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Innate Immunity Against Bacterial Infection Following Hyperoxia Exposure Is Impaired in NRF2-Deficient Mice1

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Oxygen supplementation is used as therapy to support critically ill patients with severe respiratory impairment. Although hyperoxia has been shown to enhance the lung susceptibility to subsequent bacterial infection, the mechanisms underlying enhanced susceptibility remain enigmatic. We have reported that disruption of NF-E2-related factor 2 (Nrf2), a master transcription regulator of various stress response pathways, enhances susceptibility to hyperoxia-induced acute lung injury in mice, and have also demonstrated an association between a polymorphism in the NRF2 promoter and increased susceptibility to acute lung injury. In this study, we show that Nrf2-deficient (Nrf2−/−) but not wild-type (Nrf2+/+) mice exposed to sublethal hyperoxia succumbed to death during recovery after Pseudomonas aeruginosa infection. Nrf2-deficiency caused persistent bacterial pulmonary burden and enhanced levels of inflammatory cell infiltration as well as edema. Alveolar macrophages isolated from Nrf2−/− mice exposed to hyperoxia displayed persistent oxidative stress and inflammatory cytokine expression concomitant with diminished levels of antioxidant enzymes, such as Gclc, required for glutathione biosynthesis. In vitro exposure of Nrf2−/− macrophages to hyperoxia strongly diminished their antibacterial activity and enhanced inflammatory cytokine expression compared with Nrf2+/+ cells. However, glutathione supplementation during hyperoxic insult restored the ability of Nrf2−/− cells to mount antibacterial response and suppressed cytokine expression. Thus, loss of Nrf2 impairs lung innate immunity and promotes susceptibility to bacterial infection after hyperoxia exposure, ultimately leading to death of the host. The Journal of Immunology, 2009, 183: 0000–0000.

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3 Abbreviations used in this paper: ALI, acute lung injury; Nrf2, NF-E2-related factor 2; ARE, antioxidant response element; cfu, colony forming unit; BAL, bronchoalveolar lavage; MARCO, macrophage receptor with collagenous structure; MSR1, macrophage scavenger receptor 1; GSH, glutathione.

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Oxygen supplementation, often leading to hyperoxia, is widely used to support critically ill patients with noninfectious and infectious acute lung injury (ALI) and in treating exacerbations of chronic obstructive lung disease. However, prolonged hyperoxia causes histopathological changes similar to acute lung injury in rodents. Experimental evidence obtained from various laboratories has shown that hyperoxia induces ALI by causing both lung epithelial and endothelial cell death leading to the disruption of epithelial and blood barrier integrity. Although excessive production of reactive electrophiles contributes to the development of ALI, the mechanisms underlying repair processes and inflammatory responses, as well as enhanced susceptibility to viral or bacterial infections during the recovery phase of ALI are not yet clearly understood.

NF-E2-related factor 2 (Nrf2) is a transcription factor that modulates cellular stress by regulating the expression of genes encoding several cellular detoxifying enzymes via the antioxidant response element (ARE). Nrf2 deficiency is known to cause diminished levels of basal and inducible expression of several stress response pathways crucial for both detoxification of reactive electrophiles generated by prooxidants during repair processes (see review, Ref. 7). Targeted deletion of Nrf2 enhances susceptibility of the lung to hyperoxia (8, 9). We have recently demonstrated that deletion of Nrf2 in mice leads to alveolar cell growth arrest and enhances cell sensitivity to prooxidants accompanied by a deregulated antioxidant transcriptional program both in vivo and in vitro (10, 11). In agreement with these results, administration of glutathione (GSH) to Nrf2−/− mice reverses these phenotypes. We also demonstrated persistently increased inflammation and cellular infiltration in the lungs of Nrf2−/− mice exposed to sublethal hyperoxia during recovery (12). Given that patients subjected to oxygen supplementation in intensive care units often suffer from infections such as pneumonia, which cause excess morbidity and mortality (13), we examined whether a dysfunctional Nrf2-ARE signaling enhances susceptibility to bacterial infection during recovery from hyperoxia. We provide for the first time evidence of an impairment of innate immunity against Pseudomonas aeruginosa infection following hyperoxic lung injury in Nrf2−/− mice, which occurs in part from deregulated alveolar macrophage response.

