

Conditional Deletion of *Nrf2* in Airway Epithelium Exacerbates Acute Lung Injury and Impairs the Resolution of Inflammation

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Oxidant stress, resulting from an excess of reactive electrophiles produced in the lung by both resident (epithelial and endothelial) and infiltrated leukocytes, is thought to play an obligatory role in tissue injury and abnormal repair. Previously, using a conventional (whole-body) knockout model, we showed that antioxidative gene induction regulated by the transcription factor Nrf2 is critical for mitigating oxidant-induced (hyperoxic) stress, as well as for preventing and resolving tissue injury and inflammation *in vivo*. However, the contribution to pathogenic acute lung injury (ALI) of the cellular stress produced by resident versus infiltrated leukocytes remains largely undefined *in vivo*. To address this critical gap in our knowledge, we generated mice with a conditional deletion of Nrf2 specifically in Clara cells, subjected these mice to hyperoxic insult, and allowed them to recover. We report that a deficiency of Nrf2 in airway epithelia alone is sufficient to contribute to the development and progression of ALI. When exposed to hyperoxia, mice lacking Nrf2 in Clara cells showed exacerbated lung injury, accompanied by greater levels of cell death and epithelial sloughing than in their wild-type littermates. In addition, we found that an Nrf2 deficiency in Clara cells is associated with a persistent inflammatory response and epithelial sloughing in the lungs during recovery from sublethal hyperoxic insult. Our results demonstrate (for the first time, to the best of our knowledge) that Nrf2 signaling in Clara cells is critical for conferring protection from hyperoxic lung injury and for resolving inflammation during the repair process.

Keywords: oxidative stress; lung injury and repair; inflammation

Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are common clinical syndromes caused by various injurious insults. These syndromes can lead to the development of lung pathogenesis and cause major public health problems throughout the world (1, 2). Various studies, using various experimental models of ALI/ARDS (including the hyperoxic ALI model), established that oxidants inflict damage on lung tissue by generating reactive oxygen and nitrogen

(Received in original form April 30, 2010 and in final form June 5, 2011)

This work was supported by National Institutes of Health grants HL66109, ES11863, and P50-HL073994 (S.P.R.), and GM079239 and HL081205 (S.B.).

N.M.R. and H.P.R. performed the experiments and analyzed the data. N.M.R. and S.P.R. conceived the study, designed the experiments, analyzed and interpreted the data, and wrote the manuscript. S.B. contributed to the generation of *Nrf2*^{flax/flax} mice. T.J.M. provided transgenic mice expressing Cre recombinase under the Clara cell-specific promoter.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 45, pp 1161–1168, 2011

Originally Published in Press as DOI: 10.1165/rcmb.2011-01440C on June 9, 2011

Internet address: www.atsjournals.org

CLINICAL RELEVANCE

The contribution of cellular stress, generated by the airway epithelium in response to injurious insults that promote acute lung injury, is largely undefined. Our data demonstrate that stress response-modifier Nrf2-regulated signaling in Clara cells is critical for conferring protection from oxidant-induced acute lung injury and for resolving inflammation during the repair process.

species (ROS/RNS), resulting in proteinaceous edema and inflammation and a subsequent impairment of respiratory function, as well as morbidity and mortality. The production of excessive ROS during prolonged exposure to hyperoxia can trigger damage to lung tissue and cause the death of both endothelial and alveolar Type I and Type II epithelial cells *in vitro* and *in vivo* (3–5). The infiltrated leukocytes, which are generally recruited to the lung during injury, are known to generate ROS/RNS, which contribute to or perpetuate lung injury in various experimental models of ALI/ARDS (6). However, the exact contribution of the oxidative stress generated by various cell types to ALI/ARDS and to the subsequent development of lung pathogenesis is not well-defined.

The Nrf2 transcription factor regulates the expression levels of several antioxidant enzymes and proteins that are required to protect cells against oxidant injury (7, 8). Nrf2 is ubiquitously expressed in various cell types, and is predominantly localized to the cytoplasm. However, in response to cellular stress, it is translocated to the nucleus and binds to the antioxidant response element (ARE) in the regulatory regions of various antioxidative genes, and activates their transcription. The conventional disruption of *Nrf2* leads to greater susceptibility to various injurious insults, including hyperoxia (9). When exposed to chronic levels of hyperoxic stress, Nrf2-deficient mice display greater levels of lung injury and inflammation than do Nrf2-sufficient mice (10). The lack of Nrf2 also leads to an impairment of the resolution of lung injury and inflammation after sublethal hyperoxic insults (11). The administration of the antioxidant glutathione to Nrf2-deficient mice immediately after sublethal exposure to hyperoxia can block DNA damage, restore the proliferation of endothelial and Type II alveolar cells in the lungs, and attenuate lung inflammation (11). Although studies using a whole-body Nrf2-knockout model demonstrated a protective role for Nrf2 during oxidant injury, the compartmentalized effects of lung cell type-specific Nrf2 signaling during oxidant injury and repair process remain largely undefined.

Airway epithelial cells are essential for maintaining the integrity of lung structure and function. They also serve as the first line of defense against a variety of insults, including respiratory toxicants. In response to toxicant and oxidant stress, airway epithelial cells secrete growth factors and inflammatory cytokines that regulate lung inflammation and tissue repair (12). Although

oxygen-induced cellular injury to the lung endothelium and alveolar epithelium is known to contribute to ALI and inflammation (13, 14), the contribution of cellular stress generated by the airway epithelium in response to oxidant exposure, particularly hyperoxic insult, to tissue injury and the repair process is largely undefined. To determine this contribution, we generated mice bearing a “floxed” allele of *Nrf2*, and deleted *Nrf2* specifically in Clara cells by using a Cre-loxP recombination system, and then exposed the mice to hyperoxic insult. Here, we report that the disruption of *Nrf2* in Clara cells is sufficient to exacerbate hyperoxic lung injury and to impair the resolution of inflammation during recovery.

Some of the results of these studies were previously reported in the form of an abstract at the ATS International Conference in May 2010 (15).

