ORIGINAL RESEARCH

Enhanced Resolution of Hyperoxic Acute Lung Injury as a result of Aspirin Triggered Resolvin D1 Treatment

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Abstract

Acute lung injury (ALI), which presents as acute respiratory failure, is a major clinical problem that requires aggressive care, and patients who require prolonged oxygen exposure are at risk of developing this disease. Although molecular determinants of ALI have been reported, the molecules involved in disease catabasis associated with oxygen toxicity have not been well studied. It has been reported that lung mucosa is rich in omega-3 fatty acid dicosahexanoic acid (DHA), which has antiinflammatory properties. Aspirin-triggered resolvin D1 (AT-RvD1) is a potent proresolution metabolite of DHA that can curb the inflammatory effects in various acute injuries, yet the effect of AT-RvD1 on hyperoxic acute lung injury (HALI) or in the oxygen toxicity setting in general has not been investigated. The effects of AT-RvD1 on HALI were determined for the first time in 8- to 10-week-old C57BL/6 mice that were exposed to hyperoxia ($\geq$95% O_2) for 48 hours. Mice were given AT-RvD1 (100 ng) in saline or a saline vehicle for 24 hours in normoxic ($\approx$21% O_2) conditions after hyperoxia. Lung tissue and bronchoalveolar lavage (BAL) fluid were collected for analysis associated with proinflammatory signaling and lung inflammation. AT-RvD1 treatment resulted in reduced oxidative stress, increased glutathione production, and significantly decreased tissue inflammation. AT-RvD1 treatment also significantly reduced the lung wet/dry ratio, protein in BAL fluid, and decreased apoptotic and NF-κB signaling. These results show that AT-RvD1 curbs oxygen-induced lung edema, permeability, inflammation, and apoptosis and is thus an effective therapy for prolonged hyperoxia exposure in this murine model.

Keywords: resolvin; hyperoxia; acute lung injury

Clinical Relevance

Clinically, the activation of resolvin-D1 (RvD1)-producing pathways may help disrupt the inflammatory cascade and ultimately offer a novel therapeutic strategy to help in the protection of lung tissue from acute lung injury. Further elucidation of the mechanisms of aspirin-triggered RvD1-mediated hyperoxic acute lung injury resolution is necessary to help in the development of clinical therapeutic treatments for the protection of patients requiring supplemental oxygen.

Acute lung injury (ALI) is a major clinical problem that contributes to the death of more than 70,000 people annually in the United States (1, 2). ALI is an acute respiratory syndrome hallmarked by a breakdown of the alveolar–capillary membrane barrier, lung edema formation, pulmonary epithelial cell death, and an acute inflammatory response. Although the pathogenesis is heterogeneous, extensive studies have not led to an effective treatment (3). Hyperoxic therapy remains the major form of supportive care for patients with decreased respiratory function, such as those who suffer from ALI. However, prolonged hyperoxia exposure (>65% O_2) can lead to further exacerbation of lung symptoms and cause

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hyperoxic ALI (HALI). Murine models of hyperoxia display similar symptoms and pathology to those seen in humans (4–8). Toxic concentrations of oxygen lead to the generation of reactive oxygen species, which lead to a redox imbalance and in turn cause alveolar flooding, inflammation, apoptosis, and necrotic death of alveolar epithelial cells (9, 10). Excessive hyperoxemia also leads to up-regulation of proinflammatory mediators such as IL-1β (11). It may also lead to activation of the apoptotic signaling cascades triggered by the enhanced expression of proapoptotic proteins of the BH3-only BCL-2 protein family (12, 13). Although several laboratories, including our own, have previously reported the molecular determinants that may play a role in HALI development (11, 13–15), the process of lung resolution and repair has not been well studied, especially in the oxidant toxicity setting.

HALI is usually self-limiting and resolves with minimal fibrosis in comparison to other models of ALI (16, 17); however, the molecular mechanisms involved in tissue catabasis have not been elucidated. Polyunsaturated fatty acid supplements significantly improve circulating antiinflammatory biomarkers, reduce inflammatory cytokine levels, and promote gas exchange in adolescent and adult patients with lung injury (18, 19). The polyunsaturated fatty acids generated after injury serve as key mediators of inflammation. Of these fatty acids, omega-3 derivatives, referred to as resolution-phase interaction products (resolvins), have drawn considerable interest for their potential to resolve acute injury in various disease models (20–28). These lipid mediators are antiinflammatory, promote tissue homeostasis, and help revert injured and inflamed tissue to a normal state. Resolvins, discovered in resolving inflammatory exudates in mice, are classically synthesized through a primarily lipoxygenase driven pathway. In the presence of aspirin, however, more stable and less enzymatically susceptible "aspirin-triggered" epimeric forms are generated through aspirin-induced cyclooxygenase-2 acetylation (21).

Previous reports show antiinflammatory and proresolving roles of Aspirin-Triggered Resolvin D1 (7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E19Z-docosahexaenoic acid; AT-RvD1) in the reduction of other sterile injuries, such as aspiration pneumonitis in mice (26, 27). However, oxidant stress as a result of this study was found to be very low; thus, the ability of AT-RvD1 to alleviate inflammation and injury resulting from oxygen toxicity has not been properly investigated. Therefore, in this study we investigated the effects of AT-RvD1 on the resolution of HALI. Although 24-hour treatment with vehicle allowed for modest resolution, treatment with AT-RvD1 after hyperoxia exposure resulted in a significant increase in decreased oxidative stress, increased resolution of tissue inflammation, enhanced antioxidant production, enhanced clearance of cellular infiltration, and reduction of edema formation and alveolar protein leak. AT-RvD1 treatment also resulted in a marked decrease in proinflammatory and apoptotic signaling. Thus, results also suggest that AT-RvD1 enhances HALI resolution.

Materials and Methods

Animals

The University of South Florida Institutional Animal Care and Use Committee approved the animal protocol. C57BL/6 WT mice (6–8 wk old) (Harlan Laboratories, Indianapolis, IN) were used to conduct all *in vivo* experiments. Mice were housed in isolated cages on an automatic 12-hour light to dark cycle at room temperature (25°C). Water and standard food were available *ad libitum*.

Murine Hyperoxic Injury Resolution Model

Lung injury was induced using a murine hyperoxia model as previously described (9, 29). Mice, in cages, were placed in an airtight chamber (70 × 50 × 50 cm) and exposed to hyperoxic conditions (95–100% O_2) for 48 hours, which was sufficient to induce moderate to severe ALI (30). An oxygen regulator was used to monitor and maintain the chamber's atmospheric conditions (BioSpherix, Lacona, NY). To assess the impacts of AT-RvD1 on hyperoxic lung injury resolution, mice were kept under normoxic conditions (21% O_2) after hyperoxia exposure and given either 0.5 to 0.05 μg AT-RvD1 (7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E19Z-docosahexaenoic acid, 95% purity, no LPS detected; Caymen Chemicals, Ann Arbor, MI) (Figure 1A) or vehicle (1% ethanol in saline) to serve as an indicator of normal injury resolution (control group). One hundred microliters of AT-RvD1 or saline was injected intravenously or intreaperitoneally. Mice were killed 24 hours after AT-RvD1 or vehicle treatment, and tissue samples and bronchoalveolar lavage (BAL) fluid were collected in separate experiments. The experimental outline is shown in schematic form in Figure 1B.

Lung Mechanics Analysis

In a separate experiment, mice were anesthetized with ketamine-xylazine and placed on a small rodent mechanical ventilator and respiratory mechanics apparatus (SCIREQ, Montreal, PQ, Canada). The lung resistance of mice in each group ($n \geq 8$ in each group) was measured after ventilation.

Statistical Analysis

Statistical analysis was performed using the Graphpad Prism software (GraphPad Software, Inc., San Diego, CA). Comparison of samples between two groups was completed using a standard Student's *t* test. Comparison of samples in three or more groups was calculated using a one-way ANOVA with a Tukey *post hoc* test to measure significance between groups. *P* values for all tests were calculated and labeled where significant. A *P* value <0.05 was considered significant, and all *t* tests were two-tailed.

Results

AT-RvD1 Treatment after Hyperoxic Injury Leads to Decreased Oxidative Stress and Reduced Lung Resistance

Oxidative stress is the key insult of the HALI model and thus the ability of AT-RvD1 to resolve oxidative stress in lung tissue warrants investigation. Lipid peroxidation is a well-documented indicator of oxidative stress (31–33). To test the ability of AT-RvD1 to reduce lipid peroxidation after hyperoxic injury, C57/BL6 mice were given AT-RvD1 (0.5–0.05 μg) after 48-hour hyperoxia exposure. BAL fluid was collected, and a thiobarbituric acid–reactive substances (TBARs) assay was performed (Figure 1C). Results reveal that AT-RvD1 treatment after hyperoxic injury leads to a greater than 75% decrease in lipid peroxidation as measured with the TBARs assay, in comparison to the vehicle group, which only showed a 25% decrease in lipid

