Dok-1 and Dok-2 are negative regulators of lipopolysaccharide-induced signaling

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Endotoxin, a bacterial lipopolysaccharide (LPS), causes fatal septic shock via Toll-like receptor (TLR)4 on effector cells of innate immunity like macrophages, where it activates nuclear factor κB (NF-κB) and mitogen-activated protein (MAP) kinases to induce proinflammatory cytokines such as tumor necrosis factor (TNF)-α. Dok-1 and Dok-2 are adaptor proteins that negatively regulate Ras–Erk signaling downstream of protein tyrosine kinases (PTKs). Here, we demonstrate that LPS rapidly induced the tyrosine phosphorylation and adaptor function of these proteins. The stimulation with LPS of macrophages from mice lacking Dok-1 or Dok-2 induced elevated Erk activation, but not the other MAP kinases or NF-κB, resulting in hyperproduction of TNF-α and nitric oxide. Furthermore, the mutant mice showed hyperproduction of TNF-α and hypersensitivity to LPS. However, macrophages from these mutant mice reacted normally to other pathogenic molecules, CpG oligodeoxynucleotides, poly(I:C) ribonucleotides, or Pam_3CSK_4 lipopeptide, which activated cognate TLRs but induced no tyrosine phosphorylation of Dok-1 or Dok-2. Forced expression of either adaptor, but not a mutant having a Tyr/Phe substitution, in macrophages inhibited LPS-induced Erk activation and TNF-α production. Thus, Dok-1 and Dok-2 are essential negative regulators downstream of TLR4, implying a novel PTK-dependent pathway in innate immunity.

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The innate immune response to microbial pathogens begins when pathogen-associated molecular patterns (PAMPs) meet their cognate receptors on effector cells. PAMPs are conserved motifs on pathogens that are usually not found in higher eukaryotes and include LPS, a bacterial cell wall component and the most potent stimulator in innate immunity (1). Toll-like receptors (TLRs) recognize PAMPs, and LPS stimulates the TLR4–MD-2 receptor complex, which then triggers intracellular signaling cascades (TLR4 signaling) including the activation of NF-κB and three types of mitogen-activated protein (MAP) kinases: Erk, JNK, and p38 (2, 3). These signaling molecules play indispensable roles in inducing TNF-α, a key proinflammatory cytokine for innate immunity (4). Recent studies have revealed that another LPS receptor, CD14, facilitates the binding of LPS to the TLR4–MD-2 complex and consequent intracellular signaling (5). In addition, TLR-mediated signaling depends upon adaptor molecules such as MyD88 and Toll/IL-1 receptor domain–containing adaptor-inducing IFN-β (TRIF) and is often classified into a MyD88- or TRIF-dependent pathway. In fact, TLR4 triggers both pathways, and macrophages from mice lacking these adaptors are defective in proinflammatory responses to LPS (6).

Although the innate immune response is essential for controlling the growth of pathogenic microbes, negative regulation is also critical because excessive and unleashed responses can cause inflammatory diseases such as septic shock or chronic inflammation (4, 7–10). A Toll IL-1 receptor family protein ST2 was recently reported as an inducible negative regulator of the MyD88-dependent pathway (7). Indeed, mice lacking ST2 failed to develop endotoxin tolerance a few days after primary administra-

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tion of a sublethal dose of LPS. However, due to the lag period for its induction, ST2 appeared irrelevant to primary endotoxin shock, a septic shock rapidly induced by LPS. Gene targeting studies further revealed that IL-1 receptor–associated kinase (IRAK)-M and suppressor of cytokine signaling 1 are inducible negative regulators of LPS responses (8–10). Despite these findings, very little is known about constitutively expressed negative regulator(s) of TLR4 signaling, which could work instantaneously upon LPS treatment of macrophages to control the early phase of the signaling and oppose endotoxin shock.

Dok-1 was originally identified as a major substrate of many protein tyrosine kinases (PTKs; references 11–13). When tyrosine phosphorylated, Dok-1 and its closest homologue Dok-2 work as adaptor proteins and recruit multiple SH2-containing molecules such as p120 rasGAP and Nck. These adaptors are preferentially expressed in hematopoietic cells and share structural similarities characterized by NH_2-terminal PH and PTB domains, followed by COOH-terminal SH2 target motifs (11). Experiments with mice lacking Dok-1 or Dok-2 demonstrated an indispensable role in the negative regulation of Erk downstream of PTKs in various hematopoietic cells (14–16). However, mice lacking either adaptor did not show overt defects in hematopoiesis. Although the biological significance of PTKs in TLR4 signaling is controversial, LPS activates cytoplasmic PTKs including Lyn, which is essential for the phosphorylation of Dok-1 upon B cell receptor signaling (15, 17). Here, we have studied the role of Dok-1 and Dok-2 and demonstrate that these adaptors are constitutively expressed negative regulators of TLR4 signaling.

