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Nrf2 promotes alveolar mitochondrial biogenesis and resolution of lung injury in *Staphylococcus aureus* pneumonia in mice

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Abstract

Acute lung injury (ALI) initiates protective responses involving genes downstream of the Nrf2 (Nfe2l2) transcription factor, including heme oxygenase-1 (HO-1), which stimulates mitochondrial biogenesis and related anti-inflammatory processes. We examined mitochondrial biogenesis during *Staphylococcus aureus* pneumonia in mice and the effect of Nrf2 deficiency on lung mitochondrial biogenesis and resolution of lung inflammation. *S. aureus* pneumonia established by nasal insufflation of live bacteria was studied in mitochondrial reporter (mt-COX8-GFP) mice, wild-type (WT) mice, and *Nrf2*^{-/-} mice. Bronchoalveolar lavage, wet/dry ratios, real-time RT-PCR and Western analysis, immunohistochemistry, and fluorescence microscopy were performed on the lung at 0, 6, 24, and 48 h. The mice survived *S. aureus* inoculations at 5×10^8 CFU despite diffuse lung inflammation and edema, but the *Nrf2*^{-/-} lung showed increased ALI. In mt-COX8-GFP mice, mitochondrial fluorescence was enhanced in bronchial and alveolar type II (AT2) epithelial cells. WT mice displayed rapid HO-1 upregulation and lower proinflammatory TNF- α , IL-1 β , and CCL2 and, especially in AT2 cells, higher anti-inflammatory IL-10 and suppressor of cytokine signaling-3 than *Nrf2*^{-/-} mice. In the alveolar region, WT but not *Nrf2*^{-/-} mice showed strongly induced nuclear respiratory factor-1, PGC-1 α , mitochondrial transcription factor-A, SOD2, Bnip3, mtDNA copy number, and citrate synthase. These findings indicate that *S. aureus* pneumonia induces Nrf2-dependent mitochondrial biogenesis in the alveolar region, mainly in AT2 cells. Absence of Nrf2 suppresses the alveolar transcriptional network for mitochondrial biogenesis and anti-inflammation, which worsens ALI. The findings link redox activation of mitochondrial biogenesis to ALI resolution.

Keywords

Acute lung injury; IL-10; Inflammation; Mitochondria; Oxidative stress; NRF-1; Nrf2; Free radicals

Pneumonia and acute lung injury (ALI) due to gram-positive bacteria, such as *Staphylococcus aureus*, is a major cause of in-hospital mortality from the multiple-organ dysfunction syndrome (MODS) [1]. In advanced pneumonia, alveolar neutrophil infiltration, increased cytokine synthesis, and permeability pulmonary edema lead to low lung compliance and refractory hypoxemia [2,3]. The evolution to ALI/ARDS is related to the severity and extent of inflammation and the degree of diffuse alveolar damage [4]. ALI/ARDS contributes to the pathogenesis and high mortality of MODS, which is caused in part by disturbances in mitochondrial homeostasis [5]. There is little information, however, on the molecular regulation of lung mitochondrial quality control during ALI.

The disruption of alveolar membrane integrity results in the formation of protein-rich pulmonary edema and increased lung water [6]. The pathogenic factors that damage the delicate alveolar epithelium include the influx of alveolar inflammatory cells and the overproduction of cytokines, chemokines, and reactive oxygen (ROS) and nitrogen species [7]. Type I cell destruction compromises epithelial barrier integrity [8], leading to alveolar type II cell (AT2) proliferation and differentiation and to the induction of protective epithelial genes to preserve tight junctions and restore membrane integrity [9].

These protective responses include genes regulated by the leucine zipper transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nfe2l2), or Nrf2 [10]. Nrf2 is docked constitutively to cytoplasmic Kelch-like ECH-associated protein 1 (Keap1) and ubiquitinated and degraded by the proteasome [11]. Electrophilic stress oxidizes Keap1 and blocks Nrf2 degradation, allowing its phosphorylation, nuclear translocation, and binding to antioxidant response element (ARE) motifs in the promoter regions of genes involved in oxidative and xenobiotic stress responses and in the induction of mitochondrial biogenesis [12]. Nrf2 polymorphisms have also been implicated as risk factors in ALI through interference with the oxidative stress response [13].