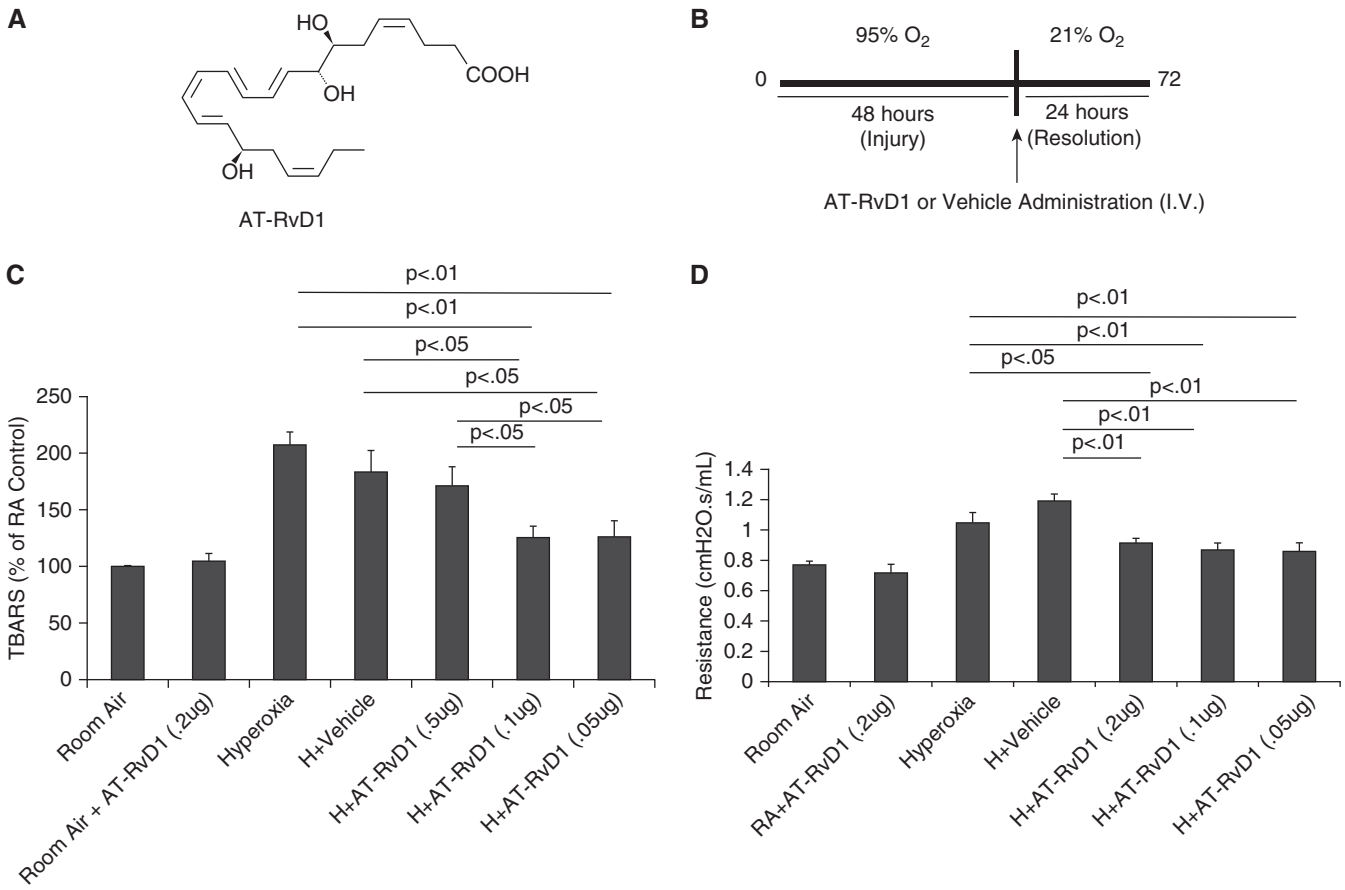

Figure 1. Aspirin-triggered resolvin D1 (AT-RvD1) treatment after hyperoxic lung injury demonstrates improved lung mechanics and decreased oxidative stress. C57BL/6 mice were subjected to hyperoxia (H) or normoxia exposure for 48 hours followed by treatment with AT-RvD1 (*A*) or saline vehicle for 24 hours administered intravenously (I.V.) (*B*). (*C*) In a separate experiment, lungs were harvested from each mouse, and lipid peroxidation was measured via the thiobarbituric acid–reactive substances assay (TBARS). (*D*) Before mice were killed, lung resistance was measured after each treatment using the flexiVent respiratory metrics apparatus (*see* MATERIALS AND METHODS). A one-way ANOVA, with a Tukey *post hoc* test, was used to determine the results, where $P < 0.05$ was deemed statistically significant ($n = 6$ for each group). RA, room air.

peroxidation. These results also suggest that AT-RvD1 provides significantly enhanced resolution of oxidative stress after HALI at an optimal dose of 0.1 μg. Antioxidant imbalance is a well-documented result of oxidative stress (34–40). Antioxidant–oxidant imbalance leads to antioxidant depletion and proinflammatory reactive oxidant–antioxidant complexes (32, 41–44). Our results reveal that mice treated with AT-RvD1 have significantly higher glutathione in lung tissues and significantly enhanced expression of antioxidant transcriptional regulator Nrf2 (*see* Figure E3 in the online supplement). Previous reports point to increased lung resistance as a result of oxidative stress (34, 45, 46). To further assess the functional contributions of AT-RvD1 (0.2–0.05 μg) to hyperoxia-induced impairment of lung mechanics, lung resistance was measured using the SCIREQ flexiVent apparatus (Figure 1D). Surprisingly, saline treatment resulted in a small increase in lung resistance after hyperoxia exposure; however, this increase was not statistically significant. This phenomenon was curtailed with AT-RvD1 treatment after injury. AT-RvD1 treatment resulted in significantly reduced lung resistance in comparison to both the hyperoxia and the hyperoxia followed by vehicle treatment groups. The decreased lung resistance in response to AT-RvD1 treatment points to improved resolution of HALI.

AT-RvD1 Treatment after Injury leads to Decreased Hyperoxia-Induced Leukocyte Infiltration

Previous reports have alluded to the increased airway resistance as a key indicator airway inflammation and an increase in inflammatory cell infiltrate (47–49). Recruitment of leukocytes to the injured area is a key hallmark of HALI, and reduction of this infiltration marks a key event in injury resolution. C57/BL6 mice were given AT-RvD1 (0.1 μg, intravenously) after 48 hours of hyperoxia exposure to determine the capacity of AT-RvD1 to reduce leukocyte infiltration after hyperoxic injury. The leukocyte accumulation in the BAL fluid was analyzed using total and differential cell counts (Figure 2). Images were taken of BAL fluid cells that were cytospun onto microscope slides and differentially stained (Figures 3A–3E). Results revealed that mice treated with AT-RvD1 had a 3-fold decrease of total cells in the BAL fluid compared with mice treated with the vehicle (Figure 2A). Differential counts of leukocytes revealed that both macrophages and neutrophils

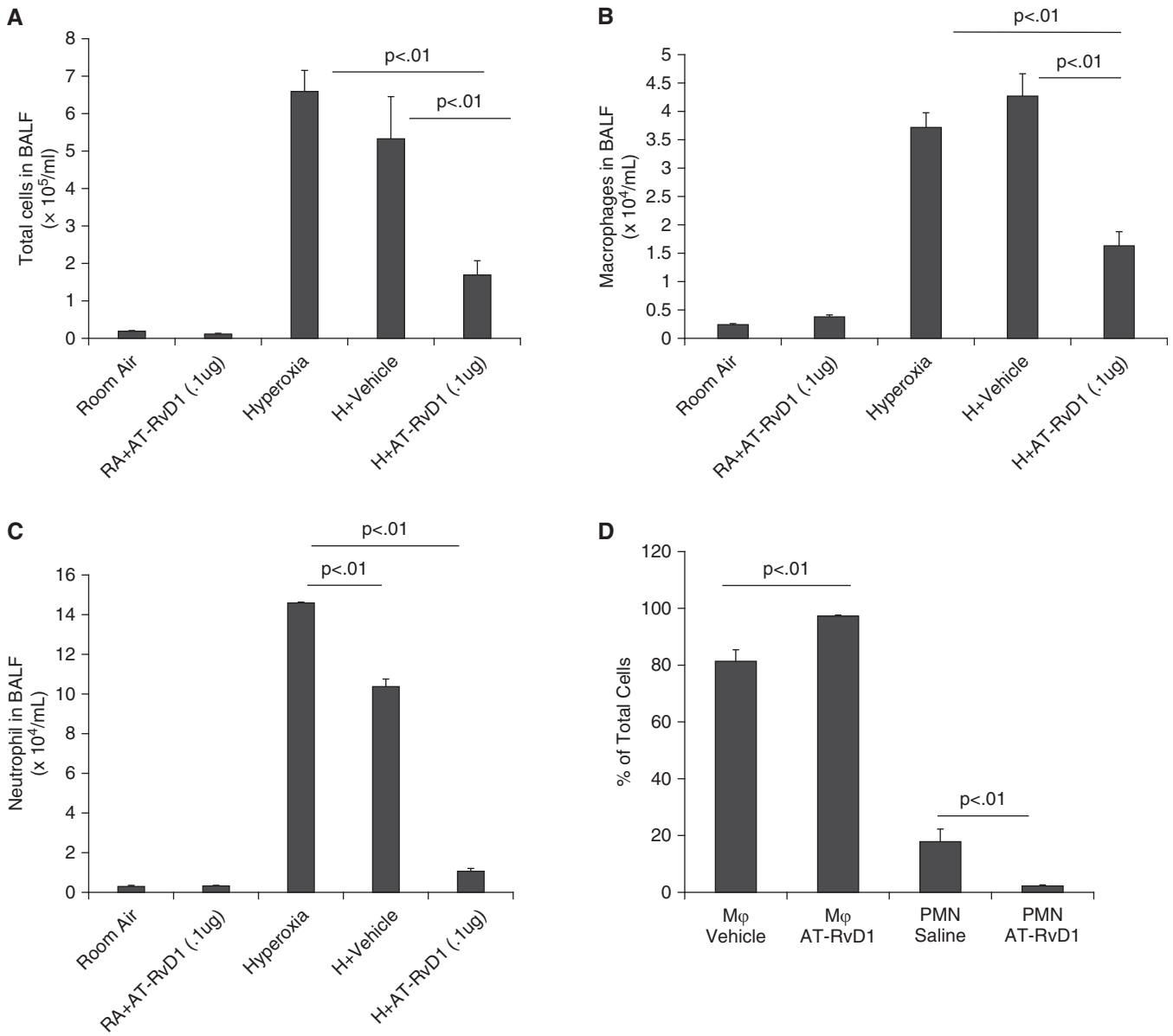

Figure 2. AT-RvD1 treatment after hyperoxic injury reduces cells in bronchoalveolar lavage fluid (BALF) and rescues leukocyte imbalance. C57BL/6 mice were subjected to normoxia or hyperoxia exposure for 48 hours followed by treatment with AT-RvD1 (100 ng) or saline vehicle for 24 hours in normoxia. An equal mixture of BALF was collected and cytospun onto microscope slides for leukocyte analysis. Cytospin smears were stained via Diffquick stain. Total (*A*) and differential (*B* and *C*) cell counts were performed. (*D*) Analysis of the BAL macrophage and neutrophil percentage was calculated. One-way ANOVA, with a Tukey *post hoc* test, was used to determine the results, where $P < 0.05$ was deemed statistically significant ($n = 10$ in each group). Mϕ, macrophages; PMN, polymorphonuclear leukocytes.

were significantly decreased in the AT-RvD1 group, with a greater than 10-fold decrease in neutrophil accumulation (Figures 2B and 2C and *black arrows* in Figures 3A–3E). AT-RvD1–treated mice show a significant decrease in comparison to mice treated with vehicle after the injury in neutrophil percentage (<3%) and a similarly significant increase in macrophage percentage (>97%) among leukocyte subsets in BAL fluid (Figure 2D).

