Adenosine receptor A$_{2b}$ on hematopoietic cells mediates LPS-induced migration of PMNs into the lung interstitium

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Submitted 13 December 2011; accepted in final form 11 June 2012

Konrad FM, Witte E, Vollmer I, Stark S, Reutershan J. Adenosine receptor A$_{2b}$ on hematopoietic cells mediates LPS-induced migration of PMNs into the lung interstitium. Am J Physiol Lung Cell Mol Physiol 303: L425–L438, 2012. First published June 15, 2012; doi:10.1152/ajplung.00387.2011.—Uncontrolled transmigration of polymorphonuclear leukocytes (PMNs) into the different compartments of the lungs (intravascular, interstitial, alveolar) is a critical event in the early stage of acute lung injury and acute respiratory distress syndrome. Adenosine receptor A$_{2b}$ is highly expressed in the inflamed lungs and has been suggested to mediate cell trafficking. In a murine model of LPS-induced lung inflammation, we investigated the role of A$_{2b}$ on migration of PMNs into the different compartments of the lung. In A$_{2b}^{-/-}$ mice, LPS-induced accumulation of PMNs was significantly higher in the interstitium, but not in the alveolar space. In addition, pulmonary clearance of PMNs was delayed in A$_{2b}^{-/-}$ mice. Using chimeric mice, we identified A$_{2b}$ on hematopoietic cells as crucial for PMN migration. A$_{2b}$ did not affect the release of relevant chemokines into the alveolar space. LPS-induced microvascular permeability was under the control of A$_{2b}$ on both hematopoietic and nonhematopoietic cells. Activation of A$_{2b}$ on endothelial cells also reduced formation of LPS-induced stress fibers, highlighting its role for endothelial integrity. A specific A$_{2b}$ agonist (BAY 60–6583) was effective in decreasing PMN migration into the lung interstitium and microvascular permeability. In addition, in vitro transmigration of human PMNs through a layer of human endothelial or epithelial cells was A$_{2b}$ dependent. Activation of A$_{2b}$ on human PMNs reduced oxidative burst activity. Together, our results demonstrate anti-inflammatory effects of A$_{2b}$ on two major characteristics of acute lung injury, with a distinct role of hematopoietic A$_{2b}$ for cell trafficking and endothelial A$_{2b}$ for microvascular permeability.

Acute lung injury; microvascular permeability; neutrophils

ALI/ARDS is characterized by a local or systemic inflammation. In the early phase, inflammatory cells migrate into the lungs, release inflammatory cytokines, perpetuating inflammatory response, and ultimately resulting in tissue damage. The lung vascular permeability increases and results in the accumulation of pulmonary edema fluid (48): as a long-term consequence, fibrotic remodeling can occur and considerably deteriorates pulmonary gas exchange.

Neutrophil Granulocytes in ALI

Recruitment of circulating polymorphonuclear neutrophil granulocytes (PMNs) is essential for host defense and initiates the following specific immune response. Impaired PMN migration into the lung leads to severe disturbances of the immune response (34). On the other hand, uncontrolled, excessive migration of PMNs results in destruction of the lung architecture, as mentioned above. Central pathomechanisms in the early stage of ALI/ARDS include uncontrolled transmigration of PMNs into the interstitium of the lung and alveolar space (1). Migration of PMNs in the different compartments of the lung as a reaction on inflammatory stimuli consists of several steps (accumulation in the capillaries, transendothelial migration into the interstitium, transepithelial migration into the alveolar space). Each migration step is regulated differently (35), and the molecular mechanisms are still incompletely understood. Experimental studies implicate that modulation of PMN trafficking improves outcome of ALI (35, 36), and persisting neutrophilia in ARDS is correlated with poor outcome (3).

Adenosine in ALI/ARDS

Adenosine is a purine nucleoside, which is released by injured or inflamed tissue and is recognized as an endogenous anti-inflammatory agent because of its potent suppressive effects on almost all cells of the immune system (18). Extracellular adenosine is a product of adenine nucleotide metabolism (ATP, ADP, and AMP) and can activate four G protein-coupled adenosine receptors: A$_{1}$, A$_{2a}$, A$_{2b}$, and A$_{3}$. The A$_{2b}$ subtype has the lowest affinity to adenosine and gets activated during inflammation with high levels of extracellular adenosine (16). A$_{2b}$ has the highest expression level in the lungs of mice (24), where it is found on lung epithelial (6), endothelial, and inflammatory cells (55). The function of A$_{2b}$ during inflammation is controversial, as both proinflammatory (45) and anti-inflammatory effects (45, 46) have been demonstrated. An important anti-inflammatory role of A$_{2b}$ in hypoxia-induced inflammation has been described (43). A recently published study demonstrated that exogenous netrin-1 attenuated LPS-induced ALI through the A$_{2b}$ receptor (27); however, compartmental leukocyte trafficking was not studied.

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We, therefore, hypothesized that activation of A2b would reduce PMN migration into the lungs and attenuate microvascular permeability. In a murine model of LPS-induced lung injury, we determined the role of A2b for the different migration steps and the release of chemotactic cytokines in the alveolar space of wild-type and A2b knockout mice.

To identify the contributing cells to A2b-dependent anti-inflammatory effects, we investigated PMN migration also in chimeric mice and in vitro transmigration assays with human pulmonary endothelial/epithelial cells and PMNs. We also studied the effect of pharmacological activation of A2b on PMN migration.

