ARTICLE

NQO1 inhibits the TLR-dependent production of selective cytokines by promoting IκB-ζ degradation

Akihiro Kimura[1], Masayuki Kitajima[1], Kyoko Nishida[1,2], Satoshi Serada[3,4], Minoru Fujimoto[3,4], Tetsuji Naka[3,4], Yoshiaki Fujii-Kuriyama[5], Satoshi Sakamoto[6], Takumi Ito[7,8], Hiroshi Handa[7], Takashi Tanaka[9], Akihiko Yoshimura[10], and Harumi Suzuki[1]

NAD(P)H:quinone oxidoreductase 1 (NQO1) protects cells against oxidative stress and toxic quinones. In this study, we found a novel role of NQO1 in suppressing Toll-like receptor (TLR)–mediated innate immune responses. NQO1-deficient macrophages selectively produced excessive amounts of IL-6, IL-12, and GM-CSF on LPS stimulation, and the deletion of NQO1 in macrophages exacerbated LPS-induced septic shock. NQO1 interacted with the nuclear IκB protein IκB-ζ, which is essential for the TLR-mediated induction of a subset of secondary response genes, including IL-6, and promoted IκB-ζ degradation in a ubiquitin-dependent manner. We demonstrated that PDLIM2, known as the ubiquitin E3 ligase, participates in NQO1-dependent IκB-ζ degradation. NQO1 augmented the association between PDLIM2 and IκB-ζ, resulting in increased IκB-ζ degradation. Collectively, this study describes a mechanism of the NQO1–PDLIM2 complex as a novel and important regulator in the innate immune signaling and suggests the therapeutic potential of NQO1 in TLR-mediated inflammation and disorders.

Introduction

The innate immune system is the initial host defense against invading pathogens. Immune cells, such as macrophages and dendritic cells, sense invading pathogens through pattern recognition receptors (PRRs), resulting in inducing the production of proinflammatory cytokines and ROS and subsequently initiating adaptive immunity (Kawai and Akira, 2011). Toll-like receptors (TLRs) are one type of PRR and recognize pathogen-associated microbial patterns (PAMPs) or danger-associated molecular patterns (DAMPs; Iwasaki and Medzhitov, 2004). Stimulation of TLRs by corresponding PAMPs or DAMPs leads to the activation of intracellular signaling pathways mediated by TLR adaptor proteins, including myeloid differentiation primary response protein 88, Toll/IL-1 receptor (TIR) domain–containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein, and TRIF-related adaptor molecule (Iwasaki and Medzhitov, 2004). The interaction between TLRs and these adaptor molecules triggers downstream signaling cascades, leading to the activation of a transcription factor, NF-κB, that induces the production of proinflammatory cytokines such as IL-6, IL-12, and TNF-α.

Although TLR-induced TNF-α production is directly regulated by NF-κB, the induction of a group of genes such as IL-6 and IL-12 is regulated by IκB-ζ that modulates NF-κB activity in the nucleus (Yamamoto et al., 2004). IκB-ζ is an atypical nuclear member of the IκB family and is rapidly induced by various proinflammatory stimuli such as LPS (Haruta et al., 2001; Eto et al., 2003; Muta, 2006). IκB-ζ induction requires NF-κB–mediated transcriptional activation, indicating that the induction of IκB-ζ–regulated genes, including IL-6 and IL-12, is governed by a two-step mechanism.

Sepsis is characterized by a system-wide amplification and dysregulation of the initial, appropriate host responses to primary bacterial infection (Cohen, 2002). Most pathogenic events and clinical signs that occur in the initial phase of sepsis are thought to be triggered by the activation of TLRs. Bacterial components activate TLRs and induce proinflammatory cytokines including IL-6, TNF-α, and IL-1β from antigen-presenting cells (APCs), which triggers the strong systemic proinflammatory responses to develop sepsis. Although the neutralization of

proinflammatory cytokines can prevent the development of septic shock in animal models (Tracey et al., 1987; Riedemann et al., 2003), the neutralizing of TNF-α or IL-1β had no sufficient clinical effects in critically ill patients (Fisher et al., 1994; Abraham et al., 1998, 2001). On the other hand, it has been reported that IL-6 is established as a clinically suitable biomarker for sepsis (Gouel-Chéron et al., 2012) and high levels of IL-6 combines with the highest risk of death in septic patients (Kellum et al., 2007). These studies suggest that it is necessary to elucidate the precise mechanisms regulating TLR-mediated IL-6 production to develop novel strategies for treating sepsis.

NAD(P)H:quinone oxidoreductase 1 (NQO1) was originally identified as a flavoenzyme that catalyzes the two-electron reduction of quinones to their hydroquinone forms, protecting cells from oxidative stress, redox cycling, and neoplastic lesions (Dinkova-Kostova and Talalay, 2000). However, recent studies have indicated that NQO1 also works as a protein stabilizer (Asher et al., 2001, 2005; Garate et al., 2008; Dinkova-Kostova and Talalay, 2010). NQO1 binds to p53 and prevents the ubiquitin-independent degradation of p53 by the 20S proteasome (Asher et al., 2001, 2005; Dinkova-Kostova and Talalay, 2010). Furthermore, NQO1 participates in the stability of other proteins such as p33ING1b and p73 (Asher et al., 2005; Garate et al., 2008), indicating that NQO1 acts as a protein chaperone. In contrast, Park et al. reported that NQO1 promotes the degradation of Aurora-A, which induces centrosome maturation, duplication, and cell cycle movement (Marumoto et al., 2005), in a ubiquitin- and proteasome-dependent manner (Park et al., 2013). These studies have suggested that NQO1 is a multifunctional protein that not only possesses antioxidant properties but also plays positive and negative roles in regulating the degradation of target proteins.

PDZ and LIM domain protein-2 (PDLIM2) is a nuclear protein containing both postsynaptic density 65-discs large-zonula occludens 1 (PDZ) and abnormal cell lineage 11-isket 1-mechanosensory abnormal 3 (LIM) domains (Torrado et al., 2004; Loughran et al., 2005; Tanaka et al., 2005). It has been reported that PDLIM2 possesses autoubiquitination activity, which is a good indicator of ligase activity, through its LIM domain and functions as an E3 ubiquitin ligase (Tanaka et al., 2005). Tanaka et al. previously demonstrated that PDLIM2 degrades the target proteins such as the p65 subunit of NF-κB and signal transducer and activator of transcription 3 (STAT3) by acting as a nuclear ubiquitin E3 ligase that subsequently inhibits NF-κB activity and T helper 17 (Th17) cell development, respectively (Tanaka et al., 2007, 2011). In addition, they found that heat shock protein of 70 kD (HSP70) is required for the PDLIM2-mediated degradation of p65 (Tanaka et al., 2014), suggesting that PDLIM2 might require chaperone proteins such as HSP70 for the ubiquitin-associated degradation of target proteins.

In the present study, we demonstrated that NQO1 selectively suppresses TLR-induced production of IL-6, but not TNF-α, in macrophages and prevents septic shock during LPS challenge in vivo. NQO1 interacts with the inducible nuclear protein IκB-ζ and subsequently leads to its ubiquitination and degradation. Furthermore, we found that PDLIM2 promotes the polyubiquitination and proteasome-dependent degradation of the IκB-ζ protein in association with NQO1. Consequently, LPS-induced

IL-6 production was selectively increased in PDLIM2-deficient macrophages as well as in NQO1-deficient macrophages. PDLIM2 associated with IκB-ζ and NQO1 through its LIM domain. NQO1 drastically enhanced the interaction between PDLIM2 and IκB-ζ, indicating that the NQO1–PDLIM2 complex determines the ubiquitin-dependent degradation of IκB-ζ in TLR-mediated responses. Collectively, our data indicate a previously undescribed function of NQO1 in cooperation with PDLIM2 in TLR-mediated immune responses and suggest the therapeutic potential of NQO1 not only in sepsis but also in TLR-mediated diseases.

Results

NQO1 expression is increased by LPS stimulation in an aryl hydrocarbon receptor (AhR)–dependent manner

To understand the role of NQO1 in TLR-mediated inflammation, we first determined whether NQO1 expression is modulated by LPS stimulation. We stimulated peritoneal macrophages with LPS and examined the expression of NQO1. Compared with unstimulated macrophages, LPS elevated the expression levels of *nqo1* mRNA and protein (Fig. 1 A).

The AhR is a ligand-activated transcription factor that belongs to the basic-helix-loop-helix-PER-ARNT-SIM family. On binding with a ligand, AhR undergoes a conformational change, translocates to the nucleus, and induces target gene expression (Fujii-Kuriyama et al., 1994). Previous studies reported that the expression of AhR is induced upon TLR stimulation and is required for induction of NQO1 by interacting with nuclear factor erythroid 2-related factor 2 (Nrf2; Ma et al., 2004; Kimura et al., 2009; Sekine et al., 2009; Wang et al., 2013). Therefore, we next examined whether AhR is responsible for NQO1 induction in macrophages. AhR deficiency suppressed *nqo1* mRNA expression in macrophages with or without LPS stimulation (Fig. 1 B). Similarly, NQO1 protein level decreased in AhR-deficient macrophages (Fig. 1 C). The dependency of AhR in NQO1 induction was more prominent in the mouse macrophage-like cell line RAW 264.7, which does not express AhR. The introduction of exogenous AhR into RAW cells restored the time-dependent induction of NQO1 (Fig. S1 A). In agreement with these results, we found that AhR ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ) and Benzo[a]pyrene (BaP), drastically induced *nqo1* expression in macrophages (Fig. 1 D). Next, we investigated whether the NQO1 gene is a direct target of AhR. Although there are several binding motifs for AhR (Boutros et al., 2004), we localized the putative xenobiotic response element (XRE) at –513 (XRE1), –5450 (XRE2), and –7583 (XRE3) upstream of the start site in the NQO1 promoter (Fig. 1 E). Chromatin immunoprecipitation (ChIP) assay confirmed that after LPS stimulation, AhR is recruited to XRE1, but not to the other XREs in the NQO1 promoter (Fig. 1 F). These data indicate that LPS increases NQO1 expression in macrophages via AhR activation.

