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MyD88 is a mediator for the activation of Nrf2

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ABSTRACT

If not controlled properly, inflammatory response is often detrimental. However, in many cases, it can be self-limited and subsides without inflicting tissue damage. In this study, we tested the hypothesis that inflammatory stimuli can trigger anti-inflammatory response, which may contribute to limiting tissue damage induced by excessive inflammation. We found that treatment of bone marrow-derived macrophages with lipopolysaccharide (LPS) activated NF-E2-related factor 2 (Nrf2), a basic leucine zipper transcription factor that regulates inflammation, leading to expression of Nrf2-regulated genes including NAD(P)H:quinine oxidoreductase 1,glutamyl cysteine ligase catalytic unit and heme oxygenase-1. Suppression of Nrf2 by siRNA significantly diminished the expression of the Nrf2-regulated genes induced by LPS. By using pharmacological, genetic and epigenetic analyses, we found that activation of Nrf2 in response to LPS is dependent on MyD88 but independent of the production of reactive oxygen species. Together, our results show that activation of Nrf2 by MyD88 dependent signaling induced by LPS is an important intrinsic mechanism that limits excessive inflammation.

1. Introduction

Inflammation is a critical component of innate immunity that protects us from infectious and non-infectious immunologic challenges. The inflammatory response must be tightly controlled to prevent tissue damage initiated by the noxious stimuli. Uncontrolled inflammation is known to be closely associated with various inflammatory diseases [1]. In general, the inflammatory response is regulated and subsides to homeostasis, suggesting that there are intrinsic mechanisms in place that prevent excessive inflammation and tissue damage. Understanding of the self-regulatory mechanisms could lead to effective therapies against various inflammatory diseases. However, these mechanisms are not well understood.

Macrophages, a key effector cell in regulation of inflammation [2], recognize pathogens, in part, through pathogen-associated molecular patterns (PAMPs) and then initiate inflammatory responses [3]. For instance, Toll-like receptor 4 (TLR4), one of the PAMPs, is predominantly expressed in macrophages [4–7] and serves as a receptor for bacterial endotoxin (lipopolysaccharide: LPS) [2]. Engagement of LPS to TLR4 initiates Toll/IL-1 receptor (TIR)-mediated signaling cascades by recruiting intracellular adaptor molecules such as MyD88 and TRIF to TLR4. The signaling cascade MyD88-dependent pathway activates canonical IκB kinase (IKK) and various MAPK to increases the transcriptional activities of NF-κB and AP-1 [8,9]. On the other hand, the signaling run through TRIF, MyD88-independent pathway, typically activates NF-κB and interferon regulatory factor-3 (IRF-3) [10–12]. Activated TLR4 signaling results in the production of various molecules that promote inflammation including cyclooxygenase-2 (COX-2), TNF-α, IL-12 and IL-1β [13]. In self-limited inflammation, the mounted inflammatory response subsides and returns to homeostasis, which may be a result of the production of anti-inflammatory mediators including prostaglandin D_2, resolvins, protectins, lipoxins, and aspirin-triggered lipoxins [14].

NF-E2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that regulates the expression of cytoprotective and detoxifying genes [15]. In unstimulated conditions, Nrf2 resides in the cytoplasmin low quantities. When activated by oxidative stress such as reactive oxygen species (ROS), Nrf2 translocates to the nucleus where it binds to the *cis*-acting antioxidant response element (ARE) sequence, and induces the expression of various phase 2 detoxification genes, such as glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H:quinine oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO-1) [16]. Notably, Nrf2 plays

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a key role in ameliorating inflammation, as evidenced by the fact that genetic ablation of *nrf2* exacerbates inflammation in animal models of acute lung injury, asthma, and smoke-induced emphysema [17–19].

