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Activation of A_1-Adenosine Receptors Promotes Leukocyte Recruitment to the Lung and Attenuates Acute Lung Injury in Mice Infected with Influenza A/WSN/33 (H1N1) Virus

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ABSTRACT

We have shown that bronchoalveolar epithelial A_1-adenosine receptors (A_1-AdoR) are activated in influenza A virus-infected mice. Alveolar macrophages and neutrophils also express A_1-AdoRs, and we hypothesized that activation of A_1-AdoRs on these cells will promote macrophage and neutrophil chemotaxis and activation and thereby play a role in the pathogenesis of influenza virus-induced acute lung injury. Wild-type (WT) C57BL/6 mice, congenic A_1-AdoR knockout (A_1-KO) mice, and mice that had undergone reciprocal bone marrow transfer were inoculated intranasally with 10,000 PFU/mouse influenza A/WSN/33 (H1N1) virus. Alternatively, WT mice underwent daily treatment with the A_1-AdoR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) from 1 day prior to inoculation. Infection increased bronchoalveolar lining fluid (BALF) adenosine comparably in WT and A_1-KO mice. Infection of WT mice resulted in reduced carotid arterial O_2 saturation (hypoxemia), lung pathology, pulmonary edema, reduced lung compliance, increased basal airway resistance, and hyperresponsiveness to methacholine. These effects were absent or significantly attenuated in A_1-KO mice. Levels of BALF leukocytes, gamma interferon (IFN-γ), and interleukin 10 (IL-10) were significantly reduced in infected A_1-KO mice, but levels of KC, IP-10, and MCP-1 were increased. Reciprocal bone marrow transfer resulted in WT-like lung injury severity, but BALF leukocyte levels increased only in WT and A_1-KO mice with WT bone barrow. Hypoxemia, pulmonary edema, and levels of BALF alveolar macrophages, neutrophils, IFN-γ, and IL-10 were reduced in DPCPX-treated WT mice. Levels of viral replication did not differ between mouse strains or treatment groups. These findings indicate that adenosine activation of leukocyte A_1-AdoRs plays a significant role in their recruitment to the infected lung and contributes to influenza pathogenesis. A_1-AdoR inhibitor therapy may therefore be beneficial in patients with influenza virus-induced lung injury.

IMPORTANCE

Because antiviral drugs are of limited efficacy in patients hospitalized for influenza virus-induced respiratory failure, there is an urgent need for new therapeutics that can limit the progression of lung injury and reduce influenza death rates. We show that influenza A virus infection results in increased production of the nucleoside adenosine in the mouse lung and that activation of A_1-subtype adenosine receptors by adenosine contributes significantly to both recruitment of innate immune cells to the lung and development of acute lung injury following influenza virus infection. We also show that treatment with an A_1-adenosine receptor antagonist reduces the severity of lung injury in influenza virus-infected mice. Our findings indicate that adenosine plays an important and previously unrecognized role in the innate immune response to influenza virus infection and suggest that drugs which can inhibit either generation of adenosine or activation of A_1-adenosine receptors may be beneficial in treating influenza patients hospitalized for respiratory failure.

Influenza A viruses cause a contagious acute respiratory disease in humans which accounts for 200,000 hospitalizations and more than 36,000 excess deaths per year in the United States alone (1). In addition, reoccurring pandemics have resulted in devastating loss of life worldwide, most notably in 1918, when approximately 50 million people may have died (2). The 2009 to 2010 H1N1 pandemic influenza virus strains are estimated to have infected around 61 million people in the United States alone within 12 months, resulting in at least 275,000 hospitalizations and 12,500 excess deaths (3, 4).

Nucleotides are present at extremely low concentrations in normal bronchoalveolar lining fluid (BALF), but large amounts of ATP are released apically by respiratory epithelial cells in response to cell stress (5). We have previously shown that influenza A virus infection resulted in an increase in BALF ATP levels in mice (*Mus musculus*) (6). This effect was reversed by treatment with the *de novo* pyrimidine synthesis inhibitor A77-1726, indicating that released ATP was derived from increased *de novo* nucleotide synthesis in response to infection (7).

The nucleoside adenosine plays a key role in regulating pulmonary fluid dynamics (8) and lung inflammation (9). Adenosine can be generated in the airspace lining fluid by sequential hydrolysis of ATP. The ecto-apyrase NTPDase 1 (CD39), which is expressed on the apical surface of respiratory epithelial cells, hydrolyzes ATP and ADP to AMP (10). AMP is then further hydrolyzed to adenosine by the ecto-5′-nucleotidase (CD73), which is also abundantly expressed in the lung (11).

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Adenosine signals through four specific adenosine receptor (AdoR) subtypes (A_1, A_{2a}, A_{2b}, and A_3) (12), all of which are expressed on respiratory epithelial cells (13), macrophages (14), and neutrophils (9). A_1-AdoRs have the highest affinity for adenosine, and A_1-AdoR signaling can promote macrophage and neutrophil activation, adhesion, and chemotaxis (15, 16), which may be important to the pathogenesis of both acute lung injury (ALI) and influenza (12, 17–20).

We previously showed that activation of bronchoalveolar epithelial A_1-AdoRs contributes to impairment of alveolar fluid clearance in influenza A virus-infected mice (6). Given the known role of A_1-AdoRs in leukocyte recruitment, we hypothesized that activation of these receptors by adenosine produced in response to influenza infection contributes significantly to the development of influenza-induced ALI by enhancing alveolar macrophage and neutrophil infiltration. We found that, compared to wild-type (WT) C57BL/6 control mice, influenza A/WSN/33 (H1N1) virus-infected, C57BL/6-congenic, A_1-AdoR-deficient (A_1-KO) mice and WT mice treated daily with the A_1-AdoR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) exhibited attenuated hypoxemia and pulmonary edema. These effects were associated with reduced alveolar macrophage and neutrophil infiltration into the lungs and lower gamma interferon (IFN-γ) and interleukin 10 (IL-10) responses to infection. Furthermore, reciprocal bone marrow transfer (BMT) studies indicated that expression of A_1-AdoRs by myeloid cells was required for leukocyte recruitment, while both stromal and myeloid cells from A_1-KO mice were necessary for full attenuation of influenza A virus-induced ALI. Our findings indicate that activation of alveolar macrophage and neutrophil A_1-AdoRs by adenosine plays a prominent role in their recruitment to the lung and contributes significantly to development of ALI following influenza A virus infection. Furthermore, they suggest that A_1-AdoR antagonists may be of therapeutic value under this condition.

(Some of the data reported herein were previously presented at the 2011 and 2012 International Conferences of the American Thoracic Society.)

MATERIALS AND METHODS

Animals. C57BL/6AnNCr WT mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6-congenic A_1-KO mice were kindly supplied by Jurgen Schnermann (NIDDK, Bethesda, MD). Offspring were genotyped as previously described (21). Animals were maintained in autoclaved microisolators and given sterile food and water *ad libitum*. All studies were performed in strict accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (22). All experimental animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee, and studies were exempted from a requirement to remove animals that had experienced greater than 20% weight loss. However, ethical considerations precluded performance of survival studies, and every effort was made to minimize animal suffering.

Preparation of viral inoculum. All studies used influenza A/WSN/33 (H1N1) virus. A/WSN/33 is a mouse-adapted influenza A strain that is pneumotropic following intranasal inoculation (23). Virus was grown in embryonated chicken eggs according to a standard protocol. Virus preparations were checked for the absence of contamination with *Mycoplasma pulmonis* by PCR (Charles River Research Animal Diagnostic Services, Wilmington, MA). The absence of endotoxin contamination was confirmed by a standard *Limulus* amebocyte assay (Lonza, Basel, Switzerland).

Mouse inoculation. Eight- to 12-week-old mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (8.7 mg/kg of body weight)-xylazine (1.3 mg/kg) and then inoculated intranasally with 10,000 PFU of influenza A/WSN/33 (H1N1) virus in 50 μl phosphate-buffered saline (PBS)–0.1% bovine serum albumin (BSA), as in our previous studies (6). In our hands, this inoculum induces severe hypoxemia and ALI in WT mice by 2 days postinfection (p.i.) and results in 100% mortality by 8 days p.i. (median time to death, 7 days) (6, 7).

Following virus inoculation, mice were individually marked and then monitored daily. Conscious mice were weighed every other day following infection, and carotid arterial O_2 saturation was recorded by pulse oximetry, as in our previous studies (7, 24). Data for each experimental group were derived from at least three independent infections.

