CUTTING EDGE

Cutting Edge: Apoptosis-Regulating Signal Kinase 1 Is Required for Reactive Oxygen Species-Mediated Activation of IFN Regulatory Factor 3 by Lipopolysaccharide[1]

Edward Chiang,[2]* Oanh Dang,[2]* Keith Anderson,* Atsushi Matsuzawa,[†] Hidenori Ichijo,[†] and Michael David[3]*

IFN regulatory factor (IRF) 3 participates in the transcriptional induction of IFN-α, IFN-β, and a subset of IFN-stimulated genes (ISGs) as a result of viral infection. In addition, bacterial cell wall components such as LPS activate IRF3 in a p38-dependent manner. In this study we show that IRF3-mediated ISG induction by LPS requires the production of reactive oxygen species (ROS) by the NADPH-dependent oxidase NOX4. Furthermore, we present evidence that LPS-mediated ROS production leads to activation of apoptosis-regulating-signal kinase (ASK) 1, a MAPK kinase kinase family member capable of activating the MAP kinase 6/p38 axis. ASK1 kinase activity proved essential for IRF3-mediated ISG induction by LPS. Thus, our results presented here suggest a novel role for ROS and ASK1 in the innate immune response as signaling intermediates in the IRF3 activation pathway.   The Journal of Immunology, 2006, 176: 5720–5724.

Innate immune recognition is mediated by germline-encoded TLRs that recognize conserved pathogen-associated molecular patterns (1). In humans, 10 TLRs have been identified. TLR4, in the presence of CD14 and MD2, mediates the host cell responses toward LPS. Ectopic expression of TLR4 can confer sensitivity toward LPS in otherwise LPS-unresponsive cells (2–4). Other TLRs are involved in the recognition of pathogen-associated molecular patterns such as lipoteichoic acid, dsRNA, glucans, or peptidoglycans (1).

Significant progress had been made in the identification of the signaling molecules involved in TLR-induced gene expression; however, much of the work focused on the MyD88-dependent activation of NF-κB.

The ubiquitously expressed transcription factor known as IFN regulatory factor (IRF3)[4] 3 emerged as an important response factor to viral infection. Infection with CMV, Newcastle disease virus, or Sendai virus causes nuclear translocation of IRF3 and cooperative DNA binding with the transcriptional coactivator CREB-binding protein/p300 (5–8). In addition, we have previously shown that IRF3 activation by LPS occurs in a p38-dependent manner (9). This activation is followed by the induction of a distinct subset of IFN-stimulated regulatory element (ISRE)-containing genes. In the last few years, several components of LPS-mediated IRF3 activation have been identified. IRF3 activation by LPS occurs in a MyD88-independent manner; however, cells deficient in the adaptor molecule Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) or the TRIF-related adaptor molecule lack IRF3 activation after TLR4 engagement (10–13). Two IκB kinase (IKK)-related proteins, IKKε and TANK-binding kinase (TBK) 1, were identified as kinases that phosphorylate IRF3 in response to viral infection or TLR3 stimulation (12, 14). Overexpression of IKKε or TBK1 induces the phosphorylation of IRF3 and activates IFN-inducible genes. Furthermore, analysis of TBK1$^{-/-}$ murine embryonic fibroblasts (MEFs) confirmed the role of TBK1 in IRF3-dependent gene expression.

Reactive oxygen species (ROS) include H_2O_2 and radicals such as superoxide anion (O_2^-), and hydroxyl radical ($OH^•$). Superoxide anions are generated by several pathways such as the mitochondrial respiratory chain, NADPH oxidase, xanthine oxidase, cyclo-oxygenase, or lipoxygenase (15). Once formed, O_2^- can be readily converted into H_2O_2 and $OH^•$ by superoxide dismutase and the Fenton/Haber-Weiss reaction. ROS production ("respiratory burst") in response to LPS has long been implicated in the bactericidal actions of macrophages. Recently,

---

ROS also received attention as intracellular signaling intermediates due to their ability to alter cytokine expression, modulate cellular proliferation, and induce apoptosis (16, 17).