In this study, we tested the hypothesis that inflammatory stimuli also trigger activation of anti-inflammatory pathways that prevent and protect from excessive inflammation. Here we show that treatment of macrophages with LPS activated Nrf2 and induced the expression of Nrf2-dependent genes, which were mediated by MyD88 without ROS, a strong Nrf2 activator. These findings suggest that Nrf2 activated in inflammation is a regulatory mechanism contributing to limiting inflammation.

2. Materials and methods

2.1. Cell culture and bone marrow-derived macrophages

A mouse macrophage cell line, RAW 264.7 (ATCC, USA), was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, and 1% penicillin/streptomycin at 37 °C under a humidified atmosphere with 5% CO_2. Bone marrow-derived macrophages (BMDM) were prepared as described elsewhere [20]. A single-cell suspension of bone marrow flushed from the femurs was cultured in 10% L929 cell conditioned medium for 7 days, prior to experiment.

2.2. Animals

Male littermate wild type (C57BL/6) and $p47^{phox-/-}$ mice were purchased from the Jackson laboratory (Bar Harbor, Maine, USA). Mice weighing 20–28 g were used for the experiments. Animal experiments were performed per the protocol approved by the Pusan National University Institutional Animal Care and Use Committee, Busan, Republic of Korea.

2.3. Reagents

TLR4-specific *E. coli* LPS (Alexis Biochemical, San Diego, CA, USA), zymosan (SIGMA, St. Louis, MO, USA), and PMA (Merck Chemicals, Whitehouse Station, NJ, USA) were used. Antibodies for Nrf2, Lamin A/C, TRIF, MyD88, and TLR4 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

2.4. Western blot analysis

Total cell extracts (5×10^6 cells) were prepared by using RIPA buffer (Thermo Scientific, IL, USA) with Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Nuclear proteins were isolated by NE-PER nuclear extraction kit as per the manufacture's protocol (Thermo Scientific). The amount of protein was measured by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amount of protein was fractionated by SDS–PAGE and then transferred to PVDF membrane (Bio-Rad). Handling of the membrane was performed as per the manufacturer's protocol. After incubation with appropriate antibodies, specific bands of interest were revealed by chemiluminescence (SuperSignal® West Femto, Thermo Scientific).

2.5. siRNA and epigenetically silencedcell lines

Nrf2 specific and control siRNA (Invitrogen, Carlsbad, CA, USA) were transfected to RAW 264.7 cells as per the manufacturer's protocol. The plasmids that encode specific siRNA for TLR4, MyD88, and TRIF (gifts from Dr. James H. Shelhamer at NIH, USA) were transfected into RAW 264.7 cells by lipofectamin LTX (Invitrogen) according to manufacturer's instructions. The transfected cells were incubated for 24 h and selected under 4 μg/ml of Puromycin (SIGMA). The silencing of protein expressions was confirmed by immunoblotting using antibodies against TLR4, MyD88 and TRIF.

2.6. Total RNA extraction and semi-quantitative RT-PCR

Three micrograms of total RNA isolated with the QIAGEN RNeasy® mini kit (Qiagen, Hilden, Germany) were reverse-transcribed by M-MLV reverse transcriptase (Promega, Madison, WI, USA). Resultant single-stranded cDNA was amplified by PCR with a set of specific primers: the forward and the reverse primers for NQO-1 were 5′-GCAGTGCTTTCCATCACCAC-3′ and 5′-TGGAGTGT GCCCAATGCTAT-3′, respectively; the primers for HO-1 were 5′-TGAAGGAGGCCACCAAGGAGG-3′ and 5′-AGAGGTCACCCAGGTAGC GGG-3′, respectively; the primers for GCLC were 5′-CACTGCCAGAA CACAGACCC-3′ and 5′-ATGGTCTGGCTGAGAAGCCT-3′, respectively; and the primers for GAPDH were 5′-GGAGCCAAAAGGGTCATCAT-3′ and 5′-GTGATGGCATGGACTGTGGT-3′, respectively. *Taq*PCRx DNA polymerase (Invitrogen) was used withthe following conditions: an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation for 40 s at 95 °C, annealing for 40 s at 55 °C and extension for 50 s at 72 °C with a final extension for 7 min at 72 °C. Amplicons were analyzed in 1.5 % agarose gels under UV light. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as internal controls to evaluate relative expressions of NQO-1, GCLC, and HO-1.