Western blot for influenza nucleoprotein. WT and A_1-KO mice were euthanized by i.p. injection of ketamine (87 mg/kg)-xylazine (13 mg/kg). Lungs and olfactory bulb/forebrain tissue were removed from each mouse and separately homogenized in cell lysis buffer 9803 (Cell Signaling Technology, Danvers, MA), using the FastPrep-24 bead beater system (MP Biomedicals, Santa Ana, CA). Lysates were centrifuged to remove debris, and 30 μg of protein per sample was loaded onto a 4-to-12% gradient polyacrylamide gel. Western blotting was performed by a standard protocol. H1N1 influenza virus nucleoprotein was detected using a rabbit polyclonal antibody (GenScript, Piscataway, NJ). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin were detected using a rabbit polyclonal antibody (sc-25778; Santa Cruz Biotechnology, Santa Cruz, CA) and a mouse monoclonal antibody (sc-47778; Santa Cruz Biotechnology), respectively. Bound primary antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (7074; Cell Signaling Technology) and anti-mouse IgG (7076; Cell Signaling Technology), as appropriate, and developed using ECL Western blotting substrate (Thermo Scientific, Rockford, IL). Densitometry of immunoreactive bands was performed on scanned film images using ImageJ software. Data were normalized to those for GAPDH, since we have previously found this to be a more consistent loading control for lung than β-actin.

Bronchoalveolar lavage. Mice were euthanized as described above, the trachea was exposed surgically, and a trimmed sterile 18-gauge intravenous catheter was inserted caudally into the lumen. The lungs were then lavaged *in situ* with 0.8 ml of sterile saline. Cell viability was determined by trypan blue exclusion, and cell types were differentiated on cytospin preparations using Wright-Giemsa stain. Cell differentials were determined from at least 200 leukocytes using standard hematological criteria.

Measurement of BALF adenosine. Lungs of euthanized mice were lavaged three times with 0.8 ml PBS containing the adenosine deaminase inhibitor EHNA [erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride; 2.5 μM] and the nucleotide transport inhibitor dipyridamole (250 μM). BALF adenosine content was measured by high-performance liquid chromatography (HPLC) (25).

Measurement of BALF mediators. BALF protein content was determined by a standard bicinchoninic acid (BCA) assay. Lactate dehydrogenase was measured by colorimetric assay (Cayman Chemical, Ann Arbor, MI). Murine IFN-γ, IL-1β, IL-6, IL-10, IL-12, KC (CXCL-1), and tumor necrosis factor alpha (TNF-α) levels were quantified by an ultrasensitive mouse proinflammatory multiplex electro-chemiluminescence assay (Meso Scale Discovery, Gaithersburg, MD). Murine IFN-α, IP-10 (CXCL-10), MCP-1 (CCL-2), and RANTES (CCL-5) levels were measured using Quantikine enzyme-linked immunosorbent assay (ELISA) kits (all from R & D Systems, Minneapolis, MN). All assays were performed in accordance with the manufacturers' instructions.

Lung wet/dry weight ratio. The lung wet/dry weight ratio was measured as previously described (26). Briefly, mice were euthanized and exsanguinated and their lungs removed, weighed, and dried in an oven at 55°C for 3 days. After drying, the lungs were weighed again. The wet weight/dry weight ratio was then calculated as an index of intrapulmonary fluid accumulation, without correction for blood content.

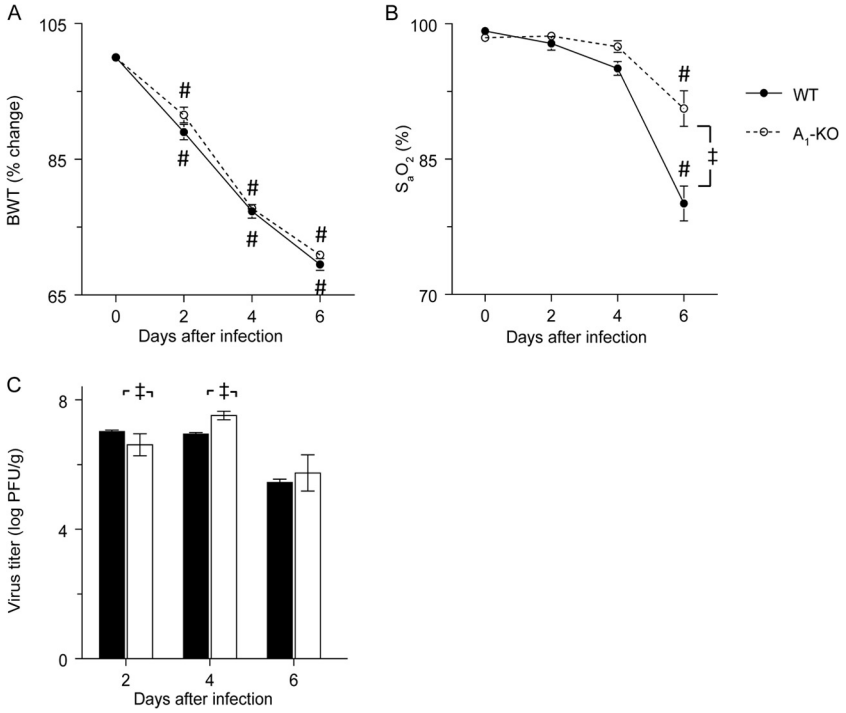

FIG 1 Hypoxemia, but not viral replication, was significantly attenuated in influenza A virus-infected A_1-KO mice. Effects of intranasal infection of wild-type C57BL/6 (WT) and A_1-adenosine receptor-knockout (A_1-KO) mice with 10,000 PFU/mouse of influenza A/WSN/33 (H1N1) virus on body weight (BWT [percent change from day 0]; $n > 25$ per group) (A), carotid arterial oxygen saturation (percent S_aO_2; $n > 10$ per group) (B), and lung homogenate viral titers (log PFU/g; $n = 5$ to 10 per group) (C). #, $P < 0.001$ versus value for uninfected WT mice; ‡, $P < 0.005$ versus value for WT mice at the same time point. Data are presented as means ± SEM.

Measurement of lung mechanics. Mechanical properties of mouse lungs were assessed using the forced-oscillation technique (27), as in our previous studies (28). Briefly, mice were anesthetized with valium (5 mg/kg, intraperitoneally [i.p.]) followed by ketamine (200 mg/kg, i.p.) 6 min later. Once the mice were at a surgical plane of anesthesia (assessed by toe pinch), the trachea was exposed, a tracheotomy performed, and a trimmed sterile 18-gauge intravenous catheter inserted caudally into the lumen. Pancuronium was then administered (0.08 μg/kg i.p.). Tracheotomized mice were then mechanically ventilated on a flexiVent computer-controlled piston ventilator (SciReq, Montreal, Canada), with an 8-ml/kg tidal volume, at a frequency of 150 breaths/minute, against 2 to 3 cm H_2O positive end-expiratory pressure (PEEP). Following two total lung capacity maneuvers to standardize volume history, volume-stepped discontinuous pressure-volume loops were implemented to derive static lung compliance values. Subsequently, pressure and flow data (reflective of airway and tissue dynamics) were collected during a series of 10 consecutive repeats of a standardized volume perturbation maneuver. These data were used to calculate lung resistance, static lung compliance, and dynamic lung compliance at baseline, using the single-compartment model (27). As in prior studies, maximal airway responsiveness to a bronchoconstrictor was subsequently measured following exposure to increasing doses of nebulized methacholine (0.1 to 50 mg/ml) (29).

Bone marrow transfer. Low-density bone marrow was isolated from hind limb long bones of euthanized donor mice by flushing them with conditioned medium. Bone marrow-recipient mice were irradiated with 1,000 cGy given in 2 doses by a ^{137}Cs irradiator, 24 h prior to transplantation. Cells (5×10^6 in 0.5 ml) were transplanted by tail vein injection into recipient mice, which were placed on Baytril water bottles 2 to 3 weeks peri-irradiation. This protocol resulted in no outward clinical signs.

DPCPX treatment regimen. Beginning 1 day prior to infection, WT mice were treated daily with 1 mg/kg of the specific A_1-AdoR antagonist DPCPX (Tocris Bioscience, Ellisville, MO) by i.p. injection in 100 μl saline (30). Controls received an equal volume of the vehicle. Preparation of tissues for histopathologic evaluation and measurement of viral titers were performed as previously described (6, 7).

Statistical analyses. Descriptive statistics (means and standard errors of the means [SEM]) were calculated using Instat software (GraphPad, San Diego, CA). Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. Between-group comparisons were made by analysis of variance (ANOVA), with a Tukey-Kramer multiple-comparison posttest. All data are presented as means ± SEM. A P of <0.05 was considered statistically significant.

