Macrophage A2A Adenosinergic Receptor Modulates Oxygen-Induced Augmentation of Murine Lung Injury

Neil R. Aggarwal¹, Franco R. D'Alessio¹, Yoshiki Eto¹, Eric Chau¹, Claudia Avalos¹, Adam T. Waickman², Brian T. Garibaldi¹, Jason R. Mock¹, Daniel C. Files^{1,3}, Venkataramana Sidhaye¹, Vsevolod Y. Polotsky¹, Jonathan Powell², Maureen Horton¹, and Landon S. King¹

¹Division of Pulmonary and Critical Care Medicine, Johns Hopkins Asthma and Allergy Center, and ²Department of Hematology–Oncology, School of Medicine, Johns Hopkins University, Baltimore, Maryland; and ³Division of Internal Medicine–Pulmonary, Critical Care, Allergy, and Immunologic Disease, School of Medicine, Wake Forest University, Winston-Salem, North Carolina

Acute respiratory distress syndrome (ARDS) causes significant morbidity and mortality. Exacerbating factors increasing the risk of ARDS remain unknown. Supplemental oxygen is often necessary in both mild and severe lung disease. The potential effects of supplemental oxygen may include augmentation of lung inflammation by inhibiting anti-inflammatory pathways in alveolar macrophages. We sought to determine oxygen-derived effects on the anti-inflammatory A2A adenosinergic (ADORA2A) receptor in macrophages, and the role of the ADORA2A receptor in lung injury. Wild-type (WT) and ADORA2A^{-/-} mice received intratracheal lipopolysaccharide (IT LPS), followed 12 hours later by continuous exposure to 21% oxygen (control mice) or 60% oxygen for 1 to 3 days. We measured the phenotypic endpoints of lung injury and the alveolar macrophage inflammatory state. We tested an ADORA2A-specific agonist, CGS-21680 hydrochloride, in LPS plus oxygen-exposed WT and ADORA2A^{-/-} mice. We determined the specific effects of myeloid ADORA2A, using chimera experiments. Compared with WT mice, ADORA2A^{-/-} mice exposed to IT LPS and 60% oxygen demonstrated significantly more histologic lung injury, alveolar neutrophils, and protein. Macrophages from ADORA2A^{-/-} mice exposed to LPS plus oxygen expressed higher concentrations of proinflammatory cytokines and cosignaling molecules. CGS-21680 prevented the oxygen-induced augmentation of lung injury after LPS only in WT mice. Chimera experiments demonstrated that the transfer of WT but not ADORA2A^{-/-} bone marrow cells into irradiated ADORA2A^{-/-} mice reduced lung injury after LPS plus oxygen, demonstrating myeloid ADORA2A protection. ADORA2A is protective against lung injury after LPS and oxygen. Oxygen after LPS increases macrophage activation to augment lung injury by inhibiting the ADORA2A pathway.

Keywords: acute lung injury; oxygen; A2A adenosinergic receptor; lung injury resolution; ARDS modifiable risk factors

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), have an annual incidence of more

CLINICAL RELEVANCE

Exacerbating factors leading to acute lung injury remain incompletely known. Supplemental oxygen may augment macrophage inflammation by inhibiting anti-inflammatory pathways. Moderate concentrations of supplemental oxygen block the tissue-protective, anti-inflammatory A2A adenosinergic (ADORA2A) receptor in macrophages, to augment their proinflammatory state. ADORA2A-deficient macrophages significantly contribute to augmentations of lung injury in an experimental model.

than 195,000 cases. Despite 35–40% mortality even in tertiary-care center intensive care units, few interventions improve survival (1). Because pulmonary inflammation precedes the onset of clinically defined ARDS, the identification of factors that can accelerate or augment inflammation in the pathogenesis of ARDS could lead to new therapeutic approaches to limit disease incidence and reduce mortality. Patients frequently develop ALI after admission to the hospital (2–5), implying a possibility that potentially modifiable in-hospital exposures could contribute to the development of ALI in predisposed hosts. Supplemental oxygen (40–60%) comprises one such exposure (4–6).

We have shown that the delivery of supplemental oxygen (60%) 12 hours after a direct instillation of low-dose intratracheal lipopolysaccharide (IT LPS) augments murine lung injury (7). Alveolar macrophages exposed to LPS plus 60% oxygen exhibited a proinflammatory phenotype, with an increased expression of cosignaling molecules and reactive oxygen species (ROS) and an increased secretion of proinflammatory cytokines, including neutrophil-recruiting chemokines. We and others have demonstrated the importance of neutrophil alveolitis in experimental models of lung injury (7–9), but factors conducive to a macrophage inflammatory state and that state's direct influence on the influx of neutrophils also comprise an important, yet understudied, area (10). Because of their ubiquitous tissue distribution, macrophages can generate a prompt, robust inflammatory response to a variety of stimuli. However, when inflammation is unregulated, excessive alveolar neutrophil accumulation and lung injury may occur (11–14).

Alveolar macrophages are activated by the engagement of pattern-recognition receptors (15, 16). Classically activated M1-type alveolar macrophages are an important component of the initial innate immune response, and they secrete the proinflammatory cytokines TNF- α and macrophage inflammatory protein 2 (MIP-2) to augment that response. They also express CD86 and CD40, costimulatory molecules capable of regulating inflammation in innate immunity via communication with lymphocytes or neutrophils (17, 18). Janssen and colleagues demonstrated that resident alveolar macrophages demonstrate plasticity through a cycle of inflammation and resolution (10) in a lung injury

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Correspondence and requests for reprints should be addressed to Neil R. Aggarwal, M.D., Division of Pulmonary and Critical Care Medicine, Johns Hopkins Asthma and Allergy Center, School of Medicine, Johns Hopkins University, 5501 Hopkins Bayview Circle, 5B-77, Baltimore, MD 21224. E-mail: naggarw1@jhmi.edu

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model, first manifesting a proinflammatory phenotype critical to the propagation of lung inflammation, but subsequently developing an anti-inflammatory profile crucial for resolution (10, 13, 14, 19, 20). The macrophage response must be tightly controlled, because an excess release of M1-type mediators can lead to host-tissue damage (21, 22). Therefore, the induction of anti-inflammatory macrophage pathways likely occurs soon after the initial inflammatory burst, as a necessary mechanism to counteract and regulate inflammation.

