Therapeutic Anti-Inflammatory Effects of Myeloid Cell Adenosine Receptor A2a Stimulation in Lipopolysaccharide-Induced Lung Injury[1]

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To determine the role of the adenosine receptor A2a in a murine model of LPS-induced lung injury, migration of polymorphonuclear leukocytes (PMNs) into the different compartments of the lung was determined by flow cytometry, microvascular permeability was assessed by the extravasation of Evans blue, and the release of chemotactic cytokines into the alveolar airspace was determined by ELISA. Measurements were performed in wild-type and A2a gene-deficient mice ($A2a^{-/-}$). To differentiate the role of A2a on hemopoietic and nonhemopoietic cells, we created chimeric mice by transfer of bone marrow (BM) between wild-type and $A2a^{-/-}$ mice and used mice that lacked A2a expression selectively on myeloid cells ($A2a^{flox/flox}$ × LysM-cre). A specific A2a receptor agonist (ATL202) was used to evaluate its potential to reduce lung injury in vivo. In wild-type mice, therapeutic treatment with ATL202 reduced LPS-induced PMN recruitment, and release of cytokines. Pretreatment, but not posttreatment, also reduced Evans blue extravasation. In the BM chimeric mice lacking A2a on BM-derived cells, PMN migration into the alveolar space was increased by ~50%. These findings were confirmed in $A2a^{flox/flox}$ × LysM-cre mice. ATL202 was only effective when A2a was present on BM-derived cells. A2a agonists may be effective at curbing inflammatory lung tissue damage. ***The Journal of Immunology,*** **2007, 179: 1254–1263.**

Acute lung injury and acute respiratory distress syndrome (ARDS)[3] are life-threatening syndromes that can develop in the course of several clinical conditions such as pneumonia, acid aspiration, major trauma, or sepsis. Recent epidemiological studies indicate that 75,000 patients die from ARDS each year in the United States (1). Numerous experimental and clinical studies have demonstrated that infiltration of polymorphonuclear leukocytes (PMNs) in the lung is a key factor in the pathophysiology of ARDS. Depletion of PMNs can curb experimental lung damage (2). Lung function in ARDS patients negatively correlates with neutrophil counts in the blood (3). Persisting pulmonary neutrophilia in ARDS is correlated with poor outcome (4). These findings suggest that modulating PMN trafficking in the lung might be an effective therapeutical option. However, no such strategies have been developed yet, because the molecular mechanisms underlying PMN trafficking in the lung remain poorly understood (5, 6). To this day, there is no specific therapy available for acute lung injury/ARDS beyond mechanical ventilation and other supportive approaches (7).

Leukocyte recruitment to the lung is initiated by activation of circulating PMNs, resulting in altered mechanical properties (8) and enhanced migratory activity (9). Release of chemotactic cytokines (chemokines) into the bronchoalveolar lavage fluid (BAL) can direct chemokine receptor-expressing PMNs to the lungs. Initial contact between PMNs and pulmonary endothelium requires adhesion molecules in some ARDS models (10), but not in others (11). Once PMNs adhere to the pulmonary vessel wall, transendothelial migration into the lung interstitium and transepithelial migration into the alveolar space are initiated. Both migration steps are regulated by adhesion molecules and chemokine receptors (10, 12) and are accompanied by cytoskeletal reorganization of PMNs, endothelial cells, and epithelial cells.

A variety of ARDS models have been described. In this study, we exposed mice to inhaled endotoxin of Gram-negative bacteria (LPS). LPS inhalation induces substantial PMN migration into the lung (10, 13) and other ARDS-typical symptoms, including increase in microvascular permeability, release of cytokines, and destruction of the pulmonary architecture (14). We used a recently developed, flow cytometry-based technique to detect PMNs in the different compartments of the lung (13). This method allows investigating molecular requirements for PMN accumulation in the pulmonary vasculature, transendothelial migration into the interstitium, and transepithelial migration into the alveolar space.

A2a is one of four G-protein-coupled adenosine receptors that are widely expressed on leukocytes (15) and nonhemopoietic cells (16). A2a signals through G_s and G_{olf}, and cellular responses include coronary vasodilatation and inhibition of platelet aggregation (17, 18). In addition, activation of A2a has been implicated in mediating anti-inflammatory effects and tissue protection (19). Consistent with this, A2a gene-deficient mice exhibit enhanced ischemia reperfusion-induced tissue damage in kidney and liver (20, 21). Activation of A2a by selective receptor agonists has been shown to reduce tissue damage and maintain organ function in

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[3] Abbreviations used in this paper: ARDS, acute respiratory distress syndrome; PMN, polymorphonuclear leukocyte; BAL, bronchoalveolar lavage fluid; BM, bone marrow.

models of cardiac (22), renal (23), and pulmonary (24) ischemia reperfusion injury. In most models, inhibition of PMN infiltration into the inflamed tissue is one of the key effects of A2a. In the kidney, protective effects require the presence of A2a on bone marrow (BM)-derived cells (25, 26).

The role of A2a in acute lung injury has yet to be defined. In hemorrhage-induced lung injury, injection of an A2a agonist reduced microvascular permeability and myeloperoxidase activity in the lung (27), suggesting an inhibitory effect on PMN recruitment. Detrimental effects of high concentrations of inspired oxygen that are often applied to overcome hypoxia in ARDS patients may disturb A2a-mediated tissue protection in the lung (28). Release of cytokines from alveolar macrophages is pivotal in many models of ARDS. Activation of A2a inhibits LPS-induced TNF-α production from peritoneal macrophages (29), suggesting that similar effects might occur in alveolar macrophages. To date, it is unknown how A2a affects PMN migration in lung injury and what cells are involved.

This study was designed to test the hypothesis that A2a plays an important role in LPS-induced lung injury. We studied the migration of PMNs into the different compartments of the lung in wild-type and global as well as myeloid-specific A2a gene-deficient mice. We used a selective A2a agonist to determine A2a-dependent effects on PMN recruitment, microvascular permeability, and cytokine release. We created BM chimeras to study A2a effects on hemopoietic vs nonhemopoietic cells. Our results demonstrate a dual role of A2a in acute lung injury and provide evidence that A2a activation on hemopoietic cells may reduce lung tissue damage.

Materials and Methods

Mice

All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia or the University of Tübingen. Mice were 8–12 wk of age. Wild-type male C57BL/6 mice were obtained from The Jackson Laboratory.