Results further show that keratinocyte chemoattractant (KC), a neutrophillic chemokine, is significantly decreased in the BAL of mice treated with AT-RvD1 after injury (Figure 3F). Myeloperoxidase, a peroxidase that is abundantly expressed in neutrophils, was significantly decreased in AT-RvD1–treated mice in comparison to vehicle control (Figure 3G). Altogether, results indicate that AT-RvD1 treatment after hyperoxic injury significantly reduces accumulation of leukocytes into the injured area, especially neutrophils.

AT-RvD1 Attenuates Hyperoxia-Induced Lung Inflammation and Permeability

The influx of inflammatory cells is a hallmark of HALI and an indicator lung inflammation (1). It was hypothesized that AT-RvD1 treatment would resolve hyperoxia-induced lung inflammation

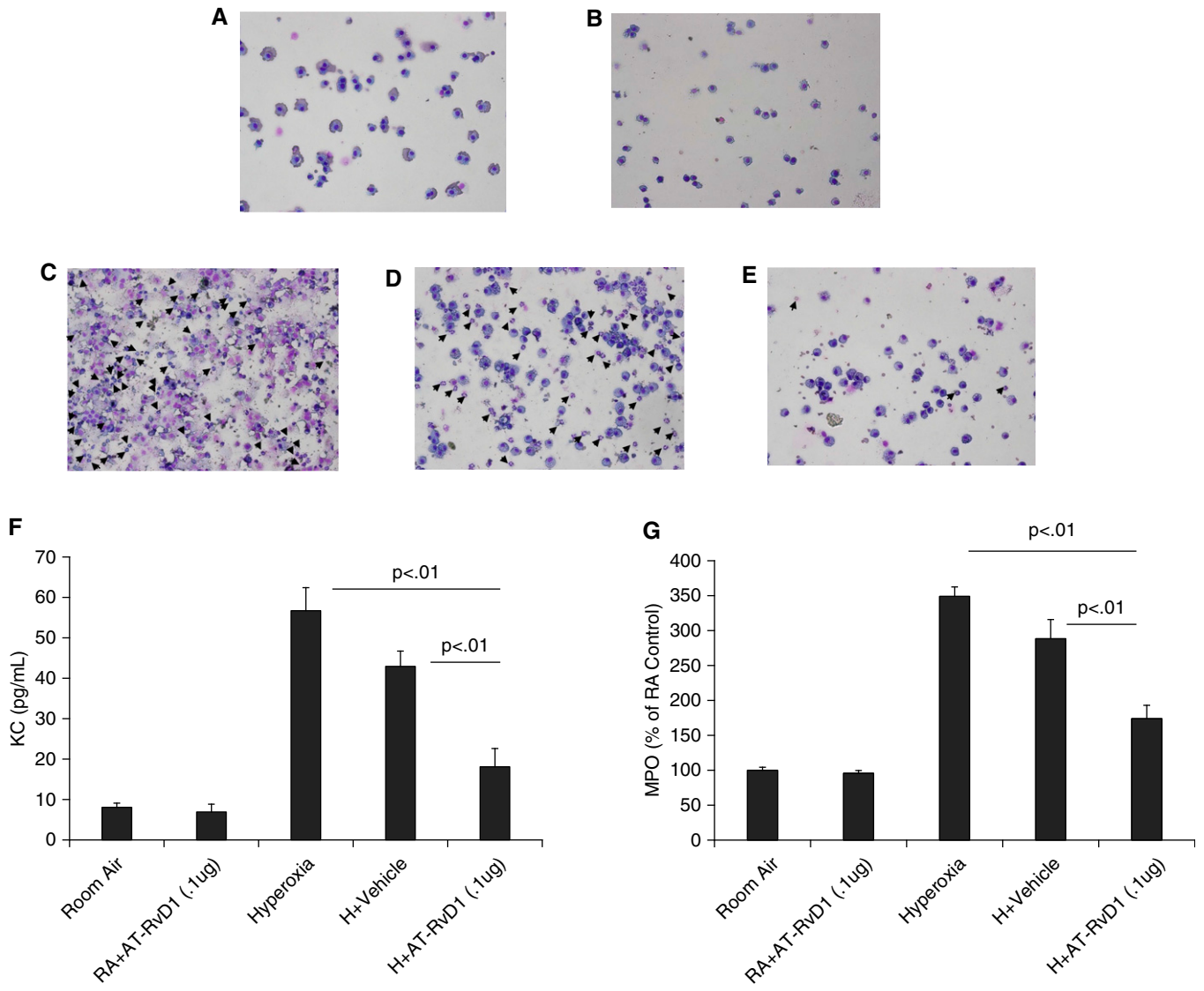

Figure 3. AT-RvD1 treatment after hyperoxic injury enhanced clearance of cellular debris and cell infiltrate in BAL fluid. C57BL/6 mice were subjected to normoxia or hyperoxia exposure for 48 hours followed by treatment with AT-RvD1 (100 ng) or saline vehicle for 24 hours in normoxia ($n = 6$ for each group). (*A–E*) An equal mixture of BAL fluid was collected and cytospun onto microscope slides for leukocyte analysis. Representative images for room air (RA) (*A*), RA + AT-RvD1 (0.1 μg) (*B*), hyperoxia (*C*), hyperoxia + vehicle (*D*), and hyperoxia + AT-RvD1 (0.1 μg) (*E*) are shown. Original magnification: ×100. Neutrophils in BALF are highlighted by *arrowheads*. Murine neutrophilic chemokine keratinocyte chemoattractant (KC) from BALF (*F*) as well as myeloperoxidase (MPO) from lung homogenates (*G*) was also measured. A one-way ANOVA was used to determine the results, where $P < 0.05$ was deemed statistically significant ($n = 10$ in each group).

because mice treated with AT-RvD1 after hyperoxic injury demonstrated reduced lipid peroxidation and showed reduced leukocytes in BAL fluids. Analysis of tissue histology, which is a direct measure of lung inflammation, revealed that AT-RvD1 treatment led to a visible decrease in alveolar thickening, congestion, and alveolar immune cell infiltration in comparison to mice treated with vehicle alone (Figures 4A–4E). Pathological severity scoring showed a small improvement in tissue inflammation after 24-hour room air treatment; however, this improvement was not significant. More importantly, administration of AT-RvD1 after injury led to a significant improvement in pathological severity in comparison to the groups that were exposed to hyperoxia alone and hyperoxia followed by saline treatments (Figure 4F). To further confirm the effects of AT-RvD1 treatment, lung edema and alveolar leak were examined by the wet to dry ratio and BAL protein analysis, respectively (Figure 5). AT-RvD1–treated mice experienced a significant decrease in lung wet-to-dry ratio and BAL protein levels after hyperoxia. This reduction in lung edema by AT-RvD1 was further confirmed by a decrease in dye extravasation into the serum and the BAL fluid (Figure 6). Altogether, these results highlight AT-RvD1's role in improved tissue histology, permeability reduction, and prevention of further lung damage after injury.

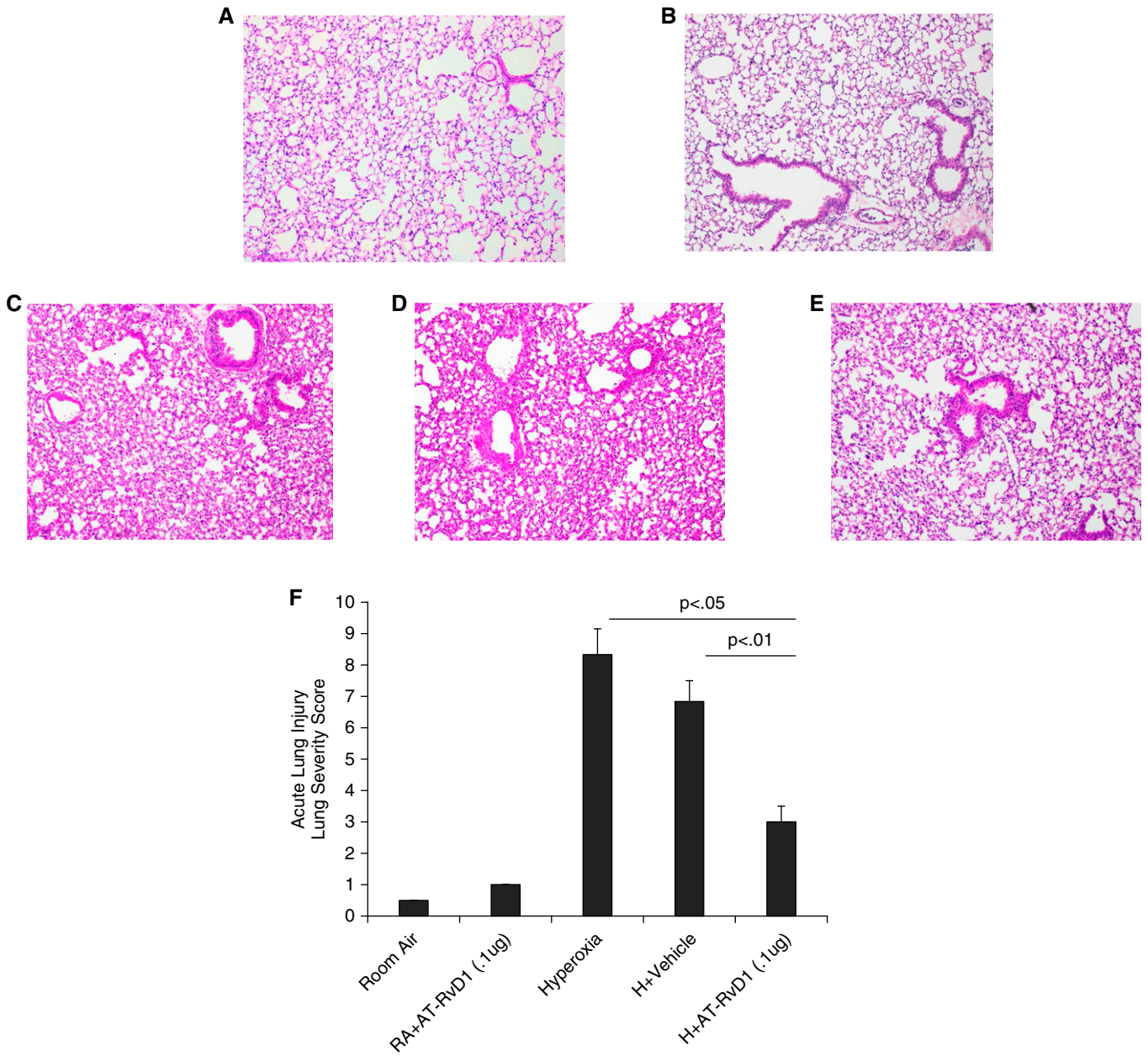

Figure 4. AT-RvD1 regulates hyperoxia enhances resolution of hyperoxia-induced tissue inflammation. C57BL/6 mice were subjected to normoxia or hyperoxia exposure for 48 hours followed by treatment with AT-RvD1 (100 ng) or saline vehicle for 24 hours in normoxia ($n = 6$ for each group) administered intravenously. (*A–E*) Lung sections were obtained and stained with hematoxylin and eosin to analyze lung histology. Original magnification: ×40. Representative images for RA (*A*), RA + AT-RvD1 (0.1 μg) (*B*), hyperoxia (*C*), hyperoxia + vehicle (*D*), and hyperoxia + AT-RvD1 (0.1 μg) (*E*) are shown. (*F*) Four independent parameters (alveolar congestion, hemorrhage, leukocyte infiltration, and alveolar wall thickness) were scored and used to determine the acute lung injury severity score. These are the cumulative results of three independent experiments ($n = 10$ in each group). A one-way ANOVA was used to determine the results, where $P < 0.05$ was deemed statistically significant.