Adenosine released by cells in response to local hypoxia and inflammation binds to adenosine receptors (8, 23–25). The activation of adenosinergic receptors, particularly A2A (ADORA2A) and A2B (ADORA2B), can modulate inflammation and promote tissue protection (8, 25). ADORA2A is one of four G-protein-coupled receptors widely expressed in leukocytes and nonhematopoietic cells, and has the highest affinity for adenosine binding (23–25). Adenosine binding to its receptor increases cyclic adenosine monophosphate (cAMP), leading to an increase in protein kinase A, which inhibits NF- κ B through its scaffold A kinase-anchoring protein 95 (26, 27). Previous work in lung-injury models focused on the protective role of neutrophil ADORA2A (8, 24, 25, 28). Thiel and colleagues demonstrated that supplemental oxygen blunts the hypoxia-induced up-regulation of neutrophil ADORA2A to propagate inflammation (8), but the role of macrophage ADORA2A in modulation of lung injury has not been investigated.

We sought to identify alveolar macrophage-associated mechanisms that regulate the intensity and duration of inflammation. We have shown that supplemental oxygen augments the macrophage M1, proinflammatory state present in LPS-stimulated macrophages. We also know that regulation of the macrophage inflammatory state is critical to controlling tissue inflammation. Because ADORA2A can prevent excessive tissue inflammation, we examine the effects of macrophage ADORA2A in regulating the oxygen-induced augmentation of the macrophage inflammatory state and subsequent lung injury. In this study, we demonstrate that macrophage ADORA2A modulates the oxygen-induced augmentation of lung injury after LPS. Exposure to continuous supplemental oxygen decreased LPS-mediated macrophage ADORA2A activation to promote a proinflammatory M1 phenotype, and to augment lung injury. Mice deficient in ADORA2A demonstrate an exaggerated oxygen-mediated effect, further demonstrating the importance of ADORA2A in regulating the macrophage inflammatory state.

MATERIALS AND METHODS

Animals

Male C57BL/6 wild-type (WT), ADORA2A^{-/-}, and B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) male mice (aged 8–10 wk) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at the Asthma and Allergy Center of Johns Hopkins University. Experiments were performed according to a protocol approved by the Animal Care and Use Committee of Johns Hopkins University.

Animal Injection and Processing

We anesthetized and injected mice as previously described (7, 17). We used *Escherichia coli* LPS (O55:B5, catalogue number L2880; Sigma Chemical Company, St. Louis, MO) at 0.3 μ g/g mouse or sterile water (vehicle control). We processed mice on Days 1 to 3 after LPS exposure, as described elsewhere (7, 17) and in the online supplement.

Oxygen Exposure

Twelve hours after exposure to IT LPS or water, mice were placed in customized, sealed cages with food and water *ad libitum*, and continuous 60% oxygen was delivered as described elsewhere (7) and in the online supplement.

Systemic Delivery of Products (Anti-Myeloid Differentiation Antigen-1 Antibody and CGS-21680)

Neutrophil depletion was performed as previously described (7). WT and ADORA2A^{-/-} animals received 125 μ g/dose/mouse of intraperitoneally injected CGS-21680 hydrochloride (Tocris Bioscience, Bristol, UK) or DMSO at 12 hours (concurrent with the initiation of oxygen exposure), 36 hours, and 60 hours after IT LPS.

Analysis of Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was obtained, total and differential cells were counted, and BAL protein was assessed as described elsewhere (7) and in the online supplement. TNF- α , keratinocyte-derived chemokine (KC), MIP-2, IL-17, and LPS-induced chemokine (LIX) were measured in BAL and the culture medium by ELISA. ROS were measured as described in the online supplement.

Histology and Lung-Injury Scoring

Lungs were processed and assessed for histologic evaluation and scoring, as described elsewhere (7) and in the online supplement.

Macrophage Isolation and Culture

Alveolar or peritoneal macrophages or transformed murine alveolar macrophages (MH-S) cells (CRL-2019; American Type Culture Collection, Manassas, VA) were exposed to LPS (100 ng/ml) and/or 60% oxygen, as described elsewhere (1) and in the online supplement. NF- κ B cell-permeable inhibitor synthetic peptide (SN-50) (P-600, 50 μ g/ml; Enzo, Farmingdale, NY) or control (P-601; Enzo), ADORA2A agonist CGS-21680 (5 μ M; Tocris Bioscience), nonselective adenosine receptor agonist (NECA, 3 μ M; Tocris Bioscience), or dimethylallyl glycine (sham) were used.

Intracellular cAMP and NF- κ B Activity

We assessed intracellular cAMP according to the cAMP enzyme immunoassay protocol (RPN225; GE Healthcare, Piscataway, NJ), and NF- κ B activity according to the Active Motif protocol (p65, 40096; Active Motif, Carlsbad, CA).

Quantitative RT-PCR

Total RNA was isolated and processed for RT-PCR using ADORA2A and ADORA2B primers, as described elsewhere (26, 29) and in the online supplement.

Bone Marrow Isolation, Adoptive Transfer, and Chimeras

We harvested bone marrow (BM) cells from ADORA2A^{-/-} and CD45.1 mice, and recipient mice (5–6 wk) were irradiated and housed as described elsewhere (17) and in the online supplement. Two hours after irradiation, we injected 1.5×10^6 BM cells intravenously (17).

Flow Cytometry

Cells were processed for flow cytometry and analyzed as described elsewhere (7) and in the online supplement, using the antibodies (or relevant isotopes) anti-annexin V-PE, anti-7-AAD, anti-LY6G-FITC, anti-IA-IE-PE, anti-CD11b-APC ϵ 780, anti-CD45.1-PE, anti-CD45.2-PerCy5.5, anti-CD14-FITC, anti-CD86-APC, anti-CD80-PE, and anti-F4/80-APC.

Statistical Analysis

All values are reported as means $\pm$ SEMs. Markers of injury were compared using the Student *t* test or Mann-Whitney rank sum test. Multiple groups were compared using one-way ANOVA or one-way ANOVA on ranks. Pairwise comparisons were performed using the Student *t* test with Bonferroni correction. Statistical analysis was performed using Sigmaplot version 11.0 (Systat Software, Chicago, IL). The survival curve was established with Kaplan-Meier survival analysis. *P* < 0.05 was considered significant.