Apoptosis-regulating signal kinase (ASK) 1 is a proapoptotic MAPK kinase kinase that actives the MAPK kinase (MKK) 3/MKK6-p38 and MKK4/MKK7-JNK kinase pathways in response to oxidative stress, anticancer drugs, growth factor deprivation, or TNF-α. In resting cells ASK1 is bound to thioredoxin (TRX), preventing its activation (18). Under oxidizing conditions, TRX forms a disulfide bridge and dissociates from ASK1, allowing ASK1 to autophosphorylate and become activated. ASK1 regulation by TRX denotes that it is regulated by ROS.

This association, in addition to being an upstream activator of p38, led us to hypothesize that LPS-stimulated ROS production could activate ASK1, leading to the activation of p38, IRF3, and subsequent IFN-stimulated gene (ISG) induction.

Materials and Methods

Cells

Human U373 astrocytoma cells (U-373 MG), stably transfected with human CD14, were generously provided by Dr. P. S. Tobias (The Scripps Research Institute, La Jolla, CA). Peritoneal macrophages were collected 5 days after i.p. administration of thioglycolate.

Reagents

LPS and allopurinol were from Sigma-Aldrich. N-acetyl-L-cysteine (L-NAC), MK886, rotenone, antimycin A3, and diphenylene iodonium chloride (DPI) were obtained from Calbiochem. IFN-β was a generous gift from Biogen.

Mice

Inducible NO synthase (iNOS), endothelial NO synthase (eNOS), and gp91^{phox}-deficient mice were purchased from The Jackson Laboratory. IRF3$^{-/-}$ mice were generously provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan).

RNase protection assay (RPA)

Total RNA was isolated using TRIzol Reagent (Invitrogen Life Technologies). Ten micrograms of RNA and ^{32}P-labeled riboprobes for ISG54, RANTES, and GAPDH were incubated in buffer (4:1 formamide and 5× stock (200 mM PIPES (pH 6.4), 2 M NaCl, and 5 mM EDTA)) overnight at 56°C before digestion with T1 RNase (Invitrogen Life Technologies). Protected fragments were solubilized in 98% formamide plus 10 mM EDTA and subjected to electrophoresis on a 4.5% polyacrylamide/urea gel.

EMSA

EMSAs were performed using a ^{32}P-labeled probe corresponding to the ISG15-ISRE (5′-GATCCATGCCTCGGGAAGGGAAACCGAAACTGAAGCC-3′). Extracts were incubated with poly(dI-dC) and labeled probes in buffer (40 mM KCl, 20 mM HEPES (pH 7.0), 1 mM MgCl$_2$, 0.1 mM EGTA, 0.5 mM DTT, 4% Ficoll, and 0.02% Nonidet P-40) and resolved by electrophoresis on a 6% Tris-borate-EDTA-polyacrylamide gel.

Western blotting

Cells were lysed in a buffer containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 mM PMSF. Cell lysates were resolved by SDS-PAGE, and blots were immunoblotted with the indicated Abs. All blots were developed with HRP-conjugated secondary Abs and ECL (Amersham Biosciences).

Luciferase assay

Cells were transfected with 5× ISRE luciferase and Renilla luciferase under the control of the thymidine kinase promoter using Superfect (Qiagen). Sixteen hours after transfection, cells were stimulated with 1 μg/ml LPS for 24 h. Luciferase activity was measured using a Dual luciferase assay kit (Promega) according to the manufacturer's instructions.

Immunofluorescence

Slides were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with PBS plus 0.2% Triton X-100, and blocked with 10% goat serum. After

rinsing, and incubation with anti-IRF3 serum in PBS, 0.05% Tween 20, and 3% BSA, cells were incubated with goat anti-rabbit IgG-tetramethylrhodamine isothiocyanate Ab conjugate (Jackson ImmunoResearch Laboratories) and overlaid with Vectashield (Vector Laboratories) after rinsing.

Real-time PCR

ISG54 expression was assayed by real-time PCR using Omniscript (Qiagen), SYBR Green (Applied Biosystems), and the primers for ISG54 (5′- TCTGAT TCTGAGGCCTTGCA-3′ and 5′-CTTGCTGACC-TCCTCCATTCTC 3′) and β-actin (5′-ACGGCCAGGTCATCACTATTG-3′ and 5′-CAA GAAGGAAGGCTGGAAAAGAG-3′).