MATERIALS AND METHODS

Mice

Mice carrying the *Nrf2* “floxed” allele (*Nrf2^{fl/f}*) were generated on a C57BL/6 background. A targeting vector of *Nrf2* was constructed by inserting LoxP sites flanking the DNA-binding domain (exon 5) of the *Nrf2* gene and a neomycin cassette flanked by flp recombinase target sites for the selection of positive clones. The linearized vector was transfected into C57BL/6J embryonic stem (ES) cells. After selection with neomycin, surviving clones were expanded for PCR analysis to identify ES clones with homologous recombination at the *Nrf2* locus. Two recombinant clones were identified and used for injection into recipient female mice. Chimeras with the targeted allele were backcrossed with C57BL/6 mice to generate the *Nrf2^{fl/fl}* (hereafter referred to as *Nrf2^{fl/f}*) and neomycin cassette (Neo)⁺ mice. Mice expressing Cre recombinase under the control of the Clara-cell secretory protein (CCSP) promoter on a C57BL/6 background were described elsewhere (16). All experimental animal protocols were performed in accordance with guidelines of the Animal Care and Use Committee at Johns Hopkins University (Baltimore, MD).

PCR Genotyping

We used N1: TCTTAGGCACCATTGGGAGAG (forward) and N2: TACAGCAGCA TACCATTGTGG (reverse) primers for the detection of “floxed” alleles. For the detection of *Nrf2* disruption, N1, N2, and N3 (TGAGAGCTCCAGACTCA CTT [forward]) primers

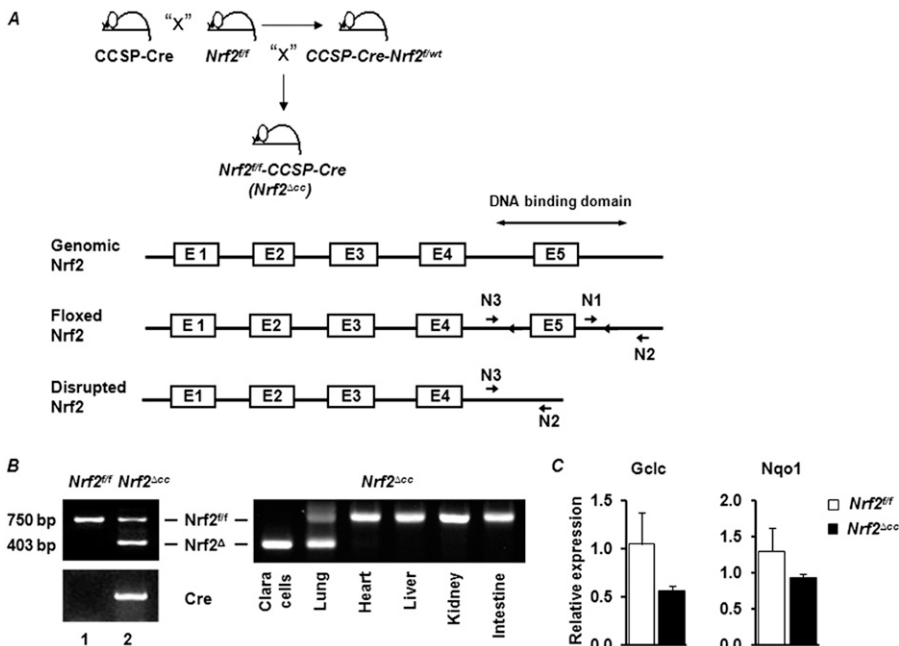


Figure 1. Generation of mice lacking *Nrf2* in Clara cells. (A) Schematic presentation shows the generation of *Nrf2^{fl/fl}*-CCSP-Cre (*Nrf2^{Δcc}*) mice. Mice with the *Nrf2* “floxed” allele (*Nrf2^{fl/f}*) were bred with Clara-cell secretory protein (CCSP)-Cre mice, and the resulting *Nrf2^{fl/fl}*/CCSP-Cre (F1) mice were backcrossed with *Nrf2^{fl/f}* mice to produce *Nrf2^{fl/fl}*/CCSP-Cre (*Nrf2^{Δcc}*) mice (top). Schematic presentation of the wild-type, floxed, and deleted locus of *Nrf2*. Location of loxP sites and PCR primer binding sites are indicated (bottom). (B) PCR amplification of genomic DNA isolated from lungs of *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice (left). PCR amplification of genomic DNA isolated from Clara cells and different tissues of *Nrf2^{Δcc}* mice (right), indicating *Nrf2* disruption only in lungs, specifically in Clara cells. bp, base pairs. (C) Relative expression of glutamate-cysteine ligase, catalytic subunit (*Gclc*) and NAD(P)H dehydrogenase, quinone 1 (*Nqo1*) in tracheal bronchial epithelial cells isolated from *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice.

were used, as outlined in Figure 1B. For the detection of CCSP-Cre, CCSP: ACTGCCATTGCCCAAACAC (forward) and Cre: ACGA ACCTGGTGCAGAAATC AGTGCG (reverse) primers were used.

Exposure to Hyperoxia and Assessment of Lung Injury and Inflammation

The *Nrf2^{fl/f}* and *Nrf2^{fl/f}*-CCSP-Cre (*Nrf2^{Δcc}*) mice (8 weeks old) were exposed to hyperoxia or room air for 48 hours or 60 hours. A separate group of mice exposed to hyperoxia for 48 hours ($n = 6$) was allowed to recover in room air for 24 or 72 hours. Lung alveolar permeability and inflammation were assessed as previously described (11). To assess vascular permeability, mice were injected intraperitoneally with Evans blue dye (EBD, 75 mg/kg body weight; Sigma, St. Louis, MO). Lungs were perfused with warm PBS containing 20 U/ml of heparin, harvested *en bloc*, rinsed in PBS, and dabbed until dry. EBD from lungs and plasma was extracted in formamide. Concentrations of EBD from lung extracts and plasma extracts were measured at 620 nm. Lungs were dried at 65°C for 72 hours, and dry weights were measured. The ratio of lung EBD/plasma EBD/lung dry weight was calculated for each sample.

Analysis of Cell Death and mRNA Expression

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and real-time PCR assays were performed as previously described (11).

Isolation of Tracheal Bronchial Epithelial Cells

Tracheal bronchial epithelial cells (TBECs) were isolated as described previously (17).