RESULTS AND DISCUSSION

Dok-1 and Dok-2 are negative regulators of TNF-α and nitric oxide (NO) production upon LPS treatment of macrophages

To understand the role of Dok-1 and Dok-2 in TLR4 signaling, we first examined the production of two major signal mediators of innate immunity, TNF-α and NO, upon LPS treatment of macrophages from mice lacking Dok-1 or Dok-2. The peritoneal resident and BM-derived macrophages from either of the mutant mice showed a larger population of TNF-α–producing cells and greater NO production than the wild-type cells, respectively (Fig. 1, A and B). However, both mutant macrophages expressed normal levels of LPS receptors, TLR4–MD-2, and CD14, indicating that loss of Dok-1 or Dok-2 does not cause down-regulation of these receptors (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041817/DC1). Thus, Dok-1 and Dok-2 are indispensable negative regulators of TNF-α and NO production downstream of TLR4.

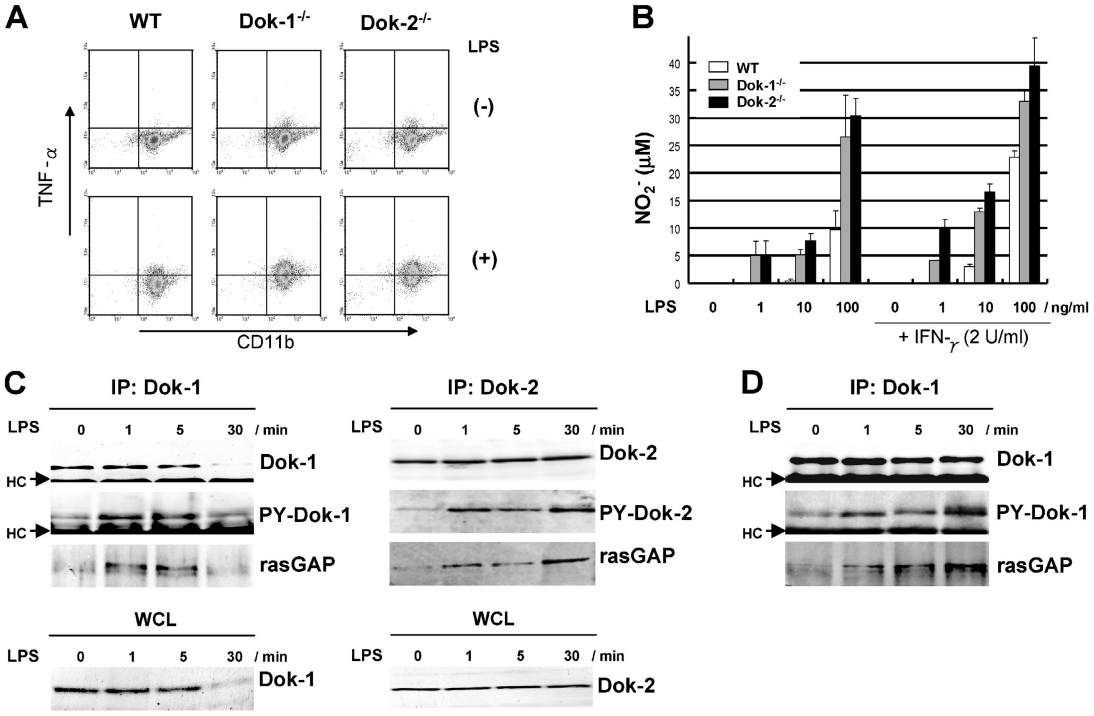

Figure 1. Dok-1 and Dok-2 are adaptors essential to the negative regulation of LPS responses. (A) Peritoneal resident macrophages from mice were treated with (+) or without (−) LPS, and then intracellular TNF-α production of $CD11b^+$ cells was examined with flow cytometry. (B) BM-derived macrophages were cultured in the indicated concentration of LPS and IFN-γ, and then NO production was evaluated. SD is from sextuplicate experiments. (C) Dok-1 or Dok2 immunoprecipitates (IP) or whole cell lysates (WCL) were subjected to immunoblotting for Dok-1, Dok-2, phosphotyrosine (PY-Dok-1 or PY-Dok-2), or p120 rasGAP upon LPS treatment of peritoneal macrophages for the indicated period. Position of immunoglobulin heavy chain (HC) is indicated. (D) Dok-1 immunoprecipitates were adjusted to contain the same levels of Dok-1 in quantity and examined as in C.

