Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis

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Host genetic factors that regulate innate immunity determine susceptibility to sepsis. Disruption of nuclear factor-erythroid 2–related factor 2 (Nrf2), a basic leucine zipper transcription factor that regulates redox balance and stress response, dramatically increased the mortality of mice in response to endotoxin- and cecal ligation and puncture–induced septic shock. LPS as well as TNF-α stimulus resulted in greater lung inflammation in Nrf2-deficient mice. Temporal analysis of pulmonary global gene expression after LPS challenge revealed augmented expression of large numbers of proinflammatory genes associated with the innate immune response at as early as 30 minutes in lungs of Nrf2-deficient mice, indicating severe immune dysregulation. The expression profile indicated that Nrf2 has a global influence on both MyD88-dependent and -independent signaling. Nrf2-deficient mouse embryonic fibroblasts showed greater activation of NF-κB and interferon regulatory factor 3 in response to LPS and polyinosinic-polycytidylic acid [poly(I:C)] stimulus, corroborating the effect of Nrf2 on MyD88-dependent and -independent signaling. Nrf2’s regulation of cellular glutathione and other antioxidants is critical for optimal NF-κB activation in response to LPS and TNF-α. Our study reveals Nrf2 as a novel modifier gene of sepsis that determines survival by mounting an appropriate innate immune response.

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Host genetic factors that regulate innate immunity determine susceptibility to sepsis. Disruption of nuclear factor-erythroid 2–related factor 2 (Nrf2), a basic leucine zipper transcription factor that regulates redox balance and stress response, dramatically increased the mortality of mice in response to endotoxin- and cecal ligation and puncture–induced septic shock. LPS as well as TNF-α stimulus resulted in greater lung inflammation in Nrf2–deficient mice. Temporal analysis of pulmonary global gene expression after LPS challenge revealed augmented expression of large numbers of proinflammatory genes associated with the innate immune response at as early as 30 minutes in lungs of Nrf2–deficient mice, indicating severe immune dysregulation. The expression profile indicated that Nrf2 has a global influence on both MyD88-dependent and -independent signaling. Nrf2–deficient mouse embryonic fibroblasts showed greater activation of NF-κB and interferon regulatory factor 3 in response to LPS and polyinosinic-polycytidylic acid [poly(I:C)] stimulus, corroborating the effect of Nrf2 on MyD88-dependent and -independent signaling. Nrf2’s regulation of cellular glutathione and other antioxidants is critical for optimal NF-κB activation in response to LPS and TNF-α. Our study reveals Nrf2 as a novel modifier gene of sepsis that determines survival by mounting an appropriate innate immune response.

Results
Disruption of Nrf2 causes drastic increase in lethality during LPS and cecal ligation and puncture–induced septic shock. First, we examined the role of Nrf2 on the survival of wild-type (Nrf2+/+) and Nrf2-deficient (Nrf2–/–) mice during an endotoxic shock. Nrf2–/– and Nrf2+/+ mice were treated i.p. with a lethal dose of LPS (0.75 and 1.5 mg/mouse), and survival was monitored for 5 days. The lower dose resulted in
the death of 50% of the Nrf2–/– mice but none of the Nrf2+/+ mice (Figure 1A). At the higher dose, 100% of the Nrf2–/– mice died within 48 hours whereas only 50% of the Nrf2–/– mice died by day 5 (Figure 1B). Next, we investigated the role of Nrf2 on survival in a clinically relevant model of septic shock induced by cecal ligation and puncture (CLP). By 48 hours after CLP, all Nrf2–/– mice died while only 20% of wild-type littermates died. After 5 days, 40% of wild-type mice still survived (Figure 1C). No death was observed in sham-operated mice of both genotypes.

**LPS elicits greater pulmonary inflammation in Nrf2-deficient mice.**

Because it is clear that Nrf2 is critical for survival during lethal septic shock, the role of this transcription factor in regulating nonlethal inflammatory stimulus was investigated. Lungs were examined after systemic (i.p. injection of 60 mg/mouse) or local (intratracheal instillation of 10 μg/mouse) administration of LPS. For both modes of LPS administration, the inflammatory response was greater in the lungs of Nrf2–/– mice than in their wild-type littermates. The influx of inflammatory cells (neutrophils and macrophages) was greater in the lungs of Nrf2–/– mice at both 6 and 24 hours after LPS challenge by either route. After i.p. administration of LPS, macrophages were the predominant cell type in bronchoalveolar lavage (BAL) fluid although both macrophages and neutrophils showed temporal increase in numbers (Figure 2, A and B). In contrast, intratracheal instillation attracted predominantly neutrophils (constituting as much as 80% of the total inflammatory cell population) in BAL fluid (Figure 2C), a finding similar to that of other investigators (13). Consistent with the BAL fluid analysis, histopathology showed a greater recruitment

**Figure 1**

Nrf2–/– mice were more sensitive to LPS and septic peritonitis–induced septic shock. (A and B) Mortality after LPS administration. Age-matched male Nrf2+/+ (n = 10) and Nrf2–/– mice (n = 10) were injected i.p. with LPS (0.75 and 1.5 mg per mouse). (C) Acute septic peritonitis was induced by CLP. CLP and sham operations were performed as described in Methods on age-matched male Nrf2+/+ (n = 10) and Nrf2–/– mice (n = 10). Mortality was assessed every 12 hours for 5 days. *Nrf2+/+ mice showed improved survival compared with Nrf2–/– mice. P < 0.05.