RESULTS

Hypoxemia, but not viral replication, was significantly attenuated in influenza A virus-infected A_1-KO mice. Uninfected adult A_1-KO mice were phenotypically normal. Following intranasal infection with influenza A/WSN/33 virus (10,000 PFU/mouse), the rates of postinfection weight loss did not differ between WT and A_1-KO mice (Fig. 1A). However, hypoxemia was less severe in A_1-KO mice at 6 days p.i. (Fig. 1B). Lung homogenate viral titers were approximately 0.5 log lower at 2 days p.i. and 0.5 log higher at 4 days p.i. in A_1-KO mice (Fig. 1C). Titers did not differ between mouse strains at 6 days p.i.

Intranasal inoculation of WT or A_1-KO mice with 10,000 PFU/mouse influenza A/WSN/33 (H1N1) virus does not result in viral replication in the brain. Influenza A/WSN/33 virus has been shown to be capable of replicating in the olfactory bulb and forebrain under some experimental conditions. However, we found by Western blotting that expression of H1N1 influenza nucleoprotein was present in lung homogenates but absent from brain homogenates in both WT and A_1-KO mice at 6 days p.i. (Fig. 2A and B).

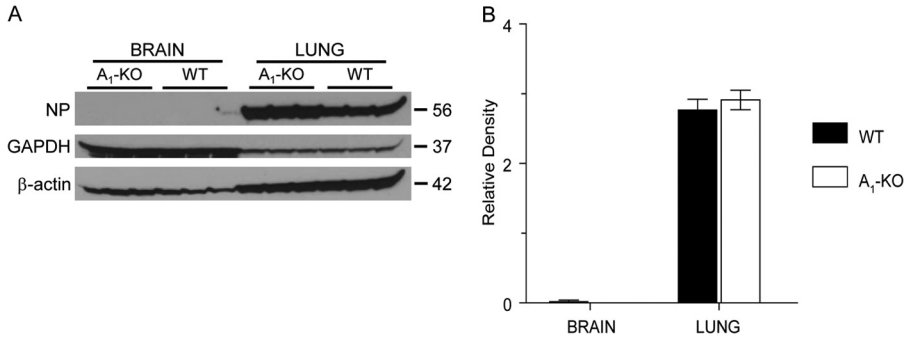

FIG 2 Intranasal inoculation of WT or A_1-KO mice with 10,000 PFU/mouse influenza A/WSN/33 (H1N1) virus does not result in viral replication in the brain. (A) Western blot for H1N1 influenza nucleoprotein (NP) in brain and lung homogenates from 3 A_1-KO mice and 3 WT mice at 6 days p.i. Probing for GAPDH and β-actin (which appear to be differentially expressed between lung and brain) demonstrated that protein loadings were comparable between groups. (B) Relative expression of NP in lung and brain homogenates from WT and A_1-KO mice (normalized to GAPDH). Data are presented as means ± SEM.

Influenza A virus infection induced an adenosine response in the lung. Previously, we showed that BALF ATP content is increased in influenza A WSN/33 (H1N1) virus-infected mice (6, 7). Using specific inhibitors, we also showed that bronchoalveolar epithelial A_1-AdoRs are activated following infection (6). However, we did not formally demonstrate that BALF adenosine levels were higher in influenza A virus-infected mice in those studies. BALF adenosine contents did not differ between uninfected WT and A_1-KO mice (Fig. 3). Infection resulted in comparable increases in BALF adenosine in both strains at 2 and 6 days p.i. BALF adenosine was higher at 6 days p.i. than at 2 days p.i. in both WT and A_1-KO mice, although this effect was not statistically significant.

Pulmonary leukocyte infiltration in response to influenza A virus infection was decreased in A_1-KO mice. Adenosine has been shown to be a potent leukocyte chemoattractant factor (16). Given the high levels of adenosine in BALF from influenza A virus-infected WT and A_1-KO mice, we investigated the role of adenosine in the pulmonary leukocyte response to influenza virus infection. BALF total cell numbers did not differ between uninfected WT and A_1-KO mice, and over 95% of cells in BALF from both mouse strains were alveolar macrophages (Fig. 4A). Neutrophil counts increased significantly at 2 days p.i. in WT mice, and a modest increase in lymphocyte numbers was found in WT mouse BALF at this time point (Fig. 4B and C, respectively). However, neutrophil and lymphocyte levels did not increase in A_1-KO mice at 2 days p.i., and both remained significantly lower than in WT mice. At 6 days p.i., there were very significant differences in BALF leukocyte numbers between strains. BALF alveolar macrophage, lymphocyte, and neutrophil counts increased dramatically from day 2 levels in WT mice. In contrast, relative to numbers in uninfected A_1-KO mice, numbers of alveolar macrophages did not change and numbers of lymphocytes increased only modestly in A_1-KO mice at 6 days p.i. In addition, BALF neutrophil counts were almost 12-fold lower than in WT controls at this time point. The difference in neutrophil responses between the two strains was reflected in measurements of whole-lung myeloperoxidase activity, which increased in WT controls by 70% at 6 days p.i. (Fig. 4D). In contrast, lung myeloperoxidase activity did not increase in A_1-KO mice at 2 days p.i. and modestly decreased at 6 days p.i., although this effect was not statistically significant. Finally, unlike BALF leukocyte counts, peripheral blood total cell, monocyte, lymphocyte, or neutrophil counts did not differ between WT and A_1-KO mice at 6 days p.i. (Table 1).

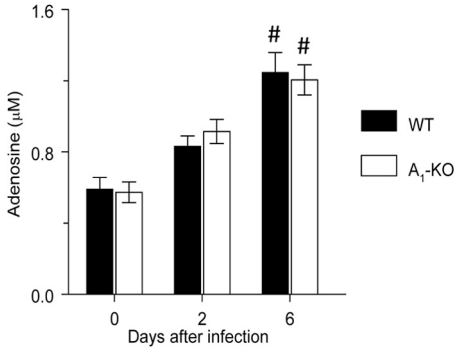

FIG 3 Influenza A virus infection induced an adenosine response in the lung. Effect of influenza A/WSN/33 (H1N1) virus infection of WT and A_1-KO mice on bronchoalveolar lavage fluid (BALF) adenosine content (μM) ($n = 5$ per group). #, $P < 0.001$ versus the value for uninfected WT mice. Data are presented as means ± SEM.

The severity of lung pathology was reduced in influenza A virus-infected A_1-KO mice. Compared to lungs from uninfected WT controls (Fig. 5A), lungs from uninfected A_1-KO mice were histologically normal (Fig. 5E). At 2 days p.i., moderate interstitial pneumonitis was present in both strains (Fig. 5B and F). By 6 days p.i., WT mice had developed severe bronchiolar and peri-bronchiolar neutrophilic inflammation, together with marked bronchiolar epithelial necrosis (Fig. 5C). Marked alveolar septal inflammation was also present in WT mice at this time point, and large numbers of neutrophils and alveolar macrophages were visible within alveoli, which were often filled with protein-rich edema fluid (Fig. 5D). Interstitial pneumonitis was significantly attenuated in lungs from A_1-KO mice at 6 days p.i., and fewer infiltrating leukocytes were visible in both the peri-bronchiolar and alveolar spaces of A_1-KO mice than in those of WT mice (Fig. 5G and H). Histopathologic findings thus support results of BALF leukocyte studies.

BALF cytokine and chemokine responses to infection with influenza A virus were significantly altered in A_1-KO mice. Minimal levels of cytokines and chemokines were detectable in BALF from uninfected WT or A_1-KO mice (Table 2). BALF IFN-α, IL-6, IP-10 (CXCL-10), KC (CXCL-1), and RANTES (CCL-5) in-