Among the Nrf2-responsive antioxidant genes is heme oxygenase-1 (*Hmox1*; HO-1) [14], which converts toxic heme to biliverdin, releasing carbon monoxide (CO) and iron [15]. HO-1 also stimulates anti-inflammatory cytokine synthesis [16], e.g., interleukin-10 (IL-10) [17], and mitochondrial biogenesis [18]. In injured tissues, maintenance of ATP production and the removal of damaged mitochondria are critical to the prevention of energy compromise and cycles of ROS overproduction that interfere with the resolution of inflammation [19]. Because cells do not make mitochondria de novo, they rely on a transcriptional network of mitochondrial biogenesis regulated by energy-sensing and redox-sensitive processes that involve, among others, the nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (Tfam), and PGC-1 α regulatory proteins [20]. Mitochondrial biogenesis along with the removal of irreparably damaged mitochondria by autophagy enhances cell survival [21].

Given that Nrf2 is so important to lung protection, we hypothesized that lack of Nrf2 transcriptional activity during *S. aureus* pneumonia would perpetuate alveolar inflammation by interfering with HO-1 induction, mitochondrial biogenesis, and the synthesis of downstream anti-inflammatory cytokines as reported in the liver for *S. aureus* peritonitis [22]. The importance of mitochondrial biogenesis to cell survival during inflammation and the paucity of information on the program in lung parenchyma [23] led us to characterize and localize alveolar mitochondrial biogenesis and anti-inflammatory cytokine production in murine *S. aureus* pneumonia and to test whether *Nrf2* gene deletion impairs the rapid induction of mitochondrial biogenesis in connection with the delayed resolution of lung inflammation.

Methods

Mice

Mouse studies were conducted in mice on protocols approved by the Duke University Institutional Animal Care and Use Committee. C57BL/6NTac mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Transgenic mice that express mitochondrial-targeted green fluorescent protein (mt-COX8-GFP) obtained from the Tokyo Metropolitan Institute of Medical Science and Nrf2^{-/-} mice purchased from the RIKEN BioResource Center (Tsukuba, Japan) were bred at our institution and the former used as mitochondrial reporter mice [24].

Bacteria

S. aureus (ssp. *aureus*, No. 25923; American Type Culture Collection, Manassas, VA, USA) was reconstituted according to the manufacturer's specifications and inoculated onto trypticase soy agar slants (BD Diagnostic Systems, Sparks, MD, USA). The slants were incubated for 18 h at 37 °C to achieve adequate log-phase growth. Bacteria were harvested sterilely and centrifuged, and the pellets were resuspended in sterile 0.9% NaCl. The suspensions were quantified on a spectrophotometer (550 nm) and stock solutions of 1×10^{10} viable colony-forming units per milliliter (CFU/ml) were generated. Serial dilutions were performed for the desired bacterial inoculations (1×10^8 , 2×10^8 , and 5×10^8 CFU).

Animal procedures

Mice of either gender of 14–18 weeks of age were anesthetized using 0.3 mg xylazine and 2.5 mg/kg ketamine ip and gently inoculated intranasally with *S. aureus*, ensuring no loss of dose. The mice were monitored daily for weight loss and signs of respiratory distress and euthanized in an isoflurane chamber 0, 6, 24, or 48 h after inoculation. The aorta was sectioned, the lungs were harvested immediately, and the parenchyma was separated at the hila and snap-frozen for RNA and protein analysis. For bronchoalveolar lavage (BAL), fresh lungs were deflated and the trachea was infused with 1 ml of cold phosphate-buffered saline (PBS), and the cells recovered from BAL fluid and the inflammatory cells (excluding red blood cells) were counted on a hemocytometer after trypan blue staining. The supernatants were used to measure protein content by bicinchoninic acid assay with a bovine serum albumin standard. For histology, the lungs were perfused with 5 ml PBS followed by inflation–fixation at 20 cm H₂O with 10% formalin. For wet/dry weight, the lungs were first gravity-perfused in situ using 5 ml PBS at a standardized 30 cm H₂O pressure via the right ventricle after opening the left atrium. The lungs were removed, the wet weight was measured, and the lungs were vacuum-dried at 60 °C and weighed daily until stable.

Assay of mRNA and mitochondrial DNA (mtDNA)

Lung RNA was extracted with TRIzol reagent (Invitrogen). RNA purity was confirmed on 1.2% agarose and the RNA converted to cDNA using oligo(dT) (ImProm-II reverse transcription system; A3800). Real-time RT-PCR was performed on an ABI Prism 7000 using gene expression assays (Applied Biosystems). A ΔC_t method was used to quantify mRNA levels for TNF- α , IL-1 β , CCL2, and IL-10. Amplification efficiency was checked with an internal 18S rRNA (AB PN 4332078) over a 0.9–90 ng range of RNA and gene expression was quantified using ABI Prism 7000 SDS and MS Excel software. Each sample was assayed in triplicate. The mtDNA content was measured as reported [22].