RESULTS

The Absence of ADORA2A Augments Early Lung Injury after LPS and 60% Oxygen

WT and ADORA2A^{-/-} mice were exposed to IT LPS (0.3 mg/kg/mouse) or water, followed 12 hours later by exposure to supplemental oxygen (60%) or room air, which was continued until the mice were killed. The time was defined by days after IT LPS or IT water. Lung injury at early time points was significantly increased in LPS plus oxygen-exposed ADORA2A^{-/-} mice, compared with

similarly treated WT mice and all other treatment groups (Figure 1). In this group, we observed a significant increase in histologic damage (Figure 1A), BAL protein (Figure 1B), and BAL neutrophils (Figure 1C), compared with all other groups. Differences appeared on Day 2, and persisted on Day 3 for BAL protein and histologic injury after LPS instillation. BAL macrophage numbers appeared to increase in all groups on Day 3, but with no differences between those groups (Figure 1E). The addition of 60% oxygen to IT water did not significantly increase the measured lung injury parameters in WT or ADORA2A^{-/-} mice. On

Figure 1. Anti-inflammatory A2A adenosinergic receptor-deficient (ADORA2A^{-/-}) mice exposed to LPS plus oxygen demonstrated a significant increase in lung injury compared with ADORA2A^{-/-} mice exposed to LPS alone, and compared with wild-type (WT) mice exposed to LPS plus oxygen. Statistics were performed by comparing all groups at the same time point. (A) Histology. Representative low-power (×4) and high-power (inset, ×40) hematoxylin and eosin-stained lung sections on Days 2 and 3 reveal increased alveolar consolidation and interstitial infiltration in ADORA2A^{-/-} mice exposed to LPS plus oxygen. Quantitative assessment using a lung-injury scoring system demonstrated a significant increase in lung injury in ADORA2A^{-/-} mice exposed to LPS plus oxygen, beginning on Day 2 and persisting until Day 3. *On Day 2, significant compared with the WT water (W) + O₂ group. ^On Day 2, significant compared with all other groups. ^On Day 3, significant compared with the WT LPS (L) + O₂ group. (B) Bronchoalveolar lavage (BAL) protein was notable for an early, pronounced increase in ADORA2A^{-/-} mice exposed to LPS plus oxygen. *On Day 1, significant compared with the WT W and ADORA2A^{-/-} W groups. ^On Day 2, significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups. ^On Day 3, significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups. (C) The BAL total cell count was increased in ADORA2A^{-/-} mice exposed to LPS plus oxygen,

compared with all other exposure groups on Day 2. *On Day 1, significant compared with the WT W and ADORA2A^{-/-} W groups. ^On Day 2, significant compared with the WT W + O₂ and ADORA2A^{-/-} W + O₂ groups. ^On Day 2, significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups. ^On Day 3, significant compared with the WT LPS and ADORA2A^{-/-} LPS groups. (D) BAL Neutrophils were also increased in ADORA2A^{-/-} mice exposed to LPS plus oxygen on Day 2. *On Day 1, significant compared with the WT W and ADORA2A^{-/-} W groups. ^On Day 2, significant compared with the WT W + O₂ and ADORA2A^{-/-} W + O₂ groups. ^On Day 2, significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups. ^On Day 3, significant compared with the WT LPS and ADORA2A^{-/-} LPS groups. (E) BAL macrophages did not increase in ADORA2A^{-/-} mice exposed to LPS plus oxygen, compared with all other groups at each time point ($n = 4$ in the W and W + O₂ groups; $n = 6-8$ in the LPS and L + O₂ groups). n.s., no significance.

Days 1 and 2, lung water accumulation was no different in WT and ADORA2A^{-/-} mice. We previously observed an oxygen-induced augmentation of lung water content no earlier than Day 4 (7). ADORA2A^{-/-} mice demonstrated a significant increase in both lung injury and mortality when treated with higher doses of LPS (1–3 mg/kg/mouse; no supplemental oxygen) (Figure E1A in the online supplement), but not at the primary study dose (0.3 mg/kg/mouse). When we depleted neutrophils in ADORA2A^{-/-} mice exposed to LPS plus oxygen, lung injury was significantly abrogated (Figure E1B), and the expression of alveolar macrophage CD86 was reduced (Figure E1C).

ADORA2A-Deficient Alveolar Macrophages Are Proinflammatory after LPS and Oxygen

To begin assessing ADORA2A-mediated differences in alveolar inflammation, we measured proinflammatory cytokines at early time points (Figure 2). BAL TNF- α and MIP-2 were significantly increased in ADORA2A^{-/-} mice exposed to LPS plus oxygen compared with WT mice exposed to LPS plus oxygen, and in comparison with other experimental groups on Day 1

(Figure 2A). MIP-2 is a potent neutrophil chemoattractant primarily secreted by macrophages. In ADORA2A^{-/-} mice exposed to LPS plus oxygen, increased BAL MIP-2 secretion occurred before the increase in alveolar neutrophils observed on Day 2. No difference in BAL MIP-2 or TNF- α was evident between WT and ADORA2A^{-/-} mice exposed to IT water plus 60% oxygen. Other neutrophil chemokines, including IL-17, KC, and LIX, were not increased in ADORA2A^{-/-} mice exposed to LPS plus oxygen; they are primarily secreted by other cell-types, and not macrophages (Figure 2B).

We also assessed ADORA2A-mediated differences in neutrophil and macrophage life spans in the inflammatory milieu of the alveolar space by measuring cell apoptosis, a process known to be crucial in the modulation of inflammation (10, 19). Compared with other groups, BAL neutrophils from ADORA2A^{-/-} mice exposed to LPS plus oxygen exhibited similar apoptotic rates on Days 2 or 3, as assessed by annexin V staining (Figure 2C). In contrast, alveolar macrophages from ADORA2A^{-/-} mice exposed to LPS plus oxygen produced significantly less activated caspase-3, a marker of macrophage apoptosis (10), compared with WT macrophages exposed to LPS plus oxygen (Figure 2D).