NQO1 deficiency enhances the selective induction of IL-6 and IL-12 in macrophages

We next compared cytokine secretion in peritoneal macrophages from WT and NQO1$^{-/-}$ mice. NQO1-deficient macrophages produced markedly higher concentrations of IL-6 and
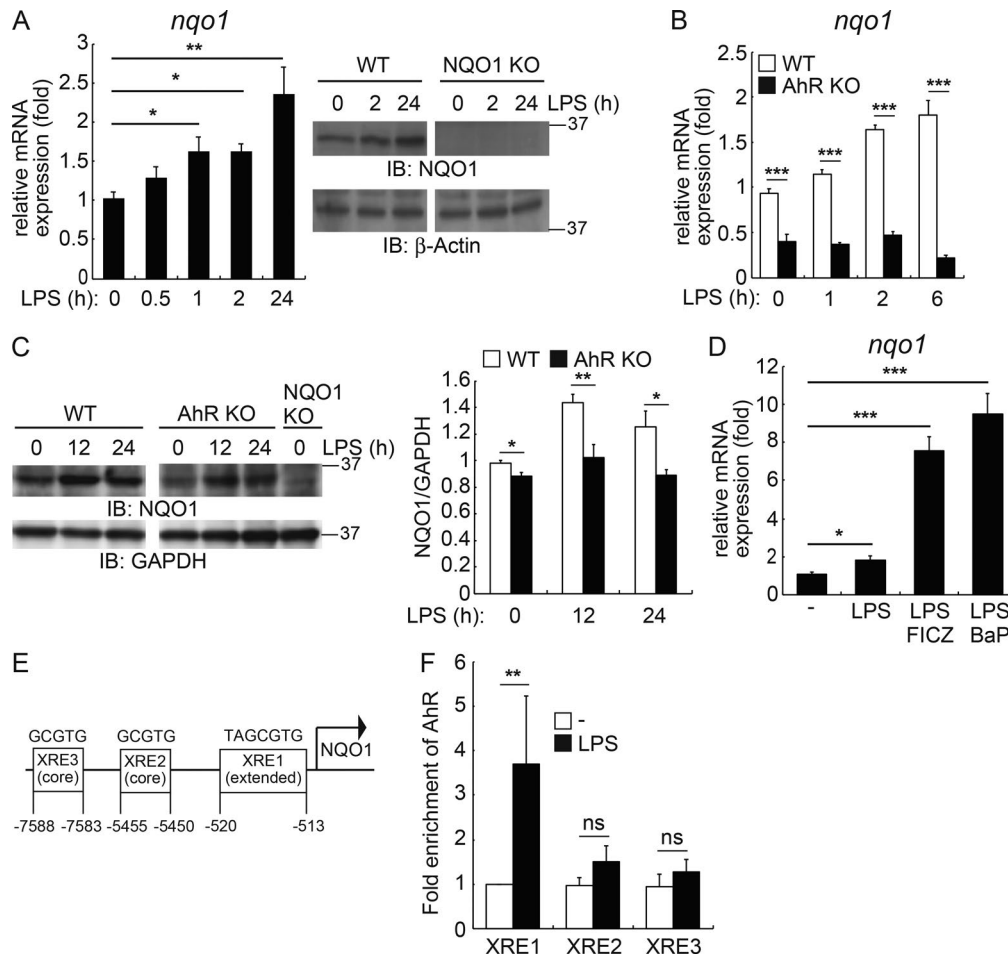
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Figure 1. AhR-dependent induction of NQO1 in macrophages activated by LPS. (A) Primary peritoneal macrophages from WT and NQO1$^{-/-}$ mice were stimulated with LPS (100 ng/ml) for the indicated times. The expression of NQO1 was analyzed by qPCR (left) and immunoblotting (IB, right). **(B and C)** Primary peritoneal macrophages from WT and AhR$^{-/-}$ mice were stimulated with LPS (100 ng/ml) for the indicated times. The expression of NQO1 was analyzed by qPCR; results are presented relative to the expression of GAPDH mRNA (B). The expression of NQO1 and GAPDH expression was detected by Western blot (C; top panel). The density of NQO1 bands was normalized to GAPDH and presented as fold change relative to nontreated control cells (C; bottom panel). IB denotes immunoblotting. **(D)** Primary peritoneal macrophages were stimulated with LPS (100 ng/ml) for 24 h in the presence or absence of the AhR ligands FICZ (100 nM) and BaP (5 μM). qPCR analysis of the expression of NQO1 in cells untreated or treated with LPS for 24 h; results are presented relative to the expression of GAPDH mRNA. **(E)** Alignment of XRE (core or extended) from the NQO1 promoter. **(F)** Peritoneal macrophages were treated with LPS (100 ng/ml) for 12 h, and ChIP was performed using anti-AhR. Purified DNA fragments were amplified using primers specific for each XRE region in the NQO1 promoter. Data are representative of two independent experiments (A and C) or of three independent experiments and are presented as mean ± SD (A, B, D, and F). *, P < 0.05; **, P < 0.005; ***, P < 0.001 (unpaired two-tailed Student's t test).

IL-12 in response to LPS than WT macrophages (Fig. 2, A and B). In addition, we found that these increases occurred even in the early phase of stimulation (2 h; Fig. S1 B). Although LPS-induced GM-CSF production also increased in NQO1-deficient macrophages, the production of TNF-α and IL-10 was equivalent in macrophages from WT and NQO1$^{-/-}$ mice (Fig. 2, C and D; and Fig. S1 C). Similar to stimulation with LPS (TLR4 ligand), CpG-oligodeoxynucleotides (ODN; TLR9 ligand) and R-848 (TLR7 ligand; Kawai and Akira, 2011) induced augmented IL-6 production in NQO1-deficient macrophages (Fig. S1, D and E). Nitric oxide is a proinflammatory mediator induced in various cell types during innate immune responses (Bogdan, 2001). Compared with WT macrophages, the LPS-induced production of nitric oxide, as well as of TNF-α, was not affected in NQO1-deficient macrophages (Fig. 2 E). Our data indicate that NQO1 shows remarkable selectivity for reducing LPS-induced production of inflammatory

mediators. To determine whether NQO1 inhibits the LPS-induced activation of the *Il6* promoter, RAW 264.7 cells were cotransfected with reporter plasmids containing the promoter of *Il6* or *Tnfa* and control or NQO1 expression vectors. NQO1 expression significantly inhibited the LPS-induced activation of the *Il6* promoter in a dose-dependent manner, whereas no effect was observed on the *Tnfa* promoter (Fig. 2 F). These data are consistent with the observation that NQO1 suppresses LPS-induced IL-6 production, but not TNF-α production.