Dok-1 and Dok-2 are essential adaptors in the negative regulation of Erk upon LPS treatment

To address the molecular mechanisms of the Dok-1– and Dok-2–mediated negative regulation of LPS-evoked responses, we examined the tyrosine phosphorylation and adaptor function in peritoneal macrophages. Antiphosphotyrosine immunoblot and coimmunoprecipitation analyses revealed that the Dok family proteins were indeed tyrosine phosphorylated as early as 1 min after LPS treatment and coimmunoprecipitated with p120 rasGAP (Fig. 1 C). Interestingly, Dok-1, but not Dok-2, decreased in quantity at least 30 min after the stimulation, indicating that the biochemical responses of these proteins differ. However, when immunoprecipitated Dok-1 was adjusted to the same quantity at each time point, its tyrosine phosphorylation and binding to rasGAP was obvious even 30 min after the stimulation with LPS (Fig. 1 D). These results indicate that Dok-1 and Dok-2 are adaptors involved in LPS-evoked signaling, which activates PTK(s) to phosphorylate them, and also suggest that these adaptors negatively regulate Erk upon TLR4 signaling, like in many other signaling situations downstream of PTKs. Thus, the activation of Erk as well as JNK and p38 MAP kinase was evaluated upon LPS treatment of BM-derived macrophages from mice lacking Dok-1 or Dok-2. Although JNK and p38 MAP kinase activation was normal in those macrophages, the activation of Erk was remarkably enhanced and sustained (Fig. 2 A). In addition, the phosphorylation and degradation of IκB-α as well as the activation of