Materials and Methods

Hyperoxia exposure, P. aeruginosa infection, and assessment of lung injury and inflammation

The wild-type (Nrf2+/+) and Nrf2-deficient (Nrf2−/−) CD1/ICR strains of mice (6–8 wk, 25–30 grams) were exposed to hyperoxia (95% oxygen) or room air as previously described (12). Mice were then infected with low (105 colony forming units (cfu)) and high (106 cfu) doses of P. aeruginosa...
O1 constitutively expressing GFP (gift from Dr. Terry Manchen, University of California, Berkeley, CA) (14) for 4 and 72 h, respectively. After infection, lung inflammation was evaluated by differential cell counts in bronchoalveolar lavage (BAL) fluid of the right lung as previously described (12). The left lung was inflated to 25 cm of water pressure and fixed with 0.8% low-melting agarose in 1.5% buffered paraformaldehyde for 24 h, and 5 μm lung sections were cut and stained with H&E. Differential cell counts were performed after staining the cells with Diff-Quik stain kit (Dade Behring). All experiments were conducted under a protocol approved by the institutional animal care use committee of the Johns Hopkins University.

**In vivo bacterial clearance**

Ampicillin-resistant *P. aeruginosa* bacteria expressing enhanced GFP, grown from a single colony, were used throughout the study. The bacteria were grown in tryptic soy broth overnight at 37°C and the bacterial numbers were determined by plating 10-fold serial dilutions on ampicillin-agar plates and counting colonies. BAL fluids and lung (right middle lobe) tissues were collected at 4 and 72 h postinfection and respiratory bacterial burdens were measured by incubating serial 10-fold dilutions of BAL fluid and lung homogenates on ampicillin-agar plates at 37°C overnight. The cfu were enumerated and the total number of bacteria present in the BAL fluid and in the lung was quantified. The results were expressed as the total cfu present both in the total BAL fluid and lung tissue.

**In vitro bactericidal assay**

Peritoneal macrophages were isolated from the peritoneal cavity of mice administered with 2% thioglycollate using a standard protocol. Cells were washed with ice-cold PBS and then immediately exposed to hyperoxia for 24 h before bacterial infection. Live bacterial numbers were determined by plating the cell lysates on to ampicillin containing agar plates.

**Real-time RT-PCR**

The expression levels of various genes were quantified in triplicate by TaqMan gene expression assays (Applied Biosystems) using glyceraldehy-3-phosphate dehydrogenase (*gapdh*) and mitochondrial ribosomal protein L32 (*Mrpl32*) as internal control genes. The absolute expression values for each gene was normalized to that of *gapdh/ Mrpl32* and values from room air samples set as one unit. Each experiment was repeated and n of at least 3 was derived from two independent experiments.

**Cytokine measurements in BAL fluids**

BAL samples were analyzed for IL-6 protein content using ELISA Quantikine Mouse IL-6 Immunoassay (R&D Systems), according to the manufacturer’s instruction. Equal protein from the BAL samples were incubated in microplates precoated with a mAb specific for mouse IL-6 for 2 h at room temperature, wells were washed and then incubated with polyclonal Ab against mouse IL-6 conjugated to HRP. After washing the wells substrate solution was added and incubated for 30 min followed by stop solution. OD was measured at 450 nm. The IL-6 concentration was quantified using a standard curve created using the mouse IL-6 standards.

**Detection of reactive electrophiles**

We used 2′,7′-dichlorodihydrofluorescein diacetate (DCF) staining, which acquires fluorescent properties upon reacting with reactive electrophiles in an intracellular environment, as per the manufacturer’s recommendations (Molecular Probes). In brief, cell cultures were washed on day 3 with PBS and the 2′,7′-dichlorodihydrofluorescein diacetate at 2 μM in PBS was added before incubation for 10 min, after which the cultures were washed with PBS and images of cells were obtained using fluorescent microscope (NIKON Eclipse, TE2000-S) with Spot software. The total number of DCF stained cells were quantified and plotted.