TLR ligands up-regulate MHC class II and costimulatory molecules, such as CD80 and CD86, on APCs (Kawai and Akira, 2011). We next investigated the influence of NQO1 on the LPS-induced expression of these surface molecules. Although the LPS-induced up-regulation of CD86 was similar between WT and NQO1-deficient macrophages, NQO1 deficiency led to the decreased expression of CD80 after LPS stimulation (Fig. 2 G).
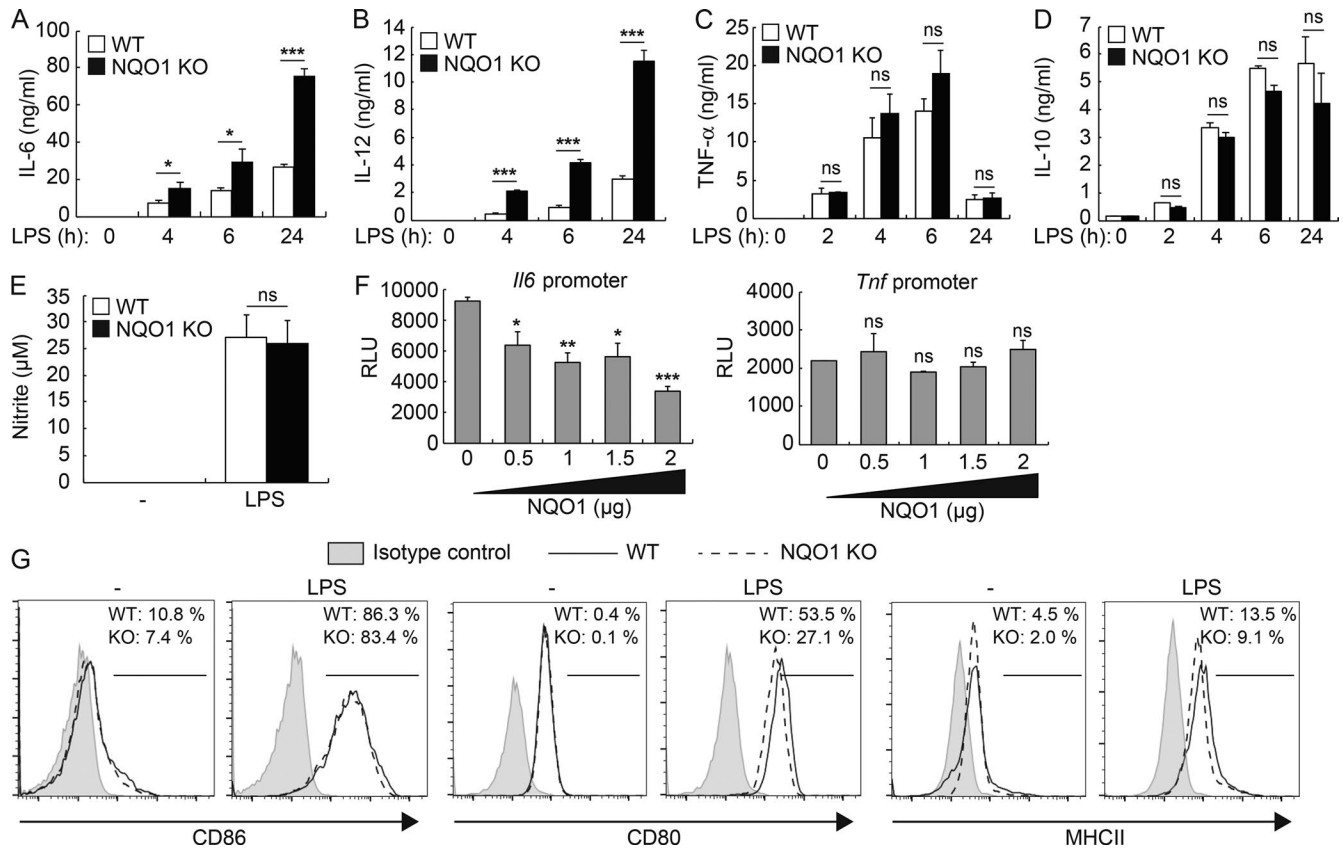
Figure 2. **NQO1 deficiency selectively increases the production of IL-6 and IL-12 induced by LPS. (A–D)** ELISA of IL-6, IL-12, TNF-α, and IL-10 in the supernatants of WT and NQO1-deficient macrophages stimulated with LPS (100 ng/ml) for the indicated times. **(E)** Peritoneal macrophages from WT and NQO1⁻/⁻ mice were stimulated with LPS (100 ng/ml) for 24 h. Nitrite production was determined in the culture medium as described in the Materials and methods section. **(F)** Luciferase activity in RAW 264.7 cells cotransfected with IL-6 or TNF-α luciferase reporters together with an NQO1 expression vector. Data in A–F are from three independent experiments and presented as mean ± SD; *, P < 0.05; **, P < 0.005; ***, P < 0.001 (unpaired two-tailed Student's *t* test). ns, not significant. **(G)** Peritoneal macrophages from WT and NQO1⁻/⁻ mice were stimulated with LPS (100 ng/ml) for 24 h. The expression of CD86, CD80, and MHCII was analyzed using a flow cytometer. Isotype-matched antibodies were used as control (in solid gray). Data are representative of three independent experiments.

Macrophages express high levels of MHC class II (Barjesteh et al., 2014); therefore, LPS stimulation did not show the strong increase of its expression in WT or NQO1-deficient macrophages (Fig. 2 G). Additionally, there was no marked difference in the expression of MHC class II between these cells with or without LPS stimulation (Fig. 2 G). Collectively, NQO1 selectively regulates the expression of cytokines and surface costimulatory molecules in macrophages.

NQO1 deficiency in macrophages enhances sensitivity to LPS-induced lethality

Because NQO1-deficient macrophages produced more LPS-induced IL-6 than WT macrophages, we asked whether NQO1⁻/⁻ mice were more susceptible to LPS-induced toxicity. WT and NQO1⁻/⁻ mice were i.p. injected with a sublethal dose of LPS. Although the administration of a sublethal dose of LPS resulted in 100% survival in WT mice, approximately half of the NQO1⁻/⁻ mice perished by 72 h (Fig. 3 A). We next measured the serum levels of IL-6, TNF-α, and IL-10 in WT and NQO1⁻/⁻ mice after the LPS challenge. Serum concentrations of IL-6 were higher in NQO1⁻/⁻ mice than in WT mice, whereas those of TNF-α and IL-10 were not significantly affected (Fig. 3 B).

To determine the functions of NQO1 in macrophages during septic shock, peritoneal macrophages from WT or NQO1⁻/⁻ mice were isolated and transferred into WT mice. After transfer, mice were i.p. challenged with PBS or a sublethal dose of LPS, and the development of sepsis and concentrations of serum cytokines were monitored. The transfer of NQO1-deficient macrophages led to 100% mortality by 24 h after administration of LPS, whereas over 60% of mice receiving WT macrophages survived under the experimental conditions (Fig. 3 C). The adoptive transfer of NQO1-deficient macrophages resulted in more severe LPS-induced endotoxin shock compared with NQO1⁻/⁻ mice injected with LPS. Serum cytokine assay revealed that the transfer of NQO1-deficient macrophages enhanced the levels of IL-6, but not of TNF-α or IL-10, during the LPS-induced septic shock experiment (Fig. 3 D). These findings indicate that macrophage-intrinsic NQO1 protects against LPS-induced sepsis through the selective inhibition of IL-6.

NQO1 promotes IκB-ζ degradation in macrophages

Our results indicate that NQO1 selectively suppresses the LPS-induced production of IL-6 and IL-12 in macrophages. We next examined the molecular mechanisms how NQO1 negatively
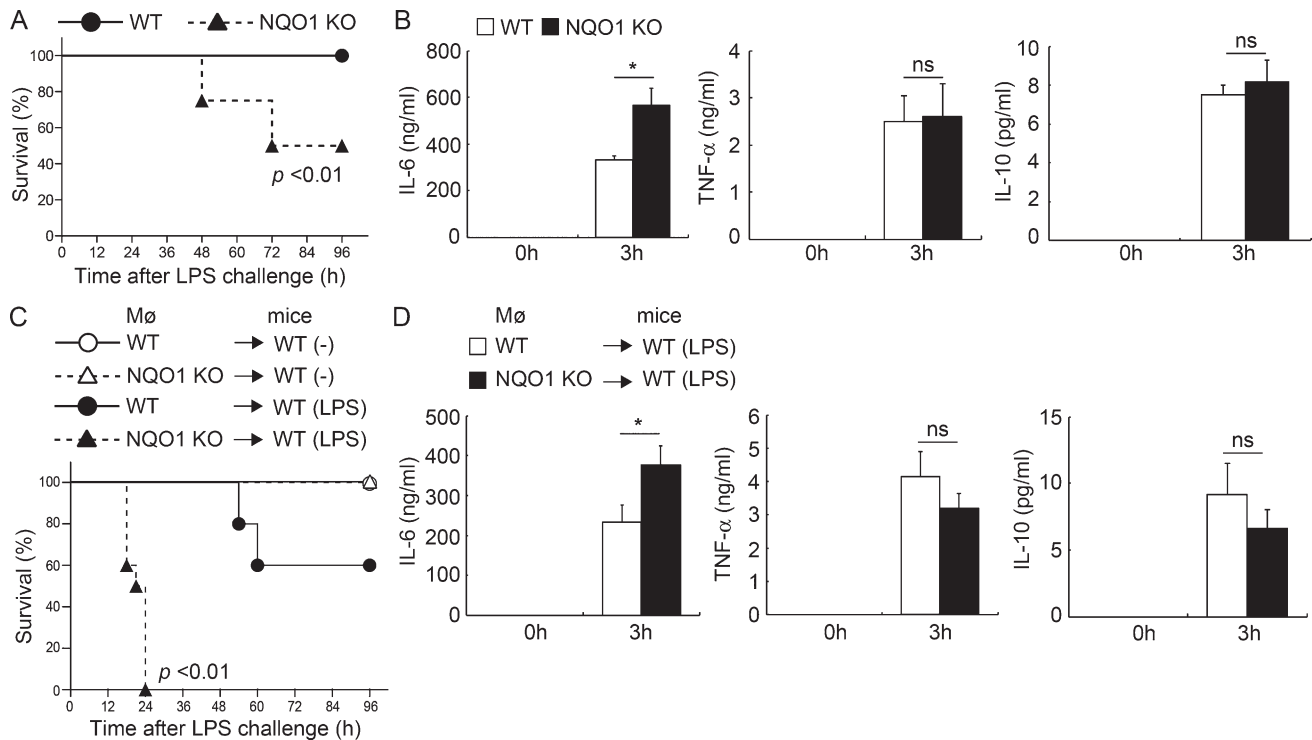
Figure 3. **NQO1 deficiency in macrophages exacerbates LPS-induced sepsis. (A)** WT and NQO1^{-/-} mice (n = 10) were i.p. injected with LPS (10 mg/kg). Survival was monitored every 12 h for 4 d. The significance of the Kaplan-Meier survival curves was assessed by the log-rank test (P < 0.01). **(B)** ELISA of serum cytokine concentrations in LPS-treated WT and NQO1^{-/-} mice. **(C)** Peritoneal macrophages (Mø) from WT (C57BL/6J) and NQO1^{-/-} mice were i.v. transferred to WT (C57BL/6J) animals (n = 10) 24 h before the i.p. injection with or without LPS (10 mg/kg). Survival was monitored every 12 h for 4 d. The significance of the Kaplan-Meier survival curves was assessed by the log-rank test (P < 0.01). **(D)** Serum cytokine concentrations 3 h after LPS administration measured from the recipients of WT (C57BL/6J) or NQO1-deficient macrophages (Mø). Data are presented as mean ± SD from five mice per group (B and D). *, P < 0.05 (unpaired two-tailed Student's t test). ns, not significant.