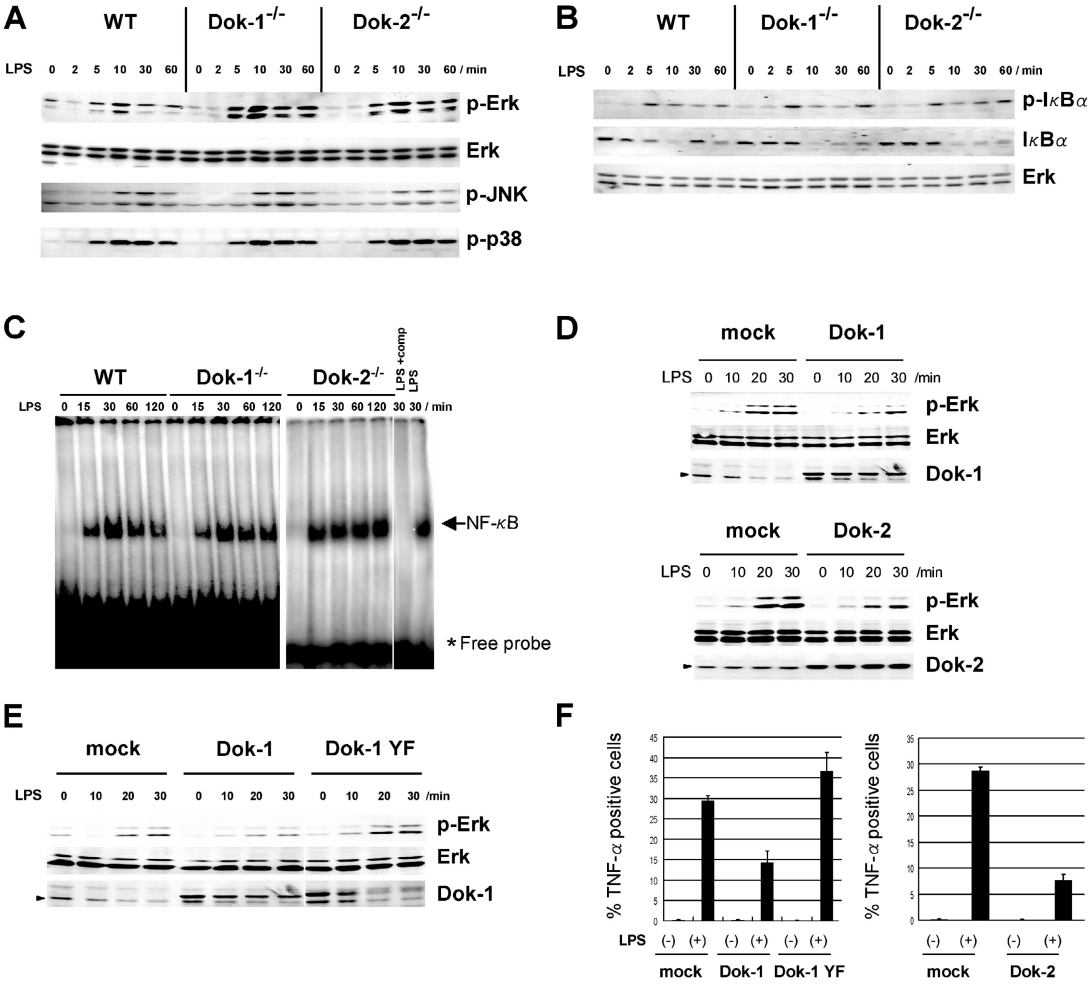

Figure 2. Dok-1 and Dok-2 are negative regulators of Erk upon TLR4 signaling. (A) Total Erk, activated Erk (p-Erk), JNK (p-JNK), or p38 MAP kinase (p-p38) was examined with immunoblotting upon LPS treatment of BM-derived macrophages from mice. (B) NF-κB activation was assessed by immunoblotting for IκBα or its phosphorylation (p-IκBα) upon LPS treatment of macrophages in A. Control immunoblotting for Erk was performed. (C) NF-κB activity was examined by gel mobility shift assay upon LPS treatment of peritoneal macrophages. Positions of the NF-κB complex and the free probes are indicated. The specificity was determined by adding excess amounts of unlabeled competitor of the probe (LPS + comp) or not (LPS) to nuclear extracts of wild-type macrophages. (D) Activated Erk (p-Erk), total Erk, Dok-1, or Dok-2 was examined with immunoblotting upon LPS treatment of RAW 264.7 cells (mock) or those expressing exogenous Dok-1 (top) or Dok-2 (bottom). An arrowhead indicates the position of endogenous Dok-1 or Dok-2. (E) RAW 264.7 cells (mock) or those expressing exogenous Dok-1 or a Dok-1 mutant (Dok-1 YF) were examined as in D. (F) RAW 264.7 cells (mock) or those expressing exogenous Dok-1, Dok-1 YF, or Dok-2 were cultured in the presence (+) or absence (−) of LPS, and then the percentage of intracellular TNF-α^+ cells was determined with flow cytometry. SD is from triplicate experiments.

NF-κB were unaffected in macrophages regardless of the mutations (Fig. 2, B and C). Together, our findings demonstrate that Dok-1 and Dok-2 are essential adaptors in the negative regulation of Erk, but not JNK, p38 MAP kinase, and NF-κB, upon TLR4 signaling. Moreover, that the expression levels of Dok-1 and Dok-2 were unchanged at least early on and the phosphorylation was very rapid upon LPS treatment indicates that both adaptors are on standby before the onset of the signaling to be rapidly activated (Fig. 1 C).

Forced expression of Dok-1 or Dok-2 inhibits LPS-induced Erk activation and TNF-α production

To confirm that Dok-1 and Dok-2 are negative regulators of Erk and TNF-α responses to LPS, we examined if forced expression in macrophages of either adaptor suffices to inhibit those responses. The control RAW 264.7 macrophages displayed an intact Erk activation, TNF-α production, and Dok-1 down-regulation upon LPS treatment (Fig. 2, D–F). However, forced expression of flag-tagged Dok-1 or Dok-2 clearly inhibited the Erk and TNF-α responses to LPS, indicating that Dok-1 and Dok-2 are potent negative regulators of the signaling. Note that the flag-tagged Dok-1, but not Dok-2, was down-regulated like the endogenous Dok-1. Recently, we identified Tyr-336 and Tyr-340 as essential residues for Dok-1 to inhibit the Ras–Erk pathway downstream of Lyn (18). Consistently, forced expression of a flag-tagged Dok-1 mutant having a Tyr/Phe substitution at these residues (Dok-1 YF) resulted in a loss of inhibitory effects on the LPS-evoked responses (Fig. 2, E and F). These results strongly suggest that tyrosine phosphorylation of Dok-1 and probably Dok-2 is essential for the inhibitory effects downstream of TLR4.