2.7. Reactive oxygen species (ROS) measurement

ROS was measured by using LumiMax superoxide anion detection kit (Agilent Technologies, Santa Clara, CA, USA) as per the manufacturer's protocol. Experiments were performed in triplicat-eat least three times independently.

2.8. Statistical analysis

For comparison among groups, paired or unpaired *t* tests or one-way analysis of variance (ANOVA) tests were used (with the assistance of InStat, Graphpad Software, Inc., San Diego, CA) (p values <0.05 were considered significant). All experiments were performed at least three times independently.

3. Results

3.1. Lipopolysaccharide induces nuclear localization of Nrf2 in macrophages

To test whether pro-inflammatory stimuli induce the activation of Nrf2, we prepared bone marrow-derived macrophages (BMDM) of C57BL/6 and treated them with either vehicle (phosphate-buffered saline: PBS) or 0.1 μg of TLR4-specific lipopolysaccharide (LPS) for 4, 8, 16, and 24 h. Nuclear extracts from the variously treated cells were prepared and analyzed by Western blotting for Nrf2. As shown in Fig. 1, the nuclear Nrf2, indicative of activated Nrf2, was minimally detectable as early as at 4 h after LPS treatment but was evident at 8 h after LPS treatment. Experiments with RAW 264.7 cells, a murine macrophage cell line, showed similar results (data not shown). These data show that treatment of BMDM with LPS results in activation of Nrf2.

3.2. LPS treatment of macrophages induces expression of genes regulated by Nrf2

Next, we examined whether Nrf2, activated by LPS treatment, leads to the expression of Nrf2-dependent genes. Similar to the experiments described above, BMDM were treated with LPS (0.1 μg/ml) for 4, 8, 16, and 24 h. Total RNA was extracted and ana-

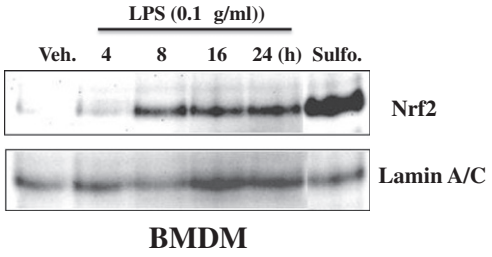

Fig. 1. LPS activates Nrf 2.5×10^5 cells of bone marrow-derived macrophages (BMDM) were treated with either vehicle (DMSO) or LPS (0.1 μg/ml) for 4, 8, 16, and 24 h. At each time points, nuclear fraction was prepared and analyzed by Western blotting for Nrf2. As a positive control for the nuclear Nrf2, BMDM were treated with well-documented inducer of Nrf2, Sulforaphane (25 mM) for 8 h. For the equal loading, the membrane was stripped and reprobed for nuclear proteins, lamin A/C. The experiment was performed at least three times independently.

lyzed by semi-quantitative RT-PCR for Nrf2-dependent genes including NQO-1, HO-1 and GCLC. As shown in Fig. 2A and B, LPS treatment induced the expression of NQO-1 (top panel), HO-1 (second panel from the top), and GCLC (third panel from the top) in a time dependent manner, suggesting that LPS activates Nrf2 with increased expression of Nrf2-dependent genes.