Western analysis

Lung proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with polyclonal rabbit anti-HO-1 (Stressgen SPA-894,

1:2000 dilution), anti-Tfam (made and characterized in our laboratory, 1:1500 dilution), anti-NRF-1 (made and characterized in our laboratory, 1:1000 dilution), anti-PGC-1 α (Santa Cruz Biotechnology, 1:500), anti-claudin-4 (sc-17664-R, 1:200), anti-PDCD2 (sc-22975, 1:200), and anti-Bnip3 (ab38621, 1:200). After incubation with the primary antibody, membranes were washed and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The membranes were developed by ECL (Santa Cruz Biotechnology) and protein bands quantified on digitized images in the mid-dynamic range. Protein loading was confirmed by reprobing stripped membranes with monoclonal rabbit antibody to β -actin (Sigma A2066, 1:1000) and by Coomassie blue staining. Three or four samples were used for densitometry at each time point.

Immunohistochemistry

Inflation-fixed lung samples were embedded in paraffin, cut into 5- μ m sections, mounted on slides, and probed with anti-IL-10 (sc-7888, 1:100 dilution), SOCS3 (sc-9023, 1:100), or citrate synthase (Chemicon MAB3087, 1:100). The slides were incubated in secondary goat anti-rabbit antibody conjugated to Alexa 594 (Invitrogen). The nuclei were stained with DAPI (Molecular Probes) and observed under a Nikon Eclipse 50i fluorescence microscope.

Statistical analysis

Grouped data are expressed as means $\pm$ SEM. Statistical differences were tested by analysis of variance (ANOVA with Fisher LSD) using JMP (SAS Institute, version 9.0.1) or with Student's *t* test (MS Excel). $P < 0.05$ compared with the appropriate control group was considered significant.

Results

Bacterial dose response

Inoculations of the mouse lung at the highest dose of *S. aureus* produced diffuse pneumonia followed by resolution. There was 100% survival at 48 h and less than 10% 7-day mortality. The *S. aureus* inoculum chosen for subsequent experiments was determined using BAL cell counts and protein concentrations for assessment of inflammation and alveolar-capillary leak at each dose (Fig. 1). BAL fluid cell counts were significantly higher than those of controls at each concentration of *S. aureus* (all postinoculation differential counts in both strains were $>80\%$ neutrophils; data not shown). Cellular inflammation was present in the lung by 6 h, peaked at 24 h, and began to resolve by 48 h postinoculation. BAL fluid protein significantly increased only at 5×10^8 CFU of *S. aureus*, at which BAL fluid protein was higher than control at each point. Because lung inflammation and BAL protein both increased significantly at 5×10^8 CFU *S. aureus*, and in preliminary studies *Nrf2*^{-/-} mice tolerated this dose for 48 h, it was used in all subsequent experiments.

Lung mitochondrial biogenesis after *S. aureus* inoculation

Areas of lung mitochondrial biogenesis were identified by fluorescence microscopy in mt-COX8-GFP reporter mice inoculated with 5×10^8 CFU *S. aureus* (Fig. 2). At 0 h, punctate green fluorescence was seen in scattered airway and lung parenchymal cells, but by 48 h, many bronchial epithelial and AT2 cells showed strong green fluorescence. In the alveolar region, mitochondrial GFP was colocalized with SP-C (Fig. 2B), identifying AT2 cells as a major site of mitochondrial biogenesis during pneumonia/ALI.

ALI, lung inflammation, and edema in wild-type (WT) and *Nrf2*^{-/-} lungs

To test the role of *Nrf2* in the regulation of lung inflammation and the resolution of lung injury, we compared WT lungs to those of *Nrf2*^{-/-} mice after inoculation with 5×10^8 *S. aureus*. *Nrf2*^{-/-} mice showed significantly higher BAL fluid cell counts and protein at 24 h, consistent with a protective role for *Nrf2* in pneumonia (Fig. 3A and B). To confirm capillary damage, we compared wet to dry weight (W/D) of WT and *Nrf2*^{-/-} mice before and after inoculation with 5×10^8 *S. aureus* (Fig. 3C). Lung W/D increased significantly in both strains after *S. aureus* inoculation, indicating pulmonary edema, but the water content of *Nrf2*^{-/-} lungs was significantly greater than that of the WT at 6 h.