**Figure 2**

Nonlethal dose of LPS induced greater lung inflammation in Nrf2-deficient lungs. (A and B) BAL fluid analysis of Nrf2+/+ and Nrf2–/– mice after 6 and 24 hours of i.p. injection of LPS (60 μg per mouse). (C) BAL fluid analysis of Nrf2+/+ and Nrf2–/– mice after 6 hours and 24 hours of LPS instillation (10 μg per mouse). (D) Histopathological analysis of lungs by H&E staining 24 hours after instillation of LPS. Arrows indicate accumulation of inflammatory cells in the alveolar spaces. Magnification, x20. (E) Immunohistology of lungs of both genotypes using anti-mouse neutrophil antibody 24 hours after LPS instillation. Sections were counterstained with hematoxylin. Arrows indicate neutrophils. Magnification, x40. (F) Myeloperoxidase (MPO) activity in lung homogenates of both genotypes 6 and 24 hours after LPS instillation. Data are presented as mean ± SEM; n = 5. *Differs from vehicle control of the same genotype. †Differs from LPS-treated wild-type mice. P < 0.05.
of inflammatory cells in perivascular, peribronchial, and alveolar spaces of Nrf2–/– mice 24 hours after LPS treatment (Figure 2D). Immunohistochemical examination of LPS-instilled lungs with anti-neutrophil antibody also confirmed a greater number of neutrophils in the lungs of Nrf2–/– mice (Figure 2E), which was further evident from myeloperoxidase activity in these lungs (Figure 2F).

As a marker of lung injury, pulmonary edema was observed to be markedly higher in Nrf2–/– mice 24 hours after LPS instillation (Figure 2G). A similar pattern of lung pathological injury was induced by systemic delivery of LPS (data not shown). Taken together, these results show that disruption of the Nrf2 gene augments the innate immune response to bacterial endotoxin.

LPS and CLP induced greater secretion of TNF-α in Nrf2-deficient mice. Since TNF-α is one of the early proinflammatory cytokines that is elevated during LPS- and CLP-induced inflammation, serum concentrations of TNF-α were measured by ELISA. After 1.5 hours of LPS challenge (1.5 mg/mouse), serum TNF-α was significantly higher in Nrf2–/– mice compared with Nrf2+/+ (Figure 3A). Similarly, after 6 hours of CLP, serum levels of TNF-α were greater in Nrf2–/– compared with Nrf2+/+ mice (Figure 3B). Furthermore, TNF-α concentrations in BAL fluid were also greater 2 hours after nonlethal LPS challenge (i.p. and intratracheal instillation) in Nrf2–/– mice compared with wild-type mice (Figure 3C). Next, we measured the concentrations of TNF receptors, TNFR1 (p55) and TNFRII (p75), in Nrf2–/– and Nrf2+/+ mice after a lethal dose of LPS. While there was no difference in the constitutive serum levels of p55 and p75, after 6 hours of LPS treatment, the serum concentrations of both receptors were increased significantly. However, there were no significant differences in the TNF receptors between the Nrf2–/– and Nrf2+/+ mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI25790DS1) after LPS challenge.

Temporal global changes in gene expression reflect the impact of Nrf2 on the innate immune response. Moderate increase in TNF-α production alone cannot explain the markedly higher CLP- and LPS-induced mortality as well as LPS-induced lung inflammation in Nrf2–/– mice (14). To systematically understand the role of Nrf2 during LPS-induced inflammation, the global gene expression profiles were examined in lungs of Nrf2–/– and Nrf2+/+ mice over time in response to a nonlethal LPS stimulus. After i.p. injection of LPS, microarray

Figure 3
LPS and CLP induce greater secretion of TNF-α in Nrf2-deficient mice. (A) Serum concentration of TNF-α in Nrf2+/+ and Nrf2–/– mice 1.5 hours after LPS injection (1.5 mg per mouse). (B) Serum concentration of TNF-α in Nrf2+/+ and Nrf2–/– mice 6 hours after CLP. (C) TNF-α levels in BAL fluid at 2 hours after LPS delivery by i.p. injection (60 μg per mouse) and/or intratracheal instillation (10 μg per mouse). TNF-α in the BAL fluid of vehicle-treated mice was not detectable. Data are presented as mean ± SEM. *Differs from vehicle control of the same genotype. †Differs from LPS-treated wild-type mice. P < 0.05. ND, not detected.

Figure 4
Greater expression of proinflammatory genes associated with the innate immune response in the lungs of Nrf2-deficient mice. (A–C) Expression of cytokines (A), chemokines (B), and adhesion molecules/receptors (C) 30 minutes after nonlethal i.p. injection of LPS (60 μg per mouse) in Nrf2-deficient and wild-type mice. Gene expression data were obtained from microarray analysis. Data are represented as mean fold change obtained from comparing LPS-challenged to vehicle-treated lungs of the same genotype on a semilog scale. All the represented fold change values of LPS-treated lungs of Nrf2–/– mice are significant compared with wild-type mice at P < 0.05.
analyses of lungs were performed at 30 minutes, 1 hour, 6 hours, 12 hours, and 24 hours. Nrf2 deficiency resulted in the enhanced expression of several clusters of genes associated with the innate immune response, even at as early as 30 minutes (Figure 4). These included specific cytokines, chemokines, and cell surface adhesion molecules and receptors, among others. Differences between genotypes in expression of most of the proinflammatory genes in the lungs of mice were significant at the early time points (30 minutes and 1 hour) following LPS challenge. At later time points, with few exceptions, there was no significant difference in expression of proinflammatory genes between the genotypes. Henceforth, unless otherwise stated, a more detailed presentation of the gene expression for cytokines such as TNF-α, IL-1α, IL-6, IL1P9, IL-10, IL-12β, IL-23p19, CSF1, and CSF2 was significantly higher in lungs of Nrf2+/− compared with Nrf2−/− mice. Among all cytokines, the expression of IL-6 was highest. Members of C-C family (CCL12 [MCP5], CCL17 [TARC], CCL2 [MCP1], CCL3 [MIP1α], CCL4 [MIP1β], and CCL6 and CCL8 [MCP2]) and C-X-C chemokines (MIP2, MIG, KC, ITAC, IP-10, and CXCL13) were greatly upregulated in LPS-challenged Nrf2+/− lungs relative to Nrf2−/− (Figure 4 and Supplemental Table 1).