Cytokine Analysis

Bronchoalveolar lavage (BAL) fluid samples from mice were analyzed according to the multiplex suspension array system, using Luminex beads (Bio-Rad Laboratories, Hercules, CA). BAL samples were thawed, diluted 1:1, and run according to the manufacturer’s protocol, and were measured as picograms per milliliter of BAL fluid ($n = 5$).

Statistical Analysis

All data involving animal experimentation were collected in a double-blinded fashion. Data were expressed as mean \pm SEM ($n = 3-6$ for each condition). ANOVA was used to compare means of multiple groups. For paired data, the Student *t* test was used. Significance was defined as $P \leq 0.05$.

RESULTS

Conditional Deletion of Nrf2 in Clara Cells

To determine the precise role of airway epithelia-specific Nrf2 signaling in providing protection against hyperoxia-induced ALI, we deleted *Nrf2* specifically in Clara cells, using a Cre-loxP recombination approach. We first generated mice bearing a “floxed” allele of *Nrf2* (*Nrf2^{fl/f}*) (Figure 1A), in which loxP sites were inserted flanking exon 5, the DNA-binding domain of Nrf2 (Figure 1A). *Nrf2^{fl/f}* mice were crossed with transgenic mice bearing cDNA encoding Cre recombinase under the control of the CCSP promoter. The resulting *Nrf2^{fl/wt}-CCSP-Cre* mice were then backcrossed to the parental *Nrf2^{fl/f}* mice to obtain *Nrf2^{fl/f}-CCSP-Cre* mice (hereafter referred to as *Nrf2^{Δcc}*). *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice were genotyped for the presence of the *Cre* gene and *Nrf2* “floxed” alleles, as outlined in Figure 1B. As anticipated, we observed both deleted and undeluted *Nrf2* “floxed” alleles in the lung tissue of *Nrf2^{Δcc}* mice (Figure 1B, left, lane 2), because the CCSP promoter drives Cre expression predominantly in Clara cells but not in other cell types in the lung. The genomic DNA from *Nrf2^{fl/f}* mice yielded only a high molecular weight band with “floxed” sequences (750 base pairs [bp]; Figure 1B, left, lane 1). To confirm the specificity of the *Nrf2* deletion further, we isolated genomic DNA from purified TBECs and other tissues such as heart, liver, kidney, and intestine from *Nrf2^{Δcc}* mice and PCR-amplified it. As anticipated, the disrupted allele was amplified (403 bp) only in Clara cells and lung tissue, whereas the floxed allele (750 bp) was amplified in other tissues (Figure 1B, right). To determine the levels of Nrf2-dependent gene expression in the airway epithelium of *Nrf2^{Δcc}* and *Nrf2^{fl/f}* mice, we analyzed glutamate-cysteine ligase, catalytic subunit (*Gclc*) and NAD(P)H dehydrogenase, quinone 1 (*Nqo1*) mRNA expression in TBECs by real-time RT-PCR analysis. As shown in Figure 1C, *Gclc* and *Nqo1* mRNA expression levels were markedly lower in *Nrf2^{Δcc}* mice than in *Nrf2^{fl/f}* mice. These results confirmed the deletion of *Nrf2* in the Clara cells of *Nrf2^{Δcc}* mice. We did not analyze the expression of *Nrf2* because the probes used in Taqman assays hybridize to both the disrupted and native forms of *Nrf2*.

Nrf2^{Δcc} Mice Exhibit Higher Levels of Hyperoxic Lung Injury Than Do Wild-Type Mice

To examine the effects of Clara cell-specific Nrf2 deficiency on oxidant-induced ALI, we exposed *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice to hyperoxia for 60 hours. This level of exposure is known to cause significant or severe forms of lung injury. In contrast, prolonged exposure (> 60 hours) causes lethality in C57BL6 mice. Lung injury was evaluated by (1) a histologic assessment of tissue sections, (2) quantifying vascular leakage with an EBD extravasation assay, and (3) analyzing alveolar permeability by measuring the protein concentration in BAL fluid. Lung inflammation was assessed by enumerating the number of inflammatory cells present in the BAL fluid obtained from the mice. As shown in Figure 2A, our histologic analysis revealed greater levels of hyperoxia-associated lung damage and epithelial sloughing in *Nrf2^{Δcc}* mice than in their *Nrf2^{fl/f}* counterparts (Figure 2A, bottom, contains enlarged images of epithelial sloughing).

The assessment of lung vascular permeability by the method of EBD extravasation revealed higher levels of vascular leakage in the lungs of *Nrf2^{Δcc}* mice than in those of their *Nrf2^{fl/f}* counterparts (Figure 2B). Consistent with these data, we found a significant increase in the accumulation of total protein in the BAL fluid of *Nrf2^{Δcc}* mice compared with *Nrf2^{fl/f}* mice (Figure 2C). Likewise, the number of total cells (Figure 3A) in BAL fluid was significantly greater in *Nrf2^{Δcc}* mice than in *Nrf2^{fl/f}* mice.