regulates the expression of these cytokines. Because IκB-ζ is indispensable for the positive regulation of a subset of TLR-mediated genes including IL-6, IL-12, and GM-CSF (Yamamoto et al., 2004), we investigated the involvement of NQO1 in the expression of IκB-ζ. We first examined the protein levels of IκB-ζ in WT and NQO1-deficient macrophages. Consistent with the results of previous studies (Totzke et al., 2006), the LPS-induced IκB-ζ protein reached a maximum level within 1–2 h and rapidly declined thereafter in WT macrophages (Fig. 4 A; and Fig. S2). Although IκB-ζ protein expression was induced in NQO1-deficient macrophages in response to LPS stimulation as well as in WT macrophages, NQO1 deficiency maintained IκB-ζ protein expression even after 24 h of LPS stimulation (Fig. 4 A and Fig. S2).

We next investigated *Ikbz* mRNA levels to determine whether the increased expression of the IκB-ζ protein in NQO1-deficient macrophages is a result of an increase in *Ikbz* transcription or a posttranscriptional mechanism. WT and NQO1-deficient macrophages without LPS stimulation had no *Ikbz* mRNA expression. After LPS stimulation, *Ikbz* mRNA induction was comparable between WT and NQO1-deficient macrophages (Fig. 4 B). Additionally, NQO1 overexpression had no influence on IκB-ζ promoter activity (Fig. 4 C), supporting no effect of NQO1 on IκB-ζ mRNA expression. These data indicate that the elevated level of the IκB-ζ protein in NQO1-deficient macrophages is not a result of an increase in *Ikbz* transcription. To assess the effects of NQO1 in LPS signal transduction, we examined the LPS-induced

degradation of IκBα and nuclear translocation of p65. There were no apparent differences in the LPS-stimulated degradation of IκBα and the translocation of p65 from the cytoplasm to the nucleus between WT and NQO1-deficient macrophages (Fig. 4, D and E), indicating that NF-κB signaling was not perturbed by NQO1 deficiency. These results are consistent with the unaltered expression of TNF-α or IL-10 between WT and NQO1-deficient macrophages. We next evaluated whether the increased expression of the IκB-ζ protein in NQO1-deficient macrophages is mediated by a posttranscriptional mechanism. The stability of the LPS-induced IκB-ζ protein in macrophages was analyzed by the cycloheximide (CHX) chase assay, which inhibits de novo protein synthesis. Although the LPS-induced IκB-ζ protein was rapidly degraded in WT macrophages in the presence of CHX, it was more stable in NQO1-deficient macrophages (Fig. 4 F). The proteasome inhibitor MG132 or cotreatment with MG132 and CHX prevented LPS-induced IκB-ζ degradation in WT macrophages, reaching levels similar to those in NQO1-deficient macrophages (Fig. 4, G and H). Together, these results indicate that NQO1 promotes IκB-ζ degradation in a proteasome-dependent manner.

To further evaluate the functions of NQO1, we examined the effect of NQO1 on IL-6 production induced by other stimuli, such as nucleotide-binding oligomerization domain (NOD) ligands and high-mobility group box 1 (HMGB1). We found that WT and NQO1-deficient macrophages produced similar amounts of IL-6 in response to NOD ligands such as C12-iE-DAP (NOD1 agonist),
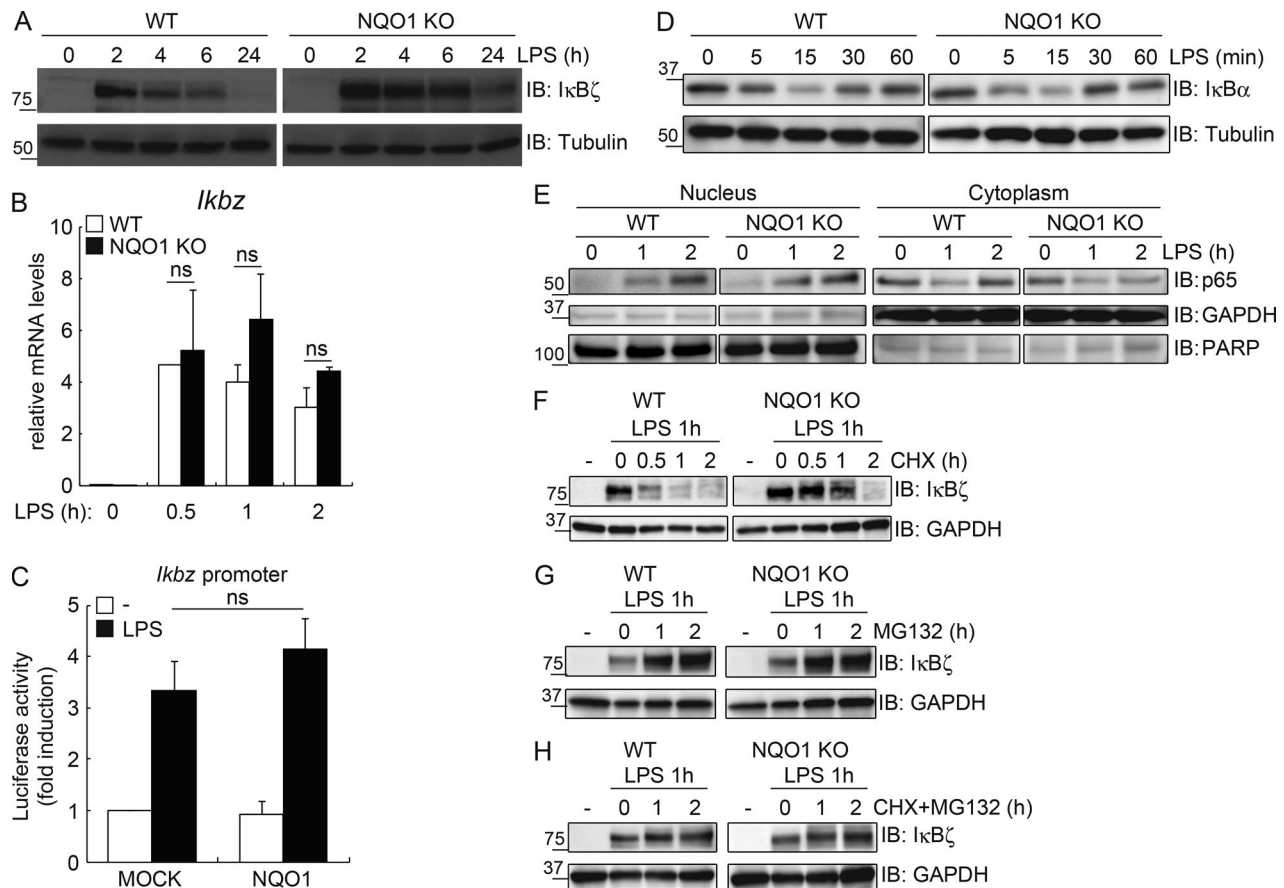
Figure 4. Stabilized IκB-ζ protein in NQO1-deficient macrophages. Primary peritoneal macrophages from WT and NQO1⁻/⁻ mice were stimulated with LPS (100 ng/ml) for the indicated times. **(A)** The expression of IκB-ζ and Tubulin was detected by Western blotting. **(B)** The expression of IκB-ζ was analyzed by qPCR; results are presented relative to the expression of GAPDH mRNA. **(C)** Luciferase activity in 293 cells stably expressing TLR4, MD2, and CD14 cotransfected with IκB-ζ luciferase reporters and NQO1 expression or control vector. Cells were stimulated with LPS for 2 h. **(D and E)** Primary peritoneal macrophages from WT and NQO1⁻/⁻ mice were stimulated with LPS (100 ng/ml) for the indicated times. The expression of IκB-α and Tubulin was detected by Western blotting (D). Cytoplasmic and nuclear p65 were detected by Western blotting. GAPDH and PARP were used to determine the purity of cytosolic and nuclear fractions, respectively (E). **(F–H)** Primary peritoneal macrophages from WT and NQO1⁻/⁻ mice were stimulated with 100 ng/ml LPS for 1 h and then treated with CHX (100 μg/ml) and/or MG132 (20 μM) for the indicated times. The expression of IκB-ζ and GAPDH was detected by Western blotting. IB denotes immunoblotting. Data are presented as mean ± SD; ns: not significant (unpaired two-tailed Student's t test) and are representative of three independent experiments (B and C) or of at least two independent experiments (A, and D–H). ns, not significant.

muramyl dipeptide (MDP; NOD2 agonist), and peptidoglycan (PGN)-ECndi (NOD2 agonist), as well as HMGB1 (Fig. S3, A and B). The major receptor for HMGB1 is the receptor for advanced glycation end-products, but it also binds to TLR2 and TLR4 (Lotze and Tracey, 2005). Therefore, it was unexpected that HMGB-1 did not induce higher levels of IL-6 in NQO1-deficient macrophages. We found that neither NOD ligands nor HMGB1 induced IκB-ζ expression in macrophages (Fig. S3, C and D). These results indicated that IL-6 production induced by NOD ligands or HMGB1 is independent of IκB-ζ, which causes the unaltered production of IL-6 induced by NOD ligands or HMGB1 between WT and NQO1-deficient macrophages.