AT-RvD1 Regulates Hyperoxia-Induced Inflammatory Mediator Production

The secretion of inflammatory mediators disrupts the immune-repressed alveolar state of resident macrophages and epithelial cells. *In vitro* and *in vivo* reports from our laboratory demonstrate that one of these mediators, IL-1β, plays an important role in the inflammatory phenotype seen in the lung after hyperoxia exposure (11, 14, 30). Therefore, the influence of AT-RvD1 treatment on IL-1β secretion after hyperoxia exposure was analyzed. In comparison to mice treated with vehicle, AT-RvD1 treatment significantly decreased IL-1β protein expression and secretion (Figure 7A). Early cytokines released after injury, such as IL-6 and MCP-1, show a similar decrease with AT-RvD1 treatment (Figures 7B and 7C). To further understand

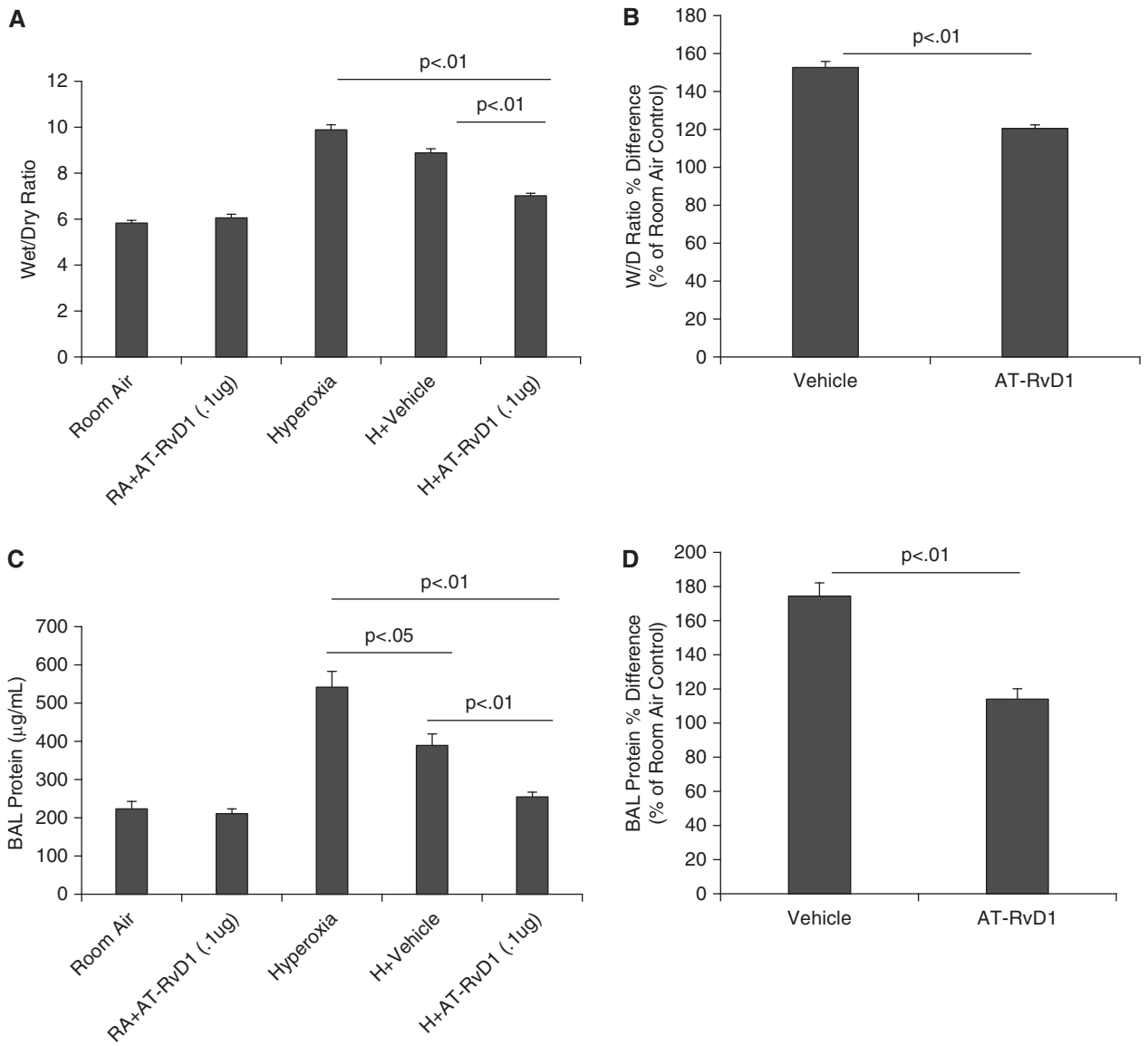

Figure 5. AT-RvD1 treatment after hyperoxia demonstrates reduced pulmonary edema and permeability. C57BL/6 mice were subjected to normoxia or hyperoxia exposure for 48 hours followed by treatment with AT-RvD1 (100 ng) or saline vehicle for 24 hours ($n = 6$ for each group) administered intravenously. (*A* and *B*) After AT-RvD1 or vehicle treatment, lung tissue was harvested, and the weight of the "wet" lungs was measured. Lungs were placed in an oven at 70°C and measured periodically until the measurement was constant ("dry weight"). The wet/dry (W/D) ratio was measured for each group. (*C* and *D*) In a separate subset of animals ($n = 6$ for each group), BALF was collected. After AT-RvD1 or vehicle treatment, total protein in BALF for each group was measured via bicinchoninic acid assay. A one-way ANOVA was used to determine the results, where $P < 0.05$ was deemed statistically significant.

the mechanism of AT-RvD1–mediated reduction of hyperoxia-induced IL-1β expression, the activity of transcriptional regulator NF-κB was analyzed. Lung tissue analysis reveals a decrease in NF-κB activity with AT-RvD1 treatment compared with mice treated with vehicle alone, as measured by the phosphorylation of the nuclear NF-κB p65 subunit (Figure 7D). Mitogen-activated protein kinases (MAPKs) are critical mediators for the activation of transcription factors, such as NF-κB, that lead to cytokine processing after inflammatory stimuli. AT-RvD1 treatment resulted in an overall decrease in phosphorylation of MAPK p38 (Figure 7E); however, no changes in the phosphorylation of p44/42 and SAPK/JNK were detected (Figure E1). These data demonstrate that AT-RvD1 treatment after hyperoxia exposure results in enhanced resolution of proinflammatory cytokine secretion and the reduction of proinflammatory signaling molecule activity.

AT-RvD1 Treatment after Injury Results in Decreased Hyperoxia-Associated Apoptotic Markers in Lung Tissue

Hyperoxia treatment leads to increased cellular apoptosis in lung tissue (9–11, 30). Caspase-3 is a well-documented indicator