Small interfering RNA (siRNA) transfection

siRNA against murine NOX4 was purchased from Dharmacon (catalog no. M-058509-00). Cells were transfected with 20 μM siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) and stimulated with LPS 48 h later.

Results and Discussion

The *ISG54* gene is under the sole control of an ISRE in its promoter region; therefore, the induction of ISG54 by IFNαβ occurs strictly in a STAT1/STAT2/IRF9-dependent manner, whereas its activation as a consequence of viral infection or LPS stimulation requires the activation of IRF3.

In our earlier studies (9) we noticed that LPS-induced, IRF3-mediated ISG54 induction is substantially delayed when compared with the rapid response elicited by the IFN-αβ-stimulated Jak/STAT pathway (Fig. 1A). This delayed activation kinetics is reminiscent of the slow activation of hypoxia-inducible factor 1, a transcription factor that is activated by ROS (19). Intriguingly, most stimuli (virus infection, bacterial cell surface contact, or environmental stresses such as DNA damage) that activate IRF3 also induce the production of ROS (20). Thus, we hypothesized that ROS might play a role in the TLR4 pathway leading to IRF3 activation.

To address this possibility, we exposed cells to the antioxidant L-NAC before stimulation with LPS or IFNβ. As shown in Fig. 1B, pretreatment of cells with L-NAC completely inhibited the LPS-mediated induction of ISG54. To exclude the possibility that L-NAC was acting in a nonspecific, potentially cytotoxic manner, we also examined its effect on ISG54 mRNA levels upon IFNβ stimulation. As anticipated, L-NAC failed to

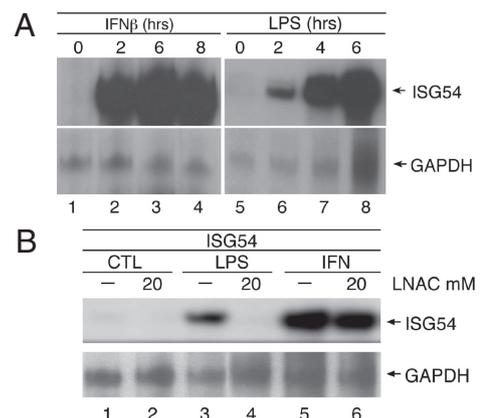**FIGURE 1.** Delayed, ROS-dependent activation of ISG induction by LPS A, U373 cells were treated with 1 μg/ml LPS or 1000 U/ml IFNβ for the indicated times in the presence of 50 μg/ml cycloheximide, and ISG54 mRNA was analyzed by RPA. B, U373 cells were pretreated for 1 h with 20 mM L-NAC (LNAC) before stimulation with 1 μg/ml LPS or 1000 U/ml IFNβ for 2 h. CTL, control.
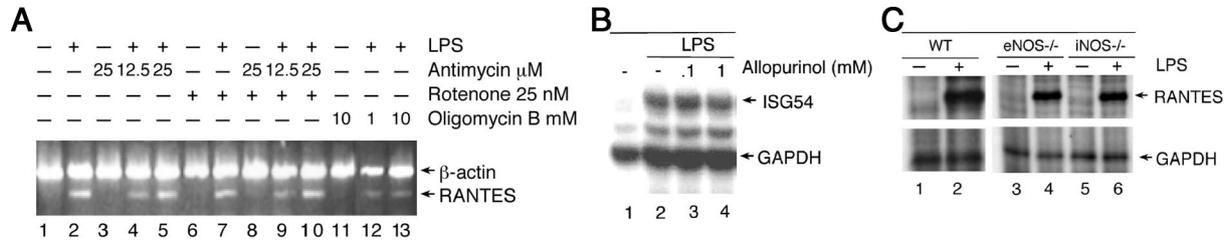
FIGURE 2. ROS-dependent activation of ISG induction by LPS. *A*, U373 cells were treated with the indicated inhibitors for 30 min before stimulation with 1 μg/ml LPS for 6 h. Human RANTES mRNA levels were analyzed by RT-PCR. *B*, Same as *A*, except that ISG54 mRNA levels were analyzed by RPA. *C*, WT, eNOS-, or iNOS-deficient peritoneal macrophages were stimulated with 1 μg/ml LPS for 6 h. Murine RANTES mRNA levels were analyzed by RPA.

exert an inhibitory effect upon the IFN-induced, Jak/STAT-mediated expression of ISG54 (Fig. 1*B*, *lanes 5* and *6*), demonstrating its specificity toward LPS-induced IRF3 activation. Similar results were seen when other antioxidants, such as butylated hydroxyanisole or nordihydroguaiaretic acid, were used. Thus, our results support the notion of a critical role for ROS in the LPS-induced activation of IRF3 and, subsequently, ISGs.