**MATERIAL AND METHODS**

**Animals**

A2b gene-deficient mice (A2b<sup>−/−</sup>) were obtained by Dr. Katya Ravid (Boston University, School of Medicine, Department of Biochemistry, Boston, MA), and corresponding wild-type mice (C57BL/6) from Charles River Laboratories (Sulzfeld, Germany). Mice were male and between 8 and 12 wk old. All animal protocols were approved by the Animal Care and Use Committee of the University of Tübingen.

**Blood Counts**

To exclude differences in the counts of leukocytes between wild-type and knockout mice, we determined absolute cell count by differential blood count from peripheral blood of the tail vein (Diff Quik, Dade Behring, Newark, DE) (n = 6).

**A2b mRNA Expression**

Real-time RT-PCR was performed to measure the transcriptional activity of A2b in the lungs. RNA from whole lung homogenates of wild-type mice was extracted 2 and 3 h after LPS inhalation (n = 6). Negative controls did not inhibit LPS. From the total RNA samples, reverse transcription was performed using SuperScript III Transcription kit (Invitrogen) and oligo(dT) primers. With A2b primers, A2b cDNA was analyzed (5′-GGGCAAGACTGAAACTT3′ and 5′-GGAAAGGTCTGCTCTCACA-3′) on an iCycler iQ Real-Time Detection System (Bio-Rad). As control for the amount of starting template, murine β-actin mRNA (primers: 5′-ACATGTGGCATGCTTTGTTT3′ and 5′-GTGTGGCTTCAACACTGCT-3′) was amplified in identical reactions.

In separate experiments, human PMNs were isolated and incubated with LPS for 2 and 4 h (n = 3). A2b mRNA was determined by RT-PCR, as described before (52).

**Western Blotting**

Wild-type mice were stimulated with aerosolized LPS; lungs were removed after 3, 6, 12, and 24 h and prepared for Western blotting, as described previously (46) (n = 4). Blots were probed with rabbit polyclonal anti-A2b (1:1,000; H-40; SC-28996; Santa Cruz Biotechnology, Santa Cruz, CA) for A2b analysis. Rabbit anti-β-actin was used for detection of β-actin (Cell Signaling Technology, Danvers, MA).

**Immunohistochemistry**

Twenty-four hours after LPS inhalation, wild-type and A2b<sup>−/−</sup> mice were euthanized (n = 4). Control mice did not receive LPS. Pulmonary circulation was perfused free of blood, the trachea was cannulated, and the lung was inflated by 4% paraformaldehyde (PFA) for 10 min at 25 cmH<sub>2</sub>O. The lungs were removed and fixed in PFA for 24 h. Using the avidin-biotin technique (Vector Laboratories, Burlingame, CA), paraffin-embedded sections (5 μm) were stained for PMNs, as described previously (32). Briefly, lung sections were deparaffinized and rehydrated, and nonspecific binding was blocked by incubation with avidin, 10% rabbit serum, and 0.5% fish skin gelatin oil. Sections were washed with PBS and incubated with a specific antibody to mouse neutrophils (rat anti-mouse neutrophils, clone 7/4; Caltag Laboratories, Burlingame, CA) over night (19). Biotinylated rabbit anti-rat IgG (5 g/ml, Vector Laboratories) was added and incubated for 1 h, followed by avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories), washed with PBS, incubated with diaminobenzidine (DAB kit; Vector Laboratories), and counterstained with hematoxylin.

**Pharmacological Activation/Inhibition of A2b**

In separate experiments, we used a selective A2b agonist (BAY 60–6583; Bayer, Wuppertal, Germany) (11), dissolved in 80% polyethylene glycol, 10% glycerin, and 10% Aqua ad injectabilia, which was a gift from Thomas Khran (Bayer). The agonist was injected intraperitoneally at a concentration of 2 mg/kg (10) in wild-type mice, 60 min before LPS inhalation, and the effects on pulmonary migration of PMNs (n = 8), microvascular permeability (n = 6), and cytoskeletal remodeling were studied. PSB 1115 (Sigma-Aldrich, Taukirchen, Germany), a selective A2b antagonist (11), was used for cytoskeletal remodeling studies.

**Generation of Chimeric Mice**

Chimeric mice that express A2b, either on hematopoietic (e.g., PMNs) or nonhematopoietic cells (e.g., endothelial and epithelial cells), were generated by transferring bone marrow (BM) between wild-type and A2b<sup>−/−</sup> mice (15).Recipient animals were irradiated with 60 Gy two times with an interval of 4 h in between. Femur and tibia were taken from the donor animal; the BM channel was flushed with HBSS+. The absolute cell count was determined, and the recipient animals obtained 3–5 × 10<sup>6</sup> cells in 300 µl via the tail vein immediately after the second irradiation. Chimeric mice that express A2b only on nonhematopoietic cells were received by transplanting BM from A2b<sup>−/−</sup> to wild-type mice (A2b<sup>−/−</sup> tissue); chimeric mice with A2b only on hematopoietic cells were received by transplanting BM from wild-type to A2b<sup>−/−</sup> mice (A2b<sup>−/−</sup> blood). Sentinel mice that did not receive BM served as an indicator for efficient radiation and died within 2 wk. In addition, we measured mRNA in lungs and BM of wild-type, A2b<sup>−/−</sup>, and both types of chimeric mice to verify expression of A2b in the specific compartments. Transplanted animals were kept sterile in individually ventilated cages, drinking water was autoclaved, and animals received antibiotics (5 mM sulfamethoxazole and 0.86 mM trimethoprim). Radiation alone did not affect inflammatory response in our model, as shown previously by our group (39). After 6- to 8-wk reconstitution was complete, mice were used for experiments (migration assay n = 4, Evans blue extravasation n = 4).