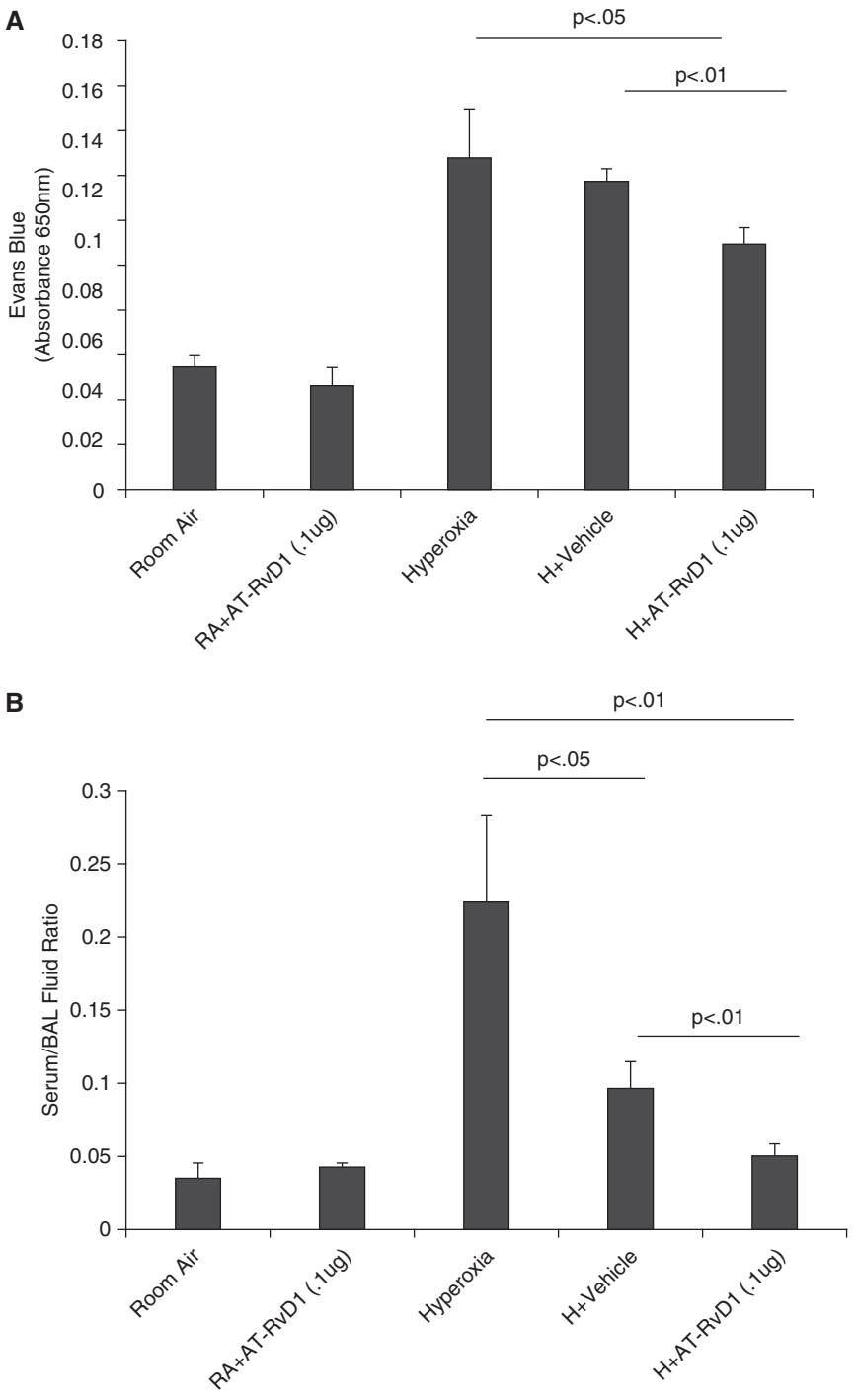

Figure 6. AT-RvD1 blunts hyperoxia-induced alveolar permeability and pulmonary edema. C57BL/6 mice were subjected to normoxia or hyperoxia exposure for 48 hours followed by treatment with AT-RvD1 (100 ng) or saline vehicle for 24 hours ($n = 6$ for each group) administered intravenously. (*A*) Endothelial barrier integrity was determined via introduction of Evans blue dye administered intravenously 30 minutes before the end of each treatment. After mice were killed, quantification of Evans blue dye in the BALF was used to examine endothelial barrier integrity. (*B*) In a separate experiment, epithelial barrier integrity was assessed by analyzing the serum:BALF ratio of extravasated 3000MW FITC dextran administered intratracheally 20 minutes before the end of each treatment. One-way ANOVA was used to determine the results, where $P < 0.05$ was deemed statistically significant.

of cellular apoptosis (50–52). Caspase-3 activity was assessed in lung tissue sections and whole lung homogenates via immunohistochemistry and Western blot, respectively, to further assess cellular apoptosis. The results demonstrated a decreased presence of active caspase-3 in lung sections and protein extracts prepared from mice given AT-RvD1 versus those given vehicle after hyperoxia exposure (Figures E2A–E2D). Proteins of the proapoptotic BCL-2 family (BH3-only proteins) regulate apoptosis through interaction and inhibition of prosurvival molecules. Expression and activation of BH3-only proteins are important hyperoxia-induced signaling events that lead to epithelial cell death in ALI (8, 13). Phosphorylation of BH3-only protein BAD abolishes its proapoptotic activity. To confirm AT-RvD1's capability to reduce hyperoxia injury–induced apoptotic markers such as BAD, phosphorylation of BAD was analyzed from each sample. AT-RvD1 treatment, in comparison to vehicle control, resulted in increased phosphorylation of BAD in mice after hyperoxia exposure (Figure E2E). No significant change in total BAD was observed between AT-RvD1 and the vehicle-treated groups. The BH3-only protein BIM also demonstrated reduced protein expression in mice given AT-RvD1 after hyperoxic injury in comparison to vehicle control (Figure E2F). These results suggest that AT-RvD1 treatment results in decreased apoptotic activity through suppression of BH3-only protein expression and signaling. These results suggest further study is needed regarding hyperoxia-induced apoptotic signaling by AT-RvD1 treatment.

Discussion

Hyperoxic therapy is a key component of supportive care for patients suffering from reduced respiratory function (53). Prolonged exposure of high concentrations of hyperoxia can lead to the generation of reactive oxygen species, which can lead to HALI, which at times presents as acute respiratory distress syndrome (54). Injury resulting from hyperoxic stress leads to decreased lung biomechanics, increased alveolar permeability, lung edema, and inflammation (30, 55). Free radical generation may also result in increased

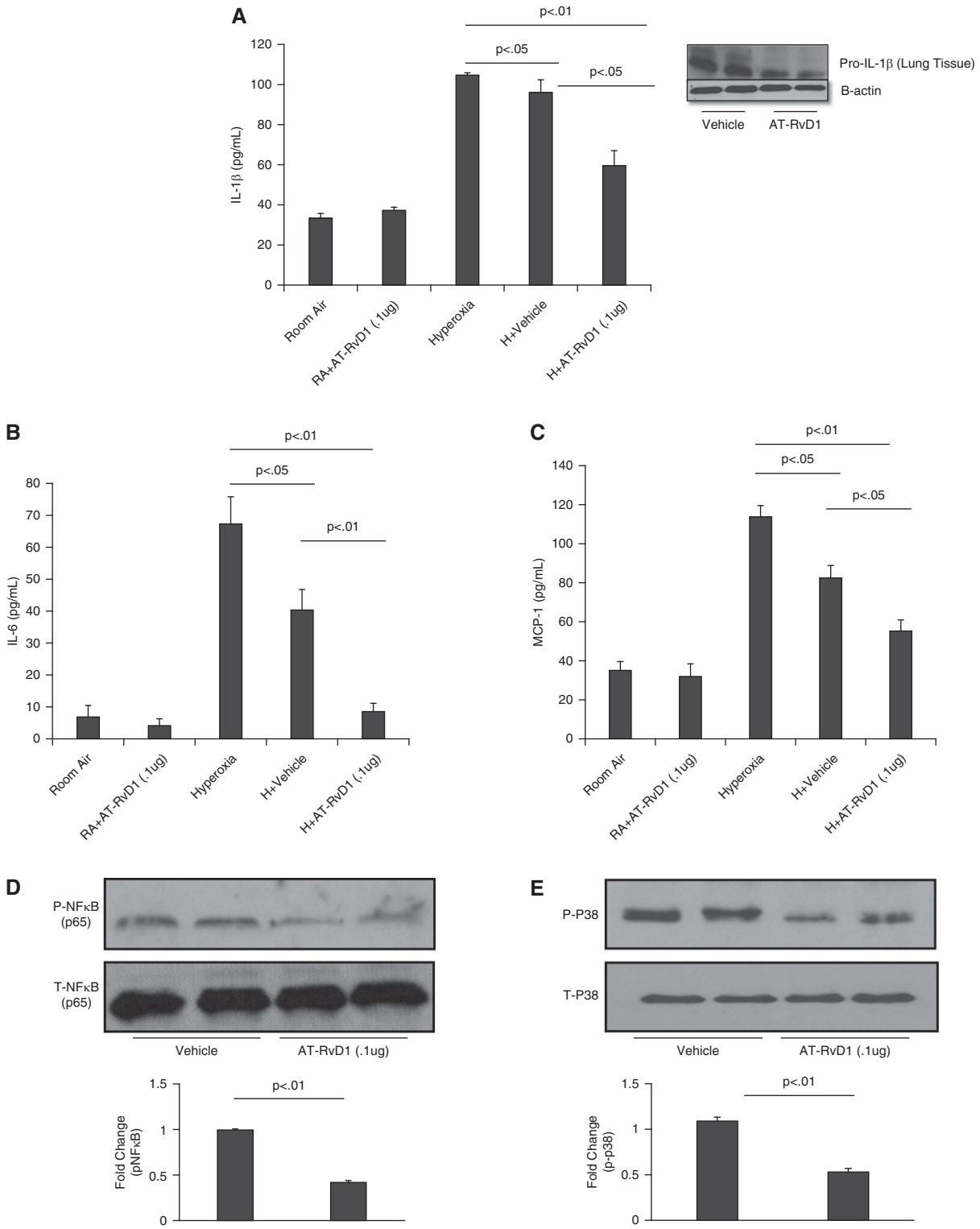

Figure 7. AT-RvD1 treatment after injury attenuates hyperoxia-induced cytokine expression and secretion. C57BL/6 mice were subjected to normoxia or hyperoxia exposure for 48 hours followed by treatment with AT-RvD1 (100 ng) or saline vehicle for 24 hours in normoxia ($n = 6$ per group). After AT-RvD1 or vehicle treatment, BALF was collected from mice in each group. ELISAs were performed using BALF to assess cytokine secretion of IL-1β (*A*), IL-6 (*B*), and monocyte chemoattractant protein-1 (MCP-1) (*C*). (*D* and *E*) In a separate experiment ($n = 6$ per group), total protein was extracted and used for Western blot analysis of phosphorylation of NF-κB P65 subunit (in the nuclear fraction) (*D*) and p38 MAP kinase (*E*). T, total; P, phosphorylated form of assayed proteins. Densitometry of each phosphorylated protein shown below the blot and is representative of three separate experiments. Representative blots of each group for each protein are shown. A one-way ANOVA (for each ELISA) or Student's *t* test (blot densitometry) was used to determine the results, where $P < 0.05$ was deemed statistically significant.

apoptotic signaling (56). Although our laboratory and others have sought to dissect the molecular determinants associated with HALI (3, 57), the molecular determinants involved in the restoration of innate tissue homeostasis and resolution of hyperoxic injury have not been clearly defined.
In this study, we hypothesized that the proresolution mediator AT-RvD1 can enhance resolution of hyperoxic lung injury through decreased oxidative stress, lung resistance, permeability, inflammation, and apoptotic marker expression (30, 58–60). Previous reports have demonstrated uniform animal death of mice exposed to 72 to 96 hours of 95% hyperoxia (6, 61); therefore, we chose to investigate at the 48-hour time point, when moderate HALI pathology occurs and resolution after 24 hours of injury can be investigated. In studying resolution, the hyperoxic model has its advantages because natural resolution takes place with mild scarring without fibrotic development (16, 17). Hyperoxia-induced reactive oxygen species generation contributes to enhanced lipid peroxidation and subsequent cellular injury (38, 52, 62, 63). Our laboratory and others have shown that hyperoxia overexposure results in the hyperactivation and ungoverned recruitment of proinflammatory leukocytes, which include neutrophils and monocytes (8, 11, 30). In the latter stages of injury, the production of inflammatory mediators and reactive species by these cells contributes to a toxic alveolar environment and further damage of healthy tissue. Results from this study demonstrate that AT-RvD1 treatment after injury shows reduced lipid peroxidation, a key indicator of oxidative stress, in comparison to both hyperoxia and hyperoxia followed by vehicle treatment. For the first time, we show that lipid peroxidation is reduced with AT-RvD1 treatment. It has been previously discussed that a diet rich in docosahexaenoic acid, a precursor of AT-RvD1, curbs oxidative stress and lipid peroxidation in rats after traumatic brain injury (64, 65). Our results further affirm these findings and point to AT-RvD1 as a key DHA metabolite in the reduction of oxidative stress after injury. DHA treatment has also been associated with increased antioxidant properties (66); however, the effects of AT-RvD1 on redox signaling have not been studied. Our results reveal increased GSH and Nrf2 expression with AT-RvD1 treatment. GSH supplementation has been demonstrated previously to enhance resolution of hyperoxic injury and is a major marker of oxidant–antioxidant imbalance (40). Nrf2, a master regulator of antioxidant gene expression, has also been demonstrated to be important in the resolution of HALI through GSH regulation (36, 40, 67). Because previous reports of AT-RvD1's beneficial action on acute injury are not a result of oxidative stress (27) and because the hyperoxia model is based on injury as a result of oxidative stress, our finding that antioxidants such as GSH and regulators such as Nrf2 are enhanced is of great significance and represents a key next step of investigation as to how AT-RvD1 can combat oxidative stress. More specifically, this paper provides a platform and a basis to assess whether increased antioxidant machinery generated by AT-RvD1 combats the antioxidant depletion and subsequent redox imbalance caused by hyperoxia (34, 38, 42) and whether or not AT-RvD1 be used as a therapeutic agent to increase antioxidant machinery and improve the effects of oxygen therapy.