**Statistical analysis**

All data involving animal experimentation were collected by an investigator or technician blinded to the specific experimental group used. Data were expressed as the mean ± SD (n = 3–5 for each condition). Student’s t test was used and the p < 0.05 was considered as significant.

**Results**

*P. aeruginosa* infection causes mortality in hyperoxia primed Nrf2-deficient mice

We have recently shown that a sublethal (48-h) hyperoxia exposure causes oxidative stress and persistent inflammation in

![FIGURE 1. Survival times of Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice infected with *P. aeruginosa* following hyperoxic insult. Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice (n = 12/genotype) were exposed to room air or hyperoxia for 48 h and then infected with 10<sup>5</sup> cfu *P. aeruginosa*. Mice were allowed to recover under normoxic condition (room air). The survival times of mice were monitored every 6–12 h thereafter for several days as indicated. *, p < 0.05 vs room air control of the same genotype (n = 5); †, p < 0.05; Nrf2<sup>-/-</sup> mice vs Nrf2<sup>+/+</sup> mice subjected to hyperoxia.

*Nrf2<sup>-/-</sup>* mice (12). To test the effect of oxidative stress caused by Nrf2-deficiency on innate immunity, we inoculated wild-type (Nrf2<sup>+/+</sup>) and Nrf2<sup>-/-</sup> mice exposed to normoxia (room air) or hyperoxia by intratracheal instillation of 10<sup>6</sup> cfu of *P. aeruginosa*, an opportunistic human pathogen commonly associated with critically ill ALI patients (13, 15). After bacterial infection, mice were allowed to recover under normoxic condition. As shown in Fig. 1, 10 of 12 (i.e., >80%) infected Nrf2<sup>-/-</sup> mice under hyperoxic stress died between 4 to 8 days. In contrast, 92% of infected Nrf2<sup>-/-</sup> mice exposed to normoxic condition survived up to 12 days. In contrast, 100% of the Nrf2<sup>+/+</sup> mice under either normoxic or hyperoxic exposure survived the infection to the end point (12 days). These observations demonstrate that Nrf2 deficiency impairs the innate immunity and greatly enhances susceptibility to bacterial infection after hyperoxic insult.

**Nrf2-deficiency impairs lung innate immunity to *P. aeruginosa* after hyperoxia exposure**

To determine the mechanisms by which Nrf2 mitigates bacterial infection after hyperoxic exposure, we infected the Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice after sublethal (48-h) hyperoxia exposure with a lower (10<sup>3</sup> cfu) dose of *P. aeruginosa* given by intratracheal instillation. Some mice exposed to hyperoxia were allowed to recover under normoxia for 72 h and were then infected with bacteria. Mice were sacrificed at 4 h postinfection and lung histology as well as bacterial clearance was assessed as detailed in Materials and Methods. Although *P. aeruginosa* infection produced a moderate and comparable degree of lung inflammation in Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice under normoxia (Fig. 2A, left), we noticed a striking difference in the lung histology between these two genotypes after hyperoxia (middle) and recovery (right) (See supplemental data for ×4 (supplemental Figure S1)) and ×10 pictures (Figure S2). *P. aeruginosa* infection caused extensive inflammatory cellular infiltration in the alveolar space of Nrf2<sup>-/-</sup> mice in the recovery period (right, bottom). In contrast, moderate cellular infiltration and intact alveolar structure was observed in the lungs of Nrf2<sup>+/+</sup> mice (right, top).