Generation of A2a receptor knockout ($A2a^{-/-}$) mice congenic with C57BL/6. $A2a^{-/-}$ mice (B6;129P-adora2atm1jfc) were bred to a C57BL/6 background by a speed congenic method. In brief, five mice with disrupted adora2a were received from Dr. J.-F. Chen (Boston University, Boston, MA) after several generations of inbreeding from (129-Adora2atm1jfc × C57BL/6)F_1 founder mice. We initiated a new line using a founder with a maximal number of informative loci (B6/129 or 129/129). Mapping consisted of 96 microsattelites covering every chromosome except Y at an average distance of 15 cM. This strategy was used effectively to move the mutant adora2a allele onto the C57BL/6 background by generation 5.

Generation of congenic lysM-Cre/adora2aflox/flox mice. Founder mice with a targeted knockin of Cre recombinase to the Lyzs (lysM) locus (Chr 10: 116.68 Mb; NCBIm36) were received from I. Foerster after multiple generations of inbreeding from (129P2- Lystm1(cre)Ifo × C57BL/6)F_1 founder mice. These mice were bred to be congenic to C57BL/6, and no residual 129 alleles were detected by microsatellite analysis. Similarly, founder mice with LoxP sites flanking the Adora2a locus were received after backcrossing and inbreeding of (129- Adora2atmJyd(flox) × C57BL/6)F_1 founder mice. These were further bred to be congenic to C57BL/6, and no residual 129 alleles were detected by microsatellite analysis. Experimental animals were generated by selective mating of the offspring of a lysM-Cre × adora2aflox/flox cross to generate lysM-Cre/adora2aflox/flox animals.

Chimeric mice

Chimeric mice were generated by transferring BM between wild-type and $A2a^{-/-}$ mice as described earlier (30). Briefly, recipient mice were lethally irradiated in two doses of 600 rad each (separated by 4 h). BM from donor mice was harvested from both femora and tibiae, and ~5 million cells were injected i.v. into recipient mice immediately upon the second irradiation. BM transplantation was performed in four groups of mice: 1) BM from $A2a^{-/-}$ into wild-type mice (chimeric mice express A2a on nonhemopoietic cells only), 2) BM from wild-type into $A2a^{-/-}$ (chimeric mice express A2a on hemopoietic cells only), 3) BM from $A2a^{-/-}$ into $A2a^{-/-}$, and 4) BM from $A2a^{+/+}$ into $A2a^{+/+}$. The latter two groups were used to control for potential effects of the radiation.

Table I. *Baseline cell counts*[a]

	wt	$A2a^{-/-}$	*p*
Leukocytes ($10^3/\mu l$)	5.4 ± 1.7	4.6 ± 1.4	NS
PMN ($10^3/\mu l$)	0.9 ± 0.3	0.7 ± 0.3	NS
Lymphocytes ($10^3/\mu l$)	4.8 ± 2.0	4.4 ± 0.9	NS
Monocytes ($10^3/\mu l$)	0.27 ± 0.10	0.14 ± 0.04	<0.05

[a] Baseline differential cell counts were performed in wild-type (wt) and A2a gene-deficient ($A2a^{-/-}$) mice using an automatic analyzer. Data are mean ± SD of four samples.

A2a mRNA expression

LPS-induced expression of A2a mRNA in whole lungs was determined by real-time RT-PCR. Wild-type mice were exposed to LPS aerosol. After 3 h, animals were euthanized, lungs were perfused free of blood, excised, and stored in RNAlater (Ambion) at −80°C. This time point was chosen because chemotactic activity of BAL peaks at 3 h (our unpublished observation). Control animals did not inhale LPS.

Total RNA was isolated from lung homogenates and reverse transcription was performed using SuperScript III Transcriptions kit (Invitrogen) and oligo(dT) primers. Reaction conditions were optimized and cDNA samples analyzed with primers for A2a (5′-ACTCCCATTGGCCCATACTC-3′ and 5′-CGTTGTCAACCCCTTCATCT-3′) on an iCycler iQ Real-Time Detection System (Bio-Rad). Values were determined using the iCycler iQ Real-Time Detection System Software, version 3.1 (Bio-Rad), and normalized to murine β-actin (primers, 5′-ACATTGGCATGGCTTTGTTT-3′ and 5′-GTTTGCTCCAACCAACTGCT-3′).

Model of acute lung injury

Up to four mice were exposed to aerosolized LPS in a custom-built cylindrical chamber (20 cm long × 9 cm diameter) connected to an air nebulizer (MicroAir; Omron Healthcare). LPS from *Salmonella enteritidis* (Sigma-Aldrich) was dissolved in 0.9% saline (500 μg/ml) and mice inhaled LPS for 30 min. As previously shown, this mimics several aspects of acute lung injury including PMN recruitment into all compartments of the lung, increase in vascular permeability (13), release of chemokines, and disruption of the pulmonary architecture (14). Control mice were exposed to saline aerosol.

Treatment with A2a agonist ATL202

N-Ethyl-2-adenosine-5′-uronamide (ATL202; 10 μg/ml; Adenosine Therapeutics) was used to selectively activate A2a receptor (31). We aerosolized ATL202 (10 μg/ml) in a nose-only inhalation system (CH Technologies) for 20 min, 30 min before, concomitant with, or 1 h after LPS exposure.

PMN recruitment into the lung

PMNs in the different compartments of the lung (pulmonary vasculature, lung interstitium, and alveolar airspace) were detected by flow cytometry using a recently developed technique (13). Briefly, 24 h after LPS exposure, intravascular PMNs were labeled by i.v. injection of Alexa 633-labeled GR-1 to murine PMN. After 5 min, mice were anesthetized and a median thoracotomy was performed. Subsequently, the right ventricle was cannulated with a 23-gauge cannula and 10 ml of PBS was injected slowly at 25 cmH_2O to remove nonadherent leukocytes from the pulmonary vasculature (13). BAL was withdrawn and lungs were removed, minced, and digested in the presence of excess unlabeled anti-GR-1 to prevent possible binding of the injected Ab to extravascular PMN. A cell suspension was prepared by passing the digested lungs through a 70-μm cell strainer (BD Falcon, Bedford, MA). Total cells in BAL and lung were counted, and percentage of PMNs was determined by flow cytometry. In the BAL, PMNs were identified by their typical appearance in the forward/sideward scatter and their expression of CD45 (clone 30-F11), 7/4 (clone 7/4), and GR-1 (clone RB6-8C5) (32, 33). In the lung, the expression of GR-1 was used to distinguish intravascular ($CD45^+7/4^+GR\text{-}1^+$) from interstitial ($CD45^+7/4^+GR\text{-}1^-$) PMNs, which were not reached by the injected Ab (13).