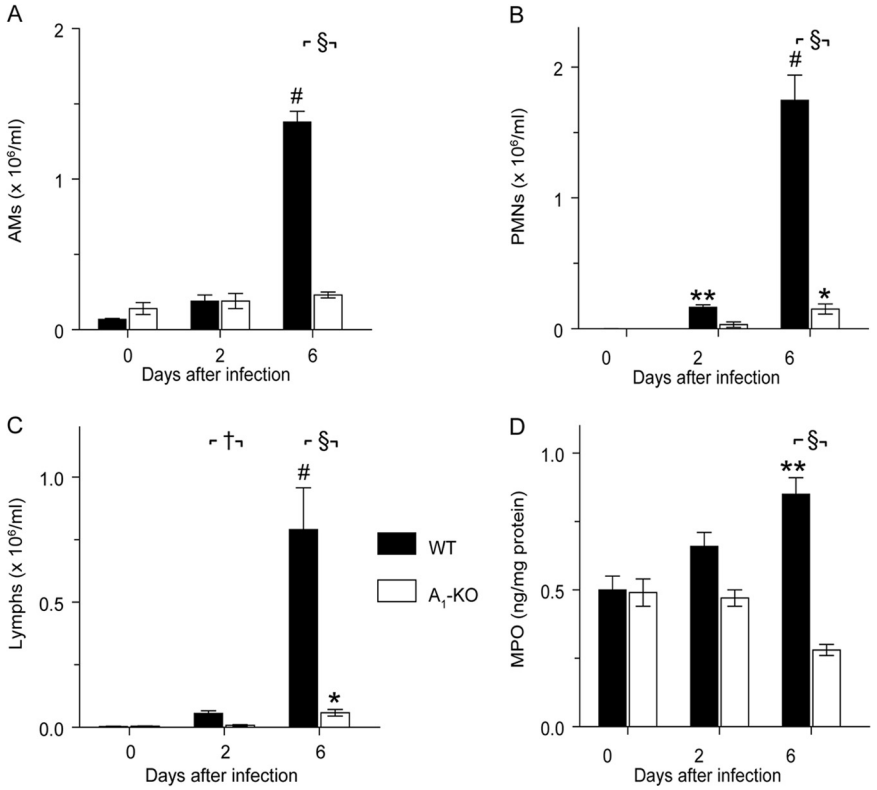

FIG 4 Pulmonary leukocyte infiltration in response to influenza A virus infection was decreased in A_1-KO mice. Effects of influenza A/WSN/33 (H1N1) virus infection of WT and A_1-KO mice on BALF alveolar macrophages (AMs) (A), BALF neutrophils (PMNs) (B), BALF lymphocytes (Lymphs) (C), and lung homogenate myeloperoxidase (MPO) activity (D) ($n = 6$ to 10 per group). *, $P < 0.05$; **, $P < 0.005$; #, $P < 0.001$ versus values for uninfected WT mice. †, $P < 0.05$; §, $P < 0.001$ versus values for WT mice at the same time point. Data are presented as means ± SEM.

creased comparably in WT and A_1-KO animals at 2 days p.i. In contrast, A_1-KO mouse BALF contained significantly more MCP-1 (CCL-2) than WT mouse BALF at 2 days p.i. Unsurprisingly, BALF IFN-γ and IL-10 levels remained very low in both WT and A_1-KO mice at 2 days p.i.

At 6 days p.i., BALF IFN-α content was very low in both strains. WT and A_1-KO mouse BALF contained high levels of IFN-γ and IL-10, but levels of both cytokines were significantly lower in A_1-KO mice than in WT controls. As at 2 days p.i., levels of BALF IL-6 and RANTES did not differ between mouse strains at 6 days p.i. However, and despite lower BALF leukocyte counts, IP-10, KC, and MCP-1 concentrations were significantly higher in BALF from A_1-KO mice than in that from WT animals at this time point.

Influenza A virus-infected A_1-KO mice were partially protected from pulmonary edema and bronchoalveolar epithelial injury. Lung water content (wet weight/dry weight ratio) was modestly but significantly elevated in WT but not in A_1-KO mice at 2 days p.i. (Fig. 6A). At 6 days p.i., a further increase in wet weight/dry weight ratios was observed in both strains, although this was attenuated in A_1-KO mice. In both WT and A_1-KO mice, infection was accompanied by an increase in BALF protein content at 6 days p.i., which is indicative of increasing damage to the bronchoalveolar epithelial barrier (Fig. 6B). However, BALF protein levels remained lower in A_1-KO mice than in WT controls at this time point.

Lung compliance and airway resistance were not altered by influenza A virus infection in A_1-KO mice. Static and dynamic lung compliance are indices of lung tissue stiffness and resistance to inflation on inspiration, which are measured at a fixed lung volume and during normal ventilation, respectively. A decrease in lung compliance is one of the defining characteristics of ALI (31). Levels of static and dynamic compliance did not differ between uninfected WT and A_1-KO mice (Fig. 7A and B, respectively). Infection resulted in a progressive decline

TABLE 1 Peripheral blood leukocyte responses to influenza virus did not differ between WT control and A_1-KO mice at 6 days p.i.[a]

Mice	No. of mice	Neutrophils		Monocytes		Lymphocytes	
		%	No. (10^3/μl)	%	No. (10^3/μl)	%	No. (10^3/μl)
WT[b]	4	47.0 ± 2.0	1.7 ± 0.1	4.5 ± 0.3	0.2 ± 0.1	45.2 ± 0.6	1.5 ± 0.1
A_1-KO[c]	6	45.2 ± 2.8	2.0 ± 0.2	6.7 ± 0.6*	0.3 ± 0.1	41.2 ± 1.9	1.9 ± 0.4

[a] Percentages and numbers of cells are means ± SEM (to the nearest significant figure). *, $P < 0.05$ versus values for WT mice (by unpaired Student's *t* test). p.i., postinfection.
[b] Wild-type C57BL/6 mice.
[c] C57BL/6-congenic A_1-adenosine receptor-knockout mice.

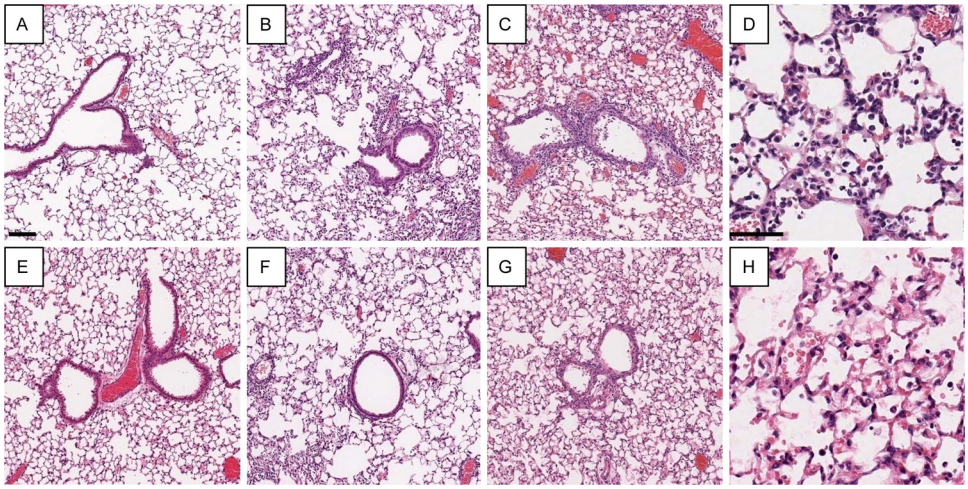

FIG 5 Severity of lung pathology was reduced in influenza A virus-infected A_1-KO mice. Representative photomicrographs of lung histology in an uninfected WT mouse (A), a WT mouse at 2 days p.i. (B), a WT mouse at 6 days p.i. (C), a WT mouse (same as in panel C) at 6 days p.i. (D), an uninfected A_1-KO mouse (E), an A_1-KO mouse at 2 days p.i. (F), an A_1-KO mouse at 6 days p.i. (G), and an A_1-KO mouse (same as in panel G) at 6 days p.i. (H). Magnifications, ×10 (A to C and E to G) and ×40 (D and H). Bars, 100 μm (A) and 50 μm (D). All sections were prepared from formalin-fixed, paraffin-embedded tissues and stained with hematoxylin and eosin by standard methods.

in both parameters in WT controls but had no effect in A_1-KO mice.

Basal airway resistance in uninfected WT mice did not differ from that in A_1-KO mice (Fig. 7C). Infection induced a significant increase in lung resistance at 6 days p.i. in WT animals but had no such effect in A_1-KO mice. Finally, airway hyperresponsiveness to escalating doses of methacholine was present at 2 days p.i. in WT controls but not in A_1-KO mice (Fig. 7D).