Hyperoxic injury results in increased lung stiffening, inflammation, and permeability (8, 45, 53, 55, 68). Whether or not AT-RvD1 could curb these hallmarks of hyperoxic lung injury needed to be investigated. AT-RvD1 treatment after injury resulted in a significant decrease in lung resistance. Lung static compliance was also improved with AT-RvD1 treatment in comparison to hyperoxia alone; however, it was not significantly different from vehicle treatment alone during the resolution recovery period (data not shown). These findings suggest that AT-RvD1 can alleviate lung stiffening and improve respiratory mechanics after hyperoxic injury. This is important because decreased respiratory function, in particular through increased airway resistance, is associated with fibrotic development (69) Results of this study demonstrated that treatment with AT-RvD1 after injury resulted in a much more significant reduction of leukocytes and tissue inflammation in the 24-hour injury resolution phase than the vehicle control. BAL fluid collection revealed decreased neutrophil counts in mice treated with AT-RvD1 after injury in comparison with hyperoxia and vehicle control. Reduced neutrophil accumulation in BAL after injury may be a result of decreased expression and secretion of neutrophil chemoattractants such as KC. Further, the peroxidase enzyme myeloperoxidase was also reduced with AT-RvD1 treatment in comparison to vehicle treatment groups. Reduced KC and myeloperoxidase have been extensively associated with reduced neutrophilia and tissue inflammation (70–77). Treatment with AT-RvD1 after hyperoxic injury also restored normal leukocyte population: the macrophage population in the BAL was increased by 17% in comparison to vehicle control, and the neutrophil population was reduced to 3% in comparison to 20% for the vehicle control. These results point to the ability of AT-RvD1 to decrease leukocyte inflammation and restore the leukocyte imbalance due to hyperoxic injury; however, whether this is attributed to decreased oxidant stress and an enhanced antioxidant profile was not thoroughly investigated. In future studies we aim to identify the link between the enhanced antioxidant activity stimulated by AT-RvD1 and the reduced inflammatory phenotype shown after AT-RvD1 treatment.

Leukocyte infiltration is a prime indicator of inflammation, which is a key hallmark of HALI, and ungoverned regulation of inflammation results in downstream fibrotic development (78). Hyperoxic injury and cell damage lead to the release of proinflammatory cytokines such as IL-1β (11, 79, 80), which has been shown to be the most bioactive cytokine in patients with lung injury (81). This laboratory has demonstrated that decreased IL-1β secretion results in improved lung pathology after hyperoxia challenge (11, 30). The results of this paper reveal that AT-RvD1 versus control treatment results in decreased expression of pro–IL-1β. Furthermore, IL-1β secretion is decreased after hyperoxia in mice given AT-RvD1 versus vehicle treatment. IL-1β, as one of the early cytokines secreted by alveolar macrophages, contributes to hypercytokinemia after injury (81, 82). Thus, it was hypothesized that AT-RvD1 treatment would result in a decrease of other proinflammatory cytokines. Analysis of proinflammatory cytokines IL-6, KC, and MCP-1 in BAL revealed a similar decrease in mice given AT-RvD1 after hyperoxia exposure in comparison to vehicle treatment. Although numerous reports from our laboratory have touched

on the proteolytic processing of pro–IL-1β that is needed for activity (11, 14, 30), we found decreased expression of IL-1β in its pro-form, which suggested that AT-RvD1 treatment resulted in reduced transcriptional activity, leading to reduced IL-1β expression. Previous reports have linked the AT-RvD1 receptor, formyl peptide receptor 2 (FPR2/ALX), with decreased activity of the proinflammatory transcription regulator NF-κB (28). NF-κB and MAPKs are important proinflammatory transcription regulators responsible for the activation of proinflammatory genes associated with the production of cytokines, adhesion molecules, and epithelial cell junction destabilization (83–86). These downstream products of NF-κB and MAPK activity contribute to the propagation of the proinflammatory cascade. Investigation into AT-RvD1's effect on NF-κB activity after hyperoxia exposure revealed a significant decrease in the phosphorylation of NF-κB after AT-RvD1 in comparison to vehicle control treatment. Phosphorylation of NF-κB is a key signaling event involved in the transcriptional activity of this molecule. Therefore, these results demonstrate that AT-RvD1 treatment significantly impairs NF-κB activation. Similar effects are seen in p38 MAPk activation; however, the phosphorylation of the SAPK/JNK and p44/42 MAPks was not altered with AT-RvD1 treatment. Data obtained from this study further demonstrate that AT-RvD1 versus control significantly resolves hyperoxia-induced inflammatory signaling and subsequent cytokine secretion. The role of FPR2/ALX in this AT-RvD1–mediated attenuation of hyperoxia-induced inflammatory signaling needs to be investigated in future studies.

The alveolar–capillary barrier plays an important homeostatic role in the regulation of immune cell infiltration (1–3, 53). However, powerful inflammatory stimuli, such as hyperoxia, cause deterioration and remodeling of this barrier, which leads to aberrant and overactive inflammatory signaling (11, 53). Reestablishment of this barrier and decreased permeability are key steps in HALI resolution. AT-RvD1 treatment after hyperoxia exposure demonstrates a significant decrease in both the lung wet to dry ratio and alveolar protein content in comparison to vehicle control. Further, AT-RvD1 treatment demonstrates reduced epithelial and vascular permeability. Altogether, these results suggest that treatment with AT-RvD1 following hyperoxic injury reduces pulmonary edema and lung permeability. More importantly, these results suggest that AT-RvD1 significantly enhances barrier integrity during the resolution phase of hyperoxic injury. Because inflammatory cytokines contribute to hyperoxia-induced alveolar permeability and barrier dysfunction, the ability of AT-RvD1 to suppress cytokine secretion may also contribute to the enhanced barrier integrity seen in this study. Tissue histology after AT-RvD1 administration was assessed because the build-up of edema fluid that results from the compromised alveolar capillary barrier can lead to further activation of the alveolar epithelium, endothelium, and macrophages (2). Pathological severity scoring from lung tissue sections further illustrated the proresolution effects of AT-RvD1 versus the control because tissue sections obtained from mice revealed improved tissue histology in areas of alveolar thickening, leukocyte infiltration, and decreased alveolar congestion. Changes in alveolar hemorrhage were not witnessed in these sections. Along with the improvement in permeability and decreased pathological severity scoring, mice administered AT-RvD1 during the resolution phase demonstrated less lung resistance and thus improved respiratory tract airflow. Taken together, these results suggest that AT-RvD1 significantly improves recovery time from injury. Results from this study demonstrate significant suppression of the ALI pathological severity. More importantly, our results suggest that AT-RvD1 may be acting on multiple protective pathways, which may point to the remarkable recovery seen in these mice treated with AT-RvD1.

Apoptotic cell death of alveolar epithelial cells is a previously described result of hyperoxic insult (6). Cellular fragments, inactivated surfactant, and cellular contents from apoptotic cells result in further secretion of noxious stimuli into the pulmonary milieu (53). Previous reports from our laboratories have shown that BH3-only proteins play a role in hyperoxia-induced apoptotic signaling (8, 13). BAD is a proapoptotic family member of the BH3-only protein faction of the Bcl-2 family. Found primarily in the cytosol, BAD interacts with antiapoptotic proteins to prevent their mechanism of inhibition of apoptosis. Phosphorylation of BAD leads to inactivation and allows the prosurvival function of antiapoptotic members of the Bcl-2 family (apoptosis). Results from this study show that treatment with AT-RvD1 versus the vehicle control significantly increased phosphorylation of BAD. More importantly, these results suggest for the first time that AT-RvD1 may affect the apoptotic signaling pathway through regulation of the proapoptotic BH3-only protein signaling cascade. Expression of the proapoptotic protein BIM showed a reduction in protein expression with AT-RvD1 treatment after hyperoxic injury in comparison to vehicle controls. Caspase-3 activation was measured to confirm the resulting decreased apoptosis. Immunohistochemistry staining showed an intense staining, localizing active caspase-3 expression mostly to the lung epithelial cells (bronchiolar and some alveolar staining) in the vehicle treatment group; however, AT-RvD1 treatment after injury resulted in a significantly decreased expression in active caspase-3 via two distinct methods. Strong staining is shown in the distal bronchiolar epithelial cells and to a lesser extent in the alveolar epithelium, which is consistent with previous reports regarding hyperoxia-induced proapoptotic signaling (87, 88). These results further support AT-RvD1's capability to resolve hyperoxia-induced apoptotic signaling.

Although resolvins demonstrate potential proresolution effects in HALI, these studies reveal an new area of beneficial effects for resolvins. For the first time, these data demonstrate that AT-RvD1 enhances resolution of injury associated with oxygen toxicity. They also demonstrate that treatment with the proresolution agonist AT-RvD1 reverses hyperoxia-mediated proinflammatory and proapoptotic signaling and aids in the homeostatic return to normal lung physiology. More importantly, our results highlight an alteration in antioxidant production and transcription factor expression coupled with decrease oxidative stress. These results provide evidence for a novel role of AT-RvD1 in regulating hyperoxia-induced lung inflammation and acute injury. Thus far, our studies and others have demonstrated a one-hit system of acute injury; however, patients also suffer from a myriad of complications that are both sterile and

pathogen induced. It is of interest to our laboratory to investigate the effects of AT-RvD1 in a two-hit system that combined sepsis and sterile injury, such as hyperoxia and LPS.