**Murine Model of ALI**

Inhalation of aerosolized LPS leads to a reproducible migration of PMNs into all compartments of the lung (36). LPS from *Salmonella enteritidis* (Sigma-Aldrich) was dissolved in sterile saline (500 µg/ml) and aerosolized by an air nebulizer that was connected to a custom-made chamber with four to eight animals. Nebulization of LPS for 30 min led to a transient pulmonal inflammation with PMN migration, release of cytokines, increase of microvascular permeability, and structural changes of the lung architecture, as seen in histology (38). Control mice were exposed to saline aerosol.

**In Vivo Migration Assay**

To determine PMN migration into the different compartments of the lung (intravascular, interstitial, alveolar space), a flow cytometry-based method was used, as described in detail before (37, 41). Briefly, a fluorescent PMN antibody (GR-1; clone RB6–8C5) was injected into the tail vein to mark all intravascular PMNs. Animals were
was also verified by measuring the total protein concentration in the BAL (53). Control animals did not receive LPS. Total protein concentration in the BAL was determined by extravasation of Evans blue (20 mg/kg; Sigma Aldrich, Steinheim, Germany), and atropine sulfate (0.025 mg/kg; B. Braun, Melsungen, Germany), and a thoracotomy was performed. To remove nonadherent leukocytes from the pulmonary vasculature, the lungs were perfused through injection of phosphate-buffered saline in the beating right ventricle of the heart. Five minutes after injection of GR-1, PMNs from the alveolar space were extracted by bronchoalveolar lavage (BAL). The lungs were homogenized and incubated with fluorescent antibodies to CD45, 7/4, and GR-1 (migration assay 8). The absolute cell count was determined in BAL and lungs, and the fraction of PMNs in the different compartments of the lung was identified by flow cytometry. BAL was stained with CD45, 7/4, and GR-1; PMNs were determined by their typical appearance in the forward/sideward scatter and the expression of CD 45, 7/4, and GR-1 (migration assay n = 8; time course migration assay over 48 h, n = 4).

Microvascular Leakage

Evans blue extravasation. LPS-induced microvascular leakage was determined by extravasation of Evans blue in wild-type and A2b+/− mice (17) (n ≥ 6). Evans blue (20 mg/kg; Sigma Aldrich, Steinheim, Germany) was injected into the tail vein 6 h after LPS exposure. After 30 min, the animals were anesthetized, and a thoracotomy was performed. To remove intravascular Evans blue, lungs were perfused through injection of phosphate-buffered saline in the beating right ventricle of the heart. Lungs were homogenized, Evans blue was extracted by formamid, and the concentration colorimetric was determined by a colorimetric method (bicinchoninic acid; Thermo Scientific, Rockford, IL), as described previously (10). The cell suspension now included leukocytes and knockout mice (A2b+/−) was injected into the tail vein 6 h after LPS exposure. After 30 min, the animals were anesthetized with ketamine (125 mg/kg; Ratiopharm, Germany), and a thoracotomy was performed. To remove nonadherent leukocytes from the pulmonary vasculature, the lungs were perfused through injection of phosphate-buffered saline in the beating right ventricle of the heart. Five minutes after injection of GR-1, PMNs from the alveolar space were extracted by bronchoalveolar lavage (BAL). The lungs were homogenized and incubated with fluorescent antibodies to CD45, 7/4, and GR-1 (migration assay 8). The absolute cell count was determined in BAL and lungs, and the fraction of PMNs in the different compartments of the lung was identified by flow cytometry. BAL was stained with CD45, 7/4, and GR-1; PMNs were determined by their typical appearance in the forward/sideward scatter and the expression of CD 45, 7/4, and GR-1 (migration assay n = 8; time course migration assay over 48 h, n = 4).

Total protein concentration in the BAL. Generation of a lung edema was also verified by measuring the total protein concentration in the BAL of wild-type and A2b+/− mice (n = 4). Six hours after LPS inhalation, total protein concentration was determined by a colorimetric method (bicinchoninic acid; Thermo Scientific, Rockford, IL), as described previously (40).

Chemokine Release

Three hours after LPS-inhalation, the release of CXCL-1 (keratinocyte-derived chemokine), CXCL-2/3 (macrophage inflammatory protein-2), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were measured in the BAL of wild-type and A2b+/− mice (enzyme-linked immunosorbent assay kits, R&D Systems, Minneapolis, MN) (n = 8). Negative controls did not receive LPS.

In Vitro PMN Migration

To evaluate the effect of A2b activation on different cell types, chemokine-induced in vitro transmigration of human PMNs across a monolayer of human microvascular endothelial cells (HMVEC-L, Lonza Walkersville, Walkersville, MD) or pulmonary epithelial cell (A549, American Type Culture Collection, Wesel, Germany) was performed (n = 3). Epithelial/endothelial cells, PNNs, or both were incubated with the A2b agonist (BAY 60–6583) for 30 min at indicated concentrations (10, 1, and 0.1 ng/ml). Human endothelial/epithelial cells were cultivated on the bottom (epithel)/top (endothel) of polycarbonate filter inserts of a Transwell system (3.0-μm pore size, 6.5-mm diameter; Costar, Cambridge, MA). After reaching confluence, isolated human PMNs (Percoll gradient, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) migrated through endothelial/epithelial covered membranes along a chemotactic gradient (CXCL-2/3; 200 ng/ml, Pepro Tech, Hamburg, Germany). After incubation of 1 h, migrated PMNs were quantified by determination of myeloperoxidase (absorbance length: 405 nm) in the bottom wells.