Reciprocal bone marrow transfer altered the outcome of influenza A virus infection. To determine whether attenuation of leukocyte recruitment and influenza A virus-induced ALI was a consequence of altered stromal or myeloid cell function in infected A_1-KO mice, we performed reciprocal BMTs between WT and A_1-KO mice and then inoculated each animal with influenza A virus at 6 weeks postengraftment. WT-to-WT BMT was performed to control for any effects of irradiation and BMT procedures. We found that WT-to-WT BMT had no significant impact on influenza outcome measures at 6 days p.i. (Fig. 8). A_1-KO–to–WT BMT did not alter the degree of postinfection weight loss, but weight loss was reduced in WT–to–A_1-KO BMT mice (Fig. 8A). Carotid arterial O_2 saturation decreased to WT levels following infection of both WT–to–A_1-KO and A_1-KO–to–WT BMT mice (Fig. 8B). Likewise, influenza A virus infection induced a significant increase in lung resistance and a significant decrease in static lung compliance at 6 days p.i. in mice that had undergone either WT–to–A_1-KO or A_1-KO–to–WT BMT (Fig. 8C and D, respectively). BALF alveolar macrophage and neutrophil counts at 6 days p.i. increased to WT levels in mice that underwent WT–to–A_1-KO BMT but did not differ significantly from A_1-KO levels in A_1-KO–to–WT BMT animals (Fig. 8E and F, respectively). However, BALF KC content (which was higher in A_1-KO mice than in WT controls) did not differ from that in WT mice in both WT–to–A_1-KO and A_1-KO–to–WT BMT mice at 6 days p.i. (Fig. 8G). Finally, neither WT–to–A_1-KO BMT nor A_1-KO–to–WT BMT had any effect on viral titers at 6 days p.i. (Fig. 8H).

Treatment with the A_1-AdoR antagonist DPCPX attenuated leukocyte infiltration and influenza-induced ALI in WT mice. To determine whether A_1-AdoR blockade might have therapeutic potential, mice were treated daily with the specific A_1-AdoR antagonist DPCPX (1 mg/kg, i.p.) from 24 h prior to infection. DPCPX significantly reduced the rate of postinfection weight loss in WT mice (Fig. 9A). Treatment with saline vehicle had no such effect. DPCPX- but not saline-treated animals also developed less severe hypoxemia than untreated animals at 6 days p.i. (Fig. 9B).

TABLE 2 Bronchoalveolar lavage fluid cytokine and chemokine responses to influenza were significantly altered in A_1-KO mice

Day p.i.[a]	Mice[b]	No. of mice	Mean concn (pg/ml) ± SEM[c]							
			IFN-α	IFN-γ	IL-6	IL-10	IP-10	KC	MCP-1	RANTES
0	WT	5	11 ± 1	<10	<10	17 ± 3	<10	<10	20 ± 1	<10
	A_1-KO	4	<10	<10	14 ± 2	25 ± 7	<10	15 ± 2	34 ± 4	<10
2	WT	8	454 ± 45	18 ± 3	560 ± 61	55 ± 8	444 ± 20	309 ± 52	824 ± 85	155 ± 23
	A_1-KO	6	470 ± 87	49 ± 8	363 ± 70	20 ± 4	529 ± 23	359 ± 76	2,289 ± 579#	126 ± 15
6	WT	6	18 ± 5	4,268 ± 1,148	476 ± 40	824 ± 72	2,921 ± 241	167 ± 16	2,323 ± 136	315 ± 29
	A_1-KO	9	28 ± 16	538 ± 83**	550 ± 121	209 ± 26#	6,047 ± 385#	725 ± 104#	3,380 ± 121#	304 ± 55

[a] p.i., postinfection.
[b] A_1-KO, C57BL/6-congenic A_1-adenosine receptor-knockout mice; WT, wild-type C57BL/6 mice.
[c] **, $P < 0.005$; #, $P < 0.001$ versus values for WT mice at the same time point (by ANOVA).

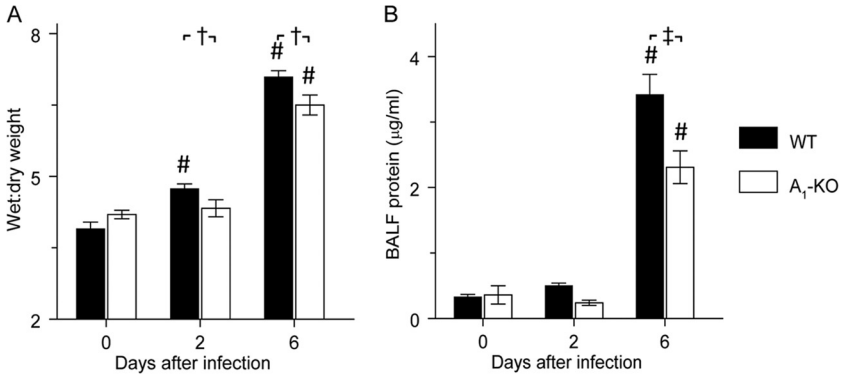

FIG 6 Influenza A virus-infected A_1-KO mice were partially protected from pulmonary edema and bronchoalveolar epithelial injury. Effects of influenza A/WSN/33 (H1N1) virus infection of WT and A_1-KO mice on lung water content (wet weight/dry weight ratio; $n = 7$ to 12 per group) (A) and BALF protein content (μg/ml; $n = 5$ to 10 per group) (B). #, $P < 0.001$ versus values for uninfected WT mice. †, $P < 0.05$; ‡, $P < 0.005$ versus values for WT mice at the same time point. Data are presented as means ± SEM.

BALF alveolar macrophage and neutrophil counts were lower in DPCPX-treated mice at both 2 and 6 days p.i., although this effect was statistically significant only at day 6 (Fig. 9C). In addition, influenza A virus-induced pulmonary edema (wet weight/dry weight) was modestly attenuated by DPCPX treatment at 2 days p.i. and more significantly so at 6 days p.i. but was unaffected by saline administration at either time point (Fig. 9D). Finally, as in A_1-KO mice, BALF IFN-γ and IL-10 levels were significantly lower in DPCPX-treated mice than in untreated controls at 6 days p.i. (Table 3). However, unlike in A_1-KO mice, BALF KC content was not higher in DPCPX-treated mice than in untreated controls. IFN-α was not measured since it is absent from the BALF of both untreated WT and A_1-KO mice at 6 days p.i.

DISCUSSION

Extracellular nucleotides and nucleosides are important signaling molecules in the lung (5, 12). We previously reported that infection with influenza A virus resulted in increased *de novo* nucleotide synthesis and channel-mediated release of ATP and UTP into the BALF (6, 7). In addition, we showed that activation of A_1-AdoRs by adenosine played a key role in the induction of alveolar fluid clearance impairment in influenza A virus-infected mice (6).

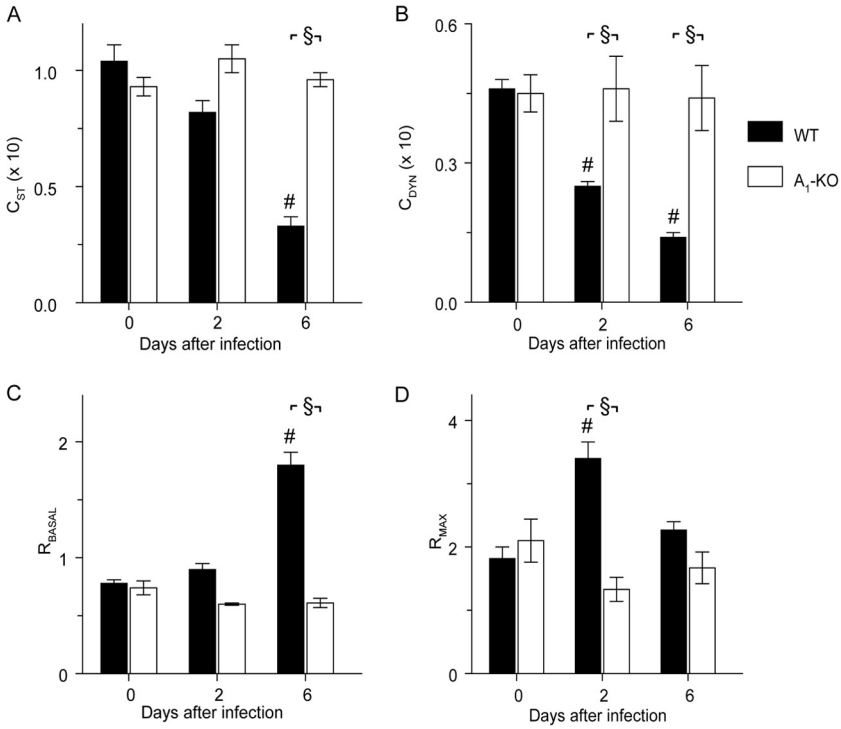

FIG 7 Lung compliance and airway resistance were not altered by influenza A virus infection in A_1-KO mice. Effects of influenza A/WSN/33 (H1N1) virus infection of WT and A_1-KO mice on static lung compliance (C_{ST}; ml/cm H_2O; $n = 6$ to 14 per group) (A), dynamic lung compliance (C_{DYN}; ml/cm H_2O; $n = 6$ to 14 per group) (B), baseline total lung resistance (R_{BASAL}, cm $H_2O \cdot$ s/ml; $n = 6$ to 14 per group) (C), and maximal lung resistance following nebulization of 50 mg/ml methacholine (R_{MAX}, cm $H_2O \cdot$ s/ml; $n = 5$ to 9 per group) (D). #, $P < 0.001$ versus values for uninfected WT mice; §, $P < 0.001$ versus values for WT mice at the same time point. Data are presented as means ± SEM.

