binding of NF-κB to the putative ADK promoter. Following this, we measured the expression level of ADK in vivo. After exposure to LPS, we also observed the repression of ADK in vivo. To further investigate the functional effects of the ADK repression, we evaluated *ADK*^{+/-} mice, which had significant repression of ADK within the pulmonary tissue. We exposed these animals to LPS inhalation and observed significantly reduced numbers of infiltrating cells and levels of cytokines and other inflammatory markers in BALF from these animals. This observation is in line with the rapid increase in extracellular adenosine and the transcriptional repression of ADK during hypoxia (22, 24), and it might be a protective adaptation to the detrimental effects of pulmonary inflammation. The ADK repression increases the extracellular adenosine concentration, which has been reported to protect tissue in several conditions (25). Extracellular adenosine signals through its known adenosine receptors (A1, A2A, A2B, A3) to ameliorate inflammation (26). We have demonstrated here that especially the adenosine 2b receptor is induced in our model of lung injury. This is in line with previous work showing the protective potential of the A2B

adenosine receptor (27). The protective role of high extracellular adenosine levels and adenosine receptor signalling were also described during myocardial reperfusion injury, several neurological conditions, arthritis and colitis (26, 28–32). To the best of our knowledge, repression of ADK during inflammatory tissue conditions, as present during lung injury, has not been shown before. This observation is novel and describes an endogenous anti-inflammatory adaptation to an acute inflammatory process.

To test the therapeutic potential of ADK inhibition for treating lung injury, we evaluated the pharmacological inhibition of this enzyme. The intracellular enzyme ADK catalyses the conversion of adenosine and ATP to AMP and ADP; as such, it plays a key role in controlling the cellular concentration of adenosine (33). Consequently, an increase in the intracellular adenosine levels as a result of ADK inhibition enhances the extracellular adenosine levels (7). To test this, we first evaluated the inhibition of ADK in vitro in epithelial cells by using ITU. In this experiment, we found a significant increase in the extracellular adenosine levels by inhibiting intracellular ADK. To evaluate the potential functional consequences we next investigated whether the increased extracellular adenosine levels, which are mediated through ADK inhibition by ITU, interfere with the migration of neutrophils across the epithelial barrier. We observed improved epithelial barrier function, as well as diminished neutrophil migration, in response to ADK inhibition. To test whether ADK inhibition has a therapeutic effect in LPS-induced acute lung injury, and whether it dampens the inflammatory response to LPS inhalation, we performed in vivo experiments using 5-iodotubercidin (ITU) administered at two concentrations after the onset of inflammation. We identified a strong inhibition of inflammation at the low concentration of ITU (0.01 mg/kg), as well as the diminished infiltration of cells, and lower protein and MPO content and lower proinflammatory cytokine levels in the BALF. Other groups found similar tissue protection and decreased inflammation after the administration of ADK inhibitors. During experimental sepsis the adenosine kinase inhibitor GP-1-515 showed to reduce the infiltration of neutrophils and the cytokine production, this was associated with improved outcome of experimental animals (34). These results were confirmed in another study of using a model of paw inflammation, where again GP-1-515 demonstrated anti-inflammatory potential through adenosine kinase inhibition (35). We used the pharmacological agent ITU here in this study through inhalation to test a lung targeted intervention with ITU which also has a low IC₅₀, that is comparable to GP-1-515. As such our results are in parts comparable to these previous studies, although genetic deletion of adenosine kinase and in vitro models for the role of ADK were not included in this work done previously. Further studies have also confirmed our findings and have shown that the tissue protective potential of ADK inhibition is also present during cerebral stroke (36, 37) and myocardial ischaemia reperfusion injury (38). As such, our findings extend the previously known protective role of ADK inhibition to the field of pulmonary inflammation.