To determine whether Nrf2-deficiency alters bacterial clearance, we quantified total cfu present both in the lung tissue and BAL fluid after a 4 h postinfection (Fig. 2B). Approximately 75% of the 10<sup>5</sup> cfu *P. aeruginosa* was cleared in Nrf2<sup>+/+</sup> mice and in Nrf2<sup>-/-</sup> mice.
mice under normoxia (compare bar 1 and bar 4). The bacterial clearance was modest in Nrf2−/− mice under hyperoxia, as 85% of the total inoculum remained in the lung (bar 2). However, 70% of the 10⁵ cfu was cleared in Nrf2+/+ mice, when infected 3 days post hyperoxia exposure (bar 3), in a manner similar to that of room air exposed control (□). In contrast, although ~70% of the 10⁵ cfu was cleared in Nrf2−/− mice under normoxia (bar 4), the bacterial cfu increased to 175% of the original inoculum under hyperoxia (bar 5). Unlike the wild-type mice, the bacterial cfu in lungs of Nrf2−/− mice was increased to 200% of the inoculum (bar 5) when infected 72 h after recovery from hyperoxia (bar 6). Thus, the ability to clear bacterial infection was significantly suppressed in the Nrf2−/− mice at 4 h postinfection, leading to high pulmonary bacterial load compared with wild-type mice.

We further investigated the histological changes and kinetics of bacterial clearance by analyzing lung tissue and BAL fluid after a 72 h infection with 10⁵ cfu (Fig. 2, C and D). This infectious period, compared with 4-h infection, caused severe cellular infiltration into alveolar spaces of hyperoxia exposed and recovered Nrf2−/− mice compared with Nrf2+/+ mice (Fig. 2C, supplemental data for ×4 (Figure S3) and ×10 pictures (Figure S4)). Similar to the 4-h infection, Nrf2+/+ mice under normoxia were able to clear more than 70% of the bacteria as assessed by the cfu present both in the lung and BAL fluid (Fig. 2D, bar 1). However, the high dose of bacteria in Nrf2−/− mice under normoxia caused bacterial outgrowth 72 h after infection (bar 4). These mice were defective at clearing bacteria and the number of bacteria rose to 125% of inoculum. Hyperoxia also inhibited bacterial clearance in Nrf2+/+ mice and 75% of the inoculum survived even after 72 h infection (bar 2), but the bacterial numbers were increased to 190% of inoculum in Nrf2−/− mice under hyperoxia (Fig. 2D, bar 5). The total cfu present in the lungs of hyperoxia-recovered Nrf2−/− mice was nearly comparable to the total inocula (bar 3), whereas the bacterial numbers rose to 260% of the original inoculum in the Nrf2−/− mice under recovery (bar 6). These results suggest that the Nrf2-regulated transcriptional response is critical for bacteria clearance following acute lung injury.

P. aeruginosa infection increases lung inflammation in Nrf2−/− mice under hyperoxia

It has been observed that an exaggerated inflammation occurs during secondary infections in injured lungs (16). To test the role of Nrf2 in controlling the inflammatory responses due to secondary infections after hyperoxia exposure, we next assessed bacteria-induced inflammation in lungs of Nrf2−/− and Nrf2+/+ mice exposed to either normoxia or hyperoxia as well as in mice undergoing recovery after hyperoxia. As a measure of lung inflammation, we quantified inflammatory cell accumulation in the BAL fluid at 4 h (Fig. 3A) and 72 h (Fig. 3B) postinfection. We observed striking differences in bacteria induced inflammatory cell accumulation in the BAL fluid between the two genotypes after hyperoxia and during recovery (Fig. 3). As shown in Fig. 3A, at 4 h postinfection severe infiltration of neutrophils and macrophages, as well as increased epithelial cell sloughing, were observed in Nrf2−/− mice exposed to hyperoxia and in the recovery period as compared with the corresponding Nrf2+/+ mice. A prolonged (72
h) infection period caused greater levels of macrophage accumulation and epithelial cell sloughing in the Nrf2+/− mice exposed to hyperoxia and in the recovery period as compared with the wild-type controls (Fig. 3B). However, as opposed to the 4 h, the 72 h infection did not cause a significant change in the numbers of neutrophils accumulated between these two genotypes of mice undergoing recovery. As a measure of lung injury, we quantified protein concentration in the BAL fluid of Nrf2+/+ and Nrf2−/− mice at 4 and 72 h postinfection with P. aeruginosa. Each bar represents the mean value with SD (n = 3–4). *p < 0.05 vs room air control of the same genotype; †, p < 0.05; Nrf2−/− mice vs Nrf2+/+.  