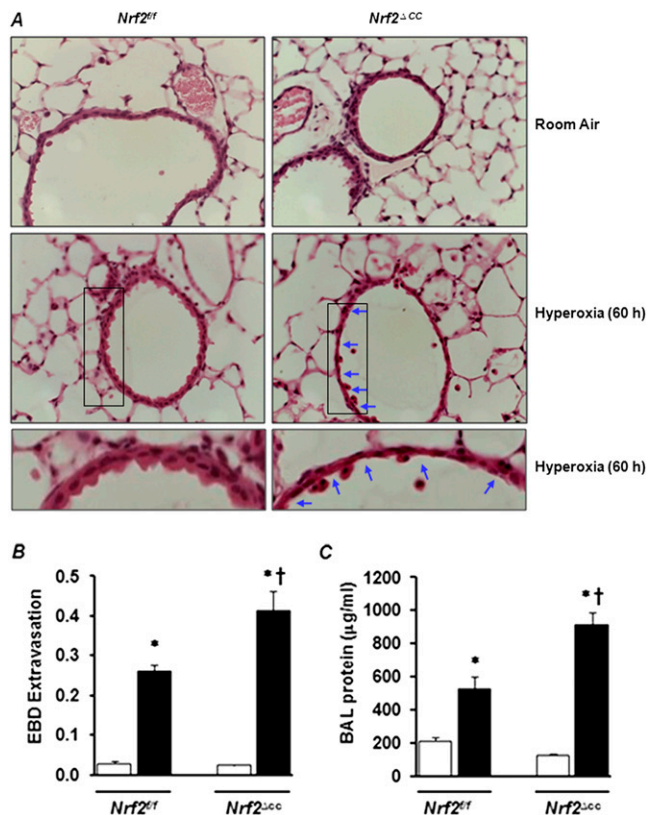


Figure 2. Hyperoxia-induced lung injury in *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice. *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice were exposed to room air (RA) or hyperoxia for 60 hours and killed, and their left lungs were inflated and fixed in 1.5% paraformaldehyde. Lung tissue sections were prepared and stained with hematoxylin and eosin (H&E). (A) Representative images of H&E-stained lung sections of three mice are shown. Bottom: Enlarged images of lung tissue sections of *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice exposed to 60 hours of hyperoxia. Arrows indicate epithelial sloughing in *Nrf2^{Δcc}* mice. (B) Evans blue dye (EBD) was injected intraperitoneally into the mice 2 hours before they were killed. Lungs were perfused, and lung EBD and serum EBD were extracted into formamide. Lungs were dried, and the ratios of lung EBD/serum EBD/dry ratios were calculated. The data shown represent means \pm SEM ($n = 5$). (C) Bronchoalveolar lavage (BAL) fluid was collected from the right lung, and total protein was estimated in BAL fluids with a Bio-Rad protein assay. Data represent means \pm SEM ($n = 6$). Significance was calculated using the Student *t* test. * $P \leq 0.05$, room air versus hyperoxia or recovery. † $P \leq 0.05$, *Nrf2^{fl/f}* mice versus *Nrf2^{Δcc}* mice. Open bars, room-air controls; solid bars, hyperoxia.

This increase in the number of total cells is mainly attributable to the increase in the accumulation of macrophages (Figure 3B) and epithelial sloughing in the lungs (Figure 3C). The hyperoxia-induced infiltration of neutrophils occurred in both genotypes, with no significant difference in concentrations of neutrophils between genotypes (Figure 3D). Thus, these results suggest that *Nrf2* disruption in Clara cells is sufficient to perpetuate lung injury in response to injurious hyperoxic exposure.

Hyperoxia Induces a Greater Level of Cell Death in *Nrf2^{Δcc}* Than in *Nrf2^{fl/f}* Mice

Because the DNA damage caused by hyperoxia can lead to cell death, we evaluated cell death by TUNEL staining in the lungs of *Nrf2^{fl/f}* and *Nrf2^{Δcc}* mice after exposure to hyperoxia for 60 hours. As expected, we found approximately equal numbers of TUNEL-positive cells in the lung parenchyma of both genotypes after exposure to hyperoxia (Figure 4A). However, we observed

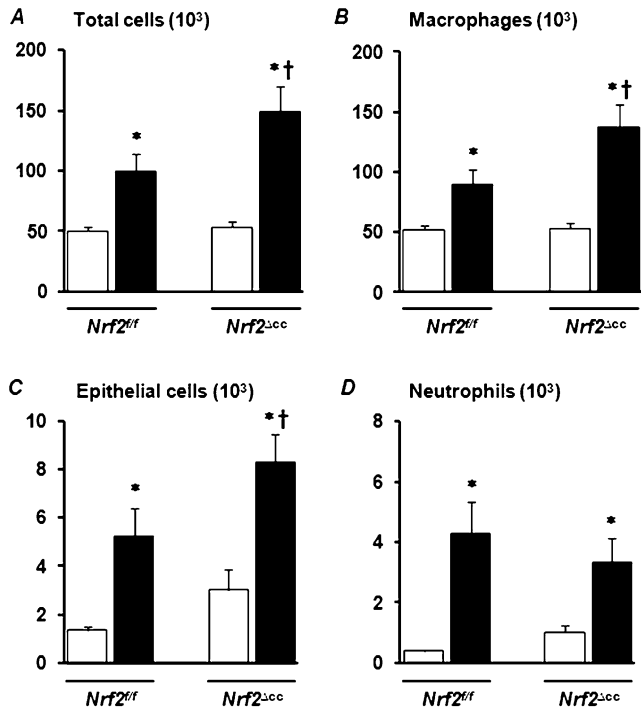


Figure 3. Deletion of *Nrf2* in Clara cells exacerbates hyperoxia-induced lung inflammation. *Nrf2^{fl/fl}* mice and *Nrf2^{Δcc}* mice were exposed to room air or hyperoxia for 60 hours. BAL fluid was collected from the right lung, centrifuged, and stained with a Diff-Quik stain kit. Differential cell counts were performed to analyze total cells (A), macrophages (B), epithelial cells (C), and neutrophils (D), and are expressed as means \pm SEM. One-way ANOVA, followed by Newman-Keuls *post hoc* analysis, was performed for multiple group comparisons, whereas two-way ANOVA with a Bonferroni correction was used to compare interactions between genotype and hyperoxia. In both cases, $P \leq 0.05$ was considered statistically significant. *Room air versus hyperoxia. †*Nrf2^{fl/fl}* versus *Nrf2^{Δcc}* counterparts. Open bars, room-air controls; solid bars, hyperoxia.

a large number of TUNEL-positive cells in the airways of *Nrf2^{Δcc}* mice (Figure 4A) because of the lack of Nrf2 in these cells. Only a very few TUNEL-positive cells (1–2 per field) were evident in the airways of *Nrf2^{fl/fl}* mice exposed to hyperoxia. The quantification of total TUNEL-positive cells revealed a 6-fold increase in cell death in *Nrf2^{Δcc}* mice compared with *Nrf2^{fl/fl}* mice (Figure 4B; compare bar 2 and bar 4). No cell death was evident in the airways of room air-exposed mice of either genotype. Taken together, these results suggest that the Nrf2 deficiency in Clara cells makes them susceptible to hyperoxia-induced damage and leads to an impairment of Clara-cell function.