Hematoxylin and eosin (H&E) staining of inflation-fixed lung showed diffuse inflammation in the alveolar region of WT and *Nrf2*^{-/-} mice after inoculation with 5×10^8 *S. aureus* (Fig. 4). By 24 h, many inflammatory cells (>80% neutrophils) had entered the lungs, and this response was exaggerated in *Nrf2*^{-/-} mice. The *Nrf2*^{-/-} mice also independently showed many intra-alveolar red blood cells, consistent with widespread alveolar–capillary damage.

Inflammatory cytokine mRNA levels

Key proinflammatory lung cytokine and chemokine mRNA levels were selected for comparison in WT and *Nrf2*^{-/-} mice before and after inoculation with 5×10^8 *S. aureus* (Fig. 5). The levels of TNF- α , IL-1 β , and CCL2 increased by 6 h, but were significantly higher in *Nrf2*^{-/-} mice. These cytokines were highest at ~6 h and returned to baseline by 48 h, except for CCL2, which remained elevated at 48 h in the *Nrf2*^{-/-} mice. The exaggeration in lung inflammatory cytokine responses in *Nrf2*^{-/-} mice after *S. aureus* indicated dampening of the inflammatory response by gene products downstream of *Nrf2*. Accordingly, anti-inflammatory IL-10 gene expression in *Nrf2*^{-/-} mice was diminished compared with WT mice.

Comparison of parenchymal proteins after ALI

To assess alveolar mitochondrial biogenesis, mitochondrial autophagy, alveolar barrier recovery, and cell death during ALI, total lung proteins were separated by SDS–PAGE and Western blots performed for PGC-1 α , the NRF-1 and Tfam transcription factors, HO-1, SOD2, the inducible Bnip3 mitochondrial autophagy marker, the alveolar integrity protein, claudin-4, and the PDCD2 cell death protein. After the primary blots were completed, the membranes were stripped and probed with β -actin as a loading control.

There was a substantial difference in the postinoculation induction of mitochondrial biogenesis in WT and *Nrf2*^{-/-} lungs (Fig. 6). The three nuclear-encoded mitochondrial regulatory proteins were rapidly upregulated in the WT lung (Fig. 6A–C). HO-1 induction was rapid in WT mice, but delayed in *Nrf2*^{-/-} mice, consistent with a secondary *Nrf2*-independent response (Fig. 6D). Mitochondrial SOD2 followed a similar pattern (Fig. 6E). The WT mice also showed increases in mtDNA copy number by 48 h, but copy number did not increase in *Nrf2*-deficient lungs, indicating impaired mitochondrial biogenesis after the *S. aureus* infection (Fig. 6F).

Claudin-4 and PDCD2 protein levels were also compared in the lungs of WT and *Nrf2*^{-/-} mice (Fig. 7). WT and *Nrf2*^{-/-} mouse lungs induced claudin-4, but the response was delayed in *Nrf2*^{-/-} mice. WT mice also displayed Bnip3 induction by 6 h, which returned to baseline by 48 h, but Bnip3 was not induced in the *Nrf2*^{-/-} lung. Both mouse strains induced PDCD2, but the response was accentuated and prolonged in the *Nrf2*^{-/-} lung.

Anti-inflammatory response in type II cells

Inflation-fixed lungs were compared for sites of anti-inflammatory cytokine production and changes in mitochondrial density at 24 h after inoculation with *S. aureus*. Lung sections were probed with antibody to citrate synthase (CS), a mitochondrial density marker, or with antibody to anti-inflammatory cytokines IL-10 or SOCS3 with Alexa 594-conjugated secondary antibodies (Fig. 8). Nuclei were counterstained with DAPI and the sections visualized by fluorescence microscopy. In control WT lung, alveolar CS was localized mainly to AT2 cells. By 24 h postinoculation, CS increased throughout the alveolar region and was sustained for 48 h, but was most apparent in AT2 cells and alveolar macrophages (Fig. 8A). The CS signal was weaker in *Nrf2*^{-/-} lungs and seen in very few cells by 24 h postinfection, suggesting depletion of the cellular mitochondrial content. Subsequent changes in CS, especially in AT2 cells, implied mitochondrial renewal in the alveolar region of WT, but not *Nrf2*^{-/-} lungs.