NQO1 translocates to the nucleus and induces IκB-ζ ubiquitination

A previous study demonstrated that NQO1 binds to Aurora-A and subsequently induces the ubiquitin–proteasome–dependent degradation of the molecule (Park et al., 2013). We hypothesized that NQO1 binds to and participates in the degradation of

IκB-ζ. To test that, we first examined whether NQO1 physically interacts with IκB-ζ. Human embryonic kidney (HEK) 293T cells were transfected with NQO1 and IκB-ζ, followed by coimmuno-precipitation, which showed the association of NQO1 with IκB-ζ (Fig. 5 A). Given that IκB-ζ is a nuclear protein, we examined whether NQO1 is localized in the nucleus. Although NQO1 is mostly located in the cytoplasm, a small, but significant, amount is distributed in the nucleus (Fig. 5 B), consistent with what was mentioned in previous studies (Winski et al., 2002; Siegel et al., 2012). The nuclear localization of NQO1 increased in the presence of IκB-ζ (Fig. 5 B). LPS stimulation also enhanced the nuclear translocation of NQO1 in association with its increased expression in macrophages (Fig. 5 C). Because the ubiquitin–proteasome system is largely involved in the regulation of transcription factor activation (Muratani and Tansey, 2003) and NQO1 induces the ubiquitin–proteasome–dependent degradation of Aurora-A (Park et al., 2013), we investigated whether the NQO1-associated degradation of IκB-ζ is caused by the ubiquitin–proteasome system. RAW 264.7 cells were transfected with
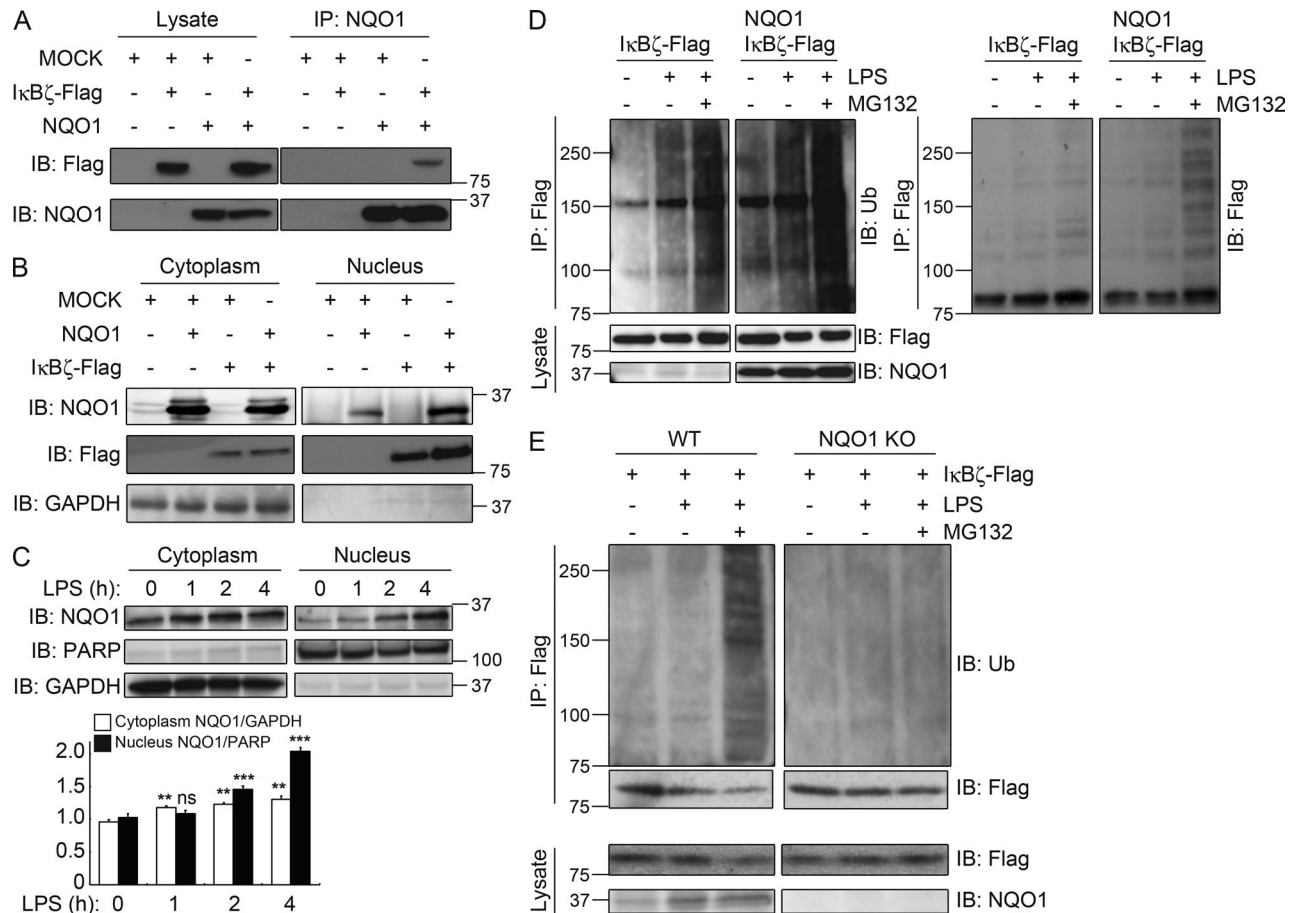
Figure 5. **NQO1 binds to and polyubiquitinates IκB-ζ. (A)** Coimmunoprecipation analysis of NQO1 and IκB-ζ in HEK-293T cells cotransfected with plasmids encoding Flag-tagged IκB-ζ and NQO1. **(B and C)** Western blot analysis of cytoplasmic and nuclear NQO1 and IκB-ζ in HEK-293T cells cotransfected with plasmids encoding Flag-tagged IκB-ζ and NQO1 (B) or peritoneal macrophages stimulated with LPS (100 ng/ml) for the indicated times (C; upper panel). GAPDH and PARP were used to determine the purity of cytosolic and nuclear fractions, respectively. The density of NQO1 bands in cytoplasm and nucleus was normalized to GAPDH and PARP, respectively, and presented as fold change relative to nontreated control cells (C; bottom panel). **(D)** Ubiquitination assay for IκB-ζ in RAW 264.7 cells cotransfected with plasmids encoding Flag-tagged IκB-ζ and NQO1 and stimulated with LPS (100 ng/ml) in the presence or absence of MG132 (20 µM) for 2 h. Flag-tagged IκB-ζ was immunoprecipitated with anti-Flag and the polyubiquitination of IκB-ζ was detected with antiubiquitin (left) or anti-Flag (right). **(E)** Ubiquitination assay for IκB-ζ in WT and NQO1-deficient macrophages transfected with plasmids encoding Flag-tagged IκB-ζ and stimulated with LPS (100 ng/ml) in the presence or absence of MG132 (20 µM) for 2 h. Flag-tagged IκB-ζ was immunoprecipitated with anti-Flag and the polyubiquitination of IκB-ζ was detected with antiubiquitin. IP and IB denote immunoprecipitation and immunoblotting, respectively. Data are representative of three independent experiments (A–E).

Flag-tagged IκB-ζ (IκB-ζ-Flag) or with both IκB-ζ-Flag and NQO1 plasmids and were treated with LPS in the presence or absence of MG132. Cell extracts were immunoprecipitated with anti-Flag antibody, followed by gel electrophoresis for detecting ubiquitin. We found that NQO1 promoted the polyubiquitination of IκB-ζ induced by LPS in RAW264.7 cells cotransfected with IκB-ζ and NQO1 in the presence of MG132 (Fig. 5 D). When these immunoprecipitated samples were blotted with an antibody against Flag, both a prominent band corresponding to IκB-ζ and a less intense smear of IκB-ζ in RAW cells transfected with NQO1 compared with that seen on the ubiquitin Western blot were detected (Fig. 5 D). We next investigated whether the ubiquitination of IκB-ζ was prevented in NQO1-deficient macrophages. WT and NQO1-deficient macrophages were transfected with IκB-ζ-Flag and were treated with LPS in the presence or absence of MG132. We found that the ubiquitination of IκB-ζ was induced in WT macrophages, but not in NQO1-deficient macrophages (Fig. 5 E).

These findings strongly suggest that NQO1 participates in the polyubiquitination of IκB-ζ.