Nrf2 Signaling in Clara Cells Is Critical to Dampening the Inflammatory Response during Recovery from Lung Injury

To determine the role of Clara cell-specific Nrf2 signaling in the resolution of the hyperoxia-induced inflammatory response, *Nrf2^{fl/fl}* and *Nrf2^{Δcc}* mice ($n = 18$) were exposed to sublethal (48-hour) hyperoxic insult. Some of these mice ($n = 6$) were immediately killed for analyses of lung injury, and some were allowed to recover in normoxia for 24 or 72 hours ($n = 6$ for each time point). Lung injury and inflammation were then assessed by histopathology and analysis of BAL fluid (Figure 5). Histopathologic examination revealed no significant difference in lung injuries between the *Nrf2^{fl/fl}* and *Nrf2^{Δcc}* mice immediately after 48-hour hyperoxia (Figure 5A). Consistent with the histopathology data, protein concentrations in BAL fluid did not differ

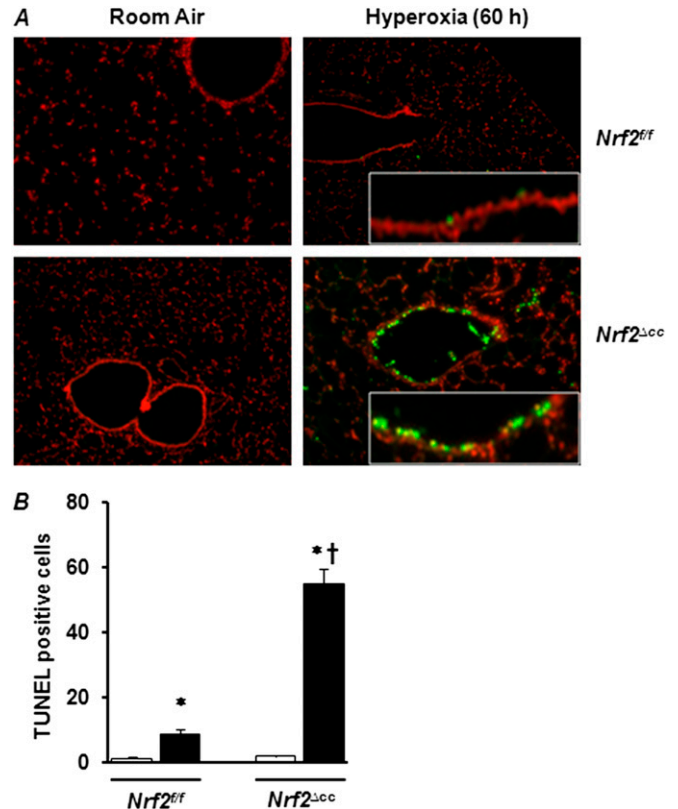


Figure 4. Hyperoxia-induced cell death in airways of *Nrf2^{fl/fl}* and *Nrf2^{Δcc}* mice. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed, using an *In Situ* Cell Death Detection Kit on lung tissues of *Nrf2^{fl/fl}* versus *Nrf2^{Δcc}* mice exposed to room air and hyperoxia for 60 hours. (A) Representative images of TUNEL staining of lung tissue from mice in room air or exposed to 60 hours of hyperoxia are shown. (B) TUNEL-positive epithelial cells were enumerated (six fields/lung section), and the data represent the average of three mice \pm SEM. Significance was calculated using the Student *t* test. * $P \leq 0.05$, room air versus hyperoxia. † $P \leq 0.05$, *Nrf2^{fl/fl}* mice versus *Nrf2^{Δcc}* counterparts. Open bars, room-air controls; solid bars, hyperoxia.

significantly between the two genotypes immediately after hyperoxic exposure (Figure 5B). The analysis of BAL fluid revealed that the number of alveolar macrophages was unaltered in both genotypes after exposure to hyperoxia (Figure 5C). However, we found greater levels of neutrophil infiltration and epithelial sloughing in both genotypes after exposure to hyperoxia, compared with room air-exposed counterparts (Figure 5C).

On the other hand, when mice were allowed to recover for 24 hours after exposure to hyperoxia, we saw an increased level of cellularity in the lungs of both genotypes, compared with their respective room air-exposed or hyperoxia-exposed littermates (Figure 5A). Although the number of total cells was comparable after 24 hours of recovery in both genotypes, an increase in epithelial sloughing occurred in the *Nrf2^{Δcc}* mice (Figure 5C). Alveolar permeability, as assessed by total protein concentration in the BAL, had increased significantly in both genotypes at 24 hours after hyperoxia (Figure 5B).

The cellular accumulation and proteinaceous edema in alveolar spaces in the lungs of *Nrf2^{Δcc}* mice persisted at 72 hours after oxidant exposure (Figure 5A). In contrast, these injury-associated phenotypes were not evident in the lungs of *Nrf2^{fl/fl}* mice. Consistent with our histologic data, the protein concentrations in BAL fluid remained high in the *Nrf2^{Δcc}* mice, but not in the *Nrf2^{fl/fl}* mice, at 72 hours after exposure (Figure 5B). Moreover, the cellular