Figure 2. Macrophages from ADORA2A^{-/-} mice secrete more neutrophil-recruiting chemokines and are less apoptotic after exposure to LPS plus oxygen, compared with WT mice. Statistics were determined by comparing all groups at the same time point. (A) BAL concentrations of macrophage inflammatory protein-2 (MIP-2) and TNF- α were increased in ADORA2A^{-/-} mice exposed to LPS plus oxygen on Day 1. [^]Significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups. (B) Other chemokines, including keratinocyte-derived chemokine (KC), IL-17, and LPS-induced chemokine (LIX), were not increased in ADORA2A^{-/-} mice exposed to LPS plus oxygen, compared with WT mice. [^]Significant compared with all other groups on BAL KC Day 1. ^{*}Significant compared with the WT W + O₂ group on BAL KC Day 1. ^{*}Significant compared with the WT and ADORA2A^{-/-} W + O₂ groups. (C) Amounts of annexin V, a marker of BAL neutrophil apoptosis, were no different between any of the assessed groups on either Day 2 or Day 3. (D) Amounts of activated caspase-3, a marker of BAL macrophage (F4-80⁺ CD11b⁺) apoptosis, were significantly lower in ADORA2A^{-/-} mice exposed to LPS plus oxygen. [^]Significant difference compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups, and with no difference between the WT LPS and ADORA2A^{-/-} LPS groups ($n = 4$ in the W and W + O₂ groups, and $n = 6-8$ in the LPS and L + O₂ groups). Macs, macrophages.

To assess the macrophage inflammatory state, we measured the intracellular mean fluorescence intensity of proinflammatory cytokines in CD11b⁺ alveolar macrophages (defined by high forward-side scatter and F4-80 positivity; see Figure E2A and Table 1). Others have demonstrated F4-80 to be a reliable alveolar macrophage marker, and CD11b to be a marker of a recruited or exudative (in contrast to resident) macrophages (10, 30, 31). We found that the percentage of F4-80 positivity according to flow cytometry correlates with BAL macrophages identified by cyto-spin and Diff-Quik staining (Figure 1E). The exposure of WT or ADORA2A^{-/-} mice to LPS plus oxygen increased the percentage of CD11b⁺ expression among F4-80⁺ alveolar macrophages, compared with LPS plus room air macrophages (Table 1). BAL macrophages (F4-80⁺ CD11b⁺) from ADORA2A^{-/-} mice exposed to LPS plus oxygen exhibited more monocyte chemoattractant protein-1 (MCP-1) and TNF- α than did WT mice exposed to LPS plus oxygen, or ADORA2A^{-/-} mice exposed to LPS plus room air (Table 1 and Figure 3A; a representative histogram is presented in Figure E2B). Adding 60% oxygen to LPS similarly increased alveolar macrophage ROS production in WT and ADORA2A^{-/-} mice (Figure 3B).

In the alveolar microenvironment, macrophage communication with other cells, including alveolar epithelial cells, recruited monocytes, or neutrophils, may influence the macrophage phenotype (18, 30). To examine specific macrophage effects, we isolated alveolar macrophages (AMs) from unchallenged WT

TABLE 1. EXUDATIVE MACROPHAGES

Day 2 Exposure	BAL Cells		F4-80 ⁺ CD11b ⁺	
	Percent F4-80 ⁺	Percent CD11b ⁺	MCP-1 MFI	TNF- α MFI
WT LPS (L)	13 (1.4)	33 (5.2)	41 (12.7)	53 (2.6)
A2AR ^{-/-} LPS	12 (2.8)	38 (6.3)	46 (3.1)	51 (3.9)
WT L + O ₂	10 (0.7)*	51 (1.7)*	63 (9.3)	68 (2.4)
A2AR ^{-/-} L + O ₂	8 (3.6)*	51 (3.1)*	90 (2.4) [†]	95 (2.9) [†]

Definition of abbreviations: ADORA2A, anti-inflammatory A2A adenosinergic receptor; A2AR^{-/-}, ADORA2A-deficient; BAL, bronchoalveolar lavage; L, lipopolysaccharide; MCP, monocyte chemoattractant protein; MFI, mean fluorescence intensity; WT, wild-type.

Day 2 BAL exudative macrophages (F4-80⁺ CD11b⁺) from ADORA2A^{-/-} mice exposed to LPS plus oxygen produced more TNF- α and MCP-1, as determined by intracellular staining using flow cytometry. Statistics were determined by comparing all groups with one-way ANOVA. Values are listed as averages (SEMs) ($n = 4-6$ in each group).

* Significant compared with the WT LPS and ADORA2A^{-/-} LPS groups.

[†] Significant compared with the WT LPS, ADORA2A^{-/-} LPS, and WT L + O₂ groups.

or ADORA2A^{-/-} mice, and then exposed them to LPS, oxygen, or a combination of both in culture for 12 to 24 hours. At 12 hours, LPS-exposed alveolar macrophages (AMs) from WT and ADORA2A^{-/-} mice produced more TNF- α than did control WT AMs (Figure 3C). The addition of oxygen 3 hours after LPS further increased TNF- α in AMs from ADORA2A^{-/-}

Figure 3. ADORA2A-deficient macrophages exposed to LPS plus oxygen demonstrate increased cytokine production as well as cosignaling molecule expression, compared with WT macrophages. Statistics were performed by comparing all groups at the same time point. (A) MCP-1 and TNF- α were increased in macrophages (F4-80⁺ CD11b⁺) from ADORA2A^{-/-} mice exposed to LPS plus oxygen. *Significant compared with the WT LPS, ADORA2A^{-/-} LPS, and WT L + O₂ groups. (B) As a marker of reactive oxygen species (ROS) production, the 2',7'-dichlorofluorescein (DCF) measured in alveolar macrophages was higher in both WT and ADORA2A^{-/-} mice exposed to LPS and oxygen. *Significant compared with all other groups, with nonsignificant differences between the WT L + O₂ and ADORA2A^{-/-} L + O₂ groups. (C) Using alveolar macrophages (Alv. Macs.) isolated from unchallenged WT or ADORA2A^{-/-} mice and exposed in culture to LPS, 60% oxygen, or the two in combination, we observed the most significant increase in TNF- α and CD86 in macrophages from ADORA2A^{-/-} mice exposed to LPS and oxygen after 12 hours. *For intracellular (IC) TNF- α , significant compared with WT media (M). [^]For IC TNF- α , significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups, with no difference between the ADORA2A^{-/-} LPS, WT LPS, or WT L + O₂ groups. [^]For CD86, significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-}