Hyperoxia induces persistent oxidative stress and inflammatory cytokine expression in Nrf2−/− alveolar macrophages

Alveolar macrophages play key roles in clearing bacteria during infections (17). Therefore, we determined the levels of oxidative stress in alveolar macrophages obtained from Nrf2+/+ and Nrf2−/− mice immediately after exposure to hyperoxia, by staining with DCF reagent as detailed in Materials and Methods. As anticipated, there was no detectable level of DCF-staining present in alveolar macrophages obtained from room air exposed mice from either genotype (Fig. 4A), see supplemental data Figure S5 for color images. We found greater levels of DCF staining in alveolar macrophages isolated from both Nrf2+/+ and Nrf2−/− mice exposed to hyperoxia (Fig. 4A, middle). However, the DCF staining of alveolar macrophages was persistent in hyperoxia-exposed Nrf2−/− mice in recovery, but not in those from the Nrf2+/+ mice (Fig. 4A, right). The total number of DCF-positive alveolar macrophages obtained from the BAL fluid was quantified and relative expression levels of DCF-positive cells were shown in the graph (Fig. 4A). Approximately 80% of total BAL macrophages of Nrf2+/+ and Nrf2−/− genotypes showed positive DCF staining following hyperoxia exposure. However, DCF staining remained persistent in Nrf2−/− alveolar macrophages during recovery from hyperoxia (bar 6), whereas counterpart Nrf2+/+ alveolar macrophages showed very low or undetectable level of DCF staining (bar 3), which was comparable to room air control group (bar 1). These results suggest that hyperoxia causes persistent oxidative stress in alveolar macrophages in the absence of Nrf2. To further correlate these results with cellular antioxidant status, we measured mRNA levels of Gclc, a classic transcriptional target of Nrf2, whose product is required for GSH biosynthesis. We found greater levels of Gclc expression in alveolar macrophages obtained from Nrf2+/+ mice exposed to hyperoxia, and the induction was persistent through the 72-h recovery compared with those of normoxic controls (Fig. 4B). The induction of Gclc following hyperoxic exposure was absent in alveolar macrophages isolated from Nrf2−/− mice, and somewhat decreased during hyperoxia and in the recovery phase.

We next examined whether these differential responses were due to production of different levels of proinflammatory cytokines. Analysis of the inflammatory cytokine expression in alveolar macrophages revealed a marked difference in the levels of II-1 and II-6 transcripts between Nrf2+/+ and Nrf2−/− mice after hyperoxia (Fig. 4C). The levels of II-1 transcript decreased in alveolar macrophages after hyperoxia in both genotypes, and were elevated during recovery. However, the magnitude of II-1 induction was 3-fold greater in Nrf2−/− macrophages during recovery compared with Nrf2+/+ macrophages (Fig. 4C, compare bars 3 and 6). II-6
mRNA expression was also increased by 20-fold in Nrf2−/− alveolar macrophages during the recovery, while it was markedly low in Nrf2+/+ alveolar macrophages. However, we found no significant differences in the expression of this cytokine between the alveolar macrophages of Nrf2+/+ and Nrf2−/− mice after hyperoxic exposure. To determine whether increased levels of II-6 mRNA expression in macrophages correlate with protein levels, we have analyzed II-6 protein in the BAL fluid of Nrf2+/+ and Nrf2−/− mice after hyperoxia and during recovery (Fig. 4D). We did not find significant change in the levels of II-6 protein in BAL fluids of Nrf2+/+ mice after hyperoxia (bar 2) and during recovery (bar 3) compared with room air control (bar 1). In contrast, II-6 protein levels were markedly increased in the BAL fluid of Nrf2−/− mice during recovery (Fig. 4D, bar 6) compared with room air (bar 4) and hyperoxia-exposed groups (bar 5) as well as counterpart Nrf2+/+ mice (bar 3). These data demonstrate that hyperoxia induces persistent oxidative stress accompanied by elevated levels of inflammatory cytokine (II-6 and II-1) expression in Nrf2−/− alveolar macrophages during recovery.