Cytoskeletal Remodeling

To characterize the effect of A2b-dependent cell remodeling, we investigated the formation of stress fibers in human endothelial and epithelial cells, as described before (39). HMVEC-L/A549 cells were plated on gelatin-coated glass slides, adhered overnight (medium with 10% FBS), incubated with the A2b-agonist (BAY 60–6583) (10 μM/A2b antagonist (PSB 1115), and then stimulated with LPS (100 ng/ml) for 5 and 15 min. Untreated cells served as controls. Cells were fixed and permeabilized (0.1% Triton X-100, Sigma, St. Louis, MO), and F-actin was stained as described (39). Coverslips were mounted on glass slides, and microscopy was performed on a confocal fluorescence microscope (LSM 510, Zeiss, Goettingen, Germany). Images are representative of three experiments with similar results.

Oxidative Burst

We evaluated the effect of A2b stimulation on the release of oxidative products by human PMNs, as described previously (30). Images are representative of three experiments with similar results. Briefly, PMNs were isolated and incubated with the A2b agonist (100 ng/ml) for 1 h at 37°C. Reaction mixture consisting of luminol, horseradish peroxidase, and cells (with or without A2b agonist) were stimulated with LPS (1 mg/ml), and luminescence was measured every 2 min, for 30 min in total.

Statistical Analysis

Values are presented as means ± SD, unless indicated otherwise. Statistical analysis was performed using GraphPad Prism version 5.3 for Windows (GraphPad Software, San Diego, CA). Differences between the groups were evaluated by one-way ANOVA, followed by Bonferroni post hoc test. P < 0.05 was considered statistically significant.

Table 1. Differential cell counts of immune cells of wild-type and knockout mice (A2b+/−)

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (×10⁶)</th>
<th>A2b−/− (×10⁶)</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Leukocytes</td>
<td>9.4 ± 0.65</td>
<td>10.0 ± 3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.2 ± 0.4</td>
<td>1.6 ± 0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>7.7 ± 0.5</td>
<td>7.6 ± 3.1</td>
<td>NS</td>
</tr>
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Values are means ± SD, n = 6. NS, not significant.

Fig. 1. Transcription levels of A2b mRNA in lungs of wild-type mice 2 and 3 h after LPS inhalation. Values are means ± SD; n = 6. *P < 0.05 compared with control.
RESULTS

No Differences in Blood Counts Between Wild-Type and Knockout Mice

No differences in the cell counts of immune cells between wild-type and knockout mice were detected (Table 1), indicating that differences in the number of immune cells in migration experiments were not affected by compensatory alterations in the blood counts of genetically modified mice.

LPS Induces A2b mRNA and Protein Expression in the Lung

Transcriptional responses of A2b following an inflammatory stimulus were evaluated by real-time RT-PCR of lung homogenates. A2b mRNA expression was determined 2 and 3 h after LPS inhalation. Two hours after LPS inhalation, the transcription level of A2b mRNA in the lung increased significantly and remained elevated for the next hour (2 h: 1.6 ± 0.05; 3 h: 1.6 ± 0.03; P < 0.05) (Fig. 1). Western blot experiments confirmed these results on protein level (Fig. 2).

Increased Infiltration of PMNs in A2b−/− Mice After LPS Stimulation in Immunohistochemistry

Lung infiltration of PMNs was demonstrated semi-quantitatively by immunohistochemistry in wild-type and A2b−/− mice. PMNs were marked with a specific antibody and appear brown in histology. Without LPS-inhalation, comparable amounts of PMNs were observed in the lungs of both groups. After LPS, more PMNs accumulated in the lungs of A2b−/− mice compared with wild-type mice (Fig. 3).

A2b−/− Mice Showed an Increase in PMN Migration into the Interstitium of the Lungs

Quantitative PMN migration in the different compartments of the lung (intravascular, interstitial, alveolar space) was determined in wild-type and A2b−/− mice. After LPS-inhalation, the number of PMNs increased in both groups, intravascular and interstitial, and in the BAL (Fig. 4). In the knockout mice, LPS-induced PMN accumulation in the interstitium was significantly higher than in wild-type mice (A2b−/−: 4.71 ± 0.56 × 10^6 vs. wild-type: 2.12 ± 0.43 × 10^6, P < 0.05). No difference was seen in the intravascular and in the BAL.

To detect differences in the time course of PMN trafficking, we evaluated in vivo migration in wild-type and A2b−/− mice over 48 h (Fig. 5). In both groups, intravascular accumulation of PMNs peaked at 3 h, stayed elevated over 24 h, and returned to baseline at 48 h. A2b−/− mice had a higher increase in intravascular PMNs after 6 (1.28 ± 0.2 × 10^6 vs. 0.56 ± 0.2 × 10^6), 12 (1.34 ± 0.2 × 10^6 vs. 0.88 ± 0.2 × 10^6), and 48 h (0.26 ± 0.1 × 10^6 vs. 0.04 ± 0.1 × 10^6, all P < 0.05). In both groups, PMNs occurred in the interstitium 3 h after LPS inhalation. After 6 h, a plateau was reached in both groups that remained for 24 h. At 24 h, significantly more PMNs migrated into the interstitium of A2b−/− animals than of wild-type controls (wild type: 3.32 ± 0.51 × 10^6, A2b−/−: 4.74 ± 0.86 × 10^6, P < 0.05). Forty-eight hours after LPS inhalation, interstitial PMN...
counts of A2b−/− mice remained high, while they returned to baseline in wild-type mice (wild-type: 0.12 ± 0.03 × 10^6, A2b−/−: 0.29 ± 0.11 × 10^6, P < 0.05).