Cell surface adhesion molecules and receptors. Disruption of Nrf2 had no effect on the expression of the LPS signaling receptor, TLR4, after LPS challenge. CD14 transcript was markedly higher in Nrf2+/− lungs. Expression of several adhesion molecules, such as PGLYRP1 (a member of the peptidoglycan recognition protein family; ref. 15), TREM-1, SELE, SELP, VCAM1, and members of the C-type lectin family (CLEC4D, CLEC4E) were highly upregulated in Nrf2+/− lungs (Supplemental Table 2). CSAR, which mediates CSA response and augments sepsis (16), was upregulated to a greater extent in Nrf2−/− mice (Supplemental Table 2). Among the cell surface adhesion molecules, TREM1 and CD14 were highly upregulated in Nrf2−/− lungs.

Regulators of cytokine signaling and transcription. Transcripts of SOCS3, which are involved in downregulating cytokine signaling, were induced to a greater extent in Nrf2+/− lungs at early time points (Supplemental Table 1). Transcription factors belonging to the NF-κB family (C-RELC, RELB, NFKB1Z, NFKB2, NFKBIB), the interferon family (IRFS, IRF1, IFI202B, IFI204, IFI1), and the early growth response family (EGR2, EGR3) as well as STAT4, which collectively regulate different inflammatory cascade pathways, were expressed to higher levels in Nrf2+/− lungs when compared with wild-type mice (Supplemental Table 3).

Immunoglobulin and MHC. Transcripts of many members of the immunoglobulin family (IGHG, IGHG-VJ558, IGH-4, IGH-6, IGJ, IGK-V21, IGK-V32, IGK-V8, IGL-V1, IGSF6, and IGJ) as well as the MHC class II family (H2-AA, H2-AB1, H2-EA, H2-DEMA, H2-DMB1, and H2-DMB2) were selectively upregulated in the lungs of Nrf2+/− mice at 30 minutes (Supplemental Table 4), indicating severe immune dysfunction.

Acute phase proteins, heat shock proteins, and other inflammation-modulating molecules and enzymes. Many genes that encode for acute phase proteins such as proteinase inhibitors (SERPINA3M,
SERPINB2, and SERPINE1), serum amyloid A (SAA2, SAA3), orosomucoid (ORM1, ORM2), and HSPA1A were markedly increased in Nrf2−/− lungs (Supplemental Table 5). Expression levels of ARG2 (an endogenous inhibitor of iNOS that regulates arginine metabolism; ref. 17), INDO (exerts immunosuppressive effects through induction of apoptosis in T cells by regulating tryptophan metabolism; ref. 18), PLEK (regulates phagocytosis activity by macrophages; ref. 19), and PFC (regulator of alternative complement system) were all higher in Nrf2−/− lungs at 30 minutes (Supplemental Table 6).

ROS/RNS generators. The expression of NCE1 (p47phox) and NCE4 (p40phox), which are members of the NADPH oxidase family involved in generation of reactive oxygen species during phagocytic activity by neutrophils and macrophages, were significantly higher in Nrf2−/− lungs at early stages (until 1 hour; Supplemental Table 6). Expression of NOS2 (iNOS), which is involved in nitric oxide generation, was induced at the 6-hour time point and was greater in the lungs of Nrf2−/− mice (Supplemental Table 6).

Antioxidants. Nrf2 is a key transcription factor for regulating the expression of antioxidative genes. Differential gene expression profiling of vehicle-treated Nrf2−/− and Nrf2+/− lungs showed constitutively elevated expression of antioxidative genes such as glutathione peroxidase 2 (GPX2), glutamate cysteine ligase catalytic subunit (GCLC), thioredoxin reductase 1, and members of the glutathione S-transferase family in wild-type mice (Supplemental Table 7). Although expression of these genes was not altered significantly in wild-type mice after LPS challenge, at all time points, transcript levels of these antioxidative genes were higher in the lungs of wild-type mice compared with Nrf2−/− mice.

Validation of microarray data by real-time quantitative PCR. Genes that were selected for validation included chemokines (MCP5, MCP1, and MIP2), cytokines (IL-6, IL-1α, TNF-α, and CSF2), an LPS membrane receptor (CD14), immunoglobulins (IGH-4 and IHSF6), an MHC class II member (H2-EA), and the transcription factor STAT4. Expression values of these genes obtained from real-time PCR were consistent with the microarray values in terms of magnitude and pattern across all time points (Supplemental Table 8).

TNF-α stimulates induction of a greater pulmonary inflammatory response in Nrf2-deficient mice. Microarray and BAL fluid analysis showed greater expression of TNF-α in the lungs of Nrf2−/− mice compared with Nrf2+/− mice in response to LPS. To characterize the effect of TNF-α-mediated inflammation, mice of both genotypes were administered TNF-α (i.p.). Following TNF-α treatment, lungs of Nrf2−/− mice showed increased infiltration of inflammatory cells as measured by BAL fluid analysis and histopathology (Figure 5, A and B) when compared with wild-type littermates. Real-time PCR analysis of selected genes (TNF-α, IL-1β, and IL-6) in the lungs of mice 30 minutes after TNF-α treatment revealed greater expression in Nrf2−/− mice compared with Nrf2+/− (Figure 5C). Taken together, as with LPS, treatment with TNF-α induced greater inflammation in Nrf2−/− lungs.