In summary, the results of our study are in line with previous investigations that demonstrated the tissue protective properties of extracellular adenosine. In this study, inflammation promoted the transcriptional and translational repression of ADK both in vitro and in vivo. The active inhibition of ADK by ITU increased adenosine levels. The naturally initiated process of ADK repression seems to be a protective tissue adaptation to an inflammatory insult. Therefore, this study improves our understanding of the mechanisms

underlying the regulation of extracellular adenosine by ADK and extends our understanding of the role of ADK during lung injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Dr. Claudia Bernardo de Oliveira Franz, Michaela Hoch-Gutbrod, Edgar Hoffmann and Alice Mager for excellent technical assistance.

Grant Support: This work was partially supported by a Grant from the Deutsche Forschungsgemeinschaft (DFG) grant DFG-MO 2252/1 to D.K and by a grant from the Deutsche Forschungsgemeinschaft (DFG) DFG-RO 3671/6-1 to P.R

References

1. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med.* 2000; 342(18): 1334–1349. [PubMed: 10793167]
2. Rubenfeld GD, Herridge MS. Epidemiology and outcomes of acute lung injury. *Chest.* 2007; 131(2):554–562. [PubMed: 17296661]
3. Wheeler AP, Bernard GR. Acute lung injury and the acute respiratory distress syndrome: a clinical review. *Lancet.* 2007; 369(9572):1553–1564. [PubMed: 17482987]
4. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med.* 2006; 354(6):610–621. [PubMed: 16467548]
5. Morote-Garcia JC, Rosenberger P, Nivillac NM, et al. Hypoxia-inducible factor-dependent repression of equilibrative nucleoside transporter 2 attenuates mucosal inflammation during intestinal hypoxia. *Gastroenterology.* 2009; 136(2):607–618. [PubMed: 19105964]
6. Kohler D, Eckle T, Faigle M, et al. CD39/Ectonucleoside Triphosphate Diphosphohydrolase 1 Provides Myocardial Protection During Cardiac Ischemia/Reperfusion Injury. *Circulation.* 2007
7. Morote-Garcia JC, Kohler D, Roth JM, et al. Repression of the equilibrative nucleoside transporters dampens inflammatory lung injury. *Am J Respir Cell Mol Biol.* 2013; 49(2):296–305. [PubMed: 23590299]
8. Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. *J Clin Invest.* 2012; 122(8):2731–2740. [PubMed: 22850883]
9. Eltzschig HK, Ibla JC, Furuta GT, et al. Coordinated Adenine Nucleotide Phosphohydrolysis and Nucleoside Signaling in Posthypoxic Endothelium: Role of Ectonucleotidases and Adenosine A2B Receptors. *The Journal of experimental medicine.* 2003; 198(5):783–796. [PubMed: 12939345]
10. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature.* 2001; 414(6866):916–920. [PubMed: 11780065]
11. Rosenberger P, Schwab JM, Mirakaj V, et al. Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia. *Nature immunology.* 2009; 10(2):195–202. [PubMed: 19122655]
12. Baldwin SA, Beal PR, Yao SY, et al. The equilibrative nucleoside transporter family, SLC29. *Pflugers Archiv: European journal of physiology.* 2004; 447(5):735–743. [PubMed: 12838422]
13. Boison D. Adenosine kinase: exploitation for therapeutic gain. *Pharmacol Rev.* 2013; 65(3):906–943. [PubMed: 23592612]
14. Peart JN, Gross GJ. Cardioprotection following adenosine kinase inhibition in rat hearts. *Basic Res Cardiol.* 2005; 100(4):328–336. [PubMed: 15795795]
15. Boison D, Scheurer L, Zumsteg V, et al. Neonatal hepatic steatosis by disruption of the adenosine kinase gene. *Proc Natl Acad Sci U S A.* 2002; 99(10):6985–6990. [PubMed: 11997462]