From a clinical perspective, activating RvD1-producing pathways by a pharmacological approach may disrupt the inflammatory cascade and ultimately may provide a novel therapeutic strategy for the protection of lung tissue from cellular injury during acute or chronic illness associated with ALI syndromes. Further understanding of the mechanisms of AT-RvD1–mediated HALI resolution may lead to the development of clinical interventions for the protection of patients requiring supplemental oxygen. ■

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

References

1. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcomes of acute lung injury. *N Engl J Med* 2005;353:1685–1693.
2. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
3. Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. *J Clin Invest* 2012;122:2731–2740.
4. Crapo JD. Morphologic changes in pulmonary oxygen toxicity. *Annu Rev Physiol* 1986;48:721–731.
5. Cacciuttolo MA, Trinh L, Lumpkin JA, Rao G. Hyperoxia induces DNA damage in mammalian cells. *Free Radic Biol Med* 1993;14:267–276.
6. Barazzone C, Horowitz S, Donati YR, Rodriguez I, Piguet PF. Oxygen toxicity in mouse lung: pathways to cell death. *Am J Respir Cell Mol Biol* 1998;19:573–581.
7. Mantell LL, Lee PJ. Signal transduction pathways in hyperoxia-induced lung cell death. *Mol Genet Metab* 2000;71:359–370.
8. Kolliputi N, Waxman AB. IL-6 cytoprotection in hyperoxic acute lung injury occurs via PI3K/Akt-mediated Bax phosphorylation. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L6–L16.
9. Waxman AB, Einarsson O, Seres T, Knickelbein RG, Warshaw JB, Johnston R, Homer RJ, Elias JA. Targeted lung expression of interleukin-11 enhances murine tolerance of 100% oxygen and diminishes hyperoxia-induced DNA fragmentation. *J Clin Invest* 1998; 101:1970–1982.
10. Crapo JD, Barry BE, Foscue HA, Shelburne J. Structural and biochemical changes in rat lungs occurring during exposures to lethal and adaptive doses of oxygen. *Am Rev Respir Dis* 1980;122: 123–143.
11. Kolliputi N, Shaik RS, Waxman AB. The inflammasome mediates hyperoxia-induced alveolar cell permeability. *J Immunol* 2010;184: 5819–5826.
12. Wu YC, O'Reilly MA. Bcl-X(L) is the primary mediator of p21 protection against hyperoxia-induced cell death. *Exp Lung Res* 2011;37:82–91.
13. Waxman AB, Kolliputi N. IL-6 protects against hyperoxia-induced mitochondrial damage via Bcl-2-induced Bak interactions with mitofusins. *Am J Respir Cell Mol Biol* 2009;41:385–396.
14. Kolliputi N, Galam L, Parthasarathy PT, Tipparaju SM, Lockey RF. NALP-3 inflammasome silencing attenuates ceramide-induced transepithelial permeability. *J Cell Physiol* 2012;227:3310–3316.
15. Bhargava M, Dey S, Becker TL, Steinbach MS, Wu B, Lee SM, Higgins L, Kumar V, Bitterman PB, Ingbar DH, *et al*. Protein expression profile of rat type two alveolar epithelial cells during hyperoxic stress and recovery. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L604–L614.
16. Martin TR, Matute-Bello G. Experimental models and emerging hypotheses for acute lung injury. *Crit Care Clin* 2011;27:735–752.
17. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L379–L399.
18. Rice TW, Wheeler AP, Thompson BT, deBoisblanc BP, Steingrub J, Rock P; Investigators NNARDSNo, Network NACT. Enteral omega-3 fatty acid, gamma-linolenic acid, and antioxidant supplementation in acute lung injury. *JAMA* 2011;306:1574–1581.
19. Barbosa VM, Miles EA, Calhau C, Lafuente E, Calder PC. Effects of a fish oil containing lipid emulsion on plasma phospholipid fatty acids, inflammatory markers, and clinical outcomes in septic patients: a randomized, controlled clinical trial. *Crit Care* 2010;14:R5.
20. Arita M, Yoshida M, Hong S, Tjonahen E, Glickman JN, Petasis NA, Blumberg RS, Serhan CN. Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proc Natl Acad Sci USA* 2005;102:7671–7676.
21. Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G, Moussignac RL. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med* 2002; 196:1025–1037.
22. Chen YF, Jiang H, Gong X, Wan JY. Resolvin E1 protects against ox-LDL-induced injury on vascular endothelial cells [article in Chinese]. *Zhonghua Xin Xue Guan Bing Za Zhi* 2011;39:1039–1043.
23. Seki H, Fukunaga K, Arita M, Arai H, Nakanishi H, Taguchi R, Miyasho T, Takamiya R, Asano K, Ishizaka A, *et al*. The anti-inflammatory and proresolving mediator resolvin E1 protects mice from bacterial pneumonia and acute lung injury. *J Immunol* 2010;184:836–843.
24. Levy BD, Kohli P, Gotlinger K, Haworth O, Hong S, Kazani S, Israel E, Haley KJ, Serhan CN. Protectin D1 is generated in asthma and dampens airway inflammation and hyperresponsiveness. *J Immunol* 2007;178:496–502.
25. Arm JP, Horton CE, Spur BW, Mencia-Huerta JM, Lee TH. The effects of dietary supplementation with fish oil lipids on the airways response to inhaled allergen in bronchial asthma. *Am Rev Respir Dis* 1989;139:1395–1400.
26. Hsiao HM, Sapinoro RE, Thatcher TH, Croasdell A, Levy EP, Fulton RA, Olsen KC, Pollock SJ, Serhan CN, Phipps RP, *et al*. A novel anti-inflammatory and pro-resolving role for resolvin D1 in acute cigarette smoke-induced lung inflammation. *PLoS One* 2013;8:e58258.
27. Eickmeier O, Seki H, Haworth O, Hilberath JN, Gao F, Uddin M, Croze RH, Carlo T, Pfeffer MA, Levy BD. Aspirin-triggered resolvin D1 reduces mucosal inflammation and promotes resolution in a murine model of acute lung injury. *Mucosal Immunol* 2013;6:256–266.
28. Levy BD. Resolvin D1 and resolvin E1 promote the resolution of allergic airway inflammation via shared and distinct molecular counter-regulatory pathways. *Front Immunol* 2012;3:390.
29. Waxman AB, Einarsson O, Seres T, Knickelbein RG, Homer R, Warshaw JB, Johnston R, Elias JA. Targeted lung expression of interleukin-11 enhances murine tolerance of 100% oxygen and diminishes hyperoxia-induced DNA fragmentation. *Chest* 1999;116: 8S–9S.
30. Fukumoto J, Fukumoto I, Parthasarathy PT, Cox R, Huynh B, Ramanathan GK, Venugopal RB, Allen-Gipson DS, Lockey RF, Kolliputi N. NLRP3 deletion protects from hyperoxia-induced acute lung injury. *Am J Physiol Cell Physiol* 2013;305:C182–C189.
31. Gupta A, Singh RL, Raghubir R. Antioxidant status during cutaneous wound healing in immunocompromised rats. *Mol Cell Biochem* 2002; 241:1–7.
32. Rasik AM, Shukla A. Antioxidant status in delayed healing type of wounds. *Int J Exp Pathol* 2000;81:257–263.
33. Mokra D, Drgova A, Pullmann R Sr, Calkovska A. Selective phosphodiesterase 3 inhibitor olprinone attenuates meconium-induced oxidative lung injury. *Pulm Pharmacol Ther* 2012;25:216–222.
34. Bargagli E, Olivieri C, Bennett D, Prasse A, Muller-Quernheim J, Rottoli P. Oxidative stress in the pathogenesis of diffuse lung diseases: a review. *Respir Med* 2009;103:1245–1256.
35. Rahman I, MacNee W. Oxidant/antioxidant imbalance in smokers and chronic obstructive pulmonary disease. *Thorax* 1996;51:348–350.
36. Fan X, Staitieh BS, Jensen JS, Mould KJ, Greenberg JA, Joshi PC, Koval M, Guidot DM. Activating the Nrf2-mediated antioxidant response element restores barrier function in the alveolar epithelium of HIV-1 transgenic rats. *Am J Physiol Lung Cell Mol Physiol* 2013; 305:L267–L277.