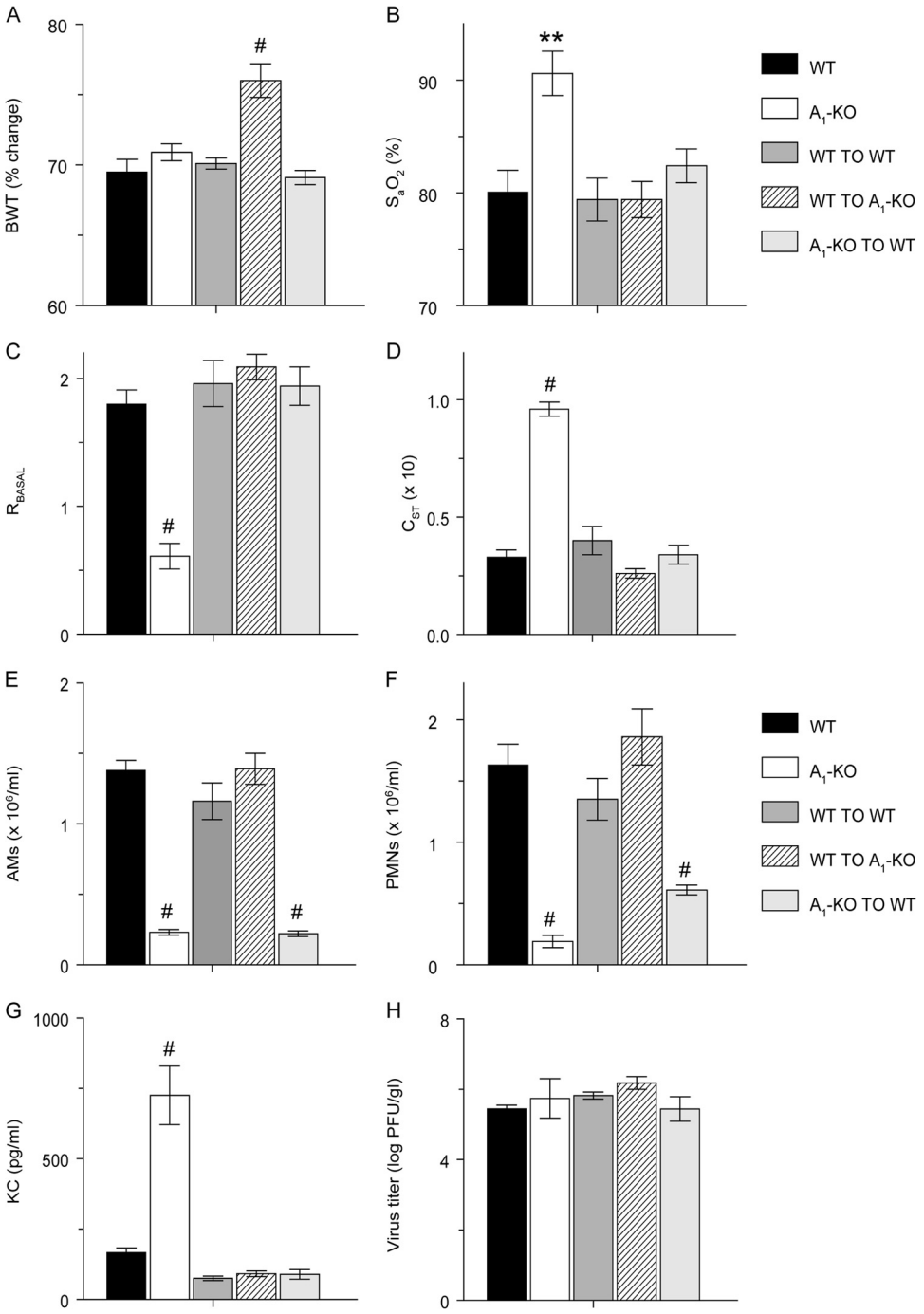

FIG 8 Reciprocal bone marrow transfer altered the outcome of influenza A virus infection. Effects of infection with influenza A/WSN/33 (H1N1) virus for 6 days following reciprocal bone marrow transfer on body weight (BWT; percent change from day 0) (A), carotid arterial oxygen saturation (percent S_aO_2) (B), baseline total lung resistance (R_{BASAL}, cm $H_2O \cdot$ s/ml) (C), static lung compliance (C_{ST}; ml/cm H_2O) (D), BALF alveolar macrophages (AMs) (E), BALF neutrophils (PMNs) (F), BALF KC content (G), and lung homogenate viral titers (H). *n*, >5 per group. **, $P < 0.005$; #, $P < 0.001$ versus values for WT mice. Data are presented as means ± SEM.

Activation of A_1-AdoRs has been shown to have both pro- and anti-inflammatory effects in different experimental models (32). In the current study, we showed for the first time that infection with influenza A virus resulted in significant but comparable increases in BALF adenosine in both WT and A_1-KO mice. However, recruitment of alveolar macrophages, neutrophils, and lymphocytes to the lung was highly attenuated in A_1-KO mice. We also found that influenza-induced hypoxemia and lung dysfunction were less severe in A_1-KO mice. These effects were not associated with significant differences in viral replication between mouse strains. In addition, reciprocal BMT studies showed that expression of A_1-AdoRs by myeloid cells was required for leuko-

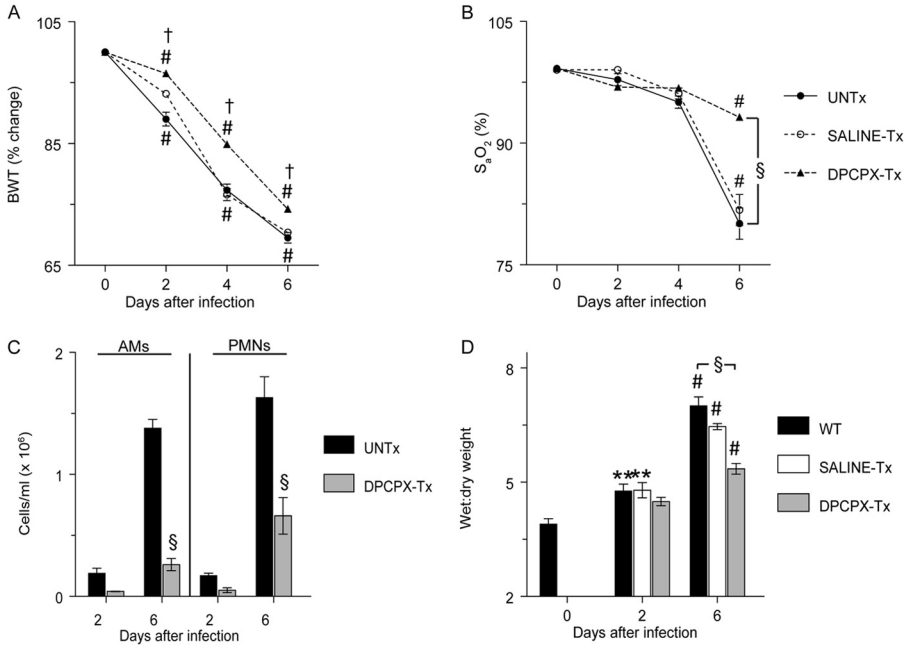

FIG 9 Treatment with the A_1-AdoR antagonist DPCPX attenuated influenza-induced ALI in WT mice. Effects of daily i.p. administration of 100 μl saline vehicle (SALINE-Tx) or 1 mg/kg 8-cyclopentyl-1,3-dipropylxanthine (DPCPX-Tx) in 100 μl saline to WT mice infected with influenza A/WSN/33 virus on body weight (BWT; percent change from day 0; $n > 10$ per treatment group) (A), carotid arterial oxygen saturation (percent S_aO_2; $n > 10$ per treatment group) (B), BALF alveolar macrophages (AMs; $n = 5$ per treatment group) and neutrophils (PMNs; $n = 5$ per treatment group) (C), and lung water content (wet weight/dry weight ratio; $n = 5$ per treatment group) (D). **, $P < 0.005$; #, $P < 0.001$ versus values for uninfected WT mice. †, $P < 0.05$; §, $P < 0.001$ versus values for untreated WT mice at the same time point. Data are presented as means ± SEM.

cyte recruitment, while attenuation of ALI in A_1-KO mice required the presence of both stromal and myeloid cells of A_1-KO origin. Finally, we found that treatment of WT mice with the A_1-AdoR antagonist DPCPX also attenuated leukocyte infiltration, improved arterial oxygenation, and reduced pulmonary edema. Our results indicate that A_1-AdoRs have a significant and previously unrecognized leukocyte chemotactic role in the influenza virus-infected lung and have potential as a therapeutic target in patients with severe influenza-induced ALI.