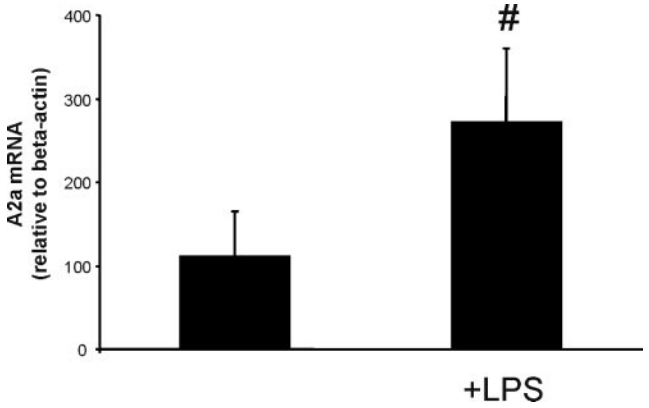

FIGURE 1. LPS-induced expression of A2a mRNA in the lung. Constitutive A2a mRNA expression was detected in lungs from healthy wild-type mice. Three hours after LPS exposure, A2a mRNA expression in the lungs increased ~3-fold. A2a mRNA levels were normalized to β-actin (=100%) and presented as mean ± SD of $n = 4$ samples; #, $p < 0.0001$ vs control.

Immunohistochemistry

Wild-type and A2a$^{-/-}$ mice were euthanized 24 h after exposure to LPS with or without pretreatment with ATL202. Control mice did not receive LPS or ATL202. The pulmonary circulation was perfused free of blood, the trachea was cannulated, and the lung was inflated with 4% paraformaldehyde for 10 min at 25 cmH_2O. The lungs were subsequently removed and fixed in paraformaldehyde for 24 h. Paraffin-embedded sections (5 μm) were stained for PMNs using the avidin-biotin technique (Vector Laboratories) as previously described (34). Briefly, deparaffinized and rehydrated sections were incubated with avidin, 10% rabbit serum, and 0.5% fish skin gelatin oil for 1 h to block nonspecific binding. After washing with PBS, a specific Ab against mouse neutrophils (clone 7/4; Caltag Laboratories) (32) was added (1 μg/ml) and incubated overnight. Sections were then washed and incubated with 5 μg/ml biotinylated rabbit anti-rat IgG (Vector Laboratories) for 1 h, followed by avidin-biotin-peroxidase complexes (Vectastain Elite ABC kit; Vector Laboratories), washed with PBS, incubated with diaminobenzidine (DAB kit; Vector Laboratories), and counterstained with hematoxylin.

Pulmonary microvascular permeability

LPS-induced microvascular permeability in the lung of wild-type and A2a$^{-/-}$ mice was determined using the Evans blue dye extravasation technique (35). Evans blue (20 mg/kg; Sigma-Aldrich) was injected i.v. 30 min before euthanasia. Lungs were perfused through the spontaneously

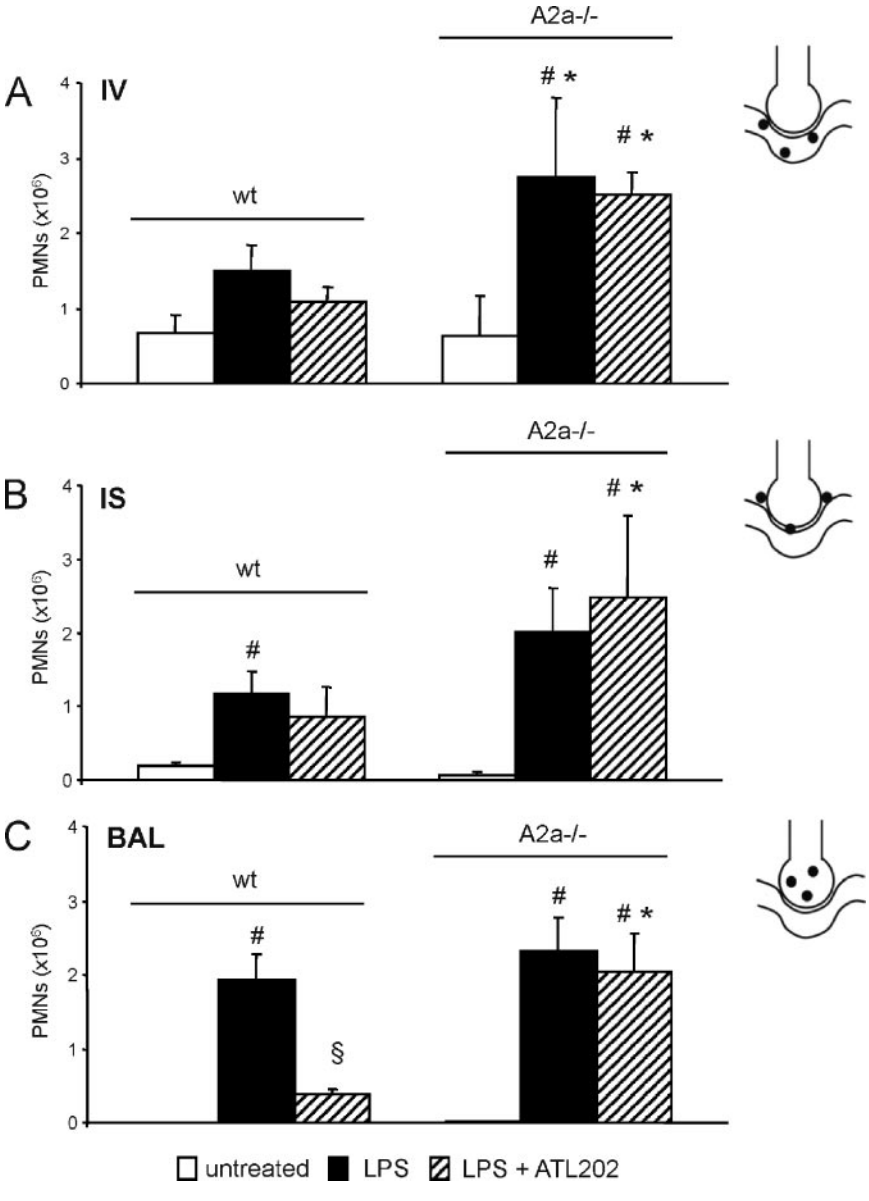

FIGURE 2. Effects of ATL202 (▨) on LPS-induced migration of PMNs (■) into the different lung compartments of wild-type and A2a$^{-/-}$ mice. □, Negative controls (no LPS). Accumulation of PMNs in the vasculature (IV) (*A*), the lung interstitium (IS) (*B*), and the bronchoalveolar space (BAL) (*C*) were analyzed. Values are means ± SD of $n = 4$ experiments. #, $p < 0.05$ vs negative control without LPS; *, $p < 0.05$ vs wild-type mice within the same treatment group; §, $p < 0.05$ vs LPS-treated mice without ATL202.