To verify that expressions of NQO-1, HO-1, and GCLC area result of activation of Nrf2, we epigenetically silenced the expression of Nrf2 in RAW 264.7 cells by using siRNA (Fig. 2C and D). RAW 264.7 cells were transfected with either scrambled, control siRNA (lanes 1 and 2) or siRNA specific for Nrf2 (lanes 3 and 4) for 24 h, and the transfected cells were treated with LPS (0.1 μg/ml). At 24 h after LPS treatment, total RNA was isolated and analyzed by semi-quantitative RT-PCR to determine the expression of NQO-1, HO-1, and GCLC. As shown in Fig. 2C and D, unlike the cells transfected with control siRNA, LPS treatment failed to induce expression of all of three genes in Nrf2 silenced RAW 264.7 cells (compare lanes 2–4), indicating that expressions of NQO-1, HO-1, and GCLC in LPS-treated macrophages were mediated by Nrf2. Together, these results suggest that LPS activates Nrf2 and induces expression of Nrf2-dependent genes.

3.3. Activation of Nrf2 by LPS treatment is not mediated by reactive oxygen species

Since reactive oxygen species (ROS) are a potent activator of Nrf2 [21,22] and LPS has been reported to induce ROS production

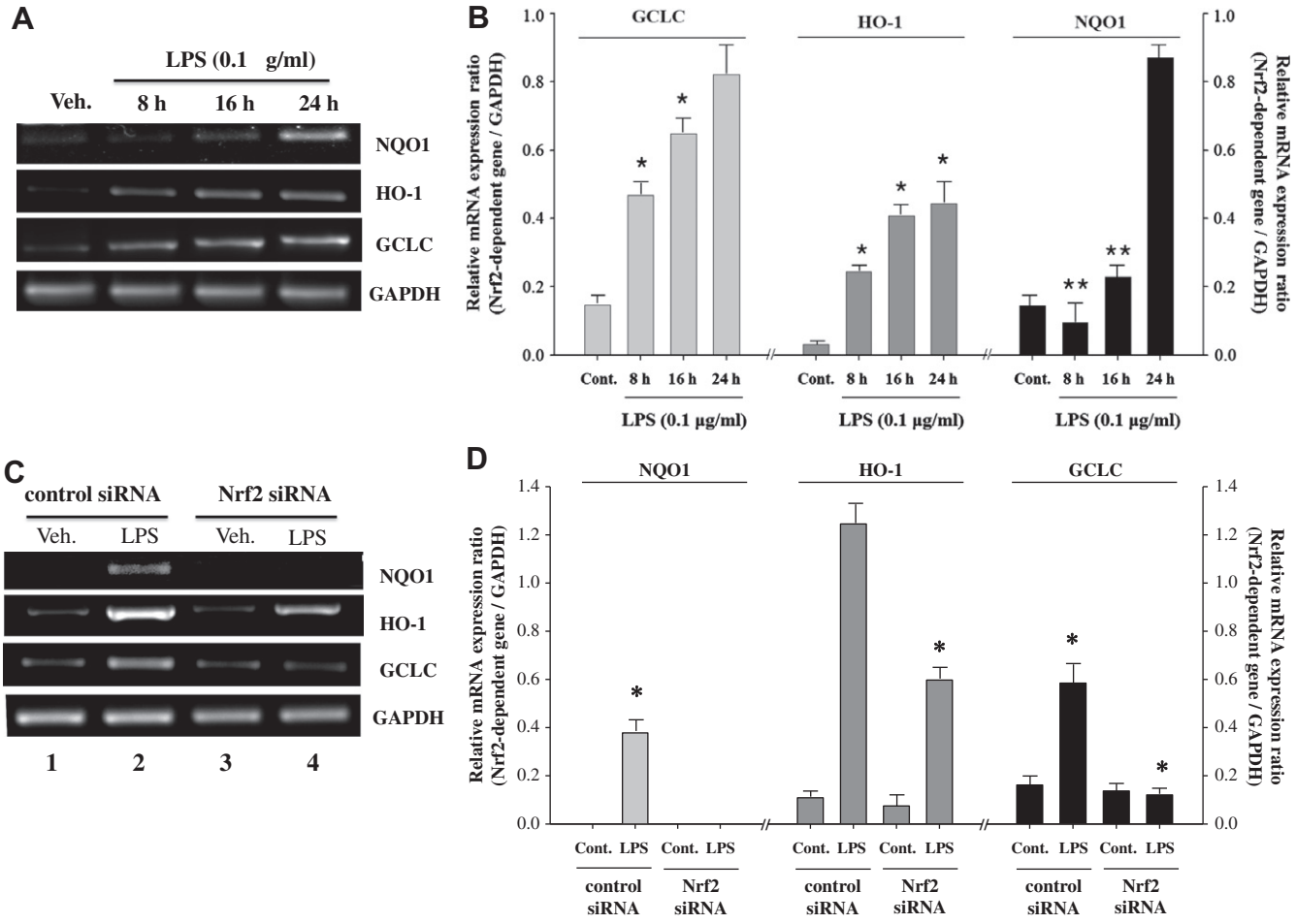

Fig. 2. LPS induces the expression of Nrf2-regulated genes. (A) 5×10^5 cells of BMDM were treated with LPS (0.1 μg/ml) for 8, 16, and 24 h, and total RNA of the variously treated cells was analyzed by semi-quantitative RT-PCR for the induction of Nrf2-dependent genes including NQO-1, HO-1, and GCLC. GAPDH was similarly prepared and analyzed for internal controls. (B) Densitometric analysis of the PCR bands of each gene was performed, and the induction of individual gene was calculated against GAPDH and shown in a graph. Data are represented as mean ± SE for three separate experiments with at least $n = 3$ for each experiment (*$p < 0.05$ and **$p < 0.01$ compared with controls). (C) For verification of the LPS-induced genes that are regulated by Nrf2, Nrf2 expression was suppressed by using siRNA. 