16. Morote-Garcia JC, Napiwotzky D, Kohler D, et al. Endothelial Semaphorin 7A promotes neutrophil migration during hypoxia. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109(35):14146–14151. [PubMed: 22891341]
17. Kohler D, Straub A, Weissmuller T, et al. Phosphorylation of vasodilator-stimulated phosphoprotein prevents platelet-neutrophil complex formation and dampens myocardial ischemia-reperfusion injury. *Circulation*. 2011; 123(22):2579–2590. [PubMed: 21606399]
18. Henes J, Schmit MA, Morote-Garcia JC, et al. Inflammation-associated repression of vasodilator-stimulated phosphoprotein (VASP) reduces alveolar-capillary barrier function during acute lung injury. *FASEB J*. 2009; 23(12):4244–4255. [PubMed: 19690214]
19. Sallusto F, Baggiolini M. Chemokines and leukocyte traffic. *Nat Immunol*. 2008; 9(9):949–952. [PubMed: 18711431]
20. Rubenfeld GD, Caldwell E, Peabody E, et al. Incidence and outcomes of acute lung injury. *N Engl J Med*. 2005; 353(16):1685–1693. [PubMed: 16236739]
21. Ware LB, Matthay MA. The Acute Respiratory Distress Syndrome. *N Engl J Med*. 2000; 342(18):1334–1349. [PubMed: 10793167]
22. Morote-Garcia JC, Rosenberger P, Kuhlicke J, et al. HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak. *Blood*. 2008; 111(12):5571–5580. [PubMed: 18309031]
23. Chen F, Castranova V, Shi X, et al. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clin Chem*. 1999; 45(1):7–17. [PubMed: 9895331]
24. Frenguelli BG, Llaudet E, Dale N. High-resolution real-time recording with microelectrode biosensors reveals novel aspects of adenosine release during hypoxia in rat hippocampal slices. *J Neurochem*. 2003; 86(6):1506–1515. [PubMed: 12950459]
25. Idzko M, Ferrari D, Riegel AK, et al. Extracellular nucleotide and nucleoside signaling in vascular and blood disease. *Blood*. 2014; 124(7):1029–1037. [PubMed: 25001468]
26. Hasko G, Linden J, Cronstein B, et al. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nature reviews Drug discovery*. 2008; 7(9):759–770. [PubMed: 18758473]
27. Schingnitz U, Hartmann K, Macmanus CF, et al. Signaling through the A2B adenosine receptor dampens endotoxin-induced acute lung injury. *J Immunol*. 2010; 184(9):5271–5279. [PubMed: 20348420]
28. Peart JN, Headrick JP. Adenosinergic cardioprotection: multiple receptors, multiple pathways. *Pharmacol Ther*. 2007; 114(2):208–221. [PubMed: 17408751]
29. Pignataro G, Maysami S, Studer FE, et al. Downregulation of hippocampal adenosine kinase after focal ischemia as potential endogenous neuroprotective mechanism. *J Cereb Blood Flow Metab*. 2008; 28(1):17–23. [PubMed: 17457365]
30. Boison D. Adenosine augmentation therapies (AATs) for epilepsy: prospect of cell and gene therapies. *Epilepsy research*. 2009; 85(2–3):131–141. [PubMed: 19428218]
31. Boison D. Adenosine as a neuromodulator in neurological diseases. *Current opinion in pharmacology*. 2008; 8(1):2–7. [PubMed: 17942368]
32. Boyle DL, Kowaluk EA, Jarvis MF, et al. Anti-inflammatory effects of ABT-702, a novel non-nucleoside adenosine kinase inhibitor, in rat adjuvant arthritis. *J Pharmacol Exp Ther*. 2001; 296(2):495–500. [PubMed: 11160636]
33. Lindberg B, Klenow H, Hansen K. Some properties of partially purified mammalian adenosine kinase. *J Biol Chem*. 1967; 242(3):350–356. [PubMed: 4290214]
34. Firestein GS, Boyle D, Bullough DA, et al. Protective effect of an adenosine kinase inhibitor in septic shock. *J Immunol*. 1994; 152(12):5853–5859. [PubMed: 8207212]
35. Rosengren S, Bong GW, Firestein GS. Anti-inflammatory effects of an adenosine kinase inhibitor. Decreased neutrophil accumulation and vascular leakage. *J Immunol*. 1995; 154(10):5444–5451. [PubMed: 7730646]
36. Jiang N, Kowaluk EA, Lee CH, et al. Adenosine kinase inhibition protects brain against transient focal ischemia in rats. *Eur J Pharmacol*. 1997; 320(2–3):131–137. [PubMed: 9059845]