NF-κB activity is greater in lungs of LPS-treated Nrf2-deficient mice. Because the lungs of Nrf2−/− mice showed greater infiltration of inflammatory cells and higher expression of largely inflammation-associated genes, we assessed NF-κB activity, which regulates the expression of several genes that are essential for initiating and promoting inflammation (20). At 30 minutes after LPS instillation, NF-κB DNA-binding activity was significantly higher in nuclear extracts from lungs of Nrf2−/− mice than in their wild-type counterparts, suggesting an inhibitory role of Nrf2 on NF-κB activation (Figure 6, A and B). Western blot analysis confirmed a greater increase in nuclear levels of p65, an NF-κB subunit, in the LPS-treated lungs of Nrf2−/− mice than in Nrf2+/− mice (Figure 6, C and D). Similarly, nuclear extracts from the lungs of Nrf2−/− mice showed increased binding of p65/κ-rel reticulendotheliosis viral oncogene homolog A (p65/RelA) subunits to NF-κB-binding sequence as measured by ELISA using Mercury TransFactor ELISA kit (Supplemental Figure 3A). A similar trend
Nrf2–/– MEFs showed greater NF-κB activity and TNF-κB secretion in peritoneal macrophages from Nrf2-deficient mice. Macrophages play a central role in immune dysfunction during endotoxic shock. To examine the effect of Nrf2 deficiency on NF-κB activation in macrophages, resident peritoneal macrophages were stimulated with LPS. After 20 minutes, the DNA-binding activity of NF-κB was substantially higher in Nrf2+/+ macrophages than in the wild-type counterparts as determined by EMSA (Figure 7A and B). The greater increase in NF-κB activity in Nrf2–/– macrophages correlated well with the increase in TNF-α levels measured 0.5 hours, 1 hour, and 3 hours after LPS treatment (Figure 7C).

Increased NF-κB activation by LPS or TNF-α in Nrf2-deficient mouse embryonic fibroblasts. To further probe the role of Nrf2 in regulating NF-κB, mouse embryonic fibroblasts (MEFs) derived from Nrf2+/+ and Nrf2–/– mice were exposed to LPS or TNF-α. Both LPS and TNF-α stimulation resulted in enhanced activation of NF-κB in Nrf2–/– MEFs compared with Nrf2+/+ cells as measured by EMSA (Figure 8A). There were 3- and 5-fold increases in NF-κB activity in Nrf2–/– MEFs relative to wild-type in response to LPS or TNF-α stimulation, respectively (Figure 8B). The specificity of NF-κB binding was assessed by adding an excess of cold mutant NF-κB oligo to the binding reactions. Supershift analysis of nuclear extracts from LPS- and TNF-α-treated Nrf2+/+ MEFs with p65 and p50 antibodies demonstrated heterodimers of p50 and p65. Nuclear extracts from the Nrf2–/– MEFs treated with LPS or TNF-α also demonstrated increased binding of p65/p50 subunits to NF-κB binding sequence as determined by an ELISA-based method of detecting NF-κB DNA-binding activity using Mercury TransFactor ELISA kit (Supplemental Figure 3B). NF-κB–mediated luciferase reporter activity was also greater in Nrf2–/– MEFs than in Nrf2+/+ MEFs in response to LPS or TNF-α (Figure 8C). In general, the Nrf2–/– MEFs showed greater NF-κB activation in response to TNF-α compared with LPS stimulation.

Nrf2 regulates NF-κB activation by modulating IκB-α degradation. To understand the mechanism of augmented NF-κB activation in Nrf2–/– MEFs, IκB-α and phosphorylated IκB-α (p-IκB-α) were measured in the whole cell extracts of Nrf2+/+ and Nrf2–/– MEFs after treatment with LPS or TNF-α. In response to LPS or TNF-α, IκB-α degradation was significantly higher in Nrf2–/– MEFs compared with wild-type cells (Figure 8, D and E). TNF-α stimulus induced greater phosphorylation of IκB-α while LPS induced moderate but statistically significant increases in phosphorylation of IκB-α in Nrf2–/– MEFs compared with Nrf2+/+ MEFs (Figure 8, D and F). Furthermore, activity of IκB kinase (IKK) kinase, which regulates phosphorylation of IκB-α, was also greater in Nrf2–/– MEFs in response to LPS or TNF-α (Figure 8, G and H).

Nrf2 affects both MyD88-dependent and MyD88-independent signaling. Microarray gene expression analysis after LPS challenge revealed that, in addition to NF-κB–regulated genes, several interferon regulatory factor 3–regulated (IRF3-regulated) genes (such as IP-10, MIG, ITAC, and ISG54; Supplemental Table 9) were expressed to a greater magnitude in the lungs of Nrf2+/+ mice. LPS via TLR4 can activate MyD88-dependent signaling leading to NF-κB activation as well as MyD88-independent signaling (TRIF/IRF3), resulting in IRF3 activation (21). As shown in Figure 8C, Nrf2 deficiency greatly upregulates NF-κB–mediated luciferase activity in MEFs in response to LPS, suggesting the effect on MyD88-dependent signaling. In order to understand the influence of Nrf2 deficiency on MyD88-independent signaling, MEFs of both genotypes were transfected with a luciferase reporter vector containing interferon-stimulated response element (ISRE) and treated with LPS or polyinosinic-polycytidylic acid [poly(I:C)]. LPS elicited greater IRF3-mediated luciferase reporter activity in Nrf2–/– MEFs compared with Nrf2+/+ MEFs (Figure 9). Similarly, in response to poly(I:C), which acts specifically via MyD88-independent signaling (22), IRF3-mediated reporter activity was significantly higher in Nrf2+/+ MEFs (Figure 9).

Glutathione levels are lower in lungs and MEFs of Nrf2-deficient mice. Nrf2 is a regulator of a battery of cellular antioxidants, including the glutathione-synthesizing (GSH-synthesizing) enzyme, glutamate cysteine ligase. Constitutive expression of GCLC was significantly lower in the lungs as well as MEFs of Nrf2–/– mice compared with Nrf2+/+ mice (Figure 10A). This difference in expression is reflected in significantly lower endogenous levels of GSH in the MEFs of Nrf2–/– mice than in Nrf2+/+ mice (Figure 10D). In response to LPS stimulus, there was a significant decrease in the levels of GSH in MEFs of both genotypes at 1 hour (Figure 10D). In contrast, after 24 hours of LPS treatment, a greater increase in GSH was observed in the lungs of Nrf2–/– mice compared with Nrf2+/+ mice (Figure 10B).
The ratio of GSH to oxidized GSH (GSSG) after LPS challenge was significantly higher in the lungs of wild-type mice, implying greater amounts of GSSG in Nrf2–/– lungs and thus a difference in redox status between the 2 genotypes (Figure 10C).