Scavenger receptor induction by hyperoxia is deregulated in Nrf2−/− macrophages

Because the macrophage receptor with collagenous structure (MARCO) and macrophage scavenger receptor (MSR1; also known as scavenger receptor SRA I) regulate phagocytosis (18, 19), we next measured their expression levels in Nrf2+/+ and Nrf2−/− alveolar macrophages obtained from hyperoxia-exposed and room air-recovered Nrf2+/+ and Nrf2−/− mice (Fig. 5). Although there was no significant difference in the expression of Marco transcripts after hyperoxia, this expression was increased significantly during recovery by 17-fold in Nrf2−/− cells. However, the magnitude of this induction was significantly diminished (~5-fold) in Nrf2−/− alveolar macrophages (left panel). As shown in the right panel, Msr1 expression was also significantly increased in Nrf2+/+ mice after hyperoxia (3.7-fold, bar 2), and during recovery (3-fold, bar 3). In contrast, Msr1 induction by hyperoxia was decreased in Nrf2−/− mice as compared with room air-exposed control (compare bars 4 and 5), but the expression of Msr1 was increased during recovery (bar 6) in Nrf2−/− mice.

GS H supplementation augments the bactericidal activity of hyperoxia-exposed peritoneal macrophages from Nrf2−/− mice

We have previously shown that administration of glutathione (GSH) to hyperoxia-exposed Nrf2−/− mice rescues the impaired resolution of lung injury and inflammation during recovery (12) as well as restores the proliferation of Nrf2−/− alveolar epithelial cells in vitro (10). To test whether glutathione supplementation...
To determine whether a diminished level of antioxidant gene expression was associated with impaired bacterial killing, we assessed induction of Gclc, the rate limiting enzyme in GSH biosynthesis, in Nrf2\(^{−/−}\) and Nrf2\(^{+/−}\) alveolar macrophages immediately after hyperoxia and/or *P. aeruginosa* exposure. The Gclc expression in Nrf2\(^{+/−}\) cells was strongly induced \(\sim 30\)-fold and \(\sim 9\)-fold by *P. aeruginosa* and hyperoxia, respectively. However, the combined exposure had either a synergistic or additive effect on the induction levels (Fig. 6B). In contrast, hyperoxia and/or *P. aeruginosa* failed to stimulate the Gclc expression in both Nrf2\(^{−/−}\) and Nrf2\(^{+/−\text{GSH}}\) macrophages compared with untreated controls (Fig. 6B).

Because oxidative stress is known to induce the expression of inflammatory cytokines, we next measured the expression levels of II-6 transcripts (Fig. 6C). Addition of *P. aeruginosa* to macrophages stimulated the II-6 expression in control and hyperoxia exposed macrophages by 305- and 217-fold, respectively (Fig. 6C, left). II-6 expression was increased 2.4-fold in Nrf2\(^{+/−}\) cells after hyperoxia compared with control macrophages. There was a robust increase in the levels of II-6 in *P. aeruginosa* infected control (4700-fold) and hyperoxia-exposed (652-fold) Nrf2\(^{−/−}\) macrophages. The induction of II-6 decreased with GSH supplementation in *P. aeruginosa* infected control and hyperoxia-exposed Nrf2\(^{+/−}\) macrophages.

**Discussion**

Collectively, our present findings demonstrate that the Nrf2-regulated transcriptional response is critical in effectively mitigating bacteria-induced lung injury and inflammation as well as mortality in mice primed with a hyperoxic insult. Although hyperoxia has been shown to impair the pulmonary innate immunity to bacterial infections (20–26), our study for the first time establishes a link between a dysfunctional Nrf2/ARE response and increased risk of opportunistic pulmonary bacterial infection during recovery from hyperoxic exposure. Overall, our findings likely have major clinical implications as bacterial infections can exacerbate preexisting lung injury and inflammation and, in some cases, cause death in critically ill patients receiving oxygen supplementation (13, 27). Importantly, several studies have shown an association between NRF2 promoter polymorphisms located at position –650 nt, –686 nt, and –684 nt and enhanced disease susceptibility (28–32). For example, we have previously shown that –650 NRF2 promoter polymorphism is associated with enhanced susceptibility to ALI in humans in a well-characterized trauma group at risk for this syndrome (28). In separate studies, Arisawa et al. (29) reported that –650 and –686 polymorphisms associate with the development of gastric mucosal inflammation induced by *Helicobacter pylori* infection, while –686 and –684 were correlated with development of ulcerative colitis and gastric ulcers in humans (32). Based on these observations, we propose that either a deregulated NRF2 expression and/or a dysfunctional Nrf2/ARE response may enhance susceptibility to opportunistic lung infections after an initial hyperoxic insult in vulnerable populations.