IκB-ζ ubiquitination and degradation require PDLIM2 associated with NQO1

NQO1 is not a ubiquitin ligase; therefore, we next attempted to identify the ubiquitin ligase responsible for the polyubiquitination of nuclear IκB-ζ. PDLIM2 is a nuclear ubiquitin E3 ligase and promotes the polyubiquitination and subsequent degradation of several transcription factors such as NF-κB p65 and STAT3 (Tanaka et al., 2007, 2011). We investigated whether PDLIM2 regulates the polyubiquitination and degradation of IκB-ζ. We stimulated macrophages from WT and PDLIM2−/− mice with LPS and analyzed the stability of the induced IκB-ζ protein. As shown in Fig. 4 A, the LPS-induced IκB-ζ protein was rapidly degraded in WT macrophages, whereas PDLIM2 deficiency stabilized the IκB-ζ protein, as observed with NQO1 deficiency (Fig. 6 A). We

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then compared LPS-induced cytokine production between WT and PDLIM2-deficient macrophages. LPS-induced IL-6 production was drastically increased in PDLIM2-deficient macrophages (Fig. 6 B). TNF-α and IL-10 production were comparable between WT and PDLIM2-deficient macrophages (Fig. 6 C; and Fig. S4 A), as observed in NQO1-deficient macrophages. We confirmed that the expression of *PDLIM2* was not affected in the absence of NQO1 (Fig. S4 B).

We further validated the association between IκB-ζ and PDLIM2 by coimmunoprecipitation analysis in HEK 293T cells. The interaction between IκB-ζ and PDLIM2 was observed (Fig. 6 D), which prompted us to clarify which domain of PDLIM2 was involved in the interaction with IκB-ζ. HEK 293T cells were cotransfected with Flag-tagged IκB-ζ and either Myc-tagged WT PDLIM2, a mutant lacking the LIM domain (PDLIM2-ΔLIM), or a mutant lacking the PDZ domain (PDLIM2-ΔPDZ). Cellular proteins were then subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting with anti-Myc antibody. PDLIM2-ΔLIM failed to interact with IκB-ζ, whereas the association between PDLIM2-ΔPDZ and IκB-ζ was detected (Fig. 6 D). Furthermore, we found that the LIM domain of PDLIM2 directly interacts with IκB-ζ (Fig. S4 C). These results indicate that IκB-ζ interacts with PDLIM2 via the LIM domain. We hypothesized that NQO1 acts as a scaffold to facilitate the association between IκB-ζ and PDLIM. To verify this, we examined whether NQO1 affects the interaction between IκB-ζ and PDLIM2. This interaction was drastically enhanced in the presence of NQO1 (Fig. 6 E). Additionally, we found that NQO1 bound to PDLIM2 through its LIM domain (Fig. S4 D). To assess whether PDLIM2 polyubiquitinates IκB-ζ proteins, we transfected HEK 293T cells with expression plasmids encoding IκB-ζ-Flag, myc-tagged PDLIM2 (WT or ΔLIM mutant), NQO1, or histidine-tagged ubiquitin. Addition of PDLIM2 augmented the polyubiquitination of IκB-ζ, which was drastically enhanced in the presence of NQO1 (Fig. 6 F). Additionally, we found that PDLIM2-ΔLIM failed to polyubiquitinate IκB-ζ proteins (Fig. 6 F). These findings suggest that NQO1 plays an important role in degrading the IκB-ζ protein by mediating the association between IκB-ζ and PDLIM2.

To determine the potential role of PDLIM2 in macrophages during LPS-induced sepsis, we adoptively transferred macrophages from WT or PDLIM2⁻/⁻ mice and challenged the transferred mice i.p. with LPS. Consistent with the transfer of NQO1-deficient macrophages, the adoptive transfer of PDLIM2-deficient macrophages augmented LPS-induced lethality (Fig. 6 G). The serum levels of IL-6, but not of TNF-α, were significantly increased in mice that received PDLIM2-deficient macrophages (Fig. 6 H). These results strongly indicated that both NQO1 and PDLIM2 play a crucial role in suppressing IκB-ζ–mediated cytokine production in TLR signaling and sepsis.

Discussion

NQO1 was originally identified as the enzyme that catalyzes the reduction of quinone substrates, but it has recently been reported to play a regulatory role in the stability of target proteins. Although NQO1 is ubiquitously expressed in almost all tissues (Lind et al., 1982), its expression is further enhanced by various xenobiotics, antioxidants, oxidants, and heavy metals (Venugopal and Jaiswal, 1998). Additionally, it has been reported that LPS induces NQO1 expression in human monocytes via Nrf2 (Rushworth et al., 2008). We observed that LPS augmented NQO1 expression in macrophages, which was dependent on AhR. NQO1 induction occurs through both antioxidant response element (ARE) and XRE, which are the binding sites of Nrf2 and AhR, respectively (Jaiswal, 2000). We showed that AhR was recruited to XRE1, which is located near the ARE, in LPS-stimulated macrophages, suggesting that LPS-induced NQO1 expression might be regulated by the interaction between the AhR–XRE and Nrf2–ARE pathways.

We demonstrated that NQO1 deficiency selectively enhanced LPS-induced IL-6 and IL-12 production, even in the early phase. These data suggest that NQO1 directly suppresses LPS-TLR4 signaling and subsequent cytokine production. A previous study demonstrated that silencing NQO1 induction in monocytes slightly increases LPS-induced TNF-α and IL-1β production (Rushworth et al., 2008). However, in the present study, NQO1 deficiency did not significantly increase LPS-induced TNF-α production in macrophages. This discrepancy may be a result of differences in LPS concentration used in this study (100 ng/ml) and the previous study (10 µg/ml). The latter concentration may lead to the excessive production of ROS and subsequent augmented inflammation.

Heme oxygenase-1 (HO-1), as well as NQO1, is one of the phase II detoxifying enzymes regulated by Nrf2 (Li et al., 2000). HO-1 suppresses LPS-induced inflammatory responses, including TNF-α production, and reprograms macrophages from the M1 to the M2 phenotype that produces antiinflammatory cytokines such as IL-10 and TGF-β (Huang et al., 2014). In contrast, we showed that NQO1 was not involved in regulating LPS-induced TNF-α and IL-10 production. These findings suggest differential regulation and function of NQO1 and HO-1 in the innate immune responses. TLR activation on APCs up-regulates CD80, CD86, and MHC II and induces inflammatory cytokine production (Kawai and Akira, 2011). These events are critical for optimal T cell activation and differentiation. Given that LPS-induced CD80 expression was down-regulated in NQO1-deficient macrophages, it is possible that NQO1 plays an important role in T cell activation.