1×10^6 cells of RAW 264.7 cells, transfected with either scrambled, control siRNA(lanes 1 and 2) or Nrf2 specific siRNA (lanes 3 and 4), were treated with LPS (0.1 μg/ml) for 24 h, and total RNA was analyzed by semi-quantitative RT-PCR for the induction of NQO-1, HO-1, and GCLC. Intensity of the PCR bands was analyzed by densitometer, calculated against GAPDH, and shown in a graph; data are represented as mean ± SE for three separate experiments with at least $n = 3$ for each experiment; *$p < 0.05$ compared with controls (D).

in macrophages [23], we examined whether Nrf2 activation after LPS treatment of macrophages is mediated by ROS. First, we measured the amount of ROS generated by macrophages. RAW 264.7 cells were treated with LPS (0.1 μg/ml) for 30 min in the presence or absence of NAC, an inhibitor of NADPH oxidase that blocks the production of ROS. Cell lysates were prepared and ROS production was measured. We found that LPS treatment for 30 min generated only a marginal amount of ROS (data not shown). Because Nrf2 activation was evident at 8 h after LPS treatment (Fig. 1), we treated RAW 264.7 cells with LPS (0.1 μg/ml) for 8 h and measured ROS. As shown in Fig. 3A, PMA treatment of RAW 264.7 cells induced the production of ROS, which was substantially diminished by pre-treatment of the cells with NAC (100 mM) for 1 h, suggesting that RAW264.7 cells are capable of producing ROS mediated by NADPH oxidase. However, LPS treatment for 8 h failed to generate a detectable level of ROS.