Pulmonary macrophages and neutrophils play key roles in clearing apoptotic and necrotic cells, as well as invading microbial pathogens, leading to a proper resolution of inflammation following toxin and oxidant exposures or lung infections (33–36). Previous studies have shown that peritoneal macrophages exposed to hyperoxia in vitro and alveolar macrophages isolated from mice exposed to hyperoxia exhibit impaired bacterial adherence, chemotaxis, phagocytosis, and pathogen killing (20, 21, 23, 24, 26, 37). Impaired clearance of bacteria after hyperoxic insult in Nrf2\(^{−/−}\) mice could be attributed to either a decline in the recruitment or impaired macrophage functions under our experimental

rescues the bactericidal activity of Nrf2\(^{−/−}\) macrophages, we isolated peritoneal macrophages from Nrf2\(^{−/−}\) and Nrf2\(^{+/−}\) mice following thioglycolate administration, and exposed them to hyperoxia *ex vivo* in the presence and absence of GSH-methyl ester for 24 h and then assessed their bactericidal activity (Fig. 6). The macrophages of Nrf2\(^{−/−}\) mice were defective in bacterial killing compared with those of the Nrf2\(^{+/−}\) mice. Hyperoxia impaired the ability to kill *P. aeruginosa* in Nrf2\(^{−/−}\) but not Nrf2\(^{+/−}\) macrophages (Fig. 6A). However, the reduction in the bactericidal activity of macrophages was more pronounced in Nrf2\(^{−/−}\) cells. Supplementation of GSH restored the bactericidal capacity in normoxia- and hyperoxia-exposed Nrf2\(^{−/−}\) mice macrophages to a level comparable to that of Nrf2\(^{+/−}\) mice.

![Image](https://example.com/image.png)

**FIGURE 6.** The effects of hyperoxia exposure on bactericidal activity of Nrf2\(^{+/−}\) and Nrf2\(^{−/−}\) peritoneal macrophages. Peritoneal macrophages isolated from Nrf2\(^{+/−}\) and Nrf2\(^{−/−}\) mice and supplemented without or with GSH were exposed to hyperoxia for 24 h and then incubated with *P. aeruginosa* at 1:100 ratio for 60 min. A. The cfu of the remaining bacteria vs Nrf2

\[\text{Nrf2}\]

![Image](https://example.com/image.png)

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\[\text{Nrf2}\]
conditions. However, despite the presence of high levels of macrophages, bacterial outgrowth in lungs of Nrf2−/- mice is remarkably higher than wild-type mice (Fig. 2), suggesting impairment of antibacterial effector function of Nrf2−/- macrophages. Our studies revealed that hyperoxia alone caused elevated levels of oxidative stress and inflammatory cytokine expression in Nrf2−/- alveolar macrophages during recovery (Fig. 4). Previous studies have shown, in agreement with our results, that exposure to particulate matter impairs macrophage effector functions in response to Streptococcus infection, which was associated with elevated levels of intracellular oxidative stress (38–40). Thus, it is likely that oxidative stress induced by hyperoxia, in the absence of a functional Nrf2-regulated ARE driven transcriptional response, might contribute to impairment of antibacterial function of alveolar macrophages in vivo, thereby resulting in lung bacterial burden and inflammation ultimately leading to death of the host.