Sepsis is a systemic inflammatory syndrome caused by microbial infection that results in multiple organ failure and subsequent death. Endotoxins and other pathogen components stimulate APCs to produce proinflammatory cytokines such as IL-6, TNF-α, and IL-1β. The excessive production of these cytokines can cause lethal systemic inflammation. Clinical trials based on the neutralization of TNF-α or IL-1β have failed to recover the pathogenesis and survival of patients with sepsis (Fisher et al., 1994; Abraham et al., 1998, 2001). In contrast, IL-6 production is elevated in patients with sepsis and its level in patients with shock is higher than that in patients without shock (Gouel-Chéron et al., 2012). Furthermore, increased IL-6 levels are associated with the highest risk of death in patients with sepsis (Kellum et al., 2007). These clinical studies suggest that the regulation of IL-6 production leads to improved survival during sepsis. In a mouse model of sepsis, we showed that NQO1⁻/⁻ mice had elevated serum levels of IL-6 and were more
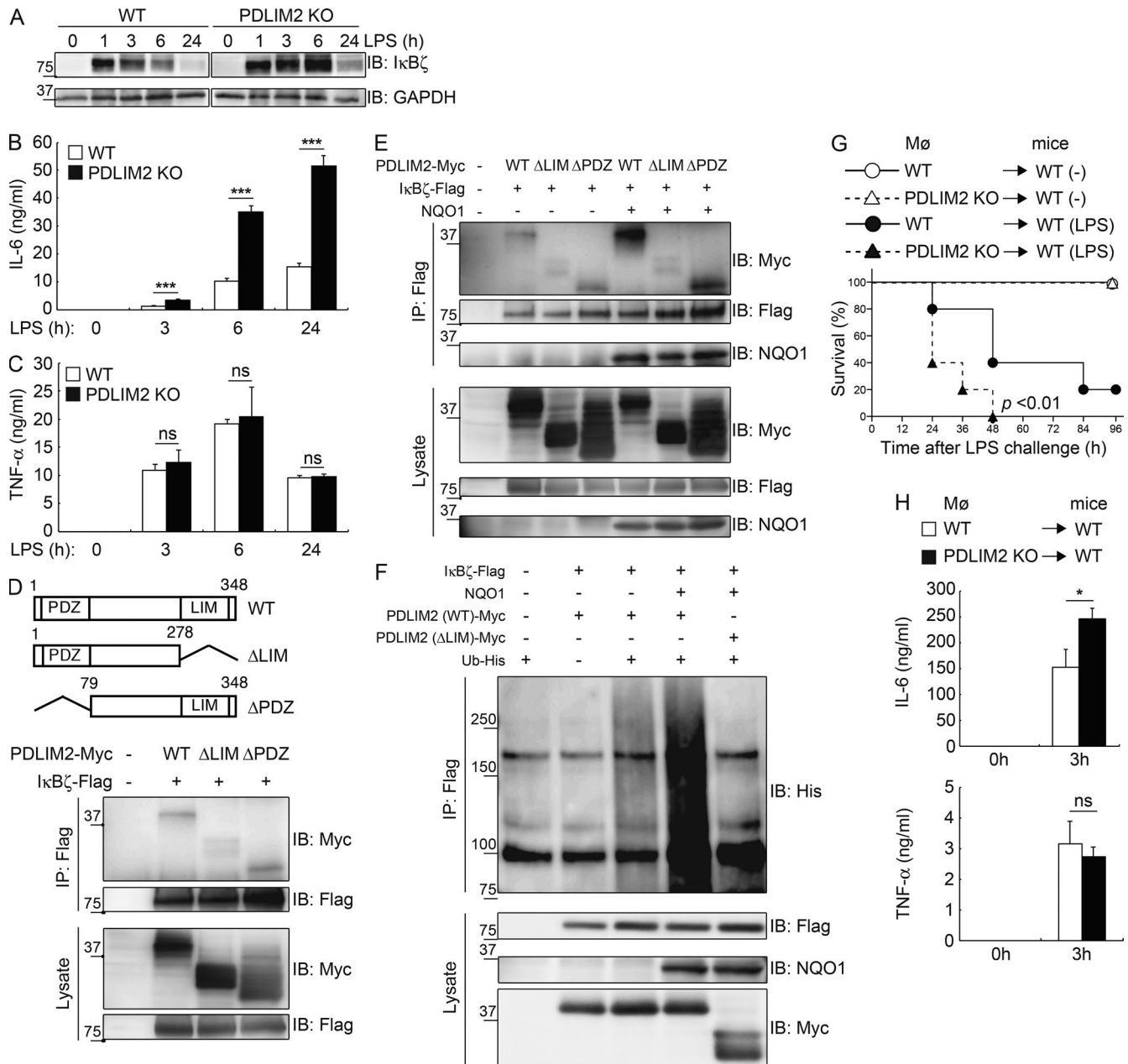
Figure 6. **The PDLIM2–NQO1 complex is required for the ubiquitination and degradation of IκB-ζ. (A)** Immunoblot analysis of IκB-ζ in lysates of WT and PDLIM2-deficient peritoneal macrophages stimulated with LPS (100 ng/ml) for the indicated times. IB denotes immunoblotting. **(B and C)** ELISA of IL-6 and TNF-α in the supernatants of WT and PDLIM2-deficient macrophages stimulated with LPS (100 ng/ml) for the indicated times. **(D)** PDLIM2 constructs of WT, LIM domain-deleted (ΔLIM), and PDZ domain-deleted (ΔPDZ) mutants. Coimmunoprecipitation analysis of PDLIM2 and IκB-ζ in HEK-293T cells cotransfected with plasmids encoding Flag-tagged IκB-ζ and Myc-tagged PDIM2 (WT or mutants). **(E)** Coimmunoprecipitation of PDLIM2 and IκB-ζ in HEK-293T cells cotransfected with plasmids encoding Flag-tagged IκB-ζ and Myc-tagged PDIM2 (WT or mutants) in the presence or absence of NQO1. **(F)** Assay for the ubiquitination of IκB-ζ in HEK 293T cells cotransfected with plasmids encoding Flag-tagged IκB-ζ, NQO1, Myc-tagged PDLIM2 (WT or ΔLIM mutant), and His-tagged ubiquitin and treated with MG132 (20 μM) for 2 h. Flag-tagged IκB-ζ was immunoprecipitated with anti-Flag, and the polyubiquitination of IκB-ζ was detected with antihistidine. IP and IB denote immunoprecipitation and immunoblotting, respectively. **(G)** Peritoneal macrophages (Mø) from WT (BALB/c) and PDLIM2$^{-/-}$ mice were i.v. transferred into WT (BALB/c) animals (n = 10) 24 h before the i.p. injection with or without LPS (10 mg/kg). Survival was monitored every 12 h for 4 d. The significance of the Kaplan-Meier survival curves was assessed by the log-rank test (P < 0.01). **(H)** Serum cytokine concentrations 3 h after LPS administration in WT (BALB/c) mice that received WT (BALB/c) or PDLIM2-deficient peritoneal macrophages (Mø). Data are representative of two or three independent experiments (A and D–F) or pooled from three independent experiments and presented as mean ± SD (B, C, and H). *, P < 0.05; ***, P < 0.001 (unpaired two-tailed Student's t test). ns, not significant.

susceptible to LPS-induced sepsis than WT mice. Our adoptive transfer experiments demonstrated that NQO1 in macrophages contributes to decreased serum levels of IL-6, but not of other cytokines, and reduces mortality. Interestingly, NQO1$^{-/-}$ mice showed milder symptoms than mice receiving NQO1-deficient macrophages in LPS-induced sepsis. Additionally, we found that

NQO1 suppressed IL-10 production in T cells (data not shown). Our data suggest that NQO1 possesses cell-type–dependent pro- and antiinflammatory functions, which may be the reason for the observed differences in the severity of sepsis. Collectively, NQO1 may be a potential target for treating sepsis as it selectively suppressed LPS-induced IL-6 production in vivo and in vitro.

We defined the mechanism underlying the selective suppression of TLR ligand-induced IL-6 production mediated by NQO1 and accentuated the role of NQO1 in IκB-ζ degradation. IκB-ζ plays a crucial role in the induction of inflammatory cytokines, including IL-6, IL-12, and GM-CSF, after LPS stimulation. The fact that NQO1 promoted the ubiquitin-dependent degradation of the IκB-ζ protein explains the specific suppression of these cytokines. Although NOD ligands and HMGB1 activate the innate immune system via NOD-like receptors and TLR2/4, respectively (Lotze and Tracey, 2005; Kufer et al., 2006), these ligands induced the same amount of IL-6 in WT and NQO1-deficient macrophages. We demonstrated that the induction of IL-6 by NOD ligands or HMGB1 was not required for the induction of IκB-ζ, which may cause the modest IL-6 production induced by these ligands compared with LPS. Collectively, we conclude that NQO1 specifically regulates IκB-ζ–dependent pathways in innate immune signaling.

Although NQO1 is known to participate in the ubiquitin-dependent degradation of Aurora-A (Park et al., 2013), the precise mechanisms underlying NQO1-mediated ubiquitination was unclear. We demonstrated that PDLIM2 facilitated degradation by direct interaction with IκB-ζ, using NQO1 as a bridge. PDLIM2 possesses autoubiquitination activity and functions as an E3 ubiquitin ligase through its LIM domain (Tanaka et al., 2005, 2011). As shown in Fig. 6 F, PDLIM2-ΔLIM mutant failed to induce the polyubiquitination of IκB-ζ, indicating that LIM domain is important for the ubiquitination of IκB-ζ. From these results, PDLIM2 is likely to be the ubiquitin ligase for IκB-ζ. However, in this study, how PDLIM2 induces the polyubiquitination of IκB-ζ remains unclear. Therefore, although further complete analysis should be required for definitive understanding of the ubiquitination of IκB-ζ, this is the first study showing that NQO1 acts as a bridge for the interaction between IκB-ζ and PDLIM2 and that PDLIM2 is required for the ubiquitination of IκB-ζ. Because PDLIM2 was reported to promote polyubiquitination of the NF-κB p65 subunit (Tanaka et al., 2007), the increased production of TNF-α was expected in PDLIM2-deficient macrophages, which was not the case (Fig. 6 C). Indeed, increased TNF-α production in PDLIM2-deficient macrophages was never reported in literatures. As shown in Fig. S5, TLR-induced TNF-α production is directly regulated by NF-κB (first step genes), whereas production of IL-6 and IL-12 is regulated by IκB-ζ–NF-κB complex (second step genes). We hypothesize that ubiquitination of NF-κB by PDLIM2 is dependent on IκB-ζ, therefore the lack of PDLIM2 (or NQO1) affects only on second step genes but not on first step genes (Fig. S5).

Much of the interest in NQO1 functions has been focused on the antioxidant system and the role as a gatekeeper by preventing the proteasomal degradation of its target proteins such as p53. In the present study, we found that NQO1 promotes the ubiquitin-dependent degradation of IκB-ζ in association with PDLIM2, thereby selectively suppressing IL-6 production induced by TLR ligands. Furthermore, our data highlighted that NQO1 and PDLIM2 might be potential targets for treating sepsis.

Materials and methods

Mice and cell culture
C57BL/6J and BALB/c mice were obtained from CLEA Japan Inc. AhR$^{-/-}$ mice (C57BL/6J background) have been described previously (Kimura et al., 2009). NQO1$^{-/-}$ mice (C57BL/6J background) were provided by A.K. Jaiswal (University of Maryland, College Park, MD). PDLIM2$^{-/-}$ mice (BALB/c background) were provided by M. Grusby (Harvard T.H. Chan School of Public Health, Boston, MA). All mice were housed under specific pathogen-free conditions. All mouse experiments (6–12 wk of age) were performed under protocols approved by the Animal Care and Use Committee of the National Center for Global Health and Medicine Research Institute.

Peritoneal macrophages were prepared as previously described (Kimura et al., 2009). The thioglycolate-elicited peritoneal macrophages and a mouse macrophage cell line (RAW 264.7 cells) were cultured in RPMI 1640 with 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin G. HEK 293T cells were cultured in DMEM with 10% FCS, 100 μg/ml streptomycin, and 100 units/ml penicillin G.