Anti-inflammatory IL-10 was seen sparingly in control alveolar tissue of both strains (0 h), but by 24 h postinoculation, IL-10 was induced in WT mice (Fig. 8B). IL-10 localized strongly to alveolar macrophages and weakly to AT2 cells at 24 h, but AT2 cell staining became heavy by 48 h. In contrast, *Nrf2*^{-/-} lungs demonstrated minimal IL-10 induction after *S. aureus*. In control WT lungs, SOCS3 was seen constitutively in resident mononuclear cells, but by 24 h after *S. aureus* AT2 cells displayed robust SOCS3 induction, and by 48 h SOCS3 had also appeared in type I and/or capillary epithelium (Fig. 8C). In control *Nrf2*^{-/-} lungs, SOCS3 was sparsely found in a few cells in the alveolar region, and after *S. aureus* inoculation increased mainly in mononuclear inflammatory cells. The induction of SOCS3 by the AT2 cell was demonstrated by immunochemical colocalization of SOCS3 with SP-C (Fig. 8D).

Discussion

Our goal was to examine the transcriptional program of mitochondrial biogenesis and its related anti-inflammatory responses in the mouse lung and test the hypothesis that this program is activated by the Nrf2 transcription factor and participates in the resolution of *S. aureus* pneumonia. Nrf2 responds rapidly to oxidative stress, an important pathogenic factor in most forms of ALI, and genes containing AREs are important in ALI resolution, for instance, in hyperoxia [25,26]. In addition to curtailing oxidative damage, Nrf2-dependent genes prevent excessive inflammation, help restore alveolar barrier integrity [27,28], and activate mitochondrial biogenesis [12].

The structural basis of ALI in *S. aureus* pneumonia involves extensive, acute alveolar type I epithelial and capillary endothelial cell damage, which physiologically disrupts the alveolar-capillary barrier. To resolve the barrier dysfunction, multiple lung cells acting together restore the alveolar liquid clearance [9,29]. In these *S. aureus* pneumonia studies, Nrf2-dependent alveolar mitochondrial biogenesis is initiated as the barrier function is restored. In particular, the AT2 cell, the source of surfactant synthesis and a critical alveolar epithelial progenitor cell [30], is seen to be a major site of lung mitochondrial biogenesis and anti-inflammatory cytokine production after *S. aureus* infection.

Using dose titrations of live *S. aureus*, the mouse lung generated a robust inflammatory response, but persistence of inflammation and significant ALI by BAL fluid protein accumulation required up to 5×10^8 CFU of bacteria. *S. aureus* is known to cause alveolar epithelial damage, particularly to alveolar type I cells, resulting in pulmonary edema [31,32], and we confirmed dose- and time-dependent lung inflammation and ALI in our model.

The effects of *S. aureus* on pulmonary mitochondrial biogenesis were monitored in transgenic mitochondrial reporter mice by tracking changes in the GFP signal by fluorescence microscopy. Before inoculation, GFP was seen in scattered lung cells in the bronchial and alveolar regions, but afterward, GFP localized strongly to bronchial epithelial cells, some endothelial cells, and AT2 cells. Lung infection does cause AT2 cell death due to mitochondrial damage and ROS production [33,34], but we also saw numerous AT2 cells exhibiting mitochondrial biogenesis, which promotes cell survival through support of energy metabolism and related antiapoptotic and anti-inflammatory functions [18].

The activation of alveolar mitochondrial biogenesis was compared in the lungs of WT and *Nrf2*^{-/-} mice after *S. aureus*, and more intense inflammatory cell infiltration and protein leak were found, as expected, in *Nrf2*^{-/-} than in WT mice [35]. Mice with pneumonia also showed prompt increases in W/D ratio, indicating impairment of epithelial integrity, which was greater in *Nrf2*-deficient than in WT lung. Thus, the absence of *Nrf2* and loss of normal upregulation of downstream antioxidant response elements aggravates ALI due to *S. aureus* pneumonia [26].

The greater alveolar membrane compromise in *Nrf2*^{-/-} than in WT mice was paralleled by higher levels of early phase inflammatory cytokines. Studies in sepsis, as well as in other inflammatory models [35,36], have shown harmful cytokine responses from *Nrf2* deletion, especially by TNF- α and IL-1 β [37]. The host response to *S. aureus* generates ample TNF- α and IL-1 β [38], but excessive cytokine levels amplify cell and organ damage [39,40]. Here TNF- α , IL-1 β , and CCL2 mRNA levels were higher in the lungs of *Nrf2*^{-/-} than those of WT mice after the bacterial challenge. In many infections, the CCL2 chemokine recruits activated monocytes, which can exacerbate cell death and tissue damage [41]. In *Nrf2*^{-/-} models of inflammation, CCL2 expression is exaggerated [42], and the postinoculation increase in CCL2 here was sustained at 48 h when early phase cytokine levels had subsided. Unremitting CCL2 or related extracellular signals for monocyte migration may account for much of the disproportionate inflammation and alveolar damage detected in *Nrf2*^{-/-} mice.