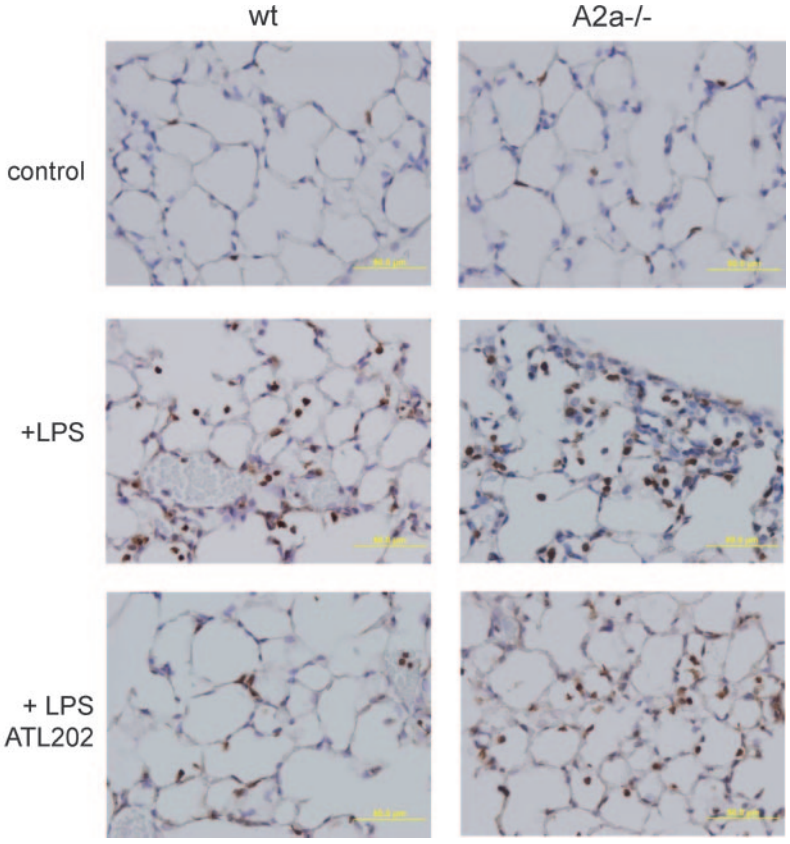

FIGURE 3. PMN infiltration into the lungs shown by immunohistochemistry. Wild-type and A2a$^{-/-}$ mice were exposed to LPS; some were pretreated with ATL202. Lung sections were stained with a specific neutrophil marker (7/4). Images are representative of $n = 4$ experiments.

beating right ventricle to remove intravascular dye. Lungs were removed, and Evans blue was extracted as described previously (36). The absorption of Evans blue was measured at 620 nm and corrected for the presence of heme pigments: A_{620} (corrected) = $A_{620} - (1.426 \times A_{740} + 0.030)$ (37). Extravasated Evans blue was determined in the different groups 6 h after LPS and calculated against a standard curve (micrograms Evans blue dye per gram lung). Some animals received ATL202 30 min before LPS exposure. Control animals received no LPS or ATL202. Pictures of representative lungs were taken in the different groups 30 min after injection of Evans blue to illustrate LPS-induced protein leakage.

Chemokine release

LPS-induced release of CXCL1 (KC), CXCL2/3 (MIP-2), TNF-α, and IL-6 were measured in the BAL of wild-type and A2a$^{-/-}$ mice using ELISA kits (R&D Systems). Some animals received ATL202 30 min before LPS exposure.

Blood counts

To reveal possible differences between wild-type and A2a$^{-/-}$ mice, baseline differential blood counts were performed (Hemavet 850 FS; CDC Technologies).

Statistical analysis

Statistical analysis was performed with JMP Statistical Software (version 5.1; SAS Institute). Differences between the groups were evaluated by one-way ANOVA followed by a post hoc Tukey's test. Data were presented as mean ± SEM, and $p < 0.05$ was considered statistically significant.

Results

Blood counts

Genetically altered mice lacking inflammatory receptors often exhibit increased leukocyte counts in the peripheral blood, which may influence cell migration to inflammatory sites. Total leukocyte, PMN, and lymphocyte counts were not different between wild-type and

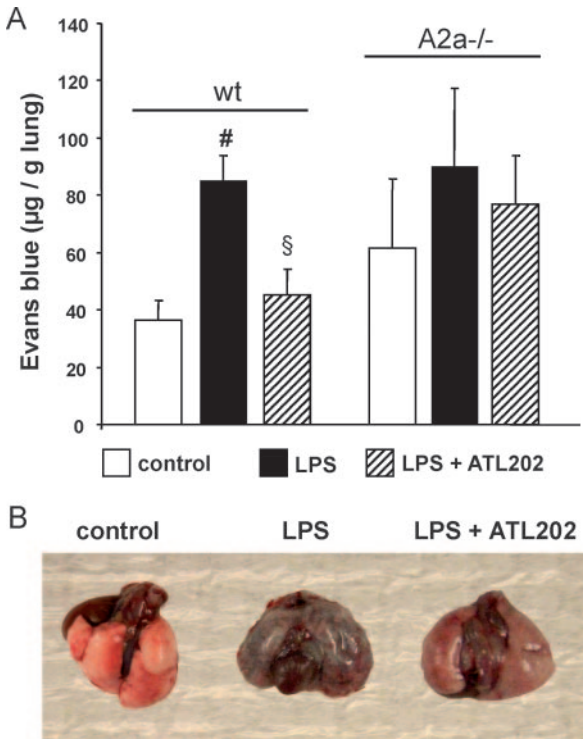

FIGURE 4. Effect of ATL202 (▨) on LPS-induced microvascular permeability (■) in the lung. *A*, Wild-type (wt) and A2a$^{-/-}$ mice were exposed to LPS with or without pretreatment with ATL202. Microvascular protein leakage was assessed by Evans blue extravasation technique 6 h after LPS exposure. Values are means ± SD of $n = 4$ experiments. #, $p < 0.05$ vs negative control without LPS; §, $p < 0.05$ vs LPS-treated mice without ATL202. *B*, Illustration of LPS-induced microvascular protein leak in the lungs of wild-type mice. Representative images of $n = 3$ experiments are shown.