A2b on Hematopoietic Cells Mediate Transmigration

To determine the role of A2b on different cell types, PMN trafficking was evaluated in chimeric mice. Wild-type mice received BM from A2b−/− mice and, therefore, expressed A2b only on nonhematopoietic cells (A2b tissue). A2b−/− mice received BM from wild-type mice and expressed A2b only on

Three hours after LPS inhalation, we detected PMNs in the BAL of wild-type, but not in knockout, mice (A2b−/−: 0.48 ± 0.2 × 10^6, wild-type: 0.05 ± 0.05 × 10^6, P < 0.05). Maximum PMN migration was reached in both groups after 24 h in the BAL. After 48 h, PMNs in the BAL of A2b−/− mice were higher than in wild-type mice (A2b−/−: 0.29 ± 0.12 × 10^6, wild-type: 0.12 ± 0.03 × 10^6, P < 0.05).

Fig. 4. In vivo migration assay of wild-type and A2b−/− mice without and 24 h after LPS inhalation. PMNs were counted in the different compartments of the lung (IV, intravascular; IS, interstitial; BAL, bronchoalveolar lavage). Values are means ± SD; n = 8. *P < 0.05 compared with control group without LPS inhalation. #P < 0.05 compared with wild type with the same LPS treatment.

Fig. 5. In vivo migration assay of wild-type and A2b−/− mice after LPS inhalation over 48 h. PMNs were counted in the different compartments of the lung (IV, IS, BAL). Values are means ± SD; n = 4. *P < 0.05 compared with wild-type control.
hematopoietic cells (A2b blood). A2b expression in the different compartments of both types of chimeric mice was confirmed by RT-PCR (data not shown). At baseline, PMN counts in all lung compartments of wild-type and chimeric mice were similar (Fig. 6). After LPS inhalation, intravascular PMN counts were without significant differences among the groups. In the interstitium of the lungs, A2b tissue behaved like knockout mice and showed a significant increase in migrated PMNs, whereas A2b blood performed corresponding to wild-type mice and demonstrated significantly less PMN migration (wild type: \(2.2 \pm 0.8 \times 10^6\), A2b \(-/-\): \(4.6 \pm 0.4 \times 10^6\), A2b blood: \(2.2 \pm 0.2 \times 10^6\), A2b tissue: \(5 \pm 0.5 \times 10^6\), \(P < 0.05\)), implicating a predominant role of A2b on hematopoietic cells (Fig. 6). These findings identify A2b as an important mediator for PMN migration into the interstitium of the lung. In the BAL, A2b blood mice had the lowest number of migrated PMNs (A2b blood: 0.8 \pm 0.3 \times 10^6 vs. wild type: \(1.9 \pm 0.4 \times 10^6\), \(P < 0.05\)); in the other three groups, no differences were detectable.

**A2b Mediates LPS-Induced Microvascular Permeability**

In addition to infiltration of PMNs into the lung, disruption of the alveolo-capillary barrier is another characteristic of acute pulmonary lung injury. The role of A2b in LPS-induced microvascular permeability was analyzed by means of BAL protein and Evans blue extravasation. LPS-induced protein leakage into the BAL of A2b \(-/-\) mice was significantly higher than in wild-type mice (Fig. 7). Accordingly, Evans blue concentration rose in wild-type, A2b \(-/-\), A2b blood, and A2b tissue mice significantly compared with control groups without LPS inhalation. Microvascular permeability was significantly higher in A2b \(-/-\) than in wild-type mice. Evans blue extravasation after LPS-inhalation in A2b blood and A2b tissue mice was comparable to the extravasation in wild-type mice, indicating that the expression of A2b on either one of the compartments, hematopoietic or nonhematopoietic, led to the same increase of permeability than in wild-type mice (wild type without LPS: \(119 \pm 36 \mu g/g\) lung, wild type + LPS: \(224 \pm 43 \mu g/g\) lung, A2b \(-/-\) control: \(169 \pm 79 \mu g/g\) lung, A2b \(-/-\) + LPS: \(315 \pm 79 \mu g/g\) lung; A2b blood: \(191 \pm 7 \mu g/g\) lung, A2b tissue: \(216 \pm 53 \mu g/g\) lung, \(P < 0.05\)) (Fig. 8). So the presence of A2b on hematopoietic and nonhematopoietic cells was protective in terms of microvascular permeability, suggesting an important role of A2b for maintaining endothelial integrity.

**Pharmacological Activation of A2b Reduced PMN Trafficking Into the Lung and Decreased Microvascular Permeability**

Inducible anti-inflammatory effects of A2b were demonstrated by the administration of the A2b agonist (BAY 60–6583) to wild-type mice before LPS inhalation. Intravascular PMNs were significantly lower in the pretreated animals (wild type: \(0.36 \pm 0.19 \times 10^6\), wild type + LPS: \(1.18 \pm 0.91 \times 10^6\), wild type + LPS + agonist: \(0.23 \pm 0.12 \times 10^6\), \(P < 0.05\)).
PMNs migrated significantly less into the lung interstitium when mice were pretreated with the A2b agonist (wild type: 0.09 ± 0.03 × 10^6, wild type + LPS: 3.29 ± 1.5 × 10^6, wild type + LPS + agonist: 0.8 ± 0.47 × 10^6, P < 0.05) (Fig. 9). Pretreatment did not alter transepithelial migration of PMNs into the alveolar space and was comparable in both groups, confirming the in vivo results with A2b−/− mice.