Numerous enzymatic processes lead to the production of ROS. To define the source of ROS generated after LPS stimulation, we tested a wide array of specific inhibitors directed against different ROS sources such as xanthine oxidase, lipoxygenase, cyclo-oxygenase, and mitochondrial respiratory chain complex I and II for their potential effect on LPS-induced ISG54 expression. However, none of these compounds were able to prevent LPS-mediated ISG induction (Fig. 2, *A* and *B*; not shown).

Because the NO synthases eNOS and iNOS are also capable of producing ROS, we tested peritoneal macrophages deficient in these enzymes for LPS induction of ISGs. As shown in Fig. 2*C*, both cell types facilitated expression of RANTES, another IRF3-dependent ISG (21), to a similar extent as that observed in wild-type (WT) cells.

The seven members of the NADPH oxidase family (Nox/Duox) share significant homology with gp91^{phox} (22). DPI has been widely used to inhibit NADPH oxidase-dependent, inducible ROS production in phagocytes (23, 24). As shown in Fig. 3*A*, pretreatment of cells with DPI before stimulation with LPS completely abrogated the induction of ISG54. Importantly, DPI had no effect on the Jak/STAT-dependent activation of ISG54 by IFNβ (Fig. 3*A*).

To confirm that DPI was indeed acting through the inhibition of IRF3 activation, we assessed IRF3 DNA binding and nuclear translocation in cell stimulated in the presence of DPI. Indeed, DPI pretreatment efficiently prohibited LPS from inducing IRF3 DNA binding (Fig. 3*B*) or its nuclear accumulation (Fig. 3*C*). The identity of the LPS-induced band was verified as IRF3 by supershift with anti-IRF3 Ab (not shown). Thus, the prevention of IRF3 binding to the ISRE and the lack of IRF3 nuclear accumulation provide additional evidence that a NADPH oxidase family member is required for the activation of IRF3 by LPS. To determine whether the NADPH oxidase was required upstream or downstream of the adaptor protein TRIF, which has been previously shown to mediate LPS-induced IRF3 activation (10, 11, 13), we tested the effect of DPI
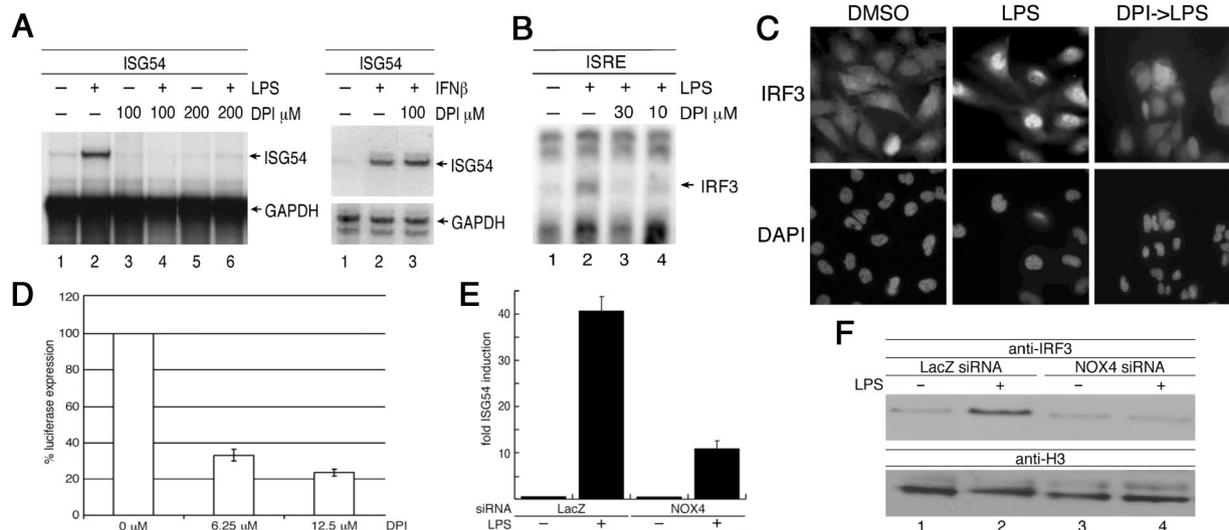