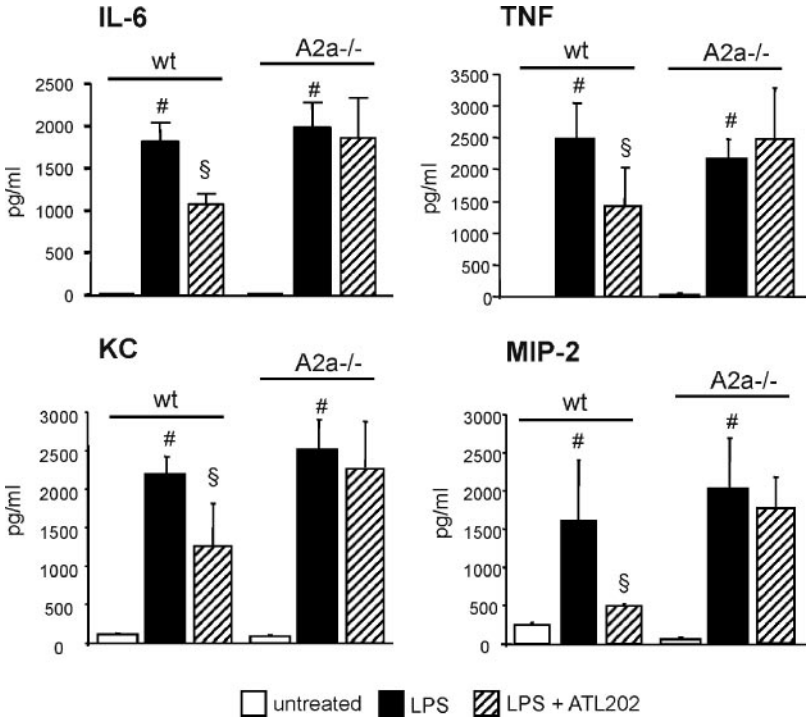

FIGURE 5. Effect of ATL202 (▨) on LPS-induced release of chemokines (■) into the alveolar airspace. Values are means ± SD of $n = 4$ experiments. #, $p < 0.05$ vs negative control without LPS (□); §, $p < 0.05$ vs LPS-treated mice without ATL202.

$A2a^{-/-}$ mice. However, $A2a^{-/-}$ mice showed significantly reduced monocyte counts (Table I).

LPS induces transcription of A2a in the lung

To test whether LPS inhalation induces transcription of A2a, real-time RT-PCR of lung homogenate was performed. A2a mRNA was expressed constitutively in control lungs. Three hours after LPS exposure, A2a mRNA expression increased ~3-fold ($p < 0.0001$; Fig. 1).

A2a activation reduces LPS-induced PMN migration into the lung

Baseline PMN counts in the lung did not differ between wild-type and $A2a^{-/-}$ mice. LPS inhalation induced PMN recruitment into all compartments of the lung of wild-type and $A2a^{-/-}$ mice. LPS-induced PMN accumulation in the pulmonary circulation (IV) was significantly higher in $A2a^{-/-}$ compared with wild-type mice ($2.8 \pm 1.0 \times 10^6$ vs $1.5 \pm 0.3 \times 10^6$; $p < 0.05$). PMN migration into interstitium (IS) and alveolar airspace (BAL) was not significantly different (Fig. 2). Pretreatment with the A2a agonist ATL202 reduced PMN migration into the alveolar space in wild-type ($0.4 \pm 0.07 \times 10^6$ vs $1.9 \pm 0.3 \times 10^6$; $p < 0.05$) but not in $A2a^{-/-}$ mice ($2.0 \pm 0.5 \times 10^6$ vs $2.3 \pm 0.4 \times 10^6$; $p =$ NS), indicating that ATL202 acts specifically through A2a receptors. PMN accumulation in the lung was also demonstrated by immunohistochemistry (Fig. 3). At baseline, alveoli were free of PMNs. LPS inhalation induced substantial infiltration of PMNs in lungs of wild-type and $A2a^{-/-}$ mice. Pretreatment with ATL202 reduced PMN infiltration in wild-type but not in $A2a^{-/-}$ mice. Similar effects were seen with a second A2a agonist, CGS-21680 (data not shown).

A2a activation reduces LPS-induced microvascular leakage

Disruption of microvascular integrity and the formation of a protein-rich edema is a key factor in acute lung injury. Baseline permeability as assessed by the extravasation of Evans blue tended to be higher in $A2a^{-/-}$ mice compared with wild type; however, this difference was not significant (Fig. 4). LPS inhalation induced an increase in microvascular permeability in both groups. This was

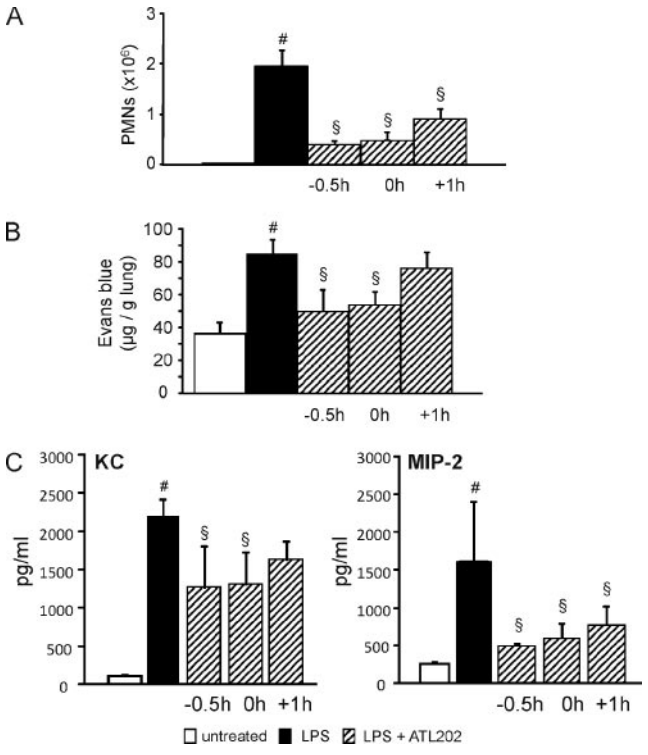

FIGURE 6. ATL202 was administered 0.5 h before, simultaneously, or 1 h after LPS exposure and PMNs in BAL (*A*); microvascular permeability (*B*) and release of the chemokines KC and MIP-2 (*C*) were determined. Values are means ± SD of $n = 4$ experiments. #, $p < 0.05$ vs negative control without LPS; §, $p < 0.05$ vs LPS-treated mice without ATL202.