LPS-induced microvascular permeability, as assessed by Evans blue extravasation, was significantly lower in wild-type mice that were pretreated with the A2b agonist (BAY 60–6583) and comparable with the control group without LPS inhalation (wild-type control: 119 ± 36 μg/g lung, wild type + LPS: 220 ± 22 μg/g lung, wild type + LPS + agonist: 74 ± 39 μg/g lung, P < 0.05) (Fig. 10).

**A2b Has No Influence on Chemokine Release**

The release of chemotactic cytokines into the alveolar space can initiate PMN recruitment to the lung. The effect of A2b on LPS-induced chemokine release was measured in the BAL of wild-type and A2b−/− mice. In both groups, baseline chemokine concentrations in the BAL were negligible (control groups). After LPS inhalation, CXCL-1, CXCL-2, IL-6, and TNF-α increased significantly compared with control groups in wild-type and A2b−/− mice (all P < 0.05). There were no significant differences detectable between wild-type and A2b−/− mice, indicating that A2b-mediated PMN trafficking was not dependent on the release of chemotactic cytokines (Fig. 11).

**Stimulation of A2b Decreased In Vitro PMN Migration**

We studied chemokine-induced migration of human PMNs in an in vitro transwell assay across a layer of human endothelial or alveolar epithelial cells to further characterize the effect of A2b on different cell types and to transfer the results of the murine model to the human system. PMNs and/or endothelium/epithelium were treated with the A2b agonist, and counts of migrated PMNs were determined. When PMNs were treated, transepithelial migration was reduced significantly (10 ng/ml: 98,249 ± 20,711; 1 ng/ml: 67,402 ± 14,445; 0.1 ng/ml: 57,427 ± 14,130 vs. control: 251,012 ± 32,446; all P < 0.05) (Fig. 12A). Treatment of the endothelium also resulted in a significant decrease of migrated PMNs (10 ng/ml: 219,754 ± 27,583; 1 ng/ml: 194,416 ± 14,541; 0.1 ng/ml: 168,257 ± 5,371 vs. control: 343,535 ± 11,396; all P < 0.05) (Fig. 12B). Simultaneous activation of A2b on PMNs and endothelial cells showed a synergistic, but not statistically significant effect (10 ng/ml: 52,289 ± 16,590; 1 ng/ml: 51,484 ± 8,798; 0.1 ng/ml: 38,332 ± 16,189 vs. control: 262,994 ± 5,895; all P < 0.05) (Fig. 12C), indicating that A2b on both PMNs and endothelial cells contributed to cell migration in vitro.

Activation of A2b on PMNs also reduced migration through a layer of epithelial cells (10 ng/ml: 117,467 ± 26,764; 1 ng/ml: 144,811 ± 68,701; 0.1 ng/ml: 139,058 ± 19,099 vs. control: 422,689 ± 7,131; all P < 0.05) (Fig. 12D); however, treatment of the epithelium alone did not lead to any changes (Fig. 12E). Treatment of both PMNs and epithelium also led to inhibition of PMN migration (10 ng/ml: 71,325 ± 6,706; 1 ng/ml: 75,925 ± 2,387; 0.1 ng/ml: 67,717 ± 17,896 vs. control: 298,087 ± 21,304; all P < 0.05) (Fig. 12F). These
data suggest a predominant role of PMNs and endothelial cells in A2b-mediated transmigration.

A2b mRNA on Human PMNs Increased After LPS Exposure

A2b mRNA concentration on human PMNs rose significantly 2 h after LPS exposure and decreased again after 4 h. These data emphasize our previous results on the importance of the A2b receptor on PMNs (Fig. 13).

A2b-Mediated Cytoskeletal Remodeling

We stained F-actin in human pulmonary endothelial and epithelial cells to evaluate the effect of A2b on cytoskeletal remodeling as one critical parameter of pulmonary barrier function. LPS exposure induced time-dependent stress fibers in both cell types. Pretreatment with A2b agonist led to a reduction of stress fibers in endothelial and epithelial cells (Fig. 14). The effect was more prominent in endothelial cells, confirming our in vivo and in vitro transmigration data. In accordance with our data, treatment with A2b antagonist induced a time-dependent increase of stress fibers in both cell types (Fig. 15), confirming the pivotal role of A2b for pulmonary barrier function in our model.

Oxidative Burst Reduced After A2b Stimulation

After LPS exposure, luminescence and therewith oxidative products rose exponentially. In A2b-stimulated cells, oxidative burst activity was reduced, highlighting antioxidant properties of A2b (Fig. 16).

DISCUSSION

This study was designed to investigate the role of A2b for multicompartmental PMN migration in a murine model of LPS-induced lung inflammation. Our data illustrate that A2b on hematopoietic cells significantly attenuates migration of PMNs into the lung interstitium, and A2b on hematopoietic and nonhematopoietic cells prevents LPS-induced microvascular permeability, both main characteristics of ALI. A specific A2b agonist was also effective in curbing PMN migration and microvascular leakage in vivo and in vitro.