Although little is known about the regulation of Erk downstream of LPS, it was reported that a MAP kinase kinase kinase, Tpl2/Cot, is required for the LPS-mediated activation of Erk in macrophages (19). The authors showed that loss of Tpl2 specifically blocks the activation of Erk among MAP kinases and NF-κB and that inhibition of MEK, and thereby inhibition of Erk, suppressed TNF-α production upon LPS signaling. Moreover, it was suggested that not only LPS but also Tpl2 requires the Ras pathway to activate Erk (20, 21). Therefore, Ras appears to be an essential element for the activation of Erk downstream of TLR4. Given that the Dok-1 YF mutant lacking residues essential for the inhibition of Ras had no inhibitory effect upon TLR4 signaling (Fig. 2, E and F), the negative signaling of Dok-1 against Erk may intersect the Tpl2-mediated positive signaling in the Ras pathway downstream of TLR4. Further studies are required to understand molecular mechanisms of the Dok-1 and Dok-2 function in the TLR4 pathway, including the negative regulation of TNF-α production and interaction with their regulators and effectors.

Dok-1 and Dok-2 are irrelevant to TLR9, TLR3, or TLR2 signaling

To delineate the Dok-1 and Dok-2 function in TLR-mediated signaling, we examined their role upon the stimulation of macrophages with CpG oligodeoxynucleotide (ODN) and poly(I:C), which mimic microbial nucleotides and induce MyD88-dependent and TRIF-dependent pathways through TLR9 and TLR3, respectively. Interestingly, both nucleotides induced normal levels of Erk, JNK, and p38 MAP kinase activation as well as TNF-α production regardless of Dok-1 or Dok-2 mutation, indicating that both adaptors are dispensable to the signaling downstream of these TLRs (Fig. 3, A and B, and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20041817/DC1). Because TLR3 and TLR9 are thought to be intracellular receptors, whereas

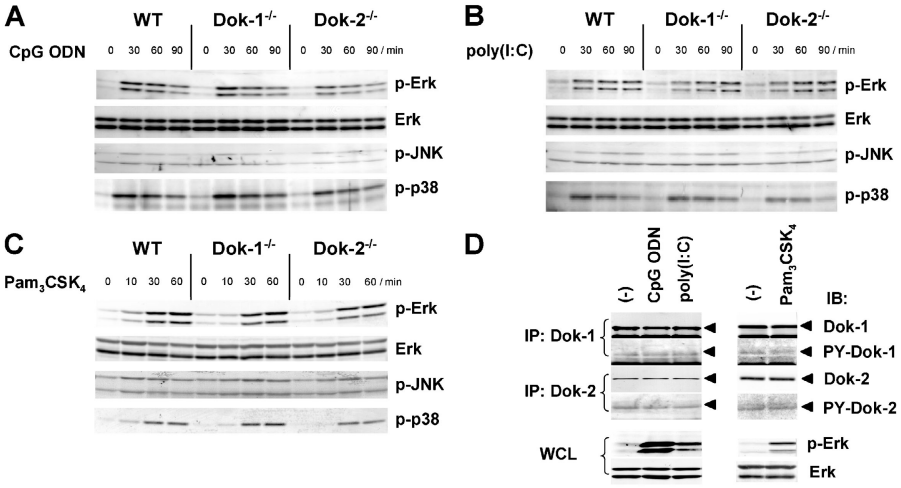

Figure 3. Dok-1 and Dok-2 are irrelevant to TLR9, TLR3, or TLR2 signaling. (A–C) Activation of each MAP kinase was examined as in Fig. 2 A upon CpG ODN, poly(I:C), or Pam_3CSK_4 treatment of BM-derived macrophages from mice. (D) Dok-1 or Dok-2 immunoprecipitates (IP) were subjected to immunoblotting (IB) for Dok-1, Dok-2, or phosphotyrosine (PY-Dok-1 or PY-Dok-2) upon CpG ODN, poly(I:C), or Pam_3CSK_4 treatment of wild-type peritoneal macrophages for 30 min. Whole cell lysates (WCL) from these macrophages were subjected to immunoblotting for activated Erk (p-Erk) or total Erk as controls.