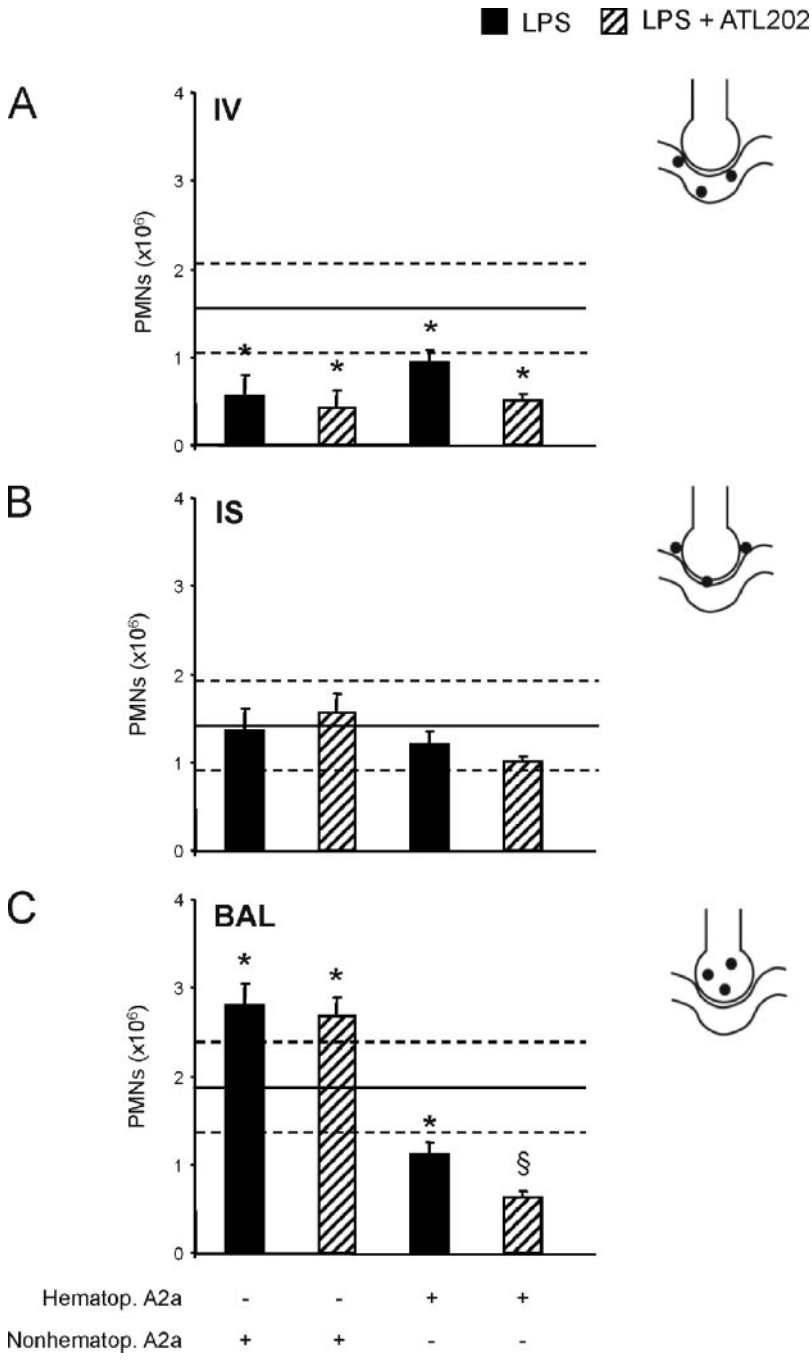

FIGURE 7. Contribution of A2a on hemopoietic and nonhemopoietic cells to LPS-induced migration of PMNs into the different compartments of the lung were analyzed by chimeric mice. Accumulation of PMNs in the vasculature (IV) (*A*), the lung interstitium (IS) (*B*), and the bronchoalveolar space (BAL) (*C*) are displayed. Means ± SD of $n = 4$ experiments of untreated (■) and ATL202-treated mice (▨). *, $p < 0.05$ vs mice expressing A2a on all cells (horizontal line ± SD indicated by dashed lines); §, $p < 0.05$ vs LPS-treated mice without ATL202.

blocked by ATL202 in wild-type (45.2 ± 8.9 vs 84.9 ± 9.1 μg/g lung; $p < 0.001$) but not in A2a$^{-/-}$ mice (76.9 ± 17.0 vs 90.0 ± 27.61 μg/g lung; p = NS). In the chimeric mice, microvascular permeability tended to be higher when A2a was selectively removed from hemopoietic cells, but differences failed to reach statistical significance (data not shown).

Effects of A2a on chemokine production

The release of cytokines and chemokines into the alveolar space initiates recruitment of inflammatory cells into the lungs. Baseline concentrations of IL-6, TNF-α, CXCL1 (KC), and CXCL2/3 (MIP-2) in the BAL of wild-type mice were negligible. LPS exposure induced significant production of all cytokines. Pretreatment with ATL202 reduced concentrations of all cytokines significantly (Fig. 5). LPS-induced cytokine production in A2a$^{-/-}$ mice was not different from wild-type mice, but ATL202 did not affect cytokine release in these mice.

Therapeutic effect of ATL202 administration

Next, we evaluated whether ATL202 maintains its protective effects when given at the same time or 1 h after LPS exposure. This mimics the clinical situation where pretreatment is not always an option. PMN migration into the BAL was significantly reduced when mice received ATL202 at the same time or 1 h after LPS exposure (Fig. 6*A*). Microvascular permeability was reduced when ATL202 was given simultaneously but not when given after LPS inhalation (Fig. 6*B*). Release of the chemokines KC and MIP-2 into the BAL was reduced when ATL202 was simultaneously administered. Posttreatment reduced release of MIP-2 but not KC (Fig. 6*C*).