N-acetyl cysteine and GSH-monoethyl ester decrease LPS- and TNF-α–induced NF-κB activation in Nrf2-deficient MEFs. To investigate whether replenishing antioxidants could suppress the enhanced NF-κB activation observed in Nrf2–/– cells, MEFs transfected with...
pretreatment with NAC (Figure 11B). Concordantly, the influx of Nrf2–4 days prior to LPS challenge (1.5 mg per mouse). All the phenotype of that exogenous antioxidants such as NAC can partially ameliorate mice with NAC provided modest protection. These results suggest pretreated with saline died within 56 hours while 40% of mice pre-
microarray in 8 μl of Lipofectamine2000. Data are mean ± SEM from 3 independent experiments; n = 3. †Differs from wild-type treatment group. *Differs from vehicle control of the same genotype; ‡Differs from wild-type treatment group.

Nrf2 deficiency resulted in augmented lung inflammation in Nrf2–/– mice and protects against lethality. Since LPS challenge enhanced the expression of several NF-κB-regulated proinflammatory genes in lungs of Nrf2+/− mice compared with wild-type littermates, we investigated whether administration of an exogenous antioxidant could attenuate this augmented proinflammatory cascade. Mice were pretreated with NAC (500 mg/kg body weight) and then challenged with nonlethal doses of LPS. After 30 minutes of LPS challenge, selected proinflammatory genes were measured by real-time PCR analysis. Transcript levels of TNF-α, IL-1β, and IL-6 were significantly reduced in the lungs of Nrf2−/− mice by pretreatment with NAC (Figure 11B). Concordantly, the influx of inflammatory cells was also significantly reduced by pretreatment of Nrf2−/− mice with NAC (Figure 11C). We next investigated to determine whether exogenous NAC supplementation could protect against LPS-induced septic shock in Nrf2−/− mice. Mice of both genotypes were pretreated with NAC (500 mg/kg body weight) for 4 days prior to LPS challenge (1.5 mg per mouse). All Nrf2−/− mice pretreated with saline died within 56 hours while 40% of mice pretreated with NAC survived (Figure 11D). Pretreatment of wild-type mice with NAC provided modest protection. These results suggest that exogenous antioxidants such as NAC can partially ameliorate the phenotype of Nrf2−/− mice.

Discussion

Deciphering novel host factors that modulate the innate immune response during sepsis and septic shock not only improves our insight into this complex disease but also provides avenues for designing novel therapies that could minimize mortality. The present study showed Nr2 to be a critical host factor for mounting an appropriate innate immune response, which determines survival during septic shock. Disruption of Nr2 in mice caused greater sensitivity to septic shock induced by either LPS or CLP. Nr2 deficiency resulted in augmented lung inflammation in response to nonlethal challenge with LPS or TNF-α. Systematic gene expression analyses by microarray and subsequent validation by real-time PCR demonstrated that the expression of effector genes (cytokines, chemokines, adhesion molecules, and receptors) of the innate immune response was dysregulated in Nrf2−/− lungs in response to LPS. Nr2 suppressed inflammation by inhibiting NF-κB activation through maintenance of redox status. Pharmacological repletion of cellular antioxidants by administering NAC partially protected Nrf2−/− mice from LPS-induced lung inflammation and lethality, indicating the importance of Nr2-mediated maintenance of antioxidants in counteracting septic shock. Collectively, the results of this study support a critical role for Nr2 in mounting an appropriate innate immune response during sepsis. Sepsis and septic shock by CLP or i.p. administration of a lethal dose of LPS induced greater mortality in Nr2-disrupted compared with wild-type mice. During a gram-negative bacterial infection, LPS initiates the innate immune response through the TLR4 signaling pathway (23). Mice with either defective or disrupted TLR4 fail to respond to LPS and are more susceptible to bacteremia (24, 25). Conversely, aberrant expression of TLR4 enhances sensitivity to LPS (26). Microarray analysis demonstrated no change in the expression of TLR4 in the lungs, constitutively and after LPS challenge between the Nr2+/+ and Nr2−/− mice. Furthermore, there were no differences in constitutive expression of TLR4 by immunoblot in peritoneal macrophages derived from Nr2+/+ and Nr2−/− mice (Supplemental Figure 2A). TNF-α is one of the early proinflammatory cytokines secreted during LPS/CLP-induced shock and is primarily secreted by macrophages and neutrophils (27–29). Lethal

Figure 9

Nrf2 deficiency increases LPS- and or poly(I:C)-induced IRF3-mediated luciferase reporter activity in MEFs. At 24 hours after transfection with ISRE-Tk-Luc vector, cells were treated with LPS and or poly(I:C) for 6 hours, and luciferase assays were performed 6 hours after treatment. For poly(I:C) stimulation, MEFs were transfected with 6 μg of poly(I:C) in 8 μl of Lipofectamine2000. Data are mean ± SEM from 3 independent experiments; n = 3. †Differs from wild-type treatment group. 