After establishing *Nrf2* as a regulator of the lung's counter-inflammatory response to *S. aureus*, we examined HO-1 induction and mitochondrial biogenesis. *S. aureus* stimulates mitochondrial biogenesis in other organs through TLR2- and NF- κ B-dependent and -independent mechanisms [43], but *Nrf2* also regulates *Hmox1* [44,45]. *S. aureus* pneumonia rapidly increased lung HO-1 protein levels in WT mice, but HO-1 upregulation was delayed in *Nrf2*^{-/-} mice and responded only later through an unidentified *Nrf2*-independent pathway.

WT mice also responded to *S. aureus* with higher levels of lung NRF-1, PGC-1 α , and Tfam, three major coordinators of the bigenomic program of mitochondrial biogenesis [20]. *Nrf2*^{-/-} mice showed no significant postinoculation increase in lung PGC-1 α or Tfam and only a slight increase in NRF-1 by 48 h. The late induction of NRF-1 occurred via an *Nrf2*-independent route that may not normally be recruited in the intact system. In the heart, *Nrf2* induces both *Hmox1* and *NRF1* [46], but these genes also respond rapidly to other regulatory factors [47].

Nrf2 was responsible for the early induction of alveolar HO-1 by *S. aureus* pneumonia in WT mice, but in *Nrf2*^{-/-} mice, in which HO-1 upregulation was delayed, activation of the lung's genetic network for mitochondrial biogenesis was likewise impaired, and *Nrf2*^{-/-} mice also failed to induce the key mitochondrial autophagy protein Bnip3. Mitochondrial autophagy directs the disposal of damaged mitochondria, and loss of Bnip3 deregulates mitochondrial turnover and leads to organ damage [21,48].

Although *Nrf2*^{-/-} mice showed impaired lung mitochondrial biogenesis, they did increase claudin-4, which is involved in epithelial tight-junction integrity and ALI resolution [9,49,50]. Claudin-4 induction was delayed in *Nrf2*^{-/-} mice, whereas PDCD2 expression persisted longer than in WT mice. Nonetheless, these mice survived these infections, indicating that the barrier damage was not severe enough to prevent their resolution.

The density of the alveolar mitochondrial population was examined by tracking the parenchymal distribution of the tricarboxylic acid cycle enzyme citrate synthase. At baseline, CS distribution was fairly homogeneous in the alveolar region in WT and *Nrf2*^{-/-} mice, but after *S. aureus* inoculation, CS protein increased strongly in WT lungs, but minimally and transiently in *Nrf2*^{-/-} lungs. The difference perhaps in part reflects the macrophage influx seen by microscopy and suggests that lung structural cells and not inflammatory cells account for the bulk of the mitochondrial biogenesis. The alveolar region in *Nrf2*^{-/-} mice showed a fairly stable mitochondrial mass 24 h after infection with only a small decline in mtDNA copy number, but mitochondrial turnover did not respond normally in the next 24 h as indicated by the delay in NRF-1 and HO-1 and lack of PGC-1 α , Tfam, and SOD2 induction and the failure to increase mtDNA copy number.

Nrf2 also induces anti-inflammatory responses, for example, through IL-10. In *Nrf2*^{-/-} lungs, peak IL-10 reached only about 15% of WT levels after *S. aureus* administration. IL-10 participates in immune modulation in ALI [51], and consistent with our data, lack of IL-10 synthesis leads to overactive proinflammatory cytokine and chemokine production, e.g., CCL2. *CCL2*^{-/-} mice have higher IL-10 and less lung inflammation after lipopolysaccharide than control mice [52], which mirrors the low IL-10 and sustained CCL2 in *Nrf2*^{-/-} mice. Moreover, in WT lung parenchyma, IL-10 was concentrated in AT2 cells, which secrete cytokines in response to paracrine signals from resident alveolar macrophages [53].

SOCS3, another anti-inflammatory cytokine, is also involved in the control of lung inflammation [54]. Alveolar SOCS3 expression was negligible in control lungs, but after *S. aureus* inoculation was strongly induced in WT mice and weakly in *Nrf2*^{-/-} mice. The distribution of SOCS3 was also different; alveolar SOCS3 in WT lungs was detected in mononuclear cells, AT2 cells, and capillary epithelial/endothelial cells, but was mainly localized to mononuclear cells in the *Nrf2*^{-/-} lung. This means that Nrf2-dependent counterinflammatory signals are arising at sites of mitochondrial biogenesis, i.e., in AT2 cells, as similarly reported, for instance, in the liver [22].