Despite significant attenuation of disease severity in influenza A virus-infected A_1-KO mice relative to the disease severity in WT controls, we found no differences in either the rates or the magnitudes of body weight loss between the two strains. In contrast, weight loss was reduced in WT–to–A_1-KO BMT mice and DPCPX-treated WT mice, although the reason for these effects is unclear. However, we believe that these commonly used outcome measures for influenza studies in the mouse model provide little information regarding the severity of influenza-induced lung dysfunction and ALI, which is the focus of our studies (7, 28). Even in the most severe cases, influenza virus-infected human subjects do not experience the extremely rapid, severe, and probably intrinsically lethal weight loss observed in influenza virus-infected mice (≥30% in 6 days). Indeed, weight loss and mortality in mice have been shown to be poorly correlated with human ALI outcomes and are often not of predictive value in determining clinical efficacy of novel ALI therapeutics (33). The rapid onset of severe cachexia following infection may therefore simply be an intrinsic limitation of the mouse as a model for studying the pathogenesis of influenza-induced ALI. Hence, we propose that assays of murine pulmonary function that reflect those used to diagnose and determine the severity of ALI in human patients are the most pertinent to understanding the pathogenesis of influenza-induced ALI.

TABLE 3 Treatment of C57BL/6 mice with the A_1-AdoR antagonist DPCPX significantly altered bronchoalveolar lavage fluid cytokine responses to influenza virus at 6 days p.i.[a]

Mice	No. of mice	Concn (pg/ml) of:			
		IFN-γ	IL-6	IL-10	KC
Untreated	4	5,560 ± 1,277	476 ± 40	936 ± 16	167 ± 16
DPCPX treated	4	1,487 ± 648*	450 ± 122	139 ± 26#	127 ± 28

[a] DPCPX treatment was 1 mg i.p. 8-cyclopentyl-1,3-dipropylxanthine/kg daily in 100 μl saline. Data are means ± SEM (to the nearest significant figure). *, $P < 0.05$; #, $P < 0.001$ versus untreated mice (by ANOVA).

The development of hypoxemia in WT mice was rapid and comparable to that in our previous experiments (6, 7). Hypoxemia was attenuated in both A_1-KO mice and DPCPX-treated WT mice at 6 days p.i. However, hypoxemia attenuation was greater in DPCPX-treated WT mice than in A_1-KO mice, and this was associated with a greater reduction in lung water content than that observed in A_1-KO mice at 6 days p.i. This suggests that the degree of impairment of gas exchange following influenza infection may depend upon the severity of interstitial pulmonary edema. However, pulmonary edema remained severe in both A_1-KO and DPCPX-treated WT mice at 6 days p.i. This finding contrasts with the results of our previous studies, in which attenuation of influenza-induced ALI was strongly correlated with reduced lung wet weight/dry weight ratios (7, 24). The disconnect between the significant improvement in peripheral oxygenation and the modest

decrease in lung edema in A_1-KO mice may reflect improved postinfection ventilation/perfusion matching relative to that in WT mice. Adenosine is known to stimulate systemic and pulmonary arterial vasodilation via A_{2a}- and A_{2b}-AdoRs (34, 35) but can also induce systemic and pulmonary arterial vasoconstriction via activation of A_1-AdoRs (36–38). Thus, genetic deletion or pharmacologic antagonism of A_1-AdoRs is predicted to promote pulmonary arterial vasodilation and thereby improve pulmonary perfusion and ventilation/perfusion matching.

As in our previous studies (7, 24), we found that attenuation of ALI in A_1-KO mice or DPCPX-treated WT mice was not associated with significant alterations in viral replication. Moreover, viral titers were unaffected by reciprocal BMT. This indicates that A_1-AdoR function does not impact viral replication and also shows that reduced leukocyte recruitment and ALI severity in A_1-KO mice or DPCPX-treated WT mice was not a result of reduced viral burden. These results are in agreement with those of several previous studies in which neutrophil depletion did not alter viral replication or body weight loss (39–42). However, macrophage depletion studies (including our own) have generally shown that macrophage depletion increases viral replication, which contrasts with our current findings (24, 43–46). The underlying reason for this discrepancy is unclear, although it may indicate that, despite being attenuated, the macrophage response to infection in A_1-KO mice may nevertheless be sufficient to control viral replication.

Activation of A_1-AdoRs by adenosine is an important signaling mechanism in the central nervous system (47), and A_1-AdoR activity can regulate blood-brain barrier permeability (48). The influenza A/WSN/33 virus strain is commonly regarded as being neurotropic, and it is therefore possible that some of the effects of A_1-AdoR knockout were an indirect consequence of altered neuronal function. However, having performed a careful review of the pertinent literature, we found that, in the majority of studies in which neural spread of the A/WSN/33 virus has been shown to occur, this was a result of i.p. infection of pregnant dams (49, 50), intranasal infection of immunodeficient mice (23, 51), direct intracerebral inoculation (52, 53), or infection of suckling mice (54–56). Indeed, we were able to identify only two studies that described spread to the brain following intranasal administration of influenza A/WSN/33 virus to adult immunocompetent mice, and in both cases, mice were infected using an inoculum 100 or 500 times greater than that used in our studies (57, 58). In contrast, viral replication could not be detected in the brains of WT mice infected with an influenza A/WSN/33 virus inoculum comparable to ours (23). Nevertheless, to exclude the possibility of a neuronal component to the differential responses of WT and A_1-KO mice to influenza, we performed Western blotting for H1N1 influenza nucleoprotein on lung and brain homogenates. We found that nucleoprotein was expressed at high levels in lung homogenates from both mouse strains but could not be detected in brain homogenates from either WT or A_1-KO mice at 6 days p.i. This finding demonstrates that neurovirulence does not contribute to WSN pathogenesis in our model and suggests that WSN is generally not neurotropic when administered directly to the respiratory tract at low titers.

The roles of alveolar macrophages and neutrophils in influenza progression remain unclear. Increased mortality following infection with highly pathogenic influenza strains has been correlated with a reduced alveolar macrophage response and/or excessive neutrophil infiltrates (19, 39, 42, 43, 59, 60). We found that the protective effects of A_1-AdoR deletion or antagonism in influenza correlated with reduced (rather than increased) BALF alveolar macrophages at 6 days p.i. This suggests that these cells were either unnecessary for protection from ALI in our model or actually contributed to the pathogenesis of ALI in WT mice. We have shown previously that alveolar macrophage depletion did not impact ALI severity in WT mice, which supports the former possibility (24). In contrast, we found that attenuation of influenza A virus-induced ALI and improved lung function in A_1-KO or DPCPX-treated mice was clearly correlated with a very significant reduction in BALF neutrophils at both 2 and 6 days p.i. This is in agreement with the findings from previous studies showing that neutrophil depletion or ablation of neutrophil recruitment to the lungs attenuates influenza-induced ALI (39, 42). Importantly, peripheral blood leukocyte numbers did not differ between WT and A_1-KO mice at 6 days p.i., which indicates that the reduction in BALF leukocytes in influenza A virus-infected A_1-KO mice could not be ascribed to either an intrinsic or an infection-induced defect in myelopoiesis in this mouse strain. Finally, we should note that BALF lymphocyte counts were also very significantly reduced in A_1-KO mice at 6 days p.i. It is therefore possible that attenuation of the lymphocyte response also contributes to protection from influenza-induced ALI, particularly given the known role of T cells in influenza immune pathology (61). Experiments to investigate this aspect of our studies are ongoing.