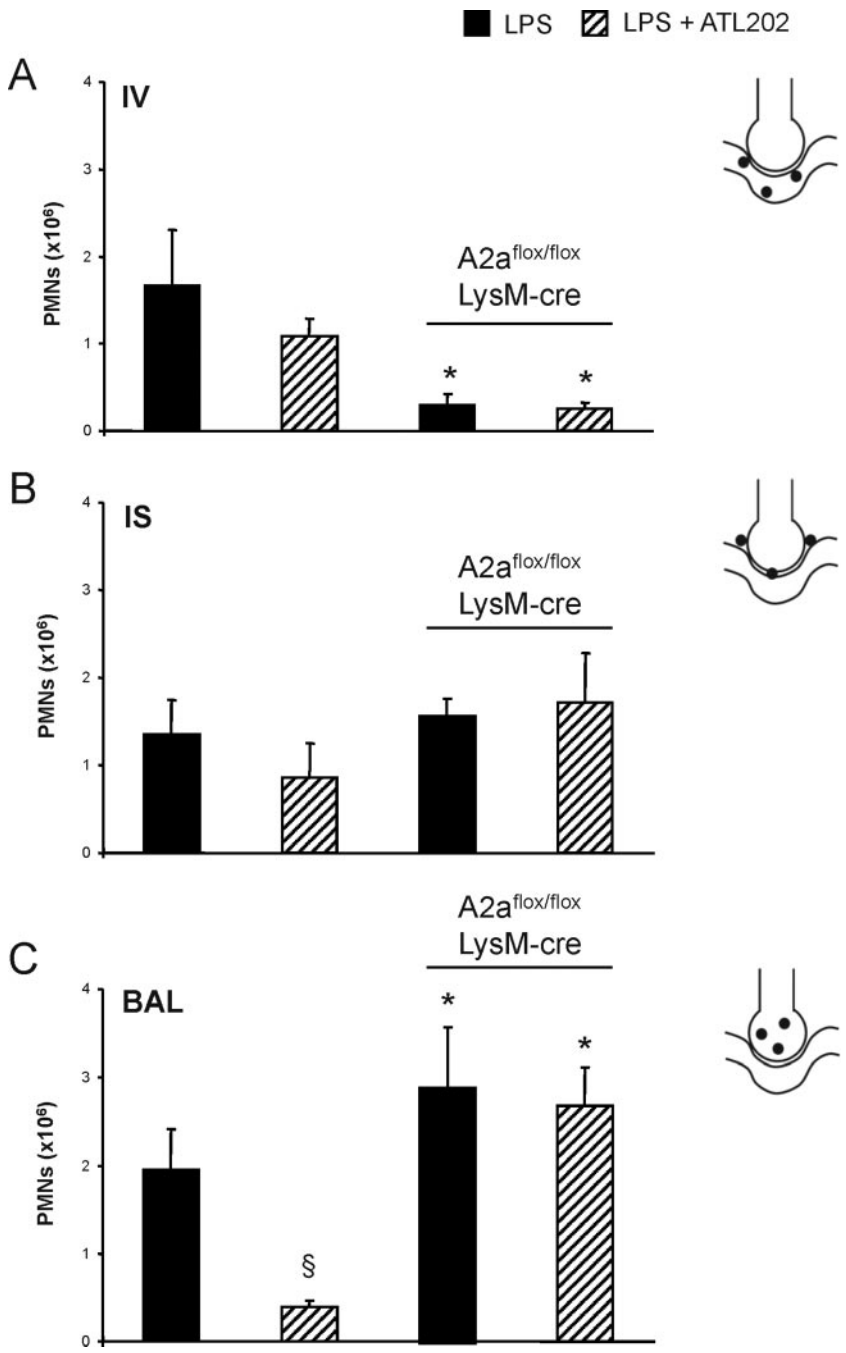

FIGURE 8. Contribution of A2a expressed by myeloid cells to LPS-induced migration of PMNs into the different compartments of the lung as analyzed in $A2a^{flox/flox}$ LysM-cre mice that selectively lack A2a in myeloid cells. Accumulation of PMNs in the vasculature (IV) (*A*), the lung interstitium (IS) (*B*), and the bronchoalveolar space (BAL) (*C*) in LPS-treated mice (■) and mice that received ATL202 (▨). Values are means ± SD of $n = 4$ experiments. *, $p < 0.05$ vs wild-type mice; §, $p < 0.05$ vs LPS-treated mice without ATL202.

Hemopoietic vs nonhemopoietic A2a effects

After we had established a role for A2a in LPS-induced PMN migration into the lung, we sought to characterize the role of A2a on BM-derived vs nonhemopoietic cells. We generated chimeric mice by transferring BM between wild-type and $A2a^{-/-}$ mice and determined LPS-induced PMN accumulation in the different lung compartments (Fig. 7). Lethally irradiated mice reconstituted with BM from the same genotype served as controls. Controls exhibited similar phenotypes compared with wild-type or $A2a^{-/-}$ mice, respectively (range indicated by dotted lines). LPS-induced accumulation of PMNs in the pulmonary vasculature was reduced when A2a was only expressed on either hemopoietic or nonhemopoietic cells (Fig. 7*A*). PMN content of the lung interstitium was similar in all groups (Fig. 7*B*).

PMN migration into the alveolar space was higher than in wild-type mice when A2a was selectively removed from hemopoietic cells (Fig. 7*C*), suggesting that leukocyte A2a has anti-inflammatory effects. In these mice, PMN counts in the BAL were higher than in mice that expressed A2a on all cells ($2.8 \pm 0.4 \times 10^6$ vs $1.9 \pm 0.4 \times 10^6$; $p < 0.05$). In contrast, migration of PMNs into the BAL was reduced when A2a was expressed on hemopoietic cells exclusively ($1.1 \pm 0.4 \times 10^6$ vs $1.9 \pm 0.4 \times 10^6$; $p < 0.05$). This suggests that, in our model, the protective role of A2a on BM-derived cells is associated with proinflammatory effects of A2a on nonhemopoietic cells.

Pretreatment with ATL202 reduced PMN migration into the BAL only when A2a was expressed on hemopoietic cells (Fig. 7*C*). This indicates that the A2a agonist acts through A2a on BM-derived rather than through nonhemopoietic cells.

To confirm these findings, we used mice that selectively lacked A2a on myeloid cells ($A2a^{flox/flox} \times$ LysM-cre). These mice showed reduced accumulation of PMNs in the pulmonary vessels

($0.3 \pm 0.1 \times 10^6$ vs $1.7 \pm 0.6 \times 10^6$; $p < 0.05$) (Fig. 8*A*). Consistent with the finding in BM chimeras lacking A2a on myeloid cells, migration of PMNs into the BAL was increased ($2.9 \pm 0.7 \times 10^6$ vs $1.9 \pm 0.3 \times 10^6$; $p < 0.05$) (Fig. 8*C*). Similar to the BM chimeras, ATL202 did not affect PMN migration in $A2a^{flox/flox}$ LysM-cre mice, suggesting that A2a on myeloid cells controls PMN recruitment in this model.