The importance of Nrf2 in the resolution of oxidative tissue damage has led to pharmacological efforts to stimulate Nrf2-responsive genes [55] and may identify agents useful in the treatment of ALI and pneumonia. The evidence linking the induction of mitochondrial biogenesis by Nrf2 to the resolution of alveolar barrier dysfunction is temporal and associative, but studies in specific lung cell types should better integrate alveolar energy metabolism with surfactant homeostasis, alveolar cell differentiation, and epithelial barrier function. On the other hand, the program of mitochondrial biogenesis directly coregulates counterinflammatory mediator production in other tissues through a redox-sensitive nuclear transcriptional network, also suggested here for the mouse lung [23,46].

Conclusions

In summary, *S. aureus* pneumonia induces mitochondrial biogenesis and counterinflammatory cytokine synthesis in the alveolar region in WT mice. This response is regulated by the redox-regulated Nrf2 transcription factor and *Nrf2*^{-/-} mice show

significantly greater lung inflammation and ALI and substantially less alveolar mitochondrial biogenesis than WT mice. The resolution mechanisms affected in Nrf2-deficient mice include delayed HO-1 and NRF-1 induction; lack of PGC-1 α , Tfam, Bnip3, and SOD2 induction; and low alveolar IL-10 and SOCS3 production. The AT2 cell is a major site of both mitochondrial biogenesis and anti-inflammatory cytokine production under the control of Nrf2. Nrf2 thus regulates the mitochondrial quality control network in the distal alveolar region, and activation of this network is accompanied by more rapid resolution of ALI.

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Fig. 1.

Dose–response curve for ALI in WT mice as a function of *S. aureus* inoculation. (A) Bronchoalveolar lavage fluid (BALF) cell concentration (cells/ml $\times 10^5$) plotted against *S. aureus* concentration. BALF cell concentration increased significantly at all levels of inoculation compared with control mice (* $P < 0.05$). (B) Total BALF protein concentration plotted against *S. aureus* concentration. Protein concentrations increased significantly in mice inoculated with *S. aureus* at 5×10^8 CFU (* $P < 0.05$ vs control). Bars are means $\pm$ SEM.

Fig. 2.

(A) Lung mitochondrial biogenesis in mt-COX8-GFP reporter mice after inoculation with 5×10^8 CFU *S. aureus* (original magnification 40 $\times$). At 0 h, minimal green fluorescence is seen in airways and lung parenchyma by fluorescence microscopy. By 48 h, increased green fluorescence is detected in bronchial epithelium and in AT2 cells and macrophages. (B) Alveolar region of control lung shows GFP mitochondria, AT2 cell staining for SP-C (red), and nuclei (DAPI; blue) (image A); image B shows lung at 24 h after *S. aureus* inoculation; image C shows lung 24 h after breathing 250 ppm CO for 1 h as a positive control. Images D–F illustrate AT2 cells for conditions in images A–C (original magnification 100 $\times$).

Fig. 3.

Alveolar inflammation, acute lung injury, and edema in WT and Nrf2^{-/-} mice after inoculation with 5×10^8 CFU *S. aureus*. (A) BALF cell concentration (cells/ml $\times 10^5$) is greater at baseline and increases more in Nrf2^{-/-} than in WT mice. (B) Postinoculation BALF protein concentration increases significantly in both strains of mice (* $P < 0.05$ vs control), but at 24 h, Nrf2^{-/-} mice show a doubling of BALF protein compared with WT mice (** $P < 0.05$ vs WT; bars are means $\pm$ SEM for $n=4$). (C) Lung wet-to-dry ratios in WT and Nrf2^{-/-} mice increase significantly postinoculation (* $P < 0.05$ vs control). At 6 h, Nrf2^{-/-} mice demonstrate significantly more edema than WT mice (** $P < 0.05$; values are means $\pm$ SEM for $n=4$ or 5 at each time point).

Fig. 4. H&E-stained sections of inflation-fixed lung in WT and *Nrf2*^{-/-} mice at 0 and 24 h postinoculation with 5×10^8 CFU *S. aureus* (original magnification 20 $\times$). Compared with control, the lung's inflammatory response is markedly enhanced in *Nrf2*^{-/-} mice at 24 h (lower right). In that photomicrograph, loss of alveolar membrane integrity is indicated by red blood cell and plasma leakage (upper arrow) and alveolar neutrophil infiltration (lower arrow).