Figure 10

Lower levels of GSH in the lungs and MEFs of Nr2-deficient mice. (A) Constitutive expression of GCLC in lungs and MEFs of Nr2+/+ and Nr2−/− mice. (B) GSH levels in the lungs of mice of both genotypes 24 hours after LPS instillation (10 μg per mouse). Data are mean ± SEM from 3 independent experiments and are expressed as percentage increases relative to vehicle-treated Nr2+/+ group. (C) Ratio of GSH to GSSG measured 24 hours after LPS instillation in the lung of Nr2+/+ and Nr2−/− mice. Data are mean ± SEM from 3 independent experiments. (D) GSH levels in Nr2+/+ and Nr2−/− MEFs at 1 hour after LPS (0.5 μg/ml) or TNF-α (10 ng/ml) stimulus. Data are presented as mean ± SEM; n = 4. †Differs from vehicle control of the same genotype. ‡Differs from wild-type treatment group. P < 0.05.
were among the main components of the innate immune response that were expressed at significantly higher levels in Nrf2−/− lungs when compared with wild-type littermates at 30 minutes after LPS challenge. CD14, the LPS-binding coreceptor that mediates LPS-TLR4 signaling, was induced to a greater extent in the lungs of Nrf2−/− mice than in the wild-type mice in response to LPS; however, constitutively, there was no difference in the expression between the genotypes (Supplemental Figure 2B). Elevated expression of CD14 has been associated with increased inflammation; neutralization of CD14 with an antibody protected primates against lethal endotoxic shock and lung inflammation (31). Collectively, the temporal analysis (0, 30 minutes, 1 hour, 6 hours, and 24 hours) of gene expression suggests the following: (a) in general, Nrf2 deficiency induced dysregulated global expression of genes that encode molecular components of innate immunity (e.g., peptidoglycan-recognition proteins, proinflammatory cytokines, chemokines, and adhesion molecules and receptors); (b) the transcriptional dysregulation of effector molecules of innate immunity in Nrf2−/− lungs was more prominent at early stages (30 minutes) of inflammation; (c) although the degree of expression of some of the inflammatory genes in wild-type lungs was either equal to or higher than that in Nrf2−/− lungs at later stages of LPS stimulation, the greater lung pathologic damage in the Nrf2−/− mice supports the conclusion that the enhanced expression of proinflammatory genes during the early stages (as early as 30 minutes) of LPS administration determines the degree of inflammatory insult. Taken together, these results suggest the critical role of Nrf2 in mounting an appropriate innate immune response by controlling an early surge of proinflammatory genes.

The innate immune response triggered by LPS-TLR4 signaling is primarily regulated by NF-κB (30). The expression of most of the LPS-induced proinflammatory genes that were upregulated to a greater extent in Nrf2−/− mice is known to be regulated by NF-κB (20). Furthermore, transcripts of several members of the NF-κB family (such as C-REL, RELB, NFKB1, NFKB2, and NFKBIE) were significantly higher in Nrf2−/− lungs after LPS challenge. NF-κB activation analyzed by EMSA in lung, peritoneal macrophages, and MEFs of Nrf2−/− mice was markedly higher after LPS stimulation as compared with wild-type. Further, NF-κB–mediated transcriptional reporter activity was greater in Nrf2−/− MEFs in response to LPS or TNF-α. NF-κB cytosolic inhibitor IκB-α showed increased degradation and phosphorylation in Nrf2−/− cells after LPS or TNF-α challenge. Phosphorylation of IκB-α is mediated by IKK, leading to ubiquitination and degradation of IκB-α. Analysis of IKK kinase activity in Nrf2−/− and Nrf2−/− MEFs in response to LPS or TNF-α demonstrated increased activity in the former group. Thus, Nrf2 regulates NF-κB activation largely by modulating its upstream signaling components.
NCF1

LPS activates NF-κB and IRF3 via MyD88-dependent and -independent signaling, respectively (21, 30). Activation of IRF3 leads to expression of interferon and interferon inducible genes through activation of TANK-binding kinase and IKKε (32). Global gene expression analysis by microarray revealed a subset of genes (IP-10, MIG, ITAC, and ISG54; Supplemental Table 9) regulated specifically by IRF3 signaling pathway (33) that were upregulated to higher levels in Nrf2−/− mice. To determine whether Nrf2 affects both MyD88-dependent and -independent signaling, an IRF3-mediated luciferase reporter assay was performed. In response to poly(I:C) stimulation, which specifically elicits inflammatory response via MyD88-independent pathways, the IRF3-mediated luciferase reporter activity was significantly higher in Nrf2+/+ cells. Similarly, in response to LPS, the IRF3 reporter activity was elevated in Nrf2−/− compared with Nrf2+/+ MEFs. These results indicate that Nrf2 may affect both MyD88-dependent and -independent signaling.

Although it is not clear how Nrf2 interferes with LPS signaling to inhibit NF-κB, several lines of evidence indicate that the redox status of the cells modulates LPS-induced NF-κB activation (34–37). LPS stimulation led to higher expression of genes involved in the production of superoxide (NCF1 and NCF4) and nitric oxide (iNOS) in Nrf2−/− lungs. Cellular antioxidant defenses can counter inflammation by limiting ROS levels. Levels of GSH and the transcript of the rate-limiting gene involved in its synthesis, GCLC, were significantly higher in lungs and MEFs of wild-type mice than of Nrf2−/− mice. Lower levels of GSH have been reported to augment NF-κB activation in response to LPS stimulation (38). Basal expression of several antioxidative enzymes, including glutathione reductase 1, thioredoxin reductase, and GPX2, were higher in lungs of Nrf2−/− mice compared with Nrf2+/+. Enhanced activation of NF-κB as early as 30 minutes in the lungs and MEFs and 20 minutes in peritoneal macrophages after LPS challenge of Nrf2−/− mice underscores the positive influence of constitutive antioxidants in modulating the ensuing inflammatory cascade. Further, pretreatment of Nrf2−/− MEFs with antioxidants such as NAC and GSH monoethyl ester greatly suppressed NF-κB activation in response to LPS or TNF-α. These results suggest that Nrf2-mediated maintenance of cellular redox status through regulation of GSH and perhaps other cellular antioxidants is crucial for regulating NF-κB activation. During the course of LPS-induced inflammation, expression levels of many antioxidative genes were significantly lower in the lungs of knockout mice compared with wild-type mice (Supplemental Table 7). Among these antioxidative enzymes, expression of GPX2 was highest in lungs of Nrf2−/− mice. A previous study showed that GPX2 is the only inducible form of GSH peroxidase in the lungs that is regulated by Nrf2 (7). A protective role of GSH peroxidase in LPS-induced inflammation has been previously reported (39). These results indicate that Nrf2-directed constitutive regulation of cellular redox status is fundamental in determining the outcome of the innate immune response. In corroboration with in vitro experiments, pretreatment of Nrf2−/− mice with NAC greatly diminished LPS-induced proinflammatory gene expression as well as inflammation in lungs. In addition, NAC pretreatment significantly reduced the LPS-induced septic mortality in Nrf2−/− mice while moderate protection was conferred in wild-type littermates. Altogether, these results imply that maintenance of redox equilibrium by regulating GSH and other antioxidative enzymes through Nrf2 may be partially responsible for alleviating LPS-induced inflammation.