LPS groups. (D) When assessed after 24 hours of exposure, macrophages from ADORA2A^{-/-} mice exposed to LPS plus oxygen secreted more TNF- α and MIP-2. *Significant compared with WT media. [^]Significant compared with the WT LPS, WT L + O₂, and ADORA2A^{-/-} LPS groups ($n = 5-6$ in each group).

mice, but not in AMs from WT mice. Cosignaling molecule CD86 expression at 12 hours after LPS plus oxygen was increased only in AMs from ADORA2A^{-/-} mice (Figure 3C). Supplemental oxygen did not increase TNF- α or CD86 in media-exposed macrophages from WT or ADORA2A^{-/-} mice. Exposing AMs from WT or ADORA2A^{-/-} mice to LPS for 24 hours significantly increased both TNF- α and MIP-2, compared with media exposure. Adding oxygen to LPS further increased TNF- α and MIP-2 only in AMs from ADORA2A^{-/-} mice (Figure 3D).

Oxygen Suppresses LPS-Induced Augmentation of ADORA2A to Promote Inflammation and Lung Injury

In ADORA2A^{-/-} mice, LPS plus oxygen augments lung injury and the M1 macrophage proinflammatory state. Therefore, we wanted to determine whether ADORA2A manipulation in WT mice might alter lung inflammation. CGS-21680 is a specific ADORA2A agonist that increases ADORA2A pathway activity (32). A daily systemic delivery of CGS-21680 (125 μ g in

100 μ l PBS) beginning at the time of oxygen exposure significantly blunted the oxygen-induced augmentation of lung injury on Day 3 after LPS (Figure 4). We assessed the effects of CGS-21680 on Day 3, when an oxygen-mediated increase in lung injury was most pronounced in WT mice (7). A marked decrease in histologic injury was evident (Figure 4A), as well as a decrease in BAL protein (Figure 4B), and the total alveolar cell count, including both neutrophils and macrophages (Figure 4C). CGS-21680 did not exert a significant effect in LPS-exposed WT mice, or in LPS plus oxygen-exposed ADORA2A^{-/-} mice. The amount of BAL MIP-2 was lower in LPS plus oxygen-exposed WT mice treated with CGS-21680 (Figure 4D), but CGS-21680 did not alter BAL KC secretion (not shown).

We isolated BAL cells to determine the importance of ADORA2A pathway activation in macrophage phenotype. Adding oxygen to LPS decreased the overall percentage of macrophages (F4-80⁺) on Day 3, an effect that was reversed with the addition of CGS-21680 (Table 2). The addition of CGS-21680 also decreased CD11b⁺ expression among F4-80⁺ macrophages (Figure E2A). Furthermore, CGS-21680 induced a significant

Figure 4. CGS-21680 hydrochloride, an ADORA2A-specific agonist, abrogates lung injury in WT mice induced by LPS plus oxygen. To do so, CGS-21680 prevents the oxygen-mediated suppression of the ADORA2A pathway. Statistics were determined by comparing groups with and without CGS-21680 treatment. (A) Representative low-power ($\times 4$) and high-power (*inset*, $\times 40$) hematoxylin and eosin-stained lung sections on Day 3 after intratracheal (IT) LPS demonstrate a CGS-21680-mediated reduction in histologic injury in WT mice exposed to LPS plus oxygen. (B) BAL protein is significantly reduced with the addition of CGS-21680 to WT mice exposed to LPS plus oxygen. *Significant compared with the WT L + O₂ group. No measurable CGS-21680-mediated difference was evident in WT mice exposed to LPS alone, or in ADORA2A^{-/-} mice exposed to LPS plus oxygen. (C) Adding CGS-21680 to LPS plus oxygen-exposed WT mice significantly reduced total BAL cells as well as BAL neutrophils and macrophages. *Significant compared with the WT L + O₂ group. (D) CGS-21680 blunted the LPS plus oxygen-mediated increase in BAL MIP-2. *Significant compared with the WT L + O₂ group. (E) We isolated naive, WT alveolar macrophages (M ϕ) and exposed them in culture to LPS, oxygen, or both in combination for 24 hours. Statistics were determined by comparing all groups at the same time point. Adding CGS-21680 to the LPS plus oxygen-exposed macrophages significantly blunted the 24-hour TNF- α and MIP-2 secretion. *Significant compared with the media (M) and M + O₂ groups. ^Significant compared with the LPS and L + O₂ + CGS-21680 groups, with no significant difference between the LPS and L + O₂ + CGS-21680 groups ($n = 5-6$ in each group).

TABLE 2. EXUDATIVE MACROPHAGES

Day 3 Exposure	BAL Cells		F4-80 ⁺ CD11b ⁺	
	Percent F4-80 ⁺	Percent CD11b ⁺	CD86 MFI	CD40 MFI
WT LPS (L)	18 (3.8)*	25 (4.1)	72 (22.9) [†]	74 (11.5)
WT L + O ₂	7 (3.5)	30 (2.1)	147 (20.4)	85 (19.2)
WT L + O ₂ + CGS	24 (0.8)*	10 (0.5) [‡]	59 (13.6) [†]	25 (3.6) [§]
A2AR ^{-/-} L + O ₂	7 (2.3)	40 (3.6)	238 (12.7) [§]	110 (6.9)
A2AR ^{-/-} L + O ₂ + CGS	7 (1.1)	36 (2.6)	240 (10.8) [§]	92 (7.5)

Definition of abbreviations: ADORA2A, anti-inflammatory A2A adenosinergic receptor; A2AR^{-/-}, ADORA2A-deficient; BAL, bronchoalveolar lavage; CGS, CGS-21680 hydrochloride; L, lipopolysaccharide; MFI, mean fluorescence intensity; WT, wild-type.

Day 3 BAL exudative macrophages (F4-80⁺ CD11b⁺) from WT mice treated with the ADORA2A agonist CGS-21680 expressed significantly less of the M1 inflammatory markers CD86 and CD40 according to flow cytometry analysis. Statistics were determined by comparing all groups with one-way ANOVA. Values are listed as averages (SEMs) (*n* = 4–6 in each group).

*In the Percent F4-80⁺ column, significant compared with the WT L + O₂, ADORA2A^{-/-} L + O₂, and ADORA2A^{-/-} L + O₂ + CGS-21680 groups.