Fig. 5.

Comparison of lung tissue cytokine mRNA levels in WT and *Nrf2*^{-/-} mice at 0 to 48 h after exposure to 5×10^8 CFU *S. aureus*. Early phase lung cytokine levels increased at 6 to 24 h postinoculation followed at 48 h by full or partial resolution, except for CCL2 expression in *Nrf2*^{-/-} mice. Compared with WT lung, the *Nrf2*^{-/-} lung showed significantly greater expression of IL-1β, CCL2, and TNF-α and less expression of IL-10 (* $P < 0.05$ compared with time 0, ** $P < 0.05$ vs WT control). Average fold change in expression is shown compared with lowest at time 0 (means $\pm$ SEM for $n=4$ samples each time point).

Fig. 6.

Pulmonary mitochondrial biogenesis after inoculation with 5×10^8 CFU *S. aureus*. (A–E) Graphs of densitometry values for immunoblot analysis of lung parenchymal homogenates from WT and *Nrf2*^{-/-} mice. (A) NRF-1 is rapidly induced in WT mice, but does not increase in *Nrf2*^{-/-} mice until 48 h. (B) PGC-1 α is rapidly induced in WT mice only. (C) Tfam is induced at 24 and 48 h in WT mice only. (D) WT lungs show early HO-1 induction, whereas *Nrf2*^{-/-} lungs show late HO-1 induction. (E) SOD2 upregulation after *S. aureus* inoculation occurs in WT lungs, but not in *Nrf2*^{-/-} lungs. All protein samples are normalized to β -actin. (F) Mitochondrial DNA copy number in the lung parenchyma after inoculation with 5×10^8 CFU *S. aureus*. Copy number fails to increase by 48 h in the *Nrf2*^{-/-} lung. For (A–F), bars are means $\pm$ SEM for $n=4$ per group per time point (* $P < 0.05$ vs timed *Nrf2*^{-/-} and the 0 time control; # $P < 0.05$ vs the 0 time control only).

Fig. 7.

Alveolar claudin-4, mitochondrial autophagy, and programmed cell death responses by Western analysis of lung homogenates of WT and *Nrf2*^{-/-} mice inoculated with 5×10^8 CFU *S. aureus*. Protein samples are normalized to β-actin. (A) WT lungs display an early postinoculation increase in claudin-4, which is delayed until 48 h in *Nrf2*^{-/-} lungs (graph of densitometry for $n=4$; * $P<0.05$ vs control and between strains). (B) *S. aureus* inoculation increases the mitochondrial autophagy protein Bnip3 in WT, but not in *Nrf2*^{-/-} lungs. Concurrently lung PDCD2 protein increases in both mouse strains, but remains strongly elevated in *Nrf2*^{-/-} mice for 48 h (graphs of densitometry for $n=4$; * $P<0.05$ vs control).

Fig. 8.

Fluorescence immunohistochemistry of WT and *Nrf2*^{-/-} mouse lung sections pre- and postinoculation with 5×10^8 CFU of *S. aureus*. Antibody-labeled protein appears in red with DAPI-stained nuclei in blue (original magnification 40 $\times$). (A) Citrate synthase (CS) staining of the mouse alveolar region. In WT lungs, CS is concentrated in cells with AT2 cell morphology (insets), especially at 24 and 48 h. In *Nrf2*^{-/-} lungs, CS is detected mainly in mononuclear cells, most probably migrating macrophages. (B) IL-10 staining in lung tissue after *S. aureus* inoculation. In WT lungs, scattered IL-10 staining is found at 0 h, but increases at 24 and 48 h. IL-10 signal localizes mainly to alveolar macrophages at 24 h and AT2 cell staining is apparent by 48 h. *Nrf2*^{-/-} lung demonstrates scattered alveolar IL-10 staining at baseline and minimal IL-10 induction after *S. aureus*. (C) SOCS3 staining in mouse alveolar region. SOCS3 is present constitutively in resident mononuclear cells in WT lung, but after *S. aureus* also appears in AT2 cells at 24 h and in type I and/or capillary epithelium at 48 h. SOCS3 is sparsely expressed by *Nrf2*^{-/-} lungs, mainly in scattered cells of the alveolar region and, after *S. aureus*, increases in mononuclear inflammatory cells at 24 and 48 h. (D) SOCS3 immunochemical localization to AT2 cells (red) using surfactant protein C (SP-C, green). AT2 cells strongly express SOCS3 (yellow) at 24 h after inoculation of the lung with 5×10^8 CFU of *S. aureus*.