Pathogens such as *Staphylococcus aureus*, *Bacillus anthracis*, enteropathogenic *Escherichia coli*, *Streptococcus suis*, and *Trichomonas vaginalis* express ectonucleotidases (62–65). Adenosine generated by these enzymes has been shown to promote pathogen survival and to suppress neutrophil activation by stimulating A_2-AdoRs. In this study, we show directly for the first time that adenosine generation can also increase following viral infection. In other models, activation of A_1-AdoRs by adenosine has been shown to enhance neutrophil adhesion to the endothelium, promote macrophage and neutrophil chemotaxis and Fc gamma receptor (FcRγ)-mediated phagocytosis, and increase neutrophil superoxide generation (15, 66). Similarly, our data indicate that activation of A_1-AdoRs by adenosine is a potent stimulus for the recruitment of alveolar macrophages and neutrophils to the lungs of influenza A virus-infected mice; although BALF adenosine levels did not differ between WT and A_1-KO mice at 2 or 6 days p.i., very few neutrophils were present in A_1-KO mouse BALF at 2 days p.i., and alveolar macrophage and neutrophil counts were, respectively, approximately 6- and 12-fold lower in A_1-KO mice than in WT controls at 6 days p.i. Similarly, BALF alveolar macrophage and neutrophil counts were significantly reduced in DPCPX-treated WT mice at 6 days p.i. BMT studies confirmed that expression of A_1-AdoRs by leukocytes was necessary for their recruitment to the lung; WT alveolar macrophages and neutrophils were recruited to the lungs of A_1-KO mice, but alveolar macrophages and neutrophils from A_1-KO mice did not home to WT lung. Most importantly, our findings suggest that A_1-AdoR activation by adenosine may actually be as important to leukocyte chemotaxis as (or more important than) those chemokines which are generally viewed as central to this process; despite attenuation of neutrophil and alveolar macrophage recruitment in A_1-KO mice at 6 days p.i., BALF KC, IP-10, and MCP-1 were actually higher than in WT controls. Likewise, BALF neutrophil counts increased to WT levels in WT–to–A_1-KO BMT mice but not in A_1-KO–

to–WT BMT mice at 6 days p.i., despite BALF from all three groups containing comparable amounts of KC. The presence of higher BALF chemokine levels in A_1-KO mice at 6 days p.i. may reflect a compensatory response to the lack of an adenosine-mediated leukocyte chemoattraction in these animals. Alternatively, BALF chemokine levels may remain high in A_1-KO mice because the reduction in BALF leukocytes effectively results in a net reduction in total lung chemokine receptor levels; since chemokines bound to their receptors are rapidly internalized (67), a reduction in receptor numbers might result in persistence of chemokines in the lung. However, it remains unclear whether adenosine can act as a chemoattractant factor by itself or whether activation of A_1-AdoRs facilitates and/or synergizes with chemokine receptor activation to promote leukocyte recruitment. Given that both receptor types are G protein coupled (68, 69), the latter is a distinct possibility. Finally, lung homogenate assay data suggest that adenosine may also be important to activation of neutrophil myeloperoxidase in influenza; despite a modest, but significant, increase in BALF neutrophils at 6 days p.i. in A_1-KO mice, lung homogenate myeloperoxidase activity was not increased. Hence, in addition to having detrimental effects on bronchoalveolar epithelial ion transport and alveolar fluid clearance in influenza virus-infected mice (6), adenosine may contribute directly to induction of ALI by promoting neutrophil recruitment and activation.

Although our data clearly demonstrated that the presence of A_1-AdoRs on alveolar macrophages and neutrophils was necessary for their recruitment to the lung, results of BMT studies also indicated that attenuation of ALI in influenza A virus-infected A_1-KO mice was not solely a result of a diminished leukocyte response. We found that hypoxemia and lung dysfunction (increased airway resistance and decreased lung compliance) developed in A_1-KO–to–WT BMT mice despite the absence of significant numbers of leukocytes in BALF. This indicates that full attenuation of influenza-induced lung dysfunction and ALI requires the presence of both stromal and myeloid cells from A_1-KO mice. It is possible that ALI developed in A_1-KO–to–WT BMT mice because bronchoalveolar epithelial cells in these animals express A_1-AdoRs, activation of which contributes to impairment of alveolar fluid clearance and development of severe pulmonary edema in influenza A virus-infected WT mice (6). Moreover, since BALF from A_1-KO mice contained as much adenosine as WT BALF at 6 days p.i., it is likely that A_{2a}-, A_{2b}-, and A_3-AdoRs expressed by lung cells were also activated following infection with influenza A virus. In some models of inflammation, A_{2a}-AdoR and A_{2b}-AdoR signaling has been shown to result in inhibition of neutrophil recruitment, degranulation, oxidative burst, and synthesis and release of inflammatory mediators (15). It is therefore possible that A_2-AdoR activation also contributed to attenuation of lung injury in A_1-KO mice. However, we have yet to explore this possibility.

Several investigators have proposed that abnormal cytokine responses and/or development of a "cytokine storm" play a significant role in the pathogenesis of severe influenza (70, 71). Type I IFNs are central to initiation of the innate immune response to influenza virus infection (72). An attenuated type I IFN response has been correlated with increased disease severity in human bronchial epithelial cells infected with a highly pathogenic H5N1 influenza A strain (73). However, BALF IFN-α responses did not differ between WT and A_1-KO mice at either 2 or 6 days p.i. In contrast, we found that ALI attenuation in both A_1-KO and DPCPX-treated mice was associated with reductions in BALF IFN-γ and IL-10, but not IL-6, at 6 days p.i. BALF KC was higher at 6 days p.i. in A_1-KO mice than in DPCPX-treated WT animals. Moreover, chemokine responses to influenza were greater in A_1-KO mice than in WT controls at 6 days p.i. This suggests that only a specific subset of cytokines and chemokines contributes directly to ALI development in influenza A virus-infected mice. Further studies are needed to determine the contribution (if any) of individual soluble mediators of protection from ALI in influenza A virus-infected A_1-KO or DPCPX-treated mice. Moreover, we cannot exclude the possibility that differences in BALF cytokine profiles between WT and A_1-KO mice at 2 and 6 days p.i. do not reflect a temporal shift in the cytokine response to infection rather than an absolute difference in its magnitudes over the entire course of infection. We have shown previously that influenza A virus infection induced *de novo* nucleotide synthesis and channel-mediated release of ATP into the BALF (6, 7). Based on our current results, we propose that released ATP is comparably metabolized in both WT and A_1-KO mice, resulting in similar increases in BALF adenosine content. In WT mice, this newly generated adenosine activates high-affinity A_1-AdoRs, resulting in increased release of proinflammatory mediators, recruitment of large numbers of leukocytes to the lung, and damage to the lung parenchyma. Because neutrophils and macrophages can themselves release ATP (74), their recruitment to the lung may perpetuate a vicious cycle of adenosine generation and A_1-AdoR-mediated inflammation, resulting in severe ALI. In contrast, genetic deletion or pharmacologic inhibition of A_1-AdoR reduces neutrophil recruitment and prevents development of influenza-induced ALI. Our findings are consistent with earlier studies showing that pretreatment with DPCPX prevented the development of ALI in feline models of sepsis and ischemia-reperfusion injury (75). However, we should note that other investigators found that treatment of A_1-KO mice with aerosolized lipopolysaccharide resulted in exaggerated neutrophil recruitment to the lung and increased pulmonary vascular leakage, suggesting an anti-inflammatory role for the A_1-AdoR in that model (76).

Vaccines and neuraminidase inhibitors are the current mainstays of influenza prophylaxis and treatment, respectively (1). However, the 2009 pandemic demonstrated that the effectiveness of annual vaccination programs is limited by difficulties with timely vaccine production and distribution (77), uptake by the public (78), and efficacy (79). Likewise, antiviral drugs are expensive (80), often induce resistance (81), and are generally less effective late in infection (82). Corticosteroids and β-adrenergic agonists generally have very limited beneficial effects on lung function in patients with severe viral ALI (83). Hence, treatment is limited to nonspecific supportive care in intensive care units (84). We are therefore faced with an urgent need for new therapeutics that can either delay the onset of influenza-induced ALI or reduce its severity. Such therapeutics would be particularly useful during pandemics, when critical care facilities are likely to be overloaded (84). Although DPCPX treatment did not reverse influenza A virus-induced pulmonary edema, the beneficial effects of this drug on arterial oxygenation suggest that A_1-AdoR antagonists such as DPCPX may be of some value in this regard, either on their own or as adjuncts to other therapeutic agents. Importantly, additional A_1-AdoR antagonists are in development for other indications, and they appear to be safe and well tolerated in humans (85).

In conclusion, our results show for the first time that activation

of respiratory epithelial and leukocyte A_1-AdoRs by adenosine plays a significant role in neutrophil recruitment to the lung following influenza A virus infection. They also indicate that influenza A virus-induced hypoxemia, pulmonary dysfunction, and ALI were either attenuated or absent in A_1-KO or A_1-AdoR antagonist-treated mice. These effects were correlated with a dramatic reduction in pulmonary neutrophils and alveolar macrophages but were not associated with altered viral replication. Moreover, our findings using DPCPX indicate that A_1-AdoR antagonists show some promise as new therapeutics to retard or ameliorate development of respiratory failure and ALI in influenza virus-infected patients.

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The authors report no conflicts of interest.

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