Extracellular adenosine is critical for the course of ventilator- and LPS-induced ALI, as demonstrated in CD39- and CD73-gene deficient mice (9, 42). Removing CD39 or CD73, two rate-limiting enzymes for extracellular adenosine generation, led to a decrease of extracellular adenosine that was associated with significant deterioration of relevant ALI parameters, such as lung edema, PMN influx, and oxygenation. Of all four adenosine receptors (A1, A2a, A2b, A3), A2b has the
lowest affinity to adenosine and requires high adenosine concentrations to get activated (4). The plasma concentration of adenosine rises during inflammation 4–10 times compared with normal physiological conditions (26), suggesting an activation of A2b in this setting. A2b is expressed on lung epithelial cells (6), on endothelial cells, and on inflammatory cells (55). The effect of A2b in lung injury is controversial and includes pro- and anti-inflammatory effects (8, 50). A new paradigm explaining these contradicting findings is based on different responses of A2b on different stimuli. A2b on tissue gets activated by a milder and rather localized inflammatory stimulus like asthma, lung ischemia-reperfusion injury, and other chronic pulmonary inflammation and provokes an inflammatory response (2, 56). When A2b on BM cells is stimulated through an acute systemic stress like endotoxin lung injury, the response of the receptors appears rather anti-inflammatory (2, 55). This theory conforms with our findings. LPS induces an acute inflammation, including elevation of cytokines and inflammatory signals. Therefore, BM cells with A2b were activated, as shown in our migration assays in chimeric mice, and led to an anti-inflammatory response with less migrated PMNs in the lung interstitium. This effect was also inducible through pharmacological activation of A2b with a specific agonist. According to these findings, in A2b−/− and A2b tissue mice, significantly more PMNs migrated into the interstitium compared with wild-type mice. Our results indicate that activation of A2b on hematopoietic cells attenuates PMN trafficking into the lung interstitium. Considering our findings in the in vitro transmigration assay and the formation of stress fibers, this might be due to A2b-mediated higher vasoendothelial integrity. In line with our findings, exposure to endotoxin resulted in less expression of vascular adhesions molecules in
A recent study (55). This effect was largely dependent on activation of A2b on hematopoietic cells.

Acute pulmonary inflammation induced by hypoxia or ventilator-induced lung injury also promoted protection through A2b (8, 10). Netrin-1, a neuronal guidance protein, dampens pulmonary inflammation during ALI through activating of A2b (27). Also, a recently published study on A2b in endotoxin-induced ALI highlighted the anti-inflammatory effects of A2b. In this study (46), a lower degree of LPS-induced IL-6 transcript in the lungs and pulmonary edema in chimeric mice with A2b expression on nonhematopoietic tissues were measured. In our study, we could not determine statistically relevant differences in cytokine levels between knockout and wild-type mice in the BAL, but our results in PMN migration assay clearly identified A2b on hematopoietic cells as necessary for attenuating pulmonary inflammation in endotoxin-induced lung inflammation. Pointing in the same direction, LPS exposure induced an increase of transcription level of A2b on human PMNs, and stimulating the A2b receptor with a specific agonist reduced oxidative burst activity.

Interestingly, A2a and A2b are both coupled to Gs protein and cause an increase in adenylyl cyclase activity (31, 33), in contrast to A1 and A3 adenosine receptors. A2a is effective in curbing LPS-mediated lung inflammation via activation of A2a receptor on hematopoietic cells (37). Activation of A2a also leads to less PMN migration into the lung interstitium and decreased microvascular permeability.

According to the above-mentioned paradigm, chronic activation of A2b would mainly stimulate A2b on tissue cells and arouse an inflammatory response. An inflammatory response of A2b in bleomycin-induced chronic lung injury and also in chronic pulmonary inflammation is described with increased pulmonary inflammation and fibrosis (50). Both were significantly attenuated after the administration of an A2b antagonist; there were no assays with chimeric mice. In addition, Anvari

![Graph](image1.png)

Fig. 13. Human PMNs were isolated and incubated with LPS for 2 and 4 h. After LPS exposure, A2b mRNA concentration rose within the first 2 h significantly and then started to decrease. Values are means ± SD; n = 3. *P < 0.05 compared with control.

![Graph](image2.png)

Fig. 14. A2b-dependent formation of F-actin (green) in HMVEC-L and in lung epithelial cells (A549). Cells were exposed to LPS (5 and 15 min). Pretreatment with A2b agonist reduced formation of F-actin, but the effect was less prominent in endothelial cells. Images are representative of 3 experiments with similar results (original magnification, ×63).
and colleagues (2) examined the effect of A2b in lung ischemia-reperfusion injury and were able to identify pulmonary tissue cells as predominant cells that contributed to A2b-dependent effects.

Adenosine desaminase (ADA) is a catalytic enzyme that degrades adenosine to inosine. ADA-deficient mice die within 3 wk from severe respiratory distress (5). A2b antagonism in ADA-deficient mice attenuated pulmonary inflammation and fibrosis (50). Surprisingly, ADA/A2b double-knockout mice exhibited enhanced pulmonary inflammation and airway destruction (57). The authors’ interpretation of their findings was that A2b has important anti-inflammatory activities during early stages of lung disease.

A2b also plays a proinflammatory role in colitis, where blocking A2b attenuates the inflammatory response (22). A2b−/− mice were compared with wild-type mice, more resistant to inflammatory stimuli like toxins or bacteria when given topical to the colon. In the same model, a systemic Salmonella infection was aggravated in A2b−/− mice. The authors explained their findings of pro- and anti-inflammatory effects of A2b by potential existence of A2b in a multiprotein complex (20, 22, 47), where diverse interactions may lead to different functional effects. Another possible explanation for the antipodal effects of A2b could be different reactions of the receptor on chronic elevation of adenosine levels in chronic diseases or rapid elevation in an acute setting (46).