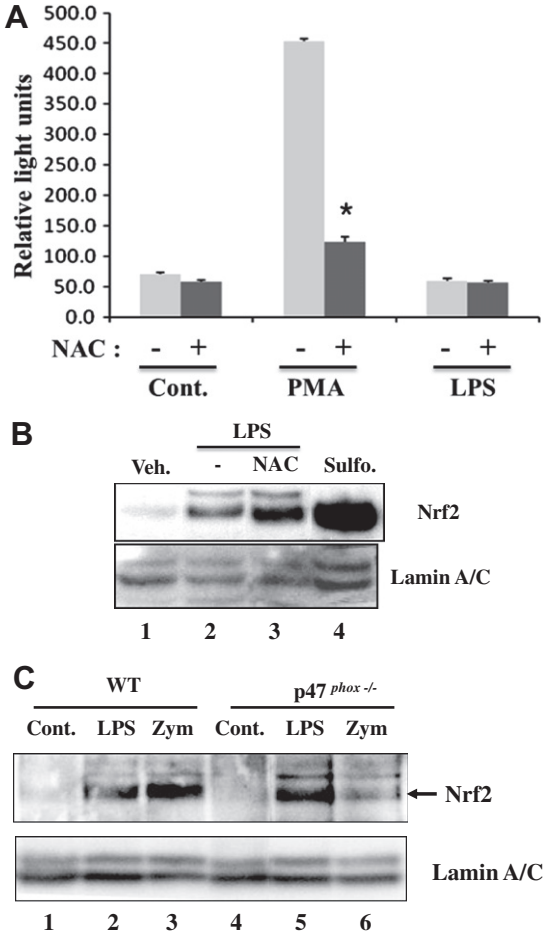

Fig. 3. Reactive oxygen species (ROS) are not a major factor in LPS-induced Nrf2 activation. (A) LPS was not a strong inducer of ROS. PMA treatment (200 ng/ml) for 8 h induced ROS production significantly, which was blunted by NAC pre-treatment (100 mM, 1 h). LPS treatment (0.1 μg/ml) for 8 h did not produce a significant amount of ROS, compared to PBS treated controls. Data are represented as mean ± SE for three separate experiments with at least $n = 3$ for each experiment; $^{*}p < 0.05$ compared with NAC-treated control. (B) LPS activated Nrf2 in the presence of an ROS blocker. RAW 264.7 cells were treated with LPS (0.1 μg/ml) for 8 h in the absence (lane 2) of presence (lane 3) of NAC, an NADPH-oxidase blocker, and the nuclear fractions of the treated cells were analyzed by Western blotting for Nrf2. As a control for Nrf2, RAW cells were treated with Sulforaphane (25 μM) for 8 h. The membrane was stripped and reprobed for lamin A/C to ensure equal loading. (C)LPS *activated Nrf2 in p47$^{phox-/-}$ mice.* BMDM of WT and p47$^{phox-/-}$ mice were prepared and treated with LPS (0.1 μg/ml) for 8 h (lanes 2 and 4), and, as for controls, similarly treated with zymosan (20 μg/ml; lanes 3 and 6). These experiments were performed at least three times independently, and representative results are shown.

Although LPS seemed to be a weak generator of ROS in the experimental settings, it is possible that low quantities of ROS could exert a cumulative effect on Nrf2 activation. To test this possibility, we treated RAW 264.7 cells with NAC (100 mM) for an hour prior to LPS treatment for 8 h. Nuclear fraction was extracted from the cells and analyzed by Western blotting for Nrf2. As shown in Fig. 3B, LPS treatment activated Nrf2 (lane 2), which was not affected by the pre-treatment of NAC (lane 3). To further confirm these results, we prepared BMDM of wild type (WT) littermate or p47$^{phox-/-}$ mice and treated them with LPS (0.1 μg/ml). At 8 h after LPS treatment, the nuclear Nrf2 was measured by Western blot analysis. As shown in Fig. 3C, similar to WT, treatment of BMDM with LPS activated Nrf2 in p47$^{phox-/-}$ (lanes 2 and 5). On the other hand, the activation of Nrf2 by zymosan (20 μg/ml) was significantly attenuated in p47$^{phox-/-}$ compared to WT (lanes 3 and 6), which is consistent with the previous findings that Nrf2 activation by zymosan is highly dependent on the production of ROS [24]. Together, these results show that LPS is able to activate Nrf2 without the involvement of ROS.