37. Geiser T, Atabai K, Jarreau PH, Ware LB, Pugin J, Matthay MA. Pulmonary edema fluid from patients with acute lung injury augments *in vitro* alveolar epithelial repair by an IL-1β-dependent mechanism. *Am J Respir Crit Care Med* 2001;163:1384–1388.
38. Nagato A, Silva FL, Silva AR, Bezerra FS, Oliveira ML, Bello-Klein A, Cristovao Porto L, Santos Valenca S. Hyperoxia-induced lung injury is dose dependent in Wistar rats. *Exp Lung Res* 2009;35:713–728.
39. Pan L, Fu JH, Xue XD, Xu W, Zhou P, Wei B. Melatonin protects against oxidative damage in a neonatal rat model of bronchopulmonary dysplasia. *World J Pediatr* 2009;5:216–221.
40. Reddy NM, Kleeberger SR, Kensler TW, Yamamoto M, Hassoun PM, Reddy SP. Disruption of Nrf2 impairs the resolution of hyperoxia-induced acute lung injury and inflammation in mice. *J Immunol* 2009; 182:7264–7271.
41. Van der Vliet A, Smith D, O'Neill CA, Kaur H, Darley-Usmar V, Cross CE, Halliwell B. Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. *Biochem J* 1994;303:295–301.
42. Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. *Am J Physiol Lung Cell Mol Physiol* 2002;283: L246–L255.
43. Bowler RP, Velsor LW, Duda B, Chan ED, Abraham E, Ware LB, Matthay MA, Day BJ. Pulmonary edema fluid antioxidants are depressed in acute lung injury. *Crit Care Med* 2003;31:2309–2315.
44. Spite M, Summers L, Porter TF, Srivastava S, Bhatnagar A, Serhan CN. Resolvin D1 controls inflammation initiated by glutathione-lipid conjugates formed during oxidative stress. *Br J Pharmacol* 2009; 158:1062–1073.
45. Sunil VR, Vayas KN, Massa CB, Gow AJ, Laskin JD, Laskin DL. Ozone-induced injury and oxidative stress in bronchiolar epithelium are associated with altered pulmonary mechanics. *Toxicol Sci* 2013;133: 309–319.
46. Goldkorn T, Filosto S, Chung S. Lung injury and lung cancer caused by cigarette smoke-induced oxidative stress: molecular mechanisms and therapeutic opportunities involving the ceramide-generating machinery and epidermal growth factor receptor. *Antioxid Redox Signal* 2014;21:2149–2174.
47. Burgel PR. The role of small airways in obstructive airway diseases. *Eur Respir Rev* 2011;20:23–33.
48. Card JW, Carey MA, Bradbury JA, Graves JP, Lih FB, Moorman MP, Morgan DL, DeGraff LM, Zhao Y, Foley JF, *et al*. Cyclooxygenase-1 overexpression decreases basal airway responsiveness but not allergic inflammation. *J Immunol* 2006;177:4785–4793.
49. Demange V, Wild P, Zmirou-Navier D, Tossa P, Bohadana A, Barbaud A, Paris C. Associations of airway inflammation and responsiveness markers in non asthmatic subjects at start of apprenticeship. *BMC Pulm Med* 2010;10:37.
50. Sakurai R, Villarreal P, Husain S, Liu J, Sakurai T, Tou E, Torday JS, Rehan VK. Curcumin protects the developing lung against long-term hyperoxic injury. *Am J Physiol Lung Cell Mol Physiol* 2013;305: L301–L311.
51. Liang X, Wei SQ, Lee SJ, Fung JK, Zhang M, Tanaka A, Choi AM, Jin Y. p62 sequestosome 1/light chain 3b complex confers cytoprotection on lung epithelial cells after hyperoxia. *Am J Respir Cell Mol Biol* 2013;48:489–496.
52. Tyurina YY, Tyurin VA, Kaynar AM, Kapralova VI, Wasserloos K, Li J, Mosher M, Wright L, Wipf P, Watkins S, *et al*. Oxidative lipidomics of hyperoxic acute lung injury: mass spectrometric characterization of cardiolipin and phosphatidylserine peroxidation. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L73–L85.
53. Kallet RH, Matthay MA. Hyperoxic acute lung injury. *Respir Care* 2013; 58:123–141.
54. Bhandari V, Choo-Wing R, Lee CG, Zhu Z, Nedrelow JH, Chupp GL, Zhang X, Matthay MA, Ware LB, Homer RJ, *et al*. Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death. *Nat Med* 2006;12:1286–1293.
55. Lagishetty V, Parthasarathy PT, Phillips O, Fukumoto J, Cho Y, Fukumoto I, Bao H, Cox R, Galam L, Lockey RF, Kolliputi N. Dysregulation of CLOCK gene expression in hyperoxia-induced lung injury. *Am J Physiol Cell Physiol* 2014;306:C999–C1007.
56. Mach WJ, Thimmesch AR, Pierce JT, Pierce JD. Consequences of hyperoxia and the toxicity of oxygen in the lung. *Nurs Res Pract* 2011;2011:260482.
57. Ware LB, Herridge M. Acute lung injury. *Semin Respir Crit Care Med* 2013;34:439–440.
58. Cox RR Jr, Phillips O, Kolliputi N. Putting the brakes on acute lung injury: can resolvins suppress acute lung injury? *Front Physiol* 2012; 3:445.
59. Lane T, Flam B, Lockey R, Kolliputi N. TXNIP shuttling: missing link between oxidative stress and inflammasome activation. *Front Physiol* 2013;4:50.
60. Sue RD, Belperio JA, Burdick MD, Murray LA, Xue YY, Dy MC, Kwon JJ, Keane MP, Strieter RM. CXCR2 is critical to hyperoxia-induced lung injury. *J Immunol* 2004;172:3860–3868.
61. Frank L, Bucher JR, Roberts RJ. Oxygen toxicity in neonatal and adult animals of various species. *J Appl Physiol* 1978;45:699–704.
62. Pietrofesa RA, Turowski JB, Arguiri E, Milovanova TN, Solomides CC, Thom SR, Christofidou-Solomidou M. Oxidative lung damage resulting from repeated exposure to radiation and hyperoxia associated with space exploration. *J Pulm Respir Med* 2013;3: 1000158.
63. Siner JM, Jiang G, Cohen ZI, Shan P, Zhang X, Lee CG, Elias JA, Lee PJ. VEGF-induced heme oxygenase-1 confers cytoprotection from lethal hyperoxia *in vivo*. *FASEB J* 2007;21:1422–1432.
64. Wu A, Ying Z, Gomez-Pinilla F. Dietary omega-3 fatty acids normalize BDNF levels, reduce oxidative damage, and counteract learning disability after traumatic brain injury in rats. *J Neurotrauma* 2004;21: 1457–1467.
65. Dharajiya N, Chisholm K, Dietz L, Sue Richards C, Kharrazi M, Schrijver I. Identification of the CFTR p.Phe508Del founder mutation in the absence of a polythymidine 9T allele in a Hispanic patient. *Clin Genet* 2013;83:598–599.
66. Yang YC, Lii CK, Wei YL, Li CC, Lu CY, Liu KL, Chen HW. Docosahexaenoic acid inhibition of inflammation is partially via cross-talk between Nrf2/heme oxygenase 1 and IKK/NF-kappaB pathways. *J Nutr Biochem* 2013;24:204–212.
67. Li N, Alam J, Venkatesan MI, Eiguren-Fernandez A, Schmitz D, Di Stefano E, Slaughter N, Killeen E, Wang X, Huang A, *et al*. Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. *J Immunol* 2004;173:3467–3481.
68. van der Vaart H, Postma DS, Timens W, ten Hacken NH. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 2004;59:713–721.
69. Bachofen H, Scherrer M. Lung tissue resistance in diffuse interstitial pulmonary fibrosis. *J Clin Invest* 1967;46:133–140.
70. Finsterbusch M, Voisin MB, Beyrau M, Williams TJ, Nourshargh S. Neutrophils recruited by chemoattractants *in vivo* induce microvascular plasma protein leakage through secretion of TNF. *J Exp Med* 2014;211:1307–1314.
71. Shah D, Romero F, Stafstrom W, Duong M, Summer R. Extracellular ATP mediates the late phase of neutrophil recruitment to the lung in murine models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2014;306:L152–L161.
72. Bhargava R, Altmann CJ, Andres-Hernando A, Webb RG, Okamura K, Yang Y, Falk S, Schmidt EP, Faubel S. Acute lung injury and acute kidney injury are established by four hours in experimental sepsis and are improved with pre, but not post, sepsis administration of TNF-alpha antibodies. *PLoS One* 2013;8:e79037.
73. Trujillo G, Habiel DM, Ge L, Ramadass M, Cooke NE, Kew RR. Neutrophil recruitment to the lung in both C5a- and CXCL1-induced alveolitis is impaired in vitamin D-binding protein-deficient mice. *J Immunol* 2013;191:848–856.
74. Chen J, Zeng T, Bi Y, Zhong Z, Xie K, Zhao X. Docosahexaenoic acid (DHA) attenuated paraquat induced lung damage in mice. *Inhal Toxicol* 2013;25:9–16.
75. Yubero S, Manso MA, Ramudo L, Vicente S, De Dios I. Dexamethasone down-regulates the inflammatory mediators but fails to reduce the tissue injury in the lung of acute pancreatitis rat models. *Pulm Pharmacol Ther* 2012;25:319–324.

76. Altintas ND, Atilla P, Iskit AB, Topeli A. Long-term simvastatin attenuates lung injury and oxidative stress in murine acute lung injury models induced by oleic acid and endotoxin. *Respir Care* 2011;56: 1156–1163.
77. Yang W, Qiang D, Zhang M, Ma L, Zhang Y, Qing C, Xu Y, Zhen C, Liu J, Chen YH. Isoforskolin pretreatment attenuates lipopolysaccharide-induced acute lung injury in animal models. *Int Immunopharmacol* 2011;11:683–692.
78. Barazzone C, Belin D, Piguet PF, Vassalli JD, Sappino AP. Plasminogen activator inhibitor-1 in acute hyperoxic mouse lung injury. *J Clin Invest* 1996;98:2666–2673.
79. Kuipers MT, Aslami H, Janczy JR, van der Sluijs KF, Vlaar AP, Wolthuis EK, Choi G, Roelofs JJ, Flavell RA, Sutterwala FS, *et al*. Ventilator-induced lung injury is mediated by the NLRP3 inflammasome. *Anesthesiology* 2012;116:1104–1115.
80. Dolinay T, Kim YS, Howrylak J, Hunninghake GM, An CH, Fredenburgh L, Massaro AF, Rogers A, Gazourian L, Nakahira K, *et al*. Inflammasome-regulated cytokines are critical mediators of acute lung injury. *Am J Respir Crit Care Med* 2012;185:1225–1234.
81. Ganter MT, Roux J, Miyazawa B, Howard M, Frank JA, Su G, Sheppard D, Violette SM, Weinreb PH, Horan GS, *et al*. Interleukin-1beta causes acute lung injury via alphavbeta5 and alphavbeta6 integrin-dependent mechanisms. *Circ Res* 2008;102:804–812.
82. Pittet JF, Koh H, Fang X, Iles K, Christiaans S, Anjun N, Wagener BM, Park DW, Zmijewski JW, Matthay MA, *et al*. HMGB1 accelerates alveolar epithelial repair via an IL-1beta- and alphavbeta6 integrin-dependent activation of TGF-beta1. *PLoS One* 2013;8:e63907.
83. Wang CC, Lin WN, Lee CW, Lin CC, Luo SF, Wang JS, Yang CM. Involvement of p42/p44 MAPK, p38 MAPK, JNK, and NF-kappaB in IL-1beta-induced VCAM-1 expression in human tracheal smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2005;288: L227–L237.
84. Lin FS, Lin CC, Chien CS, Luo SF, Yang CM. Involvement of p42/p44 MAPK, JNK, and NF-kappaB in IL-1beta-induced ICAM-1 expression in human pulmonary epithelial cells. *J Cell Physiol* 2005; 202:464–473.
85. Baldassare JJ, Bi Y, Bellone CJ. The role of p38 mitogen-activated protein kinase in IL-1 beta transcription. *J Immunol* 1999;162: 5367–5373.
86. Li N, Karin M. Is NF-κB the sensor of oxidative stress? *FASEB J* 1999; 13:1137–1143.
87. Kuwano K. Epithelial cell apoptosis and lung remodeling. *Cell Mol Immunol* 2007;4:419–429.
88. O'Reilly MA, Staversky RJ, Stripp BR, Finkelstein JN. Exposure to hyperoxia induces p53 expression in mouse lung epithelium. *Am J Respir Cell Mol Biol* 1998;18:43–50.