[†] Significant compared with the WT L + O₂ group.

[‡] Significant compared with all other groups, among F4-80⁺ CD11b⁺ inflammatory macrophages.

[§] Significant compared with all groups.

[¶] In the CD86 MFI column, significant compared with all other groups.

reduction in surface expression of cosignaling molecules CD86 and CD40 (Table 2 and Figure E2C) among F4-80⁺ CD11b⁺ cells from LPS plus oxygen-exposed mice. In isolated naive alveolar macrophages stimulated for 24 hours, adding CGS-21680 to LPS plus oxygen-treated cells significantly reduced TNF- α and MIP-2 concentrations, compared with sham treatment in the LPS plus oxygen group (Figure 4E). Therefore, CGS-21680, a specific ADORA2A agonist, significantly reduced the expression of proinflammatory markers in macrophages from WT mice exposed to LPS plus oxygen.

In WT mice, adding 60% oxygen to LPS decreased lung ADORA2A mRNA on Days 2 and 3 after IT LPS (Figure 5A). We also wanted to assess the effects of oxygen on ADORA2B in response to oxygen-mediated effects on ADORA2A. However, no differences in lung ADORA2B mRNA were evident between WT or ADORA2A^{-/-} mice exposed to LPS and oxygen for 2 or 3 days (Figure E2D). To determine whether oxygen may specifically decrease macrophage ADORA2A, we used both a macrophage cell line (MH-S) and *ex vivo* alveolar and peritoneal macrophages. LPS induced a 3- to 4-fold increase in ADORA2A mRNA in MH-S cells at 8 hours, and this increase was sustained at 24 hours (Figure 5B). Adding oxygen to LPS-treated macrophages significantly decreased ADORA2A mRNA compared with LPS alone, as we observed in lung tissue. Adding oxygen to media-treated MH-S cells did not alter ADORA2A mRNA concentrations. To determine whether an oxygen-induced decrease in ADORA2A mRNA translated to a functional effect on the macrophage ADORA2A pathway, we measured intracellular cAMP. ADORA2A activation increases cAMP, and receptor-specific manipulation alters cAMP concentrations (33). LPS significantly increased cAMP in MH-S cells (Figure 5C). Exposing LPS-stimulated macrophages to supplemental oxygen significantly reduced cAMP compared with LPS treatment alone, an effect that was negated by pretreatment with the ADORA2A agonist CGS-21680. Adding supplemental oxygen to media-exposed macrophages did not alter cAMP concentrations, and CGS-21680 exerted no effect. To assess the impact of the manipulation of ADORA2A signaling on macrophage phenotype and function, we measured cytokines (TNF- α and MCP-1) and cosignaling molecules (CD86 and CD40) in macrophages by flow cytometry. When added to LPS-exposed peritoneal macrophages, 60% oxygen significantly increased TNF- α and MCP-1 at 24 hours,

an effect blocked by pretreatment with CGS-21680 (Figure 5D). CGS-21680 also reduced the abundance of cosignaling molecules CD86 and CD40 in LPS plus oxygen-treated macrophages (Figure 5E). Adding supplemental oxygen to media-exposed peritoneal macrophages did not increase TNF- α , MCP-1, CD86, or CD40 after 24 hours. When we pretreated macrophages with a nonspecific adenosine receptor agonist, NECA (3 μ M), no additional reduction was observed in cytokines or cosignaling molecule expression compared with CGS-21680 treatment, indicating a primary role for ADORA2A signaling in our model (data not shown).

NF- κ B mediates the expression of several proinflammatory molecules, including TNF- α and MIP-2 (34, 35), and may regulate the expression of cosignaling molecules CD86 and CD40 (36). We previously observed an LPS plus oxygen-mediated increase in macrophage NF- κ B p65 activity in comparison to LPS exposure alone. Oxygen alone exerted no effect on cells exposed to media (7). In alveolar macrophages exposed to LPS, NF- κ B blockade with SN-50 (50 μ g/ml) mildly reduced TNF- α , but exerted no effect on MIP-2 (Figure 5F). In alveolar macrophages exposed to LPS plus oxygen, NF- κ B blockade using SN-50 administered at the time of oxygen exposure led to a significant, greater than 50% reduction in alveolar macrophage TNF- α and MIP-2 production. In peritoneal macrophages, the addition of oxygen to LPS increased p65 activity by 50% compared with LPS alone. Pretreatment with CGS-21680 in the group with LPS plus oxygen reduced total NF- κ B p65 activity by greater than 50% of the amount induced by oxygen plus LPS. CGS-21680 exerted no effect on cells exposed to LPS alone (Figure 5G).

Both Myeloid and Nonmyeloid ADORA2A Modulate LPS plus Oxygen-Induced Lung Injury

To determine the specific contribution of inflammatory-cell ADORA2A in modulating lung injury, we performed chimera experiments by irradiating mice and transferring bone marrow-derived cells to designated groups. We used CD45.1 (WT) mice to differentiate leukocytes from the CD45.2 leukocyte common antigen found in ADORA2A^{-/-} mice, allowing us to distinguish donor from recipient cells. Eight weeks after bone marrow cell transfer, we determined chimerism to be greater than 90% in all groups of mice (flow cytometry; data not shown). We exposed irradiated and nonirradiated age-matched WT and ADORA2A^{-/-} mice to LPS plus oxygen, and assessed the response in groups of chimeras. After 2 days of LPS plus oxygen (the earliest time point when ADORA2A^{-/-} mice first demonstrate an appreciable augmentation of lung injury compared with WT mice), injury parameters were evaluated (Figure 6). Histologic injury (Figure 6A) was most severe in ADORA2A^{-/-} mice that received ADORA2A^{-/-} myeloid cells. WT mice that received ADORA2A^{-/-} myeloid cells demonstrated the second most severe histologic injury. Histologic injury was significantly milder in ADORA2A^{-/-} mice that received WT myeloid cells and in WT mice that received WT myeloid cells. The concentration of BAL protein (Figure 6B) was higher in both chimera groups that received ADORA2A^{-/-} myeloid cells, compared with both groups that received WT myeloid cells. BAL total cells (Figure 6C) were significantly increased only in ADORA2A^{-/-} mice that received ADORA2A^{-/-} cells, compared with WT mice that received WT cells. BAL neutrophils (Figure 6D) were significantly increased only in both chimera groups that received ADORA2A^{-/-} myeloid cells. Although BAL macrophage numbers were no different between treatment groups (Figure 6D), both F4-80⁺ CD11c⁺ and F4-80⁺ CD11b⁺ macrophages from WT mice that received ADORA2A^{-/-} myeloid cells expressed significantly more TNF- α than did macrophages from both chimera groups that received WT myeloid cells (Figure 6E).