3.4. MyD88 is the major mediator in LPS-mediated activation of Nrf2

Since our results suggest that LPS signaling cascades are involved in Nrf2 activation, we determined which sub-pathway, MyD88 dependent or independent pathways, is important for Nrf2 activation. To this end, we epigenetically suppressed the expression of key molecules involved in TLR4 signaling. RAW 264.7 cells were stably transfected with a plasmid encoding scrambled siRNA, TRIF-, MyD88-, or TLR4-specific siRNA. The knock down of the respective target genes was confirmed by Western blot analysis (Fig. 4A). Each cell line was treated with LPS (0.1 μg/ml) to activate Nrf2. At 16 h after LPS treatment, the nuclear fraction of the treated cells was prepared for analysis of the nuclear Nrf2. As shown in Fig. 4B, while treatment of the cell line encoding control siRNA with LPS activated Nrf2 (lanes 1 and 2), similar treatment of the TLR4-silenced cell line failed to activate Nrf2 (lanes 7 and 8), indicating the involvement of TLR4 in Nrf2 activation. In experiments with the TRIF-silenced and the MyD88-silenced cell lines, we found that while the TRIF-silenced cell line activated Nrf2 similar to that of control cells, the MyD88-silenced cell line showed significantly less Nrf2 activation. Additional experiments were performed with other independently isolated cell lines to eliminate the findings related to cell line variations. These experiments yielded similar results (data not shown). Together, these results indicate that MyD88 plays a key role in LPS-induced Nrf2 activation.

4. Discussion

Proper regulation of inflammatory response is critical because excessive or uncontrolled inflammation is closely associated with immunologic diseases [1]. Given that in most instances inflammatory response subsides to homeostasis without significant tissue damage, we wanted to investigate the mechanisms that help limit inflammation. Our data show that treatment of macrophages with LPS activates Nrf2, a transcription factor that plays a key role in ameliorating various inflammatory diseases. Interestingly, we found that Nrf2 is activated in a MyD88 dependent fashion without the involvement of ROS. These results suggest the possibility that Nrf2 activated by inflammatory stimuli can be a mechanism that contributes to curbing excessive inflammatory response.

Nrf2 was initially known as a master regulator for the expression of detoxifying phase II enzymes, relieving oxidative stress [15]. However, a growing body of evidence shows that Nrf2 also plays a critical role in regulation of inflammation. For example,