The results of the current study suggest that Nrf2 regulates the innate immune response during sepsis and improves survival by maintaining redox homeostasis and restraint of the dysregulation of proinflammatory signaling pathways (MyD88-dependent and -independent and TNF-α signaling). A recent report by Suh et al. (40) showed that aged rats have reduced levels of GSH and diminished expression of GSH-biosynthetic enzymes (GCLC and glutamate cysteine ligase, modifier subunit [GCLM]) as a result of a decline in the transcriptional activity of Nrf2. Thus, it will be worth investigating whether variation in the Nrf2 signaling pathway mediates the increased susceptibility and severity of inflammation and septic shock in elderly patients. In addition, correction of low Nrf2-mediated transcriptional activity through the use of pharmacological activators of Nrf2 signaling may be a novel therapeutic strategy for counteracting inflammation during sepsis.

Methods

Mice. Nrf2-deficient mice (CD-1 [Institute for Cancer Research]; Nrf2−/−) were generated as described (41). Wild-type (CD-1 [Institute for Cancer Research]; Nrf2+/+) and Nrf2−/− mice were fed an AIN-76A diet and water ad libitum and were housed under controlled conditions (25 ± 2°C; 12-hour light-dark periods). All experimental protocols conducted on the mice were performed in accordance with NIH guidelines and were approved by the Johns Hopkins University Animal Care and Use Committee.

Treatment. Endotoxemic shock was induced in male mice (8 weeks old) of both genotypes by i.p. injection of LPS at doses of 0.75 or 1.5 mg per mouse (Escherichia coli, serotype O55:B5; Sigma-Aldrich) as described (42). After LPS injection, the mice were monitored for 5 days. To induce nonlethal systemic inflammation, the mice were injected with LPS (i.p., 60 μg/mouse) and recombinant hTNF-α (i.p., 10 μg/mouse) (R&D Systems). Control mice received an equivalent volume of vehicle. Intratracheal LPS instillation was used for induction of local inflammation in the lungs. Mice were first anesthetized by i.p. injection with 0.1 ml of a mixture of ketamine (10 mg/ml) and xylazine (1 mg/ml) in PBS. LPS was instilled intratracheally (10 μg in 50 μl sterile PBS) during inspiration. Control mice received an equivalent volume of vehicle.

CLP. Polymicrobial sepsis was induced by CLP. Briefly, a midline laparotomy was performed on the anesthetized mice, and the cecum was identified. The distal 50% of exposed cecum was ligated with 3-0 silk suture and punctured with 1 pass of an 18-gauge needle. The cecum was replaced in the abdomen, and the incision was closed with 3-0 suture. Postoperatively, the animals were resuscitated with 1 ml subcutaneous injection of sterile 0.9% NaCl. Mice were monitored regularly, and survival was recorded over a period of 5 days.

BAL and phenotyping. Mice were anesthetized with an overdose of sodium pentobarbital. The lungs were aspirated 3 times with 1 ml of sterile PBS to collect BAL fluid. Cells were counted by using a hemocytometer, and differential cell counts were performed on 300 cells from BAL fluid with Wright-Giemsa stain (Baxter).

Histopathology and immunohistochemistry. Lungs were inflated with 10% buffered formalin through the trachea 24 hours after the treatment and subsequently fixed for 24 hours at 4°C. After paraffin embedding, 5-μm sections were cut and stained with H&E. For identification of neutrophils, lung sections were stained by using rat IgG anti-mouse neutrophil monoclonal antibody (Serotec) followed by the secondary goat anti-rat IgG conjugated to horseradish peroxidase. Color development was performed with 3′,3′-diaminobenzidine, and the slides were counterstained with hematoxylin.
Measurement of myeloperoxidase. The activity of myeloperoxidase, an indicator of neutrophil accumulation, was measured in the supernatant fluid obtained from whole-lung homogenates as described (43).

Measurement of lung edema. Five animals per group were treated with LPS for 24 hours. Mice were sacrificed by i.p. injection of sodium pentobarbital, and the lungs were excised. All extrapulmonary tissue was cleared, weighed (wet weight), dried for 48 hours at 60°C, and then weighed again (dry weight). Lung edema was expressed as the ratio of wet weight to dry weight.

ELISA. Levels of TNF-α, TNFR1 (p55), and TNFR1 (p75) were measured by enzyme immunoassays by using murine ELISA kits (R&D Systems).

Microarray. Both genotypes were subjected to systemic inflammation by i.p. injection of LPS (60 μg per mouse). Lungs were isolated at 30 minutes, 1 hour, 6 hours, 12 hours, and 24 hours after LPS challenge. Total RNA from the lungs was extracted by using TRIZOL reagent (Invitrogen Corp.). The isolated RNA was applied to Murine Genome MOE 430A GeneChip arrays (Affymetrix) according to procedures described previously (5). This array contains probes for detecting approximately 14,500 well-characterized genes and 4371 expressed sequence tags. Scanned output files were analyzed by using Affymetrix GeneChip Operating Software version 1.3 and were independently normalized to well-characterized genes and 4371 expressed sequence tags.