Figure 5. Oxygen blunts the induction by LPS of ADORA2A and ADORA2A pathway components in macrophages to augment the proinflammatory profile. This effect is negated by the ADORA2A agonist, CGS-21680. Statistics were determined by comparing all groups at the same time point. (A) Adding oxygen to LPS reduced lung ADORA2A mRNA concentrations on Days 2 and 3. *Significant compared with LPS-treated mice at the same time point. FC, fold change. (B) LPS induced an early and sustained increase in ADORA2A mRNA in murine alveolar macrophage cell line (MH-S) cells, an effect negated by the addition of oxygen. *Significant compared with the media and M + O₂ groups. ^Significant compared with the L + O₂ 8-hour and L + O₂ 24-hour groups. (C) Oxygen significantly reduced the LPS-mediated increase in intracellular cyclic adenosine monophosphate (IC cAMP), an effect negated by treatment with CGS-21680. *Significant compared with the LPS + O₂ + CGS-21680 group. n.s., between the LPS and LPS + CGS-21680 groups. (D) Adding CGS-21680 to LPS plus oxygen-treated cells significantly reduced IC TNF-α and IC MCP-1 production to concentrations comparable to those with LPS treatment alone. *Significant compared with the L + O₂ group. (E) Adding CGS-21680 to LPS plus oxygen-treated cells significantly reduced the CD86 and CD40 mean fluorescence intensity (MFI) compared with LPS treatment alone. *Significant compared with the L + O₂ group. (F) Adding the NF-κB inhibitor, cell permeable synthetic peptide (SN-50) (50 μg/ml), at the time of oxygen exposure (3 hours after LPS) most significantly reduced the macrophage secretion of TNF-α and MIP-2 in LPS

plus oxygen-exposed cells at 24 hours. *Significant compared with the LPS + SN-50 group. ^Significant compared with all other groups for each cytokine. A small reduction in TNF-α secretion by LPS-treated cells was evident when SN-50 was added, but with no significant change in MIP-2. (G) LPS plus oxygen exposure significantly increased NF-κB activity in macrophages compared with LPS alone. Adding CGS-21680 to the LPS plus oxygen-treated group led to a 50% reduction in the increase in NF-κB activity observed with the addition of oxygen to LPS versus LPS alone. *Significant compared with the L + O₂ group, with n.s. between the L + O₂ + CGS-21680 and LPS or LPS + CGS-21680 groups ($n = 5-6$ in each group).

DISCUSSION

We conclude that macrophage ADORA2A is a critical modifier of the oxygen-induced augmentation of inflammation and lung injury after intratracheal LPS. In the absence of ADORA2A, mice exposed to LPS plus oxygen exhibit more severe lung injury, and alveolar macrophages from these mice demonstrate an augmented and sustained proinflammatory M1-phenotype, known to be associated with poor outcomes (21, 22). Furthermore, supplemental oxygen delivery after LPS augments the macrophage proinflammatory state even in WT mice, at least in part by attenuating the LPS-induced increase in anti-inflammatory ADORA2A expression. Delivery of the ADORA2A-specific agonist CGS-21680

increased ADORA2A signaling, to reduce lung injury significantly and dampen the macrophage proinflammatory state in WT mice exposed to LPS and oxygen.

The importance of the macrophage inflammatory state in animal models of lung injury has been increasingly recognized (10, 30, 31). Because of the great overlap in surface marker expression between different macrophage inflammatory subpopulations (21), we and others have sought to generate expanded marker sets to define macrophage subpopulations further. In our LPS plus oxygen lung-injury model, alveolar macrophages initiate and propagate lung inflammation, attributable in large part to an enhanced recruitment of neutrophils to the alveolar

space. In ADORA2A-deficient mice, the alveolar recruitment of neutrophils is further enhanced after LPS plus oxygen exposure because of increased chemokine secretion, predominantly from macrophages. Alveolar macrophages from ADORA2A^{-/-} mice exposed to LPS plus oxygen exhibit an increased production of MCP-1, a potent monocyte chemoattractant, indicating that

macrophages from ADORA2A^{-/-} mice promote both monocyte and neutrophil recruitment to the alveolar space.

Although we observed that moderate concentrations of oxygen augmented mild lung inflammation by reducing ADORA2A abundance or signaling through ADORA2A, the mechanisms responsible for this effect remain unknown. Oxygen blunted

Figure 6. Chimera experiments demonstrate protective effects of myeloid and nonmyeloid ADORA2A in the LPS plus oxygen lung-injury model. Statistics were determined by comparing all groups at the same time point. All mice were exposed to LPS plus oxygen for 2 days. (A) Representative low-power ($\times 4$) and high-power (*inset*, $\times 40$) hematoxylin and eosin-stained lung sections on Day 2 after delivery of IT LPS plus oxygen to defined chimera groups demonstrate increased alveolar consolidation in mice with ADORA2A^{-/-} myeloid cells. Quantitative assessment, based on a lung-injury scoring system, demonstrated maximal histologic injury in ADORA2A^{-/-} mice that received ADORA2A^{-/-} myeloid cells, with the next most severe injury in the WT mice that received ADORA2A^{-/-} myeloid cells, compared with other groups. *Significant increase in lung injury compared with the WT mice + WT cell group. ^Significant increase compared with the ADORA2A^{-/-} mice + WT cell group. #Significant increase compared with all three groups. (B) Concentrations of BAL protein were significantly higher in WT and ADORA2A^{-/-} mice that received ADORA2A^{-/-} myeloid cells. *Significant compared with the WT mice + WT cell group. ^Significant compared with the ADORA2A^{-/-} mice + WT cell group. (C) BAL total cell count was significantly increased in ADORA2A^{-/-} mice that received ADORA2A^{-/-} cells. *Significant only compared with the WT mice + WT cell and the ADORA2A^{-/-} mice + WT cell groups. (D) BAL neutrophils were significantly increased in both groups that received ADORA2A^{-/-} myeloid cells when assessed on Day 2. *Significant compared with the WT mice + WT cell group. ^Significant compared with the ADORA2A^{-/-} mice + WT cell group. (E) Among *ex vivo* alveolar macrophages (F4-80⁺ CD11c⁺ and F4-80⁺ CD11b⁺), we observed a significant increase in IC TNF- α production when assessed by flow cytometry. *Significant compared with the WT mice + WT cell group. ^Significant compared with the ADORA2A^{-/-} mice + WT cell group ($n = 4$ in each group). BM, bone marrow.