FIGURE 3. DPI inhibits LPS-mediated ISG induction and IRF3 DNA binding: *A*, U373 cells were treated with DPI for 1 h in the presence of 50 μg/ml cycloheximide and stimulated with 1 μg/ml LPS for 6 h or 1000 U/ml IFNβ for 1 h, and ISG54 mRNA levels were analyzed by RPA. *B*, Same as *A*, except that extracts were analyzed for IRF3 binding to the ISRE. *C*, U373 cells were stimulated with 1 μg/ml LPS for 6 h in the presence of 10 μM DPI before immunostaining for IRF3 (*top panel*). Nuclei were counterstained with DAPI (*bottom panel*). *D*, U373 cells transfected with TRIF and ISRE luciferase were treated with DPI at the indicated concentrations for 12 h before analysis of luciferase activity (a representative of three independent experiments is shown). *E*, RAW 264.7 cells were transfected with siRNA against either LacZ or murine NOX4. After 48 h, cells were stimulated with 100 ng/ml LPS for 6 h, and ISG54 mRNA levels were analyzed by real-time PCR. ISG54 expression was normalized using β-actin as an internal control. *F*, RAW 264.7 cells were transfected with the siRNA against LacZ or murine NOX4. After 48 h, cells were stimulated with LPS for 6 h, and nuclear extracts were analyzed for the presence of IRF3. DAPI, 4′,6′-diamidino-2-phenylindole.

FIGURE 4. Kinase activity of ASK1 is required for ISRE-mediated transcription by LPS. *A*, U373 cells were transfected with ISRE luciferase and the indicated amount of TRX plasmid. After 16 h cells were stimulated with 1 μg/ml LPS for 24 h, and luciferase production was analyzed. *B*, Cells were treated as in *A*; however, WT and kinase-inactive ASK1 plasmid was used instead of TRX. CTL, control.
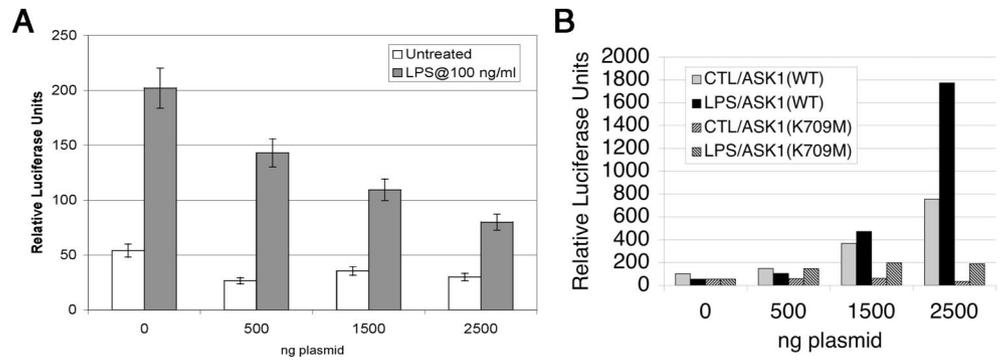
on the transcription of the ISRE luciferase when it was activated via overexpression of TRIF rather than LPS stimulation. As shown in Fig. 3*D*, DPI is still able to inhibit transcription of this reporter, suggesting that the NADPH oxidase acts downstream of TRIF. Surprisingly, NADPH oxidase gp91^{phox}-deficient mice showed no defect in IRF3-mediated transcription (data not shown). However, during the preparation of this manuscript Park et al. (25) reported the association of another NADPH oxidase family member, Nox4, with TLR4 and the requirement of NOX4 for TLR-mediated NF-κB activation. To test whether this enzyme was required for IRF3-mediated responses, we used siRNA against NOX4 as described in (25) and tested its effect on LPS-mediated induction of ISG54. Indeed, significant inhibition of ISG54 transcription (Fig. 3*E*) and IRF3 nuclear translocation (Fig. 3*F*) in response to LPS resulted from the abrogation of NOX4 expression, demonstrating that NOX4 is responsible for the generation of the ROS required for LPS-induced, IRF3-mediated transcription.