Reagents and plasmids
LPS (from *Escherichia coli* O111:B4; L-2630), CHX (C7698), and Benzo[a]pyrene (B1760) were purchased from Sigma-Aldrich. CpG-ODN (tlrl-1668), R-848 (tlrl-c97), C12-iE-DAP (tlrl-c12dap), MDP (tlrl-mdp), and PGN-ECndi (tlrl-kipgn) were purchased from InvivoGen. Recombinant HMGB1 (764004) was purchased from BioLegend. MG132 (474790) was purchased from Calbiochem. FICZ (BML-GR206) was purchased from Enzo Life Sciences. C-Myc–tagged PDLIM2 and its mutants were kindly provided by M. Grusby (Harvard T.H. Chan School of Public Health, Boston, MA). *Il6*, *Tnfa*, *Ikbz* luciferase reporter, and Flag-tagged IκB-ζ expression plasmids were kindly provided by S. Akira (Osaka University, Osaka, Japan). NQO1 expression vector (MC200234) was purchased from ORIGENE.

Animal experiments
WT and NQO1$^{-/-}$ female mice were randomly grouped and challenged with LPS (10 mg/kg) by i.p. For adoptive transfer experiments, WT mice were i.v. injected with 2 × 10^6 of WT, NQO1$^{-/-}$, or PDLIM2$^{-/-}$ peritoneal macrophages 24 h before LPS administration as previously reported (Voss et al., 2016). Survival rates of animals were monitored every 12 h for 4 d.

Immunoprecipitation and Western blotting
Peritoneal macrophages from WT, NQO1$^{-/-}$, PDLIM2$^{-/-}$ and AhR$^{-/-}$ mice were cultured with 100 ng/ml LPS for the indicated times. For the chase assay of IκB-ζ protein stability, peritoneal macrophages from WT and NQO1$^{-/-}$ mice were stimulated with 100 ng/ml LPS for 1 h and then treated with CHX (100 μg/ml) and/or MG132 (20 μM) for the indicated times. And then cells were lysed with a lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM

NaCl, 10 mM Na_2VO_4, 0.5 mM DTT, and 1/100 protease inhibitor cocktail). Cytoplasmic and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (78833; Thermo Scientific) according to manufacturer's instructions. Lysates were analyzed with Western blotting using anti-NQO1 (N5288; Sigma-Aldrich), anti–IκB-ζ (14-6801; eBioscience), anti–IκB-α (sc-371; Santa Cruz Biotechnology), anti-NFκB p65 (sc-372; Santa Cruz Biotechnology), anti-PARP (ab6079; abcam), anti-Tubulin (D-10; Santa Cruz Biotechnology), anti-Actin (A2066; Sigma-Aldrich), or anti-GAPDH (2118; Cell Signaling Technology). For coimmunoprecipitation experiments, HEK 293T cells were transfected with the indicated plasmids using X-tremeGENE HP (Roche). Whole cell lysates were incubated with anti-Flag M2 antibody (F3165; Sigma-Aldrich) or anti–Omni-probe (sc-7270; Santa Cruz) in the presence of protein G–Sepharose (Amersham Bioscience), washed four times, and subjected to Western blotting analysis with the indicated antibodies.

Ubiquitination assay
For ubiquitination assay in RAW 264.7 cells, RAW 264.7 cells were transfected with the plasmids encoding Flag-tagged IκB-ζ and NQO1 using X-tremeGENE HP (Roche). To overexpress IκB-ζ in WT and NQO1-deficient macrophages, transfection with the plasmid encoding Flag-tagged IκB-ζ was performed by using Amaxa Nucleofector kit (Lonza). Approximately 24 h after transfection, cells were treated with LPS (100 ng/ml) in the presence or absence of MG132 (20 µM) for 2 h. For denaturing immunoprecipitation, cells were lysed in a lysis buffer supplemented with SDS at a final concentration of 1% to denature and disrupt protein–protein interactions. The lysates were then sonicated for 15 s on ice and boiled at 100°C for 10 min. The boiled samples were diluted with a lysis buffer to 0.1% SDS and then centrifuged at 12,000 rpm for 5 min. The supernatants were immunoprecipitated with anti-Flag and analyzed by immunoblot with antiubiquitin (sc-8017; Santa Cruz Biotechnology). For ubiquitination assay in HEK 293Tcells, HEK 293T cells were transfected with the plasmids encoding Flag-tagged IκB-ζ, NQO1, Myc-tagged PDLIM2, and His-tagged ubiquitin using X-tremeGENE HP (Roche). Approximately 24 h after transfection, cells were treated with MG132 (20 µM) for 2 h. Denatured lysates were prepared as described above and immunoprecipitated with anti-Flag and analyzed by immunoblot with anti-Histidine (2365; Cell Signaling Technology).

Quantitative real-time PCR (qRT-PCR)
RNA was extracted from cells using the RNeasy Mini kit (Qiagen). cDNA was generated using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). RT PCR was performed on the cDNA using SYBR Premix Ex Taq II (TaKaRa) and a CFX384 RT PCR detection system (Bio-Rad). All qPCR experiments were normalized with the internal control GAPDH. Relative mRNA levels were calculated using a standard curve of a total cDNA dilution series starting with an arbitrary number and using the CFX384 software. The specific primers were as follows: NQO1, sense 5′-TTCTGTGGCTTCCAGGTCTT, antisense 5′-TCCAGACGTTTCTTCCATCC; IκB-ζ, sense 5′-GTGGAGGCAAAGGATCGTAA, antisense 5′-TCACGAAAGACAGGCAACTG; GAPDH, sense 5′-AACTTTGGCATTGTGGAAGG, antisense 5′-GGATGCAGGGATGATGTTCT.

ELISA
The concentrations of mouse IL-6, TNF-α, IL-12p40, and IL-10 from either the supernatants or the serum were measured by means of ELISA according to the manufacturer's instructions (eBioscience).

Nitrite production
Peritoneal macrophages from WT and NQO1$^{-/-}$ mice were stimulated with 100 ng/ml LPS for 24 h. Culture supernatants were incubated with Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine, and 2.5% phosphoric acid) for 10 min at room temperature and absorbance was measured at 550 nm in MIC ROPLATE READER (Model 680; Bio-Rad).

ChIP assay
The ChIP assay was performed according to Upstate Biotechnology's protocol. In brief, peritoneal macrophages were stimulated with 100 ng/ml LPS for 12 h, and then fixed with formaldehyde for 10 min. The cells were lysed, sheared by sonication, and incubated overnight with anti-AhR (SA-210; BIOMOL International) or control rabbit IgG followed by incubation with protein A–agarose saturated with salmon sperm DNA (Upstate Biotechnology). Precipitated DNA was analyzed with qPCR, and the enrichment by anti-AhR was normalized to that by IgG at the regions of interest. Primers used were as follows: XRE1 sense 5′-TGAGTCGGC AAAATTTGAGC-3′, antisense 5′-CATAATCAGCCTGTGTAGAT-3′; XRE2 sense 5′-AAGCCCCAAAACCCTAAATA-3′, antisense 5′-GCA AGAACACTATGTGTAGA-3′; XRE3 sense 5′-TACGACCTTCATTTA AATGT-3′, antisense 5′-CACAGAAGTGAAATTTACCT-3′.

Flow cytometry
Peritoneal macrophages from WT and NQO1-deficient mice were stimulated with LPS (100 ng/ml) for 24 h. Cells were washed with FACS buffer (0.2% BSA and 0.09% sodium azide in PBS, pH 7.4) and stained with the following antibodies: PE conjugated CD86 antibody (105007; BioLegend), FITC conjugated CD80 antibody (104705; BioLegend), and FITC-conjugated MHC II (107605; BioLegend). FACS analysis was performed using a FACS Canto II (BD Biosciences), and the data were analyzed with Flow Jo software, version 10.7 (Treestar).

Reporter assay
RAW 264.7 cells were transfected with the plasmids encoding the *Il6*, *Tnfa*, or *Ikbz* promoter-driven luciferase reporter and, in cotransfection experiments, together with either the control or NQO1 expression vectors in the presence of pRL-TK for use as an internal control reporter. Cells were stimulated with 100 ng/ml of LPS for 6 h and lysed with luciferase lysis reagent (Promega). Luciferase activity was determined with a commercial Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Relative light units of *Firefly* luciferase activity were normalized with *Renilla* luciferase activity.

Statistical analysis
Survival data were analyzed by using the Kaplan-Meier survival curves and the log-rank test. Data were analyzed with GraphPad Prism software. Statistical significances were

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assessed by an unpaired two-tailed Student's *t* test. P-values <0.05 were considered statistically significant. Data are presented as means ± SD.

Online supplemental material

Fig. S1 shows the expression of NQO1 in RAW/Neo and RAW/AhR cells after LPS stimulation and TLR ligands-induced cytokine production in WT and NQO1-deficient macrophages. Fig. S2 shows the expression of IκB-ζ in WT and NQO1-deficient macrophages stimulated with LPS. Fig. S3 shows the cytokine production in WT and NQO1-deficient macrophages stimulated with NOD ligands or HMGB1 and the expression of IκB-ζ in macrophages stimulated with LPS, NOD ligands, or HMGB1. Fig. S4 shows LPS-induced IL-10 production in WT and PDLIM2-deficient macrophages and LPS-induced PDLIM2 mRNA expression in WT and NQO1-deficient macrophages, and the interaction between LIM domain and IκB-ζ or NQO1 and PDLIM2 (WT and mutants). Fig. S5 describes the schematic model of the function of the NQO1-PDLIM2 complex in regulating TLR signaling.

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