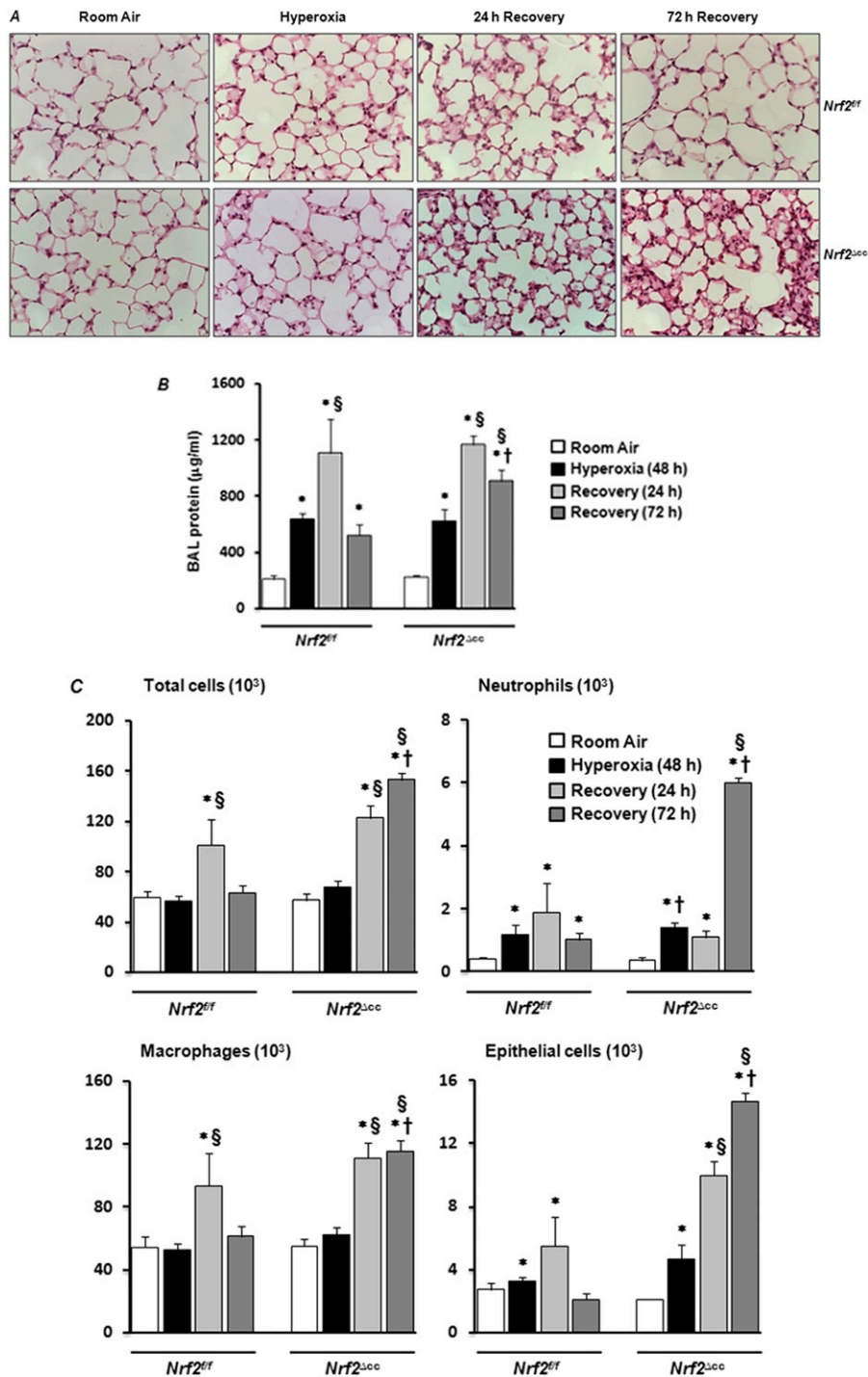


Figure 5. Effect of Nrf2-deficiency on lung injury and inflammation during recovery from hyperoxic injury. *Nrf2^{fl/fl}* and *Nrf2^{Δcc}* mice were exposed to room air or hyperoxia for 48 hours. Some mice ($n = 6$) were killed at the end of 48 hours of exposure, and some mice were allowed to recover for 24 hours ($n = 6$) or 72 hours ($n = 6$). (A) Left lungs were inflated and fixed. Lung sections were stained with H&E. Representative images of three mice are shown. (B) BAL fluid was collected from the right lung, and total protein was estimated. Data represent means \pm SEM ($n = 6$). (C) Differential cell counts were performed to analyze total cells, neutrophils, macrophages, and epithelial cells. Data are expressed as means \pm SEM. One-way ANOVA, followed by Newman-Keuls *post hoc* analysis, was performed for multiple group comparisons, whereas two-way ANOVA with a Bonferroni correction was used for comparing interactions between genotype and hyperoxia. *Room air versus 48-hour hyperoxia or recovery. §48 hours of hyperoxia versus 24 hours or 72 hours of recovery. †*Nrf2^{fl/fl}* mice versus *Nrf2^{Δcc}* counterparts.

infiltration persisted in *Nrf2^{Δcc}* mice at 72 hours after exposure, but had resolved in the *Nrf2^{fl/fl}* mice (Figure 5C). These results demonstrate that the specific disruption of Nrf2 signaling in Clara cells leads to an impairment of the resolution of tissue repair, especially inflammation, after sublethal hyperoxic insult.

A Lack of Clara Cell-Specific Nrf2 Signaling Dysregulates the Expression of Inflammatory Cytokines Induced by Hyperoxia

The inflammatory-cell influx induced by hyperoxia is orchestrated by several cytokines (18). We therefore used multiplex cytokine assays to measure the concentrations of various

cytokines in the BAL fluid obtained from *Nrf2^{fl/fl}* and *Nrf2^{Δcc}* mice after exposure to room air or 48 hours of hyperoxia, and at 72 hours after injury. Among the cytokines measured, only the concentrations of macrophage chemotactic protein (Mcp1), macrophage inflammatory protein 1 beta (Mip1β), and chemokine (C-X-C motif) ligand 1 (Cxcl1) keratinocyte-derived chemokine were significantly different between the *Nrf2^{fl/fl}* and *Nrf2^{Δcc}* mice. Basal concentrations of Mcp1 were 10-fold higher in *Nrf2^{Δcc}* mice (23 pg/ml) than in *Nrf2^{fl/fl}* mice (2 pg/ml). Hyperoxia induced a 20-fold increase in Mcp1 concentrations in *Nrf2^{fl/fl}* mice (56 pg/ml), and a modest alteration in *Nrf2^{Δcc}* mice (30 pg/ml) (Figure 6A). When mice were allowed to recover, concentrations of Mcp1 robustly increased (to 200 pg/ml) in