Real-time quantitative PCR. Total RNA was isolated by using TRIZOL reagent (Invitrogen Corp.), and reverse transcription was performed by using random hexamers and MultiScribe reverse transcriptase according to the manufacturer’s recommendations (Applied Biosystems). Quantitative PCR analyses of selected genes were performed by using primers and probe sets commercially available from Applied Biosystems. Assays were performed by using the ABI 7000 Taqman system (Applied Biosystems). GAPDH was used for normalization.

Isolation of resident peritoneal macrophages and treatment. Resident peritoneal macrophages were harvested from 4 mice of each genotype by peritoneal lavage with 5 ml of cold RPMI-1640 medium supplemented with 10% FBS. Isolated peritoneal macrophages from all mice of the same genotype were pooled and plated into 24-well plates at a density of 1 × 10⁶ cells/ml. Adherent cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin, and 1% streptomycin for 16 hours at 37°C in a CO₂ incubator. Cells were then stimulated with LPS (1 ng/ml) in serum-free medium.

Electrophoretic mobility shift assay. Nuclear proteins were isolated from tissue or cells by using the protocol described previously (7). The NF-κB probe (5’-GGTGAAGGGCAGTTTCCAGGC-3’) (Promega) was end-labeled by T4 polynucleotide kinase in the presence of [α32P] ATP. For EMSA, 5 μg of nuclear proteins was incubated with the labeled NF-κB probe in the presence of (dI-dC) in binding buffer (Promega) at 4°C for 20 minutes. The mixture was then resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel and developed by autoradiography. For supershift analysis, nuclear proteins were incubated with 1 to 2 μg of polyclonal antibody to either p65 or p50 subunit of NF-κB (Santa Cruz Biotechnology Inc.) for 30 minutes after incubation with the labeled probe.

P65/RelA DNA-binding activity. DNA-binding activity of the p65/RelA subunit of NF-κB was determined using Mercury TransFactor Kit (BD Biosciences). An equal amount of nuclear extracts was added to incubation wells precoated with the DNA-binding consensus sequence. The presence of translocated p65/RelA subunit was then assessed by using the Mercury TransFactor kit according to manufacturer instructions. Plates were read at 655 nm, and results were expressed as OD.

Western blot analysis. Western blot analysis was performed according to previously published procedures (7), using antibodies specific for p65, p50, IKκB-α, or-IκB (Santa Cruz Biotechnology Inc.), p1κB-α (Cell Signaling Technology), TLR4, and CD14 (eBioscience).

In vitro IKk kinase activity. Cytoplasmic extracts were isolated from cells using cell lysis buffer (Cell Signaling Technology), and protein was measured by BCA protein assay kit (Pierce Biotechnology). Cytoplasmic extracts (250 μg) were incubated with 1 μg IKKα monoclonal antibody (Santa Cruz Biotechnology Inc.) for 2 hours at 4°C, and then with protein A/G-conjugated Sepharose beads (Pierce Biotechnology) for 2 hours at 4°C. After washing with cell lysis buffer 5 times and 1 time with the kinase buffer (Cell Signaling Technology), the beads were incubated with 20 μM kinase buffer containing 20 μM adenosine 5′-triphosphate (ATP), 5 μCi [32P] ATP, and 1 μg glutathione S-transferase–IKBα (1-317) substrate (Santa Cruz Biotechnology Inc.) at 30°C for 30 minutes. The reaction was terminated by boiling the reaction mixture in x5 SDS sample buffer. Proteins were resolved on a 10% polyacrylamide gel under reducing conditions; the gel was dried, and the radiolabeled bands were visualized using autoradiography. To determine the total amounts of IKK in each sample, immunoblotting was performed. Proteins (30 g) from whole-cell extract were resolved on a 12% SDS-acrylamide gel then electrotransferred to a polyvinylidene difluoride and probed for IKKα (Santa Cruz Biotechnology Inc.).

Transfection and luciferase assay. MEFs from mice of both genotypes were prepared from 13.5-day embryos as described (44) and grown in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin. MEFS (60–80% confluence) were transfected with luciferase reporter genes (p–NF-κB–Luc or ISRE-Tk-Luc vector) by using Lipofectamine2000 (Invitrogen Corp.). The Renilla-luciferase reporter gene (p–RL-TK) was cotransfected for normalization. After the treatments, the reporter gene activity was measured using the Dual Luciferase Assay System (Promega). All transfection experiments were carried out in triplicate wells and were repeated separately at least 3 times.

Reduced and oxidized GSH. A Bioxytech GSH/GSSG-412 kit (Oxis International Inc.) was used to measure reduced GSH and GSSG in the lungs. Briefly, lung tissue was homogenized in cold 5% metaphosphoric acid. For measuring GSSG, 2-methyl-2-vinyl-pyridinium trifluoroethane sulfonate, a scavenger of reduced GSH, was added to an aliquot of lung homogenate. The homogenates were centrifuged at 5000 g for 5 minutes at 4°C, and the supernatant fluid was used to measure GSH and GSSG per the manufacturer’s instructions. Total GSH in MEFS was measured as previously described (45).

Intervention with NAC. To investigate the effect of replenishing antioxidant in NFκB– mice on lung inflammation induced by nonlethal dose of LPS (60 μg per mouse), mice were pretreated with NAC (Sigma-Aldrich) (500 mg/kg body weight) 3 times, 4 hours apart. One hour after the last dose of NAC, LPS was injected and BAL fluid analysis and expression of inflammatory genes were performed as described above. To determine the effect of replenishing antioxidant in NFκB– mice on LPS-induced septic shock, NAC (500 mg/kg body weight) was administered i.p. every day for 4 days. One hour after the last dose of NAC, a lethal dose of LPS (1.5 mg per mouse) was injected. Mortality was observed as described above.

Statistics. Data are expressed as mean ± SEM. Student’s 2-tailed t test was used to evaluate the differences between the control and treatment groups within a single genotype as well as between genotypes. Survival studies were analyzed by using the log rank test. Statistical significance was accepted at P < 0.05.
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