Elevated levels of inflammatory cytokines by Nrf2−/- peritoneal macrophages may promote the recruitment of other leukocytes thereby perpetuating inflammation and injury in response to bacterial infection. This notion is further supported by in vitro studies, as hyperoxia exposure diminished the ability of Nrf2−/- peritoneal macrophages to effectively clear bacterial infection, and enhanced expression of mediators of inflammation; however, GSH supplementation was able to rescue the impaired ability of the Nrf2−/- cells to clear bacteria and suppress the inflammatory cytokine expression (Fig. 6). Although defects in adherence, chemotaxis, phagocytosis, or pathogen killing can impair the ability of macrophages to effectively eliminate bacteria, it is unclear whether lack of a functional Nrf2/ARE signaling cripples one or more steps of bacterial clearance in our experimental conditions. Nonetheless, our studies reveal that Nrf2/ARE signaling is critical to counteract the effects of hyperoxia-induced oxidative stress and also for effective macrophage antibacterial function, which otherwise would impair macrophage function and enhance bacteria-induced injury and inflammation. We have observed increased levels of lung neutrophils in Nrf2+/+ mice primed with hyperoxia, both after 4 and 72 h of P. aeruginosa challenge compared with counterpart Nrf2−/- mice. Previous studies have shown that hyperoxia impairs the clearance of P. aeruginosa by decreasing the accumulation of neutrophils in lung tissue and BAL fluid by promoting their adherence to the endothelium (20). It is unclear whether apoptosis or enhanced adherence of neutrophils to endothelium contributes to lower levels of neutrophils in BAL fluid and lung tissue in Nrf2−/- mice.

Phagocytosis is an actin-dependent internalization process that requires the oxidation of actin by S-glutathionylation. S-glutathionylation of actin is essential for cell spreading and cytoskeletal reorganization and internalization of phagocytosed bacteria (41, 42). Reactive electrophiles generated during oxidant exposure consume protons in the phagosome, causing alkalinization of the phagosome and inhibition of acidic proteases (43). Although our results suggest that Nrf2-regulated, GSH-induced signaling plays an essential role in regulating bacterial clearance by macrophages; the exact mechanisms by which Nrf2-deficiency dampens the innate immunity following hyperoxic insult, as well as the means by which GSH restores this defect in vivo, remain to be investigated.

Scavenger receptors are critical for clearance of the damaged cellular organelles by toxins and oxidants as well as dead cells (18). These receptors are also critical to effectively regulate macrophage antibacterial function. Macrophages recognize and bind foreign particles and bacteria with scavenger receptors, such as MARCO and MSR1 (or SRA I) (44–46). These receptors have been shown to attenuate oxidant- and toxin-induced lung inflammation by scavenging oxidized lipids and bacteria from lung lining fluids (18, 19). Gene expression analysis revealed that both hyperoxia and P. aeruginosa strongly induce Marco and Msr1 expression in Nrf2−/- macrophages, however, their induction is markedly lower in Nrf2−/- cells (Fig. 5). Previous studies have shown that Marco and Msr1 are critical for effectively dampening bacteria-induced lung inflammation. For example, genetic disruption of Marco in mice, like that of Nrf2, causes impaired ability to clear bacteria from lungs and increased lethality (47), while disruption of Msr1 increases susceptibility to oxidant induced lung inflammation (48, 49). Thus, it is likely that diminished levels of Marco and Msr1 expression, at least in part, contribute to impairment of the antibacterial function of Nrf2−/- macrophages. Although the mechanism by which hyperoxia and P. aeruginosa regulate expression of Marco and Msr1 is unclear, murine genomic sequence analysis revealed the presence of Nrf2-binding ARE/ARE-like sites in the promoter region of Marco, but not that of Msr1. Further studies are warranted to determine whether Nrf2, directly through ARE or indirectly through ARE-regulated anti-oxidative response (GSH signaling), regulates Marco and Msr1 expression.

In summary, our studies demonstrate, for the first time, that the Nrf2-regulated transcriptional response is critical for the regulation of inflammation (especially macrophage accumulation) and in the maintenance of epithelial cell integrity during secondary microbial infection following initial injury. Because promoter polymorphisms of this transcription factor are associated with increased susceptibility to ALI and bacteria-induced inflammation in humans, our findings further support that targeting Nrf2 pathway may be a valuable therapeutic strategy in controlling lung inflammation associated with bacterial infection in critically ill patients subjected to oxygen supplementation.

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Disclosures
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References
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