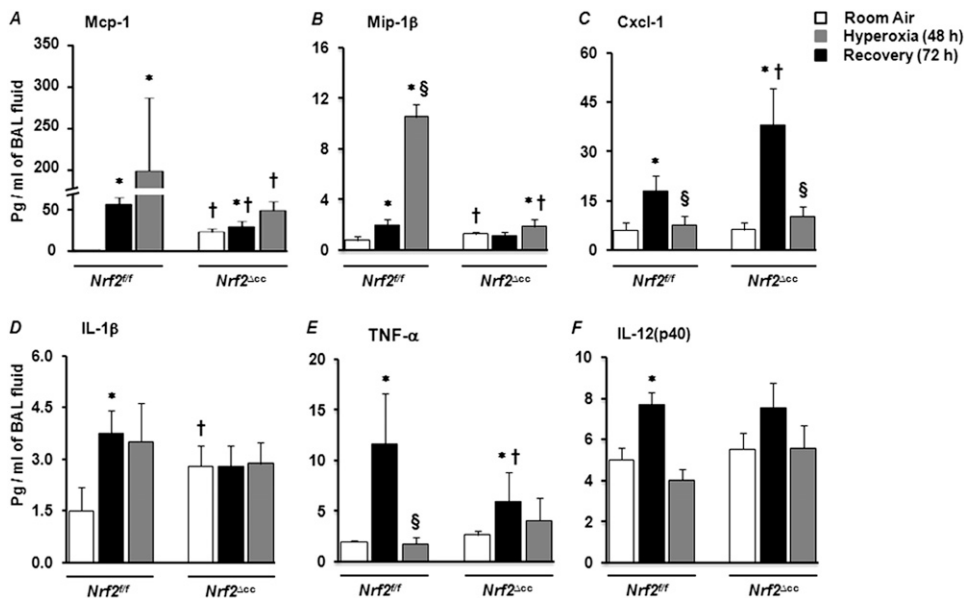


Figure 6. Expression levels of inflammatory cytokines. Cytokines were measured in BAL fluids of *Nrf2^{fl/fl}* mice versus *Nrf2^{Δcc}* mice exposed to room air, 48 hours of hyperoxia, or 72 hours after injury. Analyses of cytokines were performed with a multiplex suspension array system, using Luminex beads. Mean values \pm SEM are shown in picograms for macrophage chemotactic protein (Mcp1) (A), macrophage inflammatory protein 1 beta (Mip1 β) (B), chemokine (C-X-C motif) ligand 1 (Cxcl1) keratinocyte-derived chemokine (KC) (C), IL-1 β (D), TNF- α (E), and IL-12 (p20) (F) ($n = 5$). Significance was calculated using the Student t test. * $p \leq 0.05$, room air versus hyperoxia or recovery. § $p \leq 0.05$, hyperoxia versus recovery. † $p \leq 0.05$, *Nrf2^{fl/fl}* mice versus *Nrf2^{Δcc}* counterparts.

Nrf2^{fl/fl} mice, whereas a 2-fold increase was observed in *Nrf2^{Δcc}* mice (49 pg/ml) compared with room air-exposed control mice (Figure 6A). Similarly, concentrations of Mip1 β increased 2-fold and 11-fold in the lungs of *Nrf2^{fl/fl}* mice during exposure to hyperoxia and recovery, respectively. In contrast, concentrations of Mip1 β in *Nrf2^{Δcc}* mice were unaltered during hyperoxia, and only modestly increased (2-fold) during recovery (Figure 6B).

Concentrations of Cxcl1, a neutrophil chemoattractant, in *Nrf2^{fl/fl}* and *Nrf2^{Δcc}* mice had significantly increased after 48 hours of hyperoxia, compared with those of room air-exposed mice (Figure 6C). In agreement with the enhanced infiltration of neutrophils, concentrations of Cxcl1 were approximately 2-fold higher in *Nrf2^{Δcc}* mice than in *Nrf2^{fl/fl}* mice. Concentrations of Cxcl1 had decreased to basal level in both genotypes at 72 hours of recovery. Concentrations of IL-1 β were 2-fold higher in room air-exposed *Nrf2^{Δcc}* mice than in *Nrf2^{fl/fl}* mice. No induction of IL-1 β was evident during hyperoxia or recovery in *Nrf2^{Δcc}* mice. In contrast, concentrations of IL1 β protein increased by 2.5-fold after exposure, and remained high (a 2-fold increase) during recovery in *Nrf2^{fl/fl}* mice (Figure 6D). Hyperoxia significantly induced TNF- α in both wild-type and *Nrf2* mutant mice. However, the increase in TNF- α was significantly higher in *Nrf2^{fl/fl}* mice than in *Nrf2^{Δcc}* mice (Figure 6E). Concentrations of TNF- α decreased to basal level in both genotypes during recovery. Concentrations of IL-12p40 did not differ between genotypes in room air-exposed or hyperoxia-exposed mice, either immediately after hyperoxia or after recovery (Figure 6F). These results indicate that the disruption of *Nrf2* in Clara cells alters the expression levels of a variety of important cytokines.

DISCUSSION

The oxidative stress produced by resident (lung epithelial and endothelial) cells and infiltrating leukocytes appears to play a major role in the development and perpetuation of oxidant-induced ALI, but the individual contributions of these cell types to pathogenic ALI remain largely undefined *in vivo*. In the present study, we provide experimental (genetic) evidence to support a pivotal role for airway epithelial (Clara) cell-specific Nrf2 signaling in conferring protection from oxidant-induced ALI. Dysfunction in the Nrf2-regulated antioxidant defense system

in CCSP-expressing cells exacerbated lung injury (vascular and alveolar permeability) and increased the infiltration of inflammatory leukocytes into the lung in response to hyperoxic insult. These phenotypic changes were coincident with greater levels of apoptotic death, predominantly in Clara cells, and altered cytokine expression in the lung. Furthermore, a lack of Nrf2 signaling in Clara cells perpetuated lung injury and inflammation when mice were allowed to recover from sublethal hyperoxic insult, whereas these processes resolved in wild-type mice. Thus, our data illuminate a novel role for Clara cell-specific Nrf2 signaling, both during and after hyperoxic lung injury and repair.

Reactive electrophiles, generated during prolonged exposure to hyperoxia, induce apoptotic death in both endothelial cells and alveolar Type I and Type II epithelial cells (19, 20). However, the lung's epithelial lining fluid is known to possess a superior antioxidant system, and epithelial cells are relatively resistant to oxidant stress (21). Consistent with this notion, TUNEL staining revealed moderate levels of cell death in the airways of wild-type mice exposed to hyperoxia. However, we observed higher numbers of TUNEL-positive cells in the airways of *Nrf2* mutant mice (Figure 4). The DNA damage induced by hyperoxia was reported to promote growth arrest in Type II alveolar epithelial cells *in vivo* and *in vitro* (22, 23). We previously showed that a deficiency of Nrf2 causes cellular stress and DNA damage, and impairs the proliferation of Type II alveolar cells as a result of a deregulation of expression of genes involved in cell-cycle progression (24). Moreover, we found an up-regulation of cytokine expression in Nrf2-deficient Type II epithelial cells (25). Thus, the lack of an Nrf2 transcription factor in Clara cells caused an increase in cellular stresses, as a result of their insufficient antioxidant defense system. This vulnerability led to cell death and a deregulated inflammatory response, which ultimately impaired the resolution of inflammation and processes of tissue repair in *Nrf2^{Δcc}* mice.