37. Miller LP, Jelovich LA, Yao L, et al. Pre- and peristroke treatment with the adenosine kinase inhibitor, 5'-deoxyiodotubercidin, significantly reduces infarct volume after temporary occlusion of the middle cerebral artery in rats. *Neurosci Lett*. 1996; 220(2):73–76. [PubMed: 8981476]
38. Peart J, Matherne GP, Cerniway RJ, et al. Cardioprotection with adenosine metabolism inhibitors in ischemic-reperfused mouse heart. *Cardiovasc Res*. 2001; 52(1):120–129. [PubMed: 11557240]

Figure 1. Adenosine kinase is repressed during LPS-induced inflammation in vivo
(A) ADK mRNA concentrations in the lung tissue from mice 4 hours after NaCl or LPS inhalation (mean $\pm$ SEM, n=4 per group; *P<0.05 as indicated). **(B)** Analysis of murine pulmonary ADK protein concentration 4 hours after NaCl or LPS inhalation. Protein samples were pooled from n=7 per group; β -Actin levels were used to control the protein loading conditions. **(C)** Immunohistochemistry of ADK in murine lungs using cytokeratin as a structural epithelial marker. Tissue sections were stained with antibodies specific for ADK (green) and epithelial cytokeratin (red). The colocalisation of ADK with cytokeratin is shown in yellow in the overlaid image. DAPI (blue) served as a nuclear identifier (magnification $\times$ 400, n=3/group). IgG and negative control staining are shown in Supplemental Figure E1B (magnification $\times$ 400, n=3/group).

Figure 2. Adenosine kinase is repressed under inflammatory conditions in vitro

(A) Confluent pulmonary A549 epithelial cells were exposed to TNF- α (100 ng/ml) or IL-6 (20 ng/ml) for 2, 4, 8, and 24 hours or (B) A549 cells were stimulated for 24 hours with increasing concentrations of TNF- α (0, 1, 10 and 100 ng/ml) or IL-6 (0, 0.2, 2 and 20 ng/ml). Data are analysed and displayed as the fold change in transcripts relative to the baseline of nonstimulated control samples (mean $\pm$ SEM, n = 4 per group; *P<0.05, **P<0.01 and ***P<0.001 as indicated using Dunnett's multiple comparison). (C) Expression of ADK protein in A549 epithelial cells exposed to increasing concentrations of TNF- α (0, 1, 10 and 100 ng/ml) or IL-6 (0, 0.2, 2 and 20 ng/ml). (D) Immunofluorescence staining for human ADK in nonstimulated confluent pulmonary A549 cells compared with cells exposed to

TNF- α (100 ng/ml) for 24 h. Sections were stained using an antibody specific for human actin (Alexa 594; red) for structural depiction and DAPI (blue) as a nuclear marker. To specify the repression of ADK protein after inflammatory stimulation of these A549 cells, we used Alexa 488 (green). IgG and negative control staining is shown in Supplemental Figure E1A (magnification $\times 400$, $n=3$ /group).

Figure 3. Analysis of potential NF-κB binding to the ADK promoter region

(A) Graphical depiction of the putative NF-κB binding site in the promoter region of ADK. The potential NF-κB binding site was identified in the promoter region at -309 to -318 relative to the transcription start site (TSS). (B) Chromatin immunoprecipitation (ChIP) assay to determine whether the ADK promoter binds NF-κB. ChIP analysis of nuclear DNA. The IgG control and positive control are shown. (C) A549 cells were transfected with the full-length (FL) promoter or the indicated constructs containing mutations in the potential NF-κB binding site and then a reporter assay for luciferase activity was conducted after

exposure to 100 ng/ml TNF- α for 24 hours. The results reflect the fold change in luminescence relative to nonstimulated control samples. To control for the transcriptional activity of NF- κ B, cells were transfected with pNF- κ B-Luc. Data are normalised to the background vector (pGL4.17) and total protein (mean $\pm$ SEM, n=4/group, ***P<0.001 as indicated).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Inhibition of adenosine kinase results in increased intracellular and extracellular adenosine levels