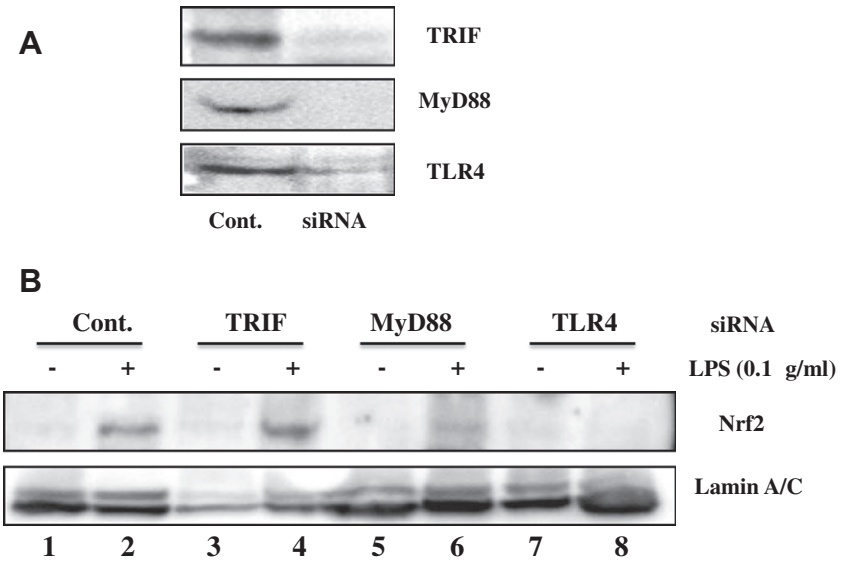

Fig. 4. MyD88 is a key molecule in LPS-mediated Nrf2 activation. (A) RAW 264.7cells were stably transfected with a plasmid encoding either scrambled, TRIF-, MyD88-, or TLR4-specific siRNA. Each cell line, in which the expression of one of three genes was silenced, was treated with LPS (0.1 μg/ml) for 24 h to activate Nrf2, along with the control siRNA cell line that was stably transfected with a plasmid encoding scrambled siRNA. The nuclear Nrf2 in each cell line was determined by Western blot analysis (B) Compared to the expressions of TRIF, MyD88, or TLR4 in the control siRNA cell line, the expression level of a silenced gene product in the cognate cell line was determined by Western blot analysis.

in acute lung injury animal studies, mice with *nrf2*$^{-/-}$(*nrf2* knockout) are more susceptible to inflammation in the lung caused by butylated hydroxytoluene [17] and hypoxia [25]. The *nrf2* knockout mice develop asthma [19] more readily and are more susceptible to emphysema induced by cigarette-smoking [18] than wild type mice. In addition, Nrf2 suppresses pleurisy induced by carrageenan [26] and pulmonary fibrosis [27]. Therefore, it is highly likely that Nrf2 activated by LPS, as demonstrated in this study, is involved in regulation of inflammation, contributing to curbing excessive inflammatory response.

The transcriptional activity of Nrf2 is regulated by Keap1, a key regulatory protein that mediates ubiquitination and degradation of Nrf2 in the cytoplasm [28]. Oxidative or electrophilic stress caused by, for example, ROS induces formation of a Keap1 dimer through disulfide bonds between two Keap1 molecules. The dimer no longer mediates degradation of Nrf2, which results in accumulation of Nrf2 in the nucleus and Nrf2-dependent gene expression [29]. It was reported that MyD88 in TLR2 mediated signaling is physically and functionally associated with NADPH oxidase [30], suggesting that MyD88 is involved in Nrf2 activation via the production of ROS by NADPH oxidase. Consistent with these results, our results show that MyD88 in TLR4 signaling is also involved in Nrf2 activation. However, our data indicate that MyD88 is able to activate Nrf2 without the involvement of ROS. These results suggest that an unidentified pathway, which is uncoupled with NADPH oxidase, mediates Nrf2 activation.

The mechanisms by which Nrf2 is activated during inflammation and controls inflammatory responses are largely unknown. Based on our results, we speculated the mechanisms. It is conceivable that ROS, a key arsenal to kill bacteria, produced by neutrophils generates an oxidative environment and activates Nrf2 [21,22]. Given that macrophages are a key player in regulation of neutrophilic inflammation [3,31–33], generated ROS activates Nrf2 in macrophages, which in turn helps macrophages to scavenge ROS, preventing exacerbating inflammatory response and collateral tissue damage. Since Nrf2 is ubiquitously expressed [34], it is also possible that Nrf2 in neutrophils is activated by ROS in an autocrine fashion, controlling neutrophil activities. On the other hand, Nrf2 in macrophages and neutrophils is activated by an MyD88-dependent mechanism, further contributing to regulation of inflammation. During inflammation, prostaglandins including prostaglandin D_2 (PGD_2) [20] and its derivative, 15-deoxy-$\Delta^{12,14}$-PGJ_2, (15-deoxy-PGJ_2) are produced, and down-regulate inflammation [14]. Since 15-deoxy-PGJ_2, in particular, is capable of activating Nrf2 [35], it is possible that these prostaglandins contribute to curbing excessive inflammation through activation of Nrf2.

In this study, we investigated if Nrf2 is activated by an inflammatory stimulus and further determined the mechanisms involved. Our results show that LPS activated Nrf2 and induced the expression of Nrf2-dependent genes through MyD88 signaling without the help of ROS, a strong Nrf2 activator. Given the well-documented role of Nrf2 in suppression of inflammation, these findings suggest that Nrf2 activated by inflammatory stimuli serves as a regulatory mechanism to limit the tissue damage induced by excessive inflammation.

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