Next, we decided to explore the connection between free ROS and the phosphorylation events that lead to IRF3-mediated ISG induction. LPS-induced activation of p38 was reported to require ROS production (26) as well as ASK1 (27). We had previously found that IRF3 activation by LPS requires the activity of MKK6 and p38 (9, 28) and therefore hypothesized that ASK1 might be the link between LPS-induced ROS production and activation of the MKK6/p38/IRF3 cascade. Indeed, ASK1$^{-/-}$ was recently shown to be significantly less susceptible to LPS-induced septic shock (29).

To examine a potential role of ASK1 in LPS-induced, IRF3-mediated transcription, we first tested whether ectopic expression of the ASK1 inhibitor TRX would attenuate the LPS-induced activation of an ISRE luciferase reporter. Indeed, ectopic expression of TRX caused a dose-dependent decrease in the induction of ISRE luciferase by LPS (Fig. 4*A*); however, no such inhibition was seen when the constitutively active IRF3-5D mutant was used to activate the reporter construct (data not shown). Although these results were indicative of an ASK1 requirement in LPS-induced ISG induction, we also decided to test whether ASK1 was sufficient to induce the ISRE luciferase. Ectopic expression of WT ASK1 resulted in a dose-dependent increase in luciferase production after LPS stimulation, whereas the kinase-dead ASK1(K709M) mutant failed to promote such a response (Fig. 4*B*). These findings further indicated a role of ASK1 in the LPS signaling pathway leading to the induction of ISGs.

ASK1 activation in response to oxidative stress requires phosphorylation of T845 in its activation loop (30). We therefore used phosphotyrosine 845-ASK1 Abs to examine the phosphorylation state of ASK1 after LPS stimulation. ASK1 was clearly phosphorylated on T845 for an extended period of time in response to LPS to a similar extent as observed after TNF-α treatment (Fig. 5*A*).

To definitively determine whether ASK1 is essential in the LP- induced transcription of ISGs, we analyzed LPS responses in ASK1$^{-/-}$ peritoneal macrophages. Although LPS stimulation of WT macrophages yielded a clear induction of ISG54, a
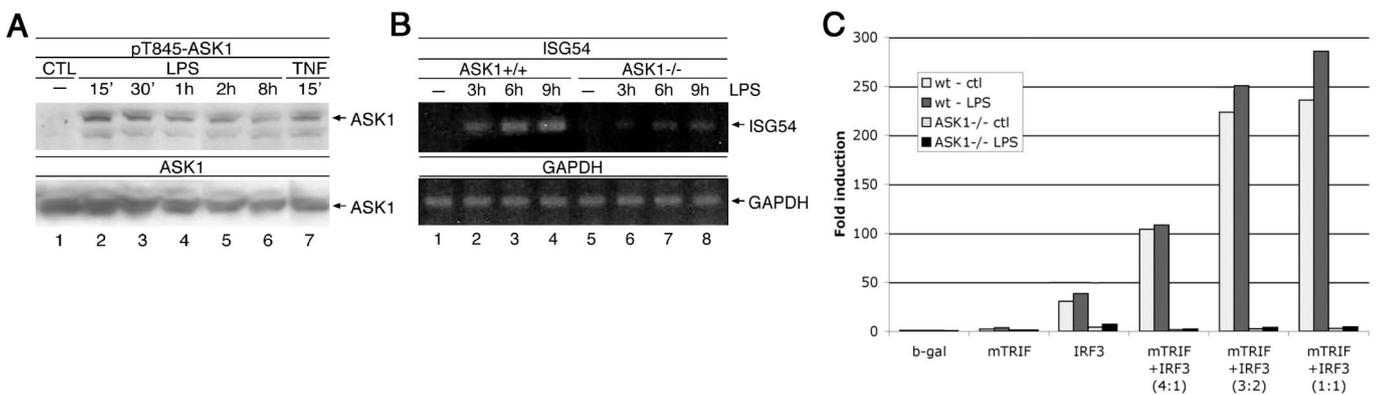
FIGURE 5. Activation and requirement of ASK1 in LPS signaling: *A*, U373 cells were stimulated with 1 μg/ml LPS or TNF-α for the indicated times, and blots were probed with anti-phospho-T845 ASK1 or ASK1 Abs. CTL, control. *B*, Peritoneal macrophages from WT and ASK1$^{-/-}$ mice were stimulated with 1 μg/ml LPS for the indicated times, and ISG54 and GAPDH mRNA levels were analyzed by RT-PCR. *C*, WT and ASK1$^{-/-}$ MEFs were transfected with murine TRIF (mTRIF) and/or IRF3 and ISRE luciferase. After 24 h, luciferase activity was analyzed. A representative of three independent experiments is shown. b-gal, β-galactosidase.