(A) Confluent A549 cells were treated with different concentrations (10 μ M and 25 μ M) of the ADK inhibitor ITU. To detect the intracellular adenosine production, we used the adenosine uptake inhibitor dipyridamole. The intracellular adenosine levels were determined by HPLC. Both of the ITU concentrations led to a continuous increase in the intracellular adenosine levels over time ($n=5-6$). (B) A549 cells were treated with the indicated concentrations of ITU, and adenosine transport/uptake was quantified. Control experiments were performed in the presence of dipyridamole (20 μ M) (mean $\pm$ SEM, $n=4-6$).

Figure 5. Inhibition of adenosine kinase results in decreased transepithelial neutrophil migration and decreased transepithelial flux

(A) PMN transmigration experiments were performed in transwells using fMLP as a chemoattractant in the lower compartment. The epithelium was pre-incubated with ITU (25 μM) prior to transmigration. A blank control without the chemoattractant fMLP and a DMSO control (the solvent for ITU) with fMLP were evaluated. (B) Passive flux of FITC Dextran across A549 epithelial monolayers. Flux was determined by measuring the FITC Dextran that migrated into the basal compartment (mean±SEM, n=4/condition; ***P<0.001 as indicated)

Figure 6. Inflammatory changes are attenuated in *ADK*^{+/-} mice during lung injury
Mice were exposed to LPS through inhalation, and the (A) cell count; (B) protein and (C) MPO content and (D) TNF-α, (E) IL-1β and (F) IL-6 levels were measured in the bronchoalveolar lavage fluid (BALF) of ADK heterozygous (*ADK*^{+/-}) and WT mice 4 hours after NaCl or LPS inhalation. (mean ± SEM; n = 5/group, **P<0.01 and ***P<0.001 according to 1-way ANOVA testing). (G) Staining of infiltrating PMN cells in pulmonary histological tissue sections of *ADK*^{+/-} and WT mice 4 hours after exposure to LPS or NaCl

through inhalation (magnification 400×). Representative images of histological lung sections, which were selected from 3 examined mice per group, are shown.

Figure 7. Pharmacological inhibition of ADK decreases pulmonary inflammation

Mice were exposed to LPS through inhalation, and the (A) cell count; (B) protein and (C) MPO content; and (D) TNF- α , (E) IL-1 β , and (F) IL-6 levels were measured in the bronchoalveolar lavage fluid (BALF) with and without pharmacological inhibition of ADK by ITU in WT animals 4 hours after NaCl or LPS inhalation (mean $\pm$ SEM; n = 5/group, *P<0.05, **P<0.01 and ***P<0.001 according to 1-way ANOVA testing). (G) Staining of infiltrating PMN cells in pulmonary histological tissue sections of WT mice at 4 h after LPS or NaCl inhalation, mice were also treated with or without ITU in different concentrations

for 30 min following LPS inhalation (magnification 400×). Representative images of histological lung sections selected from 3 mice per group are shown.

Figure 8. Model of adenosine kinase repression and pharmacological inhibition

1.) Intracellular adenosine kinase (ADK) converts adenosine and adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) and as such regulates intracellular adenosine levels **2.)** transcriptional repression of ADK results in a significant increase of intracellular adenosine levels which results in increased extracellular adenosine through the equilibrative nucleoside transporters (ENTs) and as such promotes the anti-inflammatory effects of extracellular adenosine **3.)** pharmacological repression of ADK through ITU increases intracellular adenosine levels which also results in anti-inflammatory effects through extracellular adenosine