To our knowledge, our study is the first to demonstrate that activation of A2b on hematopoietic cells attenuates the migration of PMNs from intravascular to interstitial space of the lung. This finding is supported by the demonstration of increased leukocyte adhesion to the vasculature in A2b−/− mice with increased adhesion molecules and augmented number of leukocytes rolling at a slower velocity (55). After stimulation of A2b, we also detected increased cell integrity of endothelium in
our in vitro transmigration assay, and this phenomenon was also apparent in our cytoskeletal remodeling studies.

Our results implicate an A2b\textsuperscript{-independent} emigration of PMNs from the interstitium into the alveolar space. In our in vivo assays, the amount of alveolar PMNs was similar in wild-type, A2b\textsuperscript{+/−}, and A2b\textsuperscript{−/−} mice. Considering higher PMN counts in the interstitium of A2b\textsuperscript{+/−} and A2b\textsuperscript{−/−} mice, there was actually less transepithelial PMN migration in these mice. Consistent with these findings, there was no difference in alveolar PMN counts after treatment with the A2\textsubscript{a} agonist, even though less PMNs had migrated into the interstitium. Only in A2b\textsuperscript{+/−} blood mice was less PMNs accumulated in the BAL compared with all other groups, suggesting an involvement of A2b on nonhematopoietic cells.

For the recruitment of PMNs from the interstitial to the alveolar space, chemotactic cytokines are critical (21, 39, 49). Corresponding with our in vivo migration studies, we did not detect differences in cytokine levels in the BAL between wild-type, A2b\textsuperscript{+/−}, and A2b\textsuperscript{−/−} mice. At first sight, this is in contrast with a recently published study on A2b in endotoxin-induced ALI (46). The authors found elevated transcription levels of IL-1\beta, IL-6, and TNF-\alpha in the lungs of A2b\textsuperscript{+/−} mice 30 min after LPS exposure. We measured cytokine levels (CXCL-1, CXCL-2/3, IL-6, and TNF-\alpha) in the BAL 6 h after LPS inhalation (30, 52) and found no differences between wild-type and A2b\textsuperscript{−/−} mice. In our in vivo assay, we found an increased number of migrated PMNs into the interstitium of the lung in A2b\textsuperscript{−/−} mice, which would correspond with higher cytokine levels in the interstitium and a higher transcription level of cytokines in the lung. Additionally, increased transcription levels of cytokines do not automatically imply an elevation of cytokine protein.

Our in vitro results also implicate an A2b\textsuperscript{-independent} migration of PMNs from the interstitium into the alveolar space (transepithelial step). Stimulation with the A2b agonist led to a reduced number of migrated PMNs through the endothelial layer, but the stimulation had no effect on the migration through an epithelial layer. In our cytoskeletal remodeling studies, the protective effect of the A2b agonist was also much more prominent in endothelial cells.

Recently, attention has been drawn to the cross talk between hypoxia and inflammation (8, 13): tissue hypoxia leads to inflammation and vice versa, i.e., inflamed tissue often becomes hypoxic (13). Among other stimuli, hypoxia induces the release of extracellular adenosine by \textit{1)} enhancing the generation by epithelial cells (12), and \textit{2)} repression of equilibrative nucleoside transporters-2, which regulates extracellular adenosine uptake (29). Further, adenosine kinase (AK) lowers intracellular adenosine concentration by converting intracellular adenosine to AMP. Hypoxia was found to repress AK transcript, protein, and function (28), again resulting in elevated intra- and extracellular adenosine levels. Both hypoxia-induced suppression of equilibrative nucleoside transporter-2 and AK are hypoxia-inducible factors (HIF-1) dependent, as demonstrated in HIF-1 mutant mice (28, 29). HIF-1 has also been shown to induce the transcription of the A2b\textsubscript{a} receptor (23), and hypoxia-induced vascular leakage was attenuated with an A2b agonist (8), supporting the findings of our Evans blue studies and highlighting the cross talk between hypoxia and the adenosine-dependent inflammatory response. In addition, in ischemia-reperfusion-induced injury, adenosine, through A2\textsubscript{a} and A2b, is a critical mediator in limiting the inflammatory response and promoting the resolution of tissue injury (14).

Conclusion

Our data demonstrate anti-inflammatory effects of A2b in endotoxin-induced lung injury. The anti-inflammatory effects affect the two main characteristics of ALI: PMN migration and microvascular permeability. PMN migration into the interstitium of the lung was mediated through A2b on hematopoietic cells. Cell integrity of the endothelium and, therefore, attenuated microvascular permeability was mediated through A2b on hematopoietic and nonhematopoietic cells. In our model, an A2b agonist was effective in decreasing PMN migration, both in vivo and in vitro, and microvascular permeability.

ACKNOWLEDGMENTS

A2b gene-deficient mice were provided by Dr. Katya Ravid (Boston University, School of Medicine, Department of Biochemistry, Boston, MA). The A2b agonist (BAY 60-6583) was used with permission of Thomas Krahn (Bayer HealthCare, Leverkusen, Germany).

GRANTS

This work was supported by German Research Foundation Grant RE 1683/3-1 (to J. Reutershan).

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

F.M.K., E.W., and J.R. conception and design of research; F.M.K., E.W., I.V., and S.S. performed experiments; F.M.K., E.W., I.V., S.S., and J.R. analyzed data; F.M.K. and J.R. interpreted results of experiments; F.M.K. and J.R. prepared figures; F.M.K. and J.R. drafted manuscript; J.R. edited and revised manuscript; J.R. approved final version of manuscript.

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