substantially weaker response was observed in their ASK$^{-/-}$ counterparts (Fig. 5B). Matsuzawa et al. (29) recently reported similar induction of *IP10*, another MyD88-independent gene, in WT and ASK1$^{-/-}$ dendritic cells or splenocytes in response to 10 μg/ml LPS. It is possible that tissue-specific differences are responsible for this observation; in addition, because ASK1$^{-/-}$ cells are not completely deficient in LPS-mediated ISG induction, it is possible that the use of the higher 10 μg/ml LPS concentration masks any differences that exist between WT and ASK1$^{-/-}$ cells. To determine where ASK1 was required in the activation cascade relative to TRIF, we ectopically expressed TRIF, IRF3, and the ISRE luciferase in WT and ASK1$^{-/-}$ MEFs. As shown in Fig. 5C, expression of TRIF together with IRF3 in WT MEFs is sufficient to strongly activate the ISRE luciferase independently of LPS. When ASK1$^{-/-}$ MEFs were used for transfection, however, no induction of the reporter construct could be seen regardless of LPS stimulation, suggesting that ASK1 in required downstream of TRIF.

IRF3 activation is a crucial part of the innate immune response as evidenced by the fact that IRF3$^{-/-}$ mice fail to produce type I IFNs, as well as other proinflammatory cytokines and chemokines, in response to TLR ligation (31, 32). Recent reports have demonstrated a role for TBK1 and IKKε in IRF3 activation in response to viral infection or dsRNA (12, 14), which requires the phosphorylation of IRF3 on S396 (33). The notion that phosphorylation of this residue occurs in response to LPS is somewhat controversial, offering the possibility that a distinct pathway mediates IRF3 activation via TLR2 and TLR4 ligands. In the present study we show that ISG induction by LPS, which occurs in an IRF3-dependent manner, requires the generation of ROS by the NADPH-dependent oxidase NOX4. Subsequent activation of ASK1 links LPS-induced ROS production to the activation of MKK6 and p38, two kinases that we had previously identified as components of the LPS-induced IRF3 activation cascade. It remains to be determined whether ASK1 is also a contributor to the activation of IRF3 in response to other stimuli such as viral infection or DNA damaging agents.

Disclosures

The authors have no financial conflict of interest.