Discussion

This study was designed to characterize the role of A2a adenosine receptors in LPS-induced lung injury. We focused our efforts on defining how activation of A2a affected migration of PMNs into the different compartments of the lung, and which cell types responded to an A2a agonist. We found that A2a on BM-derived cells limited PMN recruitment and adhesion to the pulmonary microvasculature. LPS-induced PMN accumulation in the BAL was highest when A2a was selectively removed from leukocytes. This is consistent with the known anti-inflammatory effect of A2a on leukocytes (38). Inhalation with the selective A2a agonist ATL202 reduced LPS-induced PMN migration, microvascular permeability, and chemokine release. The protective effects of ATL202 were largely maintained when ATL202 was given up to 1 h after LPS exposure, suggesting that this might be a promising therapeutical substance class in acute lung injury.

Adenosine receptors are a critical part of the physiological negative-feedback mechanism for limitation and termination of tissue-specific and systemic inflammatory responses. A2a activation is known to reduce expression of adhesion molecules and release of proinflammatory mediators. The cellular responses seem to be mediated predominately by cAMP and result in inhibition of oxidative burst in neutrophils (39), reduced cytokine release (40), and inhibition of leukocyte activation (41). All of these mechanisms have been suggested to contribute to the A2a-mediated protection that is seen in LPS-induced inflammation (19).

Previous research on anti-inflammatory properties of A2a mainly focused on ischemia reperfusion-induced tissue injury (22, 24, 42). In renal reperfusion injury, A2a activation significantly reduced PMN recruitment and maintained organ function (42). Reduction of PMN infiltration was accompanied by decreased expression of endothelial P-selectin and ICAM-1, both of which mediate cell adhesion. Consistent with these results, we found that, in $A2a^{-/-}$ mice, LPS-induced PMN accumulation was increased within the pulmonary microvasculature but not within the lung tissue or the alveolar space. Elevated PMN accumulation in $A2a^{-/-}$ mice also suggests that endogenous adenosine is at least partially involved in limiting the inflammatory response. The molecular requirements for intravascular PMN accumulation in our model are not completely understood. Activation of PMNs is sufficient to induce their accumulation in the small lung capillaries. In our model, LFA-1 and ICAM-1 account for ~50% of PMN trafficking (10).

PMN accumulation in the pulmonary microvasculature was reduced when A2a was only expressed on either hemopoietic or nonhemopoietic cells in the chimeric mice. As previously demonstrated, LPS-induced migration of PMN into the different lung compartments is time dependent (13). Accumulation in the pulmonary vasculature occurs rapidly with a peak at 4 h. Twenty-four hours after LPS exposure, the majority of PMNs have left the pulmonary vasculature, either by transmigration into the lung or by release into the systemic circulation. When A2a was expressed on nonhemopoietic cells only (Fig. 7, *left bars*), we observed an increased migratory activity into the alveolar airspace. This effect may explain why PMN counts in the intravascular space were lower in these mice. When A2a was expressed on hemopoietic cells only, accumulation of PMNs in the vasculature was similar to that seen in wild-type mice and reduced by ~50% by pretreatment with ATL202.

$A2a^{-/-}$ mice in our study had significantly lower blood monocyte counts (Table I) and tended to have lower PMN counts in the blood. There are reports that adenosine suppresses the proliferation of murine BM-derived macrophages (43), but this effect is mediated by the A2b receptor. A2a stimulation promotes internalization of chemokine receptors (44), thereby limiting emigration of leukocytes from the blood into the tissue. This effect could also impact on monocyte and neutrophil efflux from the BM, a process controlled by chemokine receptors. Increased apoptotic cell death in $A2a^{-/-}$ mice might also cause reduced leukocyte counts (45).

In addition to local effects within the inflamed tissue, A2a has been demonstrated to mediate protection in remote organs, suggesting that circulating leukocytes may be involved (46). The relative contribution of A2a on BM-derived cells has been discussed controversially. Detrimental effects have been described in a model of cerebral ischemia where A2a activation on BM-derived cells led to increased brain tissue damage (47). However, in most other models, anti-inflammatory effects of A2a agonists predominate (38, 48).

In lung injury, alveolar macrophages significantly contribute to tissue damage and recruitment of inflammatory cells (49). In our study, A2a activation reduced release of cytokines into the BAL. Although other cells in the alveolar space, e.g., type II pneumocytes, might be involved in chemokine production (50), alveolar macrophages seem to be the major source. PMNs also are a potent chemokine source that perpetuates lung inflammation (51, 52). A2a activation reduced transcription of relevant chemoattractants on PMNs recruited into an air pouch (53). Transcriptional effects may also contribute to the A2a-mediated decrease in chemokine concentration in the BAL.

LPS induced up-regulation of A2a mRNA in the lung. Although we did not specifically evaluate the A2a-expressing cell types in our study, this finding is consistent with previous reports demonstrating that LPS preferentially up-regulates A2a mRNA in inflammatory macrophages (29).

Although the role of other adenosine receptors has been discussed controversially (54–56), A2a appears to exhibit predominantly anti-inflammatory effects. Other A2a agonists, including CGS-21680, have been used to evaluate protective effects in different models of acute lung injury. In hemorrhage-induced lung injury, injection of CGS-21680 limited accumulation of myeloperoxidase activity in the lung, which is indicative of reduced PMN recruitment (27). Application of high concentrations of inspired oxygen, in ARDS patients often required to overcome hypoxia, can exacerbate lung injury (57). There is evidence that disturbance of A2a-mediated lung tissue protection occurs during hyperoxia, a phenomenon that can be reversed by treatment with CGS-21680 (28). In a murine model of OVA-induced chronic obstructive pulmonary disease, A2a activation reduced influx of leukocytes into the BAL. Similar to our findings, pretreatment with CGS-21680 also reduced LPS-induced PMN infiltration (58, 59) and cytokine release into the alveolar airspace, although not statistically significant in one study (59).

In the present study, LPS-induced transmigration into the lung was enhanced above the level seen in $A2a^{-/-}$ mice when A2a was selectively removed from leukocytes, either by BM transfer or by tissue-specific deletion. This finding was unexpected and suggests a proinflammatory role of endothelial and/or epithelial A2a in the lung. A2a stimulation may result in migration of pulmonary endothelial and/or epithelial cells (60) or may induce the release of

vascular endothelial growth factor (61, 62), which can enhance transmigration of inflammatory cells.

In conclusion, we have identified opposing roles of A2a in LPS-induced lung injury. Whereas A2a activation on BM-derived cells reduces recruitment and adhesion of PMNs to pulmonary endothelial cells, A2a on lung cells promotes cell migration through the alveolo-capillary barrier. Selective A2a agonists like ATL202 delivered to BM-derived cells may be a promising approach to treat acute lung injury and ARDS.

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J. Linden owns stock in Adenosine Therapeutics (ATL).

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