the LPS-mediated increase in ADORA2A mRNA. The transcription factor hypoxia-inducible factor-1 (HIF-1) is increased after LPS and stabilized under hypoxic conditions, and is known to induce the transcription of ADORA2A (37, 38). The degradation of HIF-1 requires the activity of prolyl hydroxylases (PHDs), which use oxygen as a substrate with a Michaelis-Menten constant known to demonstrate peak activity at 45% oxygen (39). One possibility, as Chandel and Budinger proposed, involves supranormal oxygen (60%) maximizing PHD activity in excess of that which is present in ambient air (21%) (40). If this is the case, then macrophages exposed to oxygen after LPS in our model would manifest relatively lower HIF-1 concentrations compared with macrophages exposed to LPS alone, which could decrease ADORA2A mRNA.

Adding 60% oxygen also decreased the LPS-induced increase in macrophage cAMP, indicative of functional ADORA2A pathway blockade. This may result from reduced ADORA2A total protein or reduced ADORA2A surface receptor protein. Collins and colleagues recently showed that hyaluronic acid (HA) prevented the cycling of ADORA2A to the cell membrane, but exerted no effect on total ADORA2A protein concentrations (29). The mechanisms by which oxygen alters ADORA2A signaling after LPS exposure remain unknown, but possibilities include the production of reactive oxygen species (41), direct binding to a cell surface receptor/coreceptor, or indirect interaction through HA/Toll-like receptor (TLR) signaling pathways (42–44).

ADORA2A is an important regulator of inflammation in other models of injury, yet the actions of ADORA2A in macrophages remain largely unknown (25, 29, 45–48). Because CGS-21680 does express weak affinity for another adenosine receptor, ADORA2B (1/100 potency compared with ADORA2A), we used NECA as a nonselective AR agonist to assess the potential additional contribution of ADORA2B to the observed response in our model (11). We observed no additional anti-inflammatory benefit of NECA over CGS-21680, reinforcing the principal role of ADORA2A.

The inhibitory effect of ADORA2A in macrophages has been identified in models of inflammation involving signaling through several TLRs, including 2, 3, 7, and 9 (49). Therefore, ADORA2A binding likely inhibits a common proinflammatory activator (11, 26). Multiple mechanisms surely exist, but fundamental to the anti-inflammatory effects of the ADORA2A pathway is its inhibition of the transcription factor NF- κ B (50, 51). CGS-21680, a specific ADORA2A agonist, blocked 50% of the oxygen-induced increase in NF- κ B activity in macrophages exposed to LPS. However, as others have observed with adenosine (52), CGS-21680 exerted no effect on NF- κ B activity in macrophages exposed to LPS alone, suggesting distinct oxygen-mediated effects on NF- κ B compared

with LPS. One possibility involves a differential effect on interleukin-1 receptor-associated kinase M, a known repressor of NF- κ B signaling that directly associates with CD86 according to coimmunoprecipitation experiments (53). CD86 expression in macrophages was also down-regulated with CGS-21680 treatment.

Macrophages from ADORA2A^{-/-} mice may promote inflammation by interactions with other cell types, including neutrophils and epithelial cells. Others have shown that the neutrophil activation of macrophages occurs via CD86 (36, 48). When we systemically depleted neutrophils, the expression of CD86 in F4-80⁺ CD11b⁺ alveolar macrophages was reduced, suggesting a possible neutrophil-mediated effect on macrophage CD86 expression (Figure E1C). In isolated macrophages, the antibody-mediated blockade of CD86 did not alter the abundance of other cosignaling molecules or intracellular cytokines in the context of LPS, oxygen, or the two in combination (data not shown). However, we did not test this effect in the presence of neutrophils.

Epithelial ADORA2A has been shown to modify lung water accumulation (54, 55) and prevent allograft airway rejection (49, 56). Our chimera experiments demonstrated that the absence of either myeloid or epithelial/endothelial ADORA2A augments lung injury after LPS plus oxygen exposure, and the absence of both exerts an additive effect on lung injury. In contrast to our findings, Reutershan and colleagues concluded that epithelial or endothelial ADORA2A may be proinflammatory (25). The harmful effect of lacking epithelial ADORA2A may be particularly revealing in our lung injury model, in which lung epithelial cells are constantly exposed to elevated oxygen tensions. Therefore, the addition of supplemental oxygen may constitute a pertinent difference in our model. Adding 60% oxygen to LPS-treated alveolar epithelial cells (murine lung epithelial-12 cell line) in contact with macrophages synergistically increased the secretion of neutrophil-recruiting chemokines, and subsequently enhanced neutrophil migration (unpublished observations by Neil R. Aggarwal and Landon S. King). In the absence of epithelial ADORA2A, the synergistic proinflammatory effect of this cell–cell interaction may be even more pronounced.

Macrophage ADORA2A prevents the augmentation of lung injury after LPS and oxygen. Without ADORA2A, macrophages exposed to LPS plus oxygen displayed both an increased and sustained proinflammatory M1 phenotype, leading to an enhanced recruitment of alveolar neutrophils and the augmentation of lung injury. In WT mice, supplemental oxygen appears to blunt LPS-induced increases in cAMP, consistent with an ADORA2A pathway blockade. The effects of oxygen in macrophages are clearly multifactorial. Our experiments demonstrate that supplemental oxygen, as a modifiable exposure, augments lung inflammation through the down-regulation of the ADORA2A pathway. Although we do not understand the specific oxygen-induced effects

in humans with acute lung inflammation and injury, the protective effects of the ADORA2A agonist CGS-21680 on the oxygen-mediated augmentation of LPS-induced murine lung injury may provide an avenue for investigation in human trials of ARDS.

Author disclosures are available with the text of this article at www.atsjournals.org.

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