References

1. Kopp, E. B., and R. Medzhitov. 1999. The Toll-receptor family and control of innate immunity. *Curr. Opin. Immunol.* 11: 13–18.
2. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388: 394–397.
3. Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4. *J. Exp. Med.* 189: 615–625.
4. Yang, R. B., M. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 395: 284–288.
5. Navarro, L., K. Mowen, S. Rodems, B. Weaver, N. Reich, D. Spector, and M. David. 1998. Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon-stimulated response element-binding complex. *Mol. Cell. Biol.* 18: 3796–3802.
6. Lin, R., C. Heylbroeck, P. M. Pitha, and J. Hiscott. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol. Cell. Biol.* 18: 2986–2996.
7. Yoneyama, M., W. Suhara, Y. Fukuhara, M. Fukuda, E. Nishida, and T. Fujita. 1998. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J.* 17: 1087–1095.
8. Wathelet, M., C. H. Lin, B. S. Parekh, L. V. Ronco, P. M. Howley, and T. Maniatis. 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-β enhancer in vivo. *Mol. Cell* 1: 507–518.
9. Navarro, L., and M. David. 1999. p38-dependent activation of interferon regulatory factor 3 by lipopolysaccharide. *J. Biol. Chem.* 274: 35535–35538.
10. Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll adapters TRAM and TRIF. *J. Exp. Med.* 198: 1043–1055.
11. Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, et al. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424: 743–748.
12. Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis. 2003. IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* 4: 491–496.
13. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 301: 640–643.
14. Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 300: 1148–1151.
15. Kamata, H., and H. Hirata. 1999. Redox regulation of cellular signalling. *Cell. Signal.* 11: 1–14.
16. Forman, H. J., and M. Torres. 2002. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am. J. Respir. Crit. Care Med.* 166: S4–S8.
17. Rhee, S. G., Y. S. Bae, S. R. Lee, and J. Kwon. 2000. Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci. STKE* 2000: PE1.
18. Saitoh, M., H. Nishitoh, M. Fujii, K. Takeda, K. Tobiume, Y. Sawada, M. Kawabata, K. Miyazono, and H. Ichijo. 1998. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase 1 (ASK1). *EMBO J.* 17: 2596–2606.
19. Gao, N., M. Ding, J. Z. Zheng, Z. Zhang, S. S. Leonard, K. J. Liu, X. Shi, and B. H. Jiang. 2002. Vanadate-induced expression of hypoxia-inducible factor 1 α and vascular endothelial growth factor through phosphatidylinositol 3-kinase/Akt pathway and reactive oxygen species. *J. Biol. Chem.* 277: 31963–31971.
20. Gate, L., J. Paul, G. N. Ba, K. D. Tew, and H. Tapiero. 1999. Oxidative stress induced in pathologies: the role of antioxidants. *Biomed. Pharmacother.* 53: 169–180.
21. Lin, R., C. Heylbroeck, P. Genin, P. M. Pitha, and J. Hiscott. 1999. Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. *Mol. Cell. Biol.* 19: 959–966.
22. Lambeth, J. D. 2002. Nox/Duox family of nicotinamide adenine dinucleotide (phosphate) oxidases. *Curr. Opin. Hematol.* 9: 11–17.
23. Stuehr, D. J., O. A. Fasehun, N. S. Kwon, S. S. Gross, J. A. Gonzalez, R. Levi, and C. F. Nathan. 1991. Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *FASEB J.* 5: 98–103.
24. Hancock, J. T., and O. T. Jones. 1987. The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages. *Biochem. J.* 242: 103–107.
25. Park, H. S., H. Y. Jung, E. Y. Park, J. Kim, W. J. Lee, and Y. S. Bae. 2004. Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-κB. *J. Immunol.* 173: 3589–3593.
26. Hsu, H. Y., and M. H. Wen. 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.* 277: 22131–22139.
27. Tobiume, K., A. Matsuzawa, T. Takahashi, H. Nishitoh, K. Morita, K. Takeda, O. Minowa, K. Miyazono, T. Noda, and H. Ichijo. 2001. ASK1 is required for sustained activation of JNK/p38 MAP kinases and apoptosis. *EMBO Rep.* 2: 222–228.
28. Dang, O., L. Navarro, K. Anderson, and M. David. 2004. Cutting edge: anthrax lethal toxin inhibits activation of IFN-regulatory factor 3 by lipopolysaccharide. *J. Immunol.* 172: 747–751.
29. Matsuzawa, A., K. Saegusa, T. Noguchi, C. Sadamitsu, H. Nishitoh, S. Nagai, S. Koyasu, K. Matsumoto, K. Takeda, and H. Ichijo. 2005. ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. *Nat. Immunol.* 6: 587–592.
30. Tobiume, K., M. Saitoh, and H. Ichijo. 2002. Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. *J. Cell. Physiol.* 191: 95–104.
31. Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Distinct and essential roles of transcription factors IRF3 and IRF7 in response to viruses for IFNα/β gene induction. *Immunity* 13: 539–548.
32. Sakaguchi, S., H. Negishi, M. Asagiri, C. Nakajima, T. Mizutani, A. Takaoka, K. Honda, and T. Taniguchi. 2003. Essential role of IRF-3 in lipopolysaccharide-induced interferon-β gene expression and endotoxin shock. *Biochem. Biophys. Res. Commun.* 306: 860–866.
33. Servant, M. J., N. Grandvaux, B. R. tenOever, D. Duguay, R. Lin, and J. Hiscott. 2003. Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. *J. Biol. Chem.* 278: 9441–9447.