Resident epithelial cells secrete various chemotactic inflammatory cytokines in response to pro-oxidant exposure. These cytokines play an important role in the augmentation and the resolution of inflammation (26, 27). The activation of circulating neutrophils and their subsequent transmigration into the alveolar air space after an injurious insult were linked to the development of ALI. The transmigration of neutrophils into the alveolar compartment of the lung is regulated by Cxcl1 through receptor-mediated

signaling (i.e., binding to its receptor, CXCR2) (28). Consistent with this mechanism, our analysis of cytokines revealed higher concentrations of Cxcl1 in the BAL fluid obtained from *Nrf2^{Δcc}* mice after hyperoxic injury, which was correlated with the increased concentrations of neutrophils seen in these mice when compared with their wild-type littermates (*Nrf2^{fl/fl}*) (Figure 6). We also found that *Mcp1*, a chemokine required for the resolution of inflammation (29, 30), was induced several-fold in *Nrf2^{fl/fl}* mice compared with *Nrf2^{Δcc}* mice. *Mcp1* is expressed in airway epithelial cells in response to oxidant stress, and regulates the production of mucus in lungs (31). Similarly, concentrations of *Mip1β* protein were not induced in *Nrf2^{Δcc}* mice, but were elevated in *Nrf2^{fl/fl}* mice during exposure to hyperoxia and recovery. *Mip1β* is mainly produced by hematopoietic cells and to a lesser extent by epithelial cells, and it regulates a wide range of target-cell functions, including chemotaxis, degranulation, and phagocytosis (32). Nrf2 binds to the ARE, and activates transcription after challenge with a pro-oxidant or toxic stimulus. The analysis of transcription factor motifs revealed the presence of consensus AREs in both murine and human *Mcp1* and *Mip1β* gene promoters. Although our results suggest that the expression of *Mcp1* and *Mip1β* is regulated by Nrf2, whether these genes are direct or indirect targets of Nrf2 and their exact roles in resolving inflammation in our experimental conditions warrant further study.

Macrophages regulate both the progression and resolution of inflammation. They are also essential for the clearance of apoptotic granulocytes and cell debris and the resolution of lung injury and inflammation (33, 34). However, the accumulation of macrophages releases additional ROS and inflammatory cytokines, which contribute to the development of acute lung injury (35). We found no change in the number of macrophages in *Nrf2^{fl/fl}* mice immediately after 48 hours of hyperoxia or during recovery, when compared with room air-exposed controls. Although the number of macrophages in *Nrf2^{Δcc}* mice immediately after 48 hours of hyperoxia was comparable to that in *Nrf2^{fl/fl}* mice, we observed a greater level of macrophage influx into the BAL fluid and interstitium of the lungs in *Nrf2^{Δcc}* mice. Notably, our data revealed an increased level of cellular infiltration in *Nrf2^{Δcc}* mice during recovery after injury, although concentrations of cytokines had decreased to basal level by this time. The increased level of cellular inflammation in *Nrf2^{Δcc}* mice could be attributed either to the initial release of chemokines in the lungs of these mice in response to hyperoxia, or to a lack of the specific cytokines, such as *Mcp1*, required for the resolution of macrophage inflammation. Nonetheless, our results suggest that the Clara cell-specific Nrf2 transcriptional response may play a role in limiting the inflammatory response in the lung, most likely by modulating macrophage functions.

Clara cells are critical for airway repair after injury as well for the modulation of airway inflammation (36, 37). The CCSP secreted by Clara cells attenuates the inflammatory response induced by various stimuli by modulating the activity of leukocytes through the regulation of inflammatory cytokine expression. For example, CCSP increases neutrophil phagocytosis and decreases the oxidative activity of neutrophils (38), and the deletion of CCSP augments LPS-induced ALI and inflammation in mice (37). The activation of NF-κB in airway epithelia in response to endotoxin and nitric oxide toxicity was attributed to lung injury and inflammation (39–41). The overexpression of transcription factors such as signal-transducer and activator of transcription 3C and CCAAT/enhancer binding protein alpha in airway epithelia protects mice from hyperoxia-induced ALI (42, 43), whereas the deletion of signal-transducer and activator of transcription 3 specifically in Clara cells impairs epithelial repair after exposure to naphthalene *in vivo* (44). Although our data demonstrate that the Nrf2 transcriptional response in

Clara cells is critical for mitigating hyperoxia-induced ALI, whether Nrf2 acts as a functional effector in regulating these proteins remains to be investigated under our experimental conditions. Our data suggest that Nrf2 signaling in Clara cells is critical in conferring protection from hyperoxia-induced ALI and in the resolution of inflammation during recovery from injury. Under physiological conditions, other transcriptional factors such as Nrf3 and the activator protein-1 family of proteins, such as c-Jun and Jun-D, may compensate for the functions of Nrf2, to maintain a basal level of expression in the antioxidant defense system (45, 46). However, the deletion of *Nrf2* is unlikely to lead to an up-regulation of a compensatory pathway to mitigate cellular stress in Clara cells in response to oxidant stress, because the specific deletion of *Nrf2* resulted in a greater level of cellular damage, specifically to airway epithelia, after exposure to hyperoxia.

In conclusion, our findings indicate that airway epithelial (Clara cell)-specific Nrf2 signaling is important for mitigating oxidant (hyperoxia)-induced ALI as well as for resolving lung inflammation after injury. Activation of the Nrf2 pathway in airway epithelia may offer a promising approach to attenuating the lung injury and inflammatory responses seen in ALI/ARDS.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank the Pathology Core of the Acute Lung Injury-Specialized Centers of Clinically Oriented Research (through National Institutes of Health grant P50-HL073994) for providing their services on a charge basis, Dr. Steven Kleeberger (National Institute of Environment Health Sciences, National Institutes of Health) for his continued interest in and support of this work, and Deborah McClellan for editorial assistance.

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