TLR4 is present on the cell surface (22), we further examined the role of Dok-1 and Dok-2 upon the stimulation of macrophages with Pam_3CSK_4, which is an analogue of bacterial outer membrane lipoproteins and activates the MyD88-dependent pathway through a cell surface receptor, TLR2. However, Pam_3CSK_4 induced normal levels of MAP kinase activation and TNF-α production regardless of Dok-1 or Dok-2 mutation, indicating that both adaptors are dispensable to TLR2 signaling (Fig. 3 C and Fig. S2). Consistently, CpG-ODN, poly(I:C), or Pam_3CSK_4 treatment did not induce tyrosine phosphorylation of Dok-1 and Dok-2 or down-regulation of Dok-1, indicating that these adaptors are irrelevant to TLR9, TLR3, or TLR2 signaling (Fig. 3 D). Together, Dok-1 and Dok-2 are essential adaptors for the negative regulation of Erk specifically upon LPS treatment, likely because LPS, but not CpG ODN, poly(I:C), or Pam_3CSK_4, induces their tyrosine phosphorylation.

Mice lacking Dok-1 or Dok-2 are hypersensitive to LPS

Our in vitro and ex vivo findings suggest that mice lacking Dok-1 or Dok-2 are hypersensitive to LPS; therefore, we examined TNF-α production upon i.p. administration of LPS to such mutant mice. Because overproduction of TNF-α due to excessive inflammatory responses to LPS is a cause of endotoxin shock or lethality, we also examined the survival of LPS-injected mice. As expected, the serum concentration of TNF-α was increased three- to fourfold in mice lacking Dok-1 or Dok-2 as early as 1 h after injection as compared with the wild-type controls (Fig. 4 A). Consistently, the mutant mice displayed severe responses to LPS injection at a dose sublethal to the wild-type controls (Fig. 4 B). These results demonstrate that Dok-1 and Dok-2 are negative regulators of innate immunity, at least in the early inflammatory responses to LPS in vivo. Because the Dok-1 or Dok-2 deficiency did not influence TNF-α receptor–mediated activation of MAP kinases and NF-κB in peritoneal macrophages (unpublished data), such a mutation causes hypersensitivity to LPS, but not to TNF-α induced by LPS. It is of note that augmented production of NO, another cause of septic shock (23), was seen in macrophages lacking Dok-1 or Dok-2 (Fig. 1 B).

The recognition of microbial pathogens by cognate TLRs triggers the innate immune response. TLR-mediated signaling involves at least four crucial adaptors, MyD88, TRIF, TIRAP/Mal, and TRAM, having a Toll IL-1 receptor domain, which has the capability to bind an appropriate Toll IL-1 receptor domain in the cytoplasmic region of TLR(s) (24). Recent studies demonstrated that TIRAP and TRAM are essential for TLR4 to recruit MyD88 and TRIF, respectively (25–27). TLR2 also requires TIRAP to recruit MyD88. IRAK-M is a negative regulator of the MyD88-dependent pathway forming a complex with IRAK and IRAK4 to prevent phosphorylation of IRAK and its dissociation from the MyD88–TLR complex, thereby inhibiting NF-κB activation (8). MyD88s acts similarly by blocking the access of IRAK-4 to IRAK (28), and ST2 sequestrates MyD88 and TIRAP from TLR signaling (7). Although these negative regulators play important roles in LPS-mediated signaling in macrophages, there is an inevitable lag period for their induction upon LPS treatment of TLR4 as mentioned earlier. Here, we demonstrated that Dok-1 and Dok-2 are expressed at functional levels before LPS treatment and thus on standby to negatively regulate Erk immediately after the onset of TLR4 signaling. Interestingly, these adaptors are irrelevant to TLR2, TLR3, and TLR9, indicating the specificity of Dok-1 and Dok-2 to TLR4 signaling evoked by LPS, the most potent stimulator in innate immunity. Because TLR2 or TLR9 triggers the MyD88-dependent pathway and TLR3 triggers the TRIF-dependent pathway, each pathway does not suffice to induce the negative function of these adaptors. Although studies are underway to clarify the molecular basis for the Dok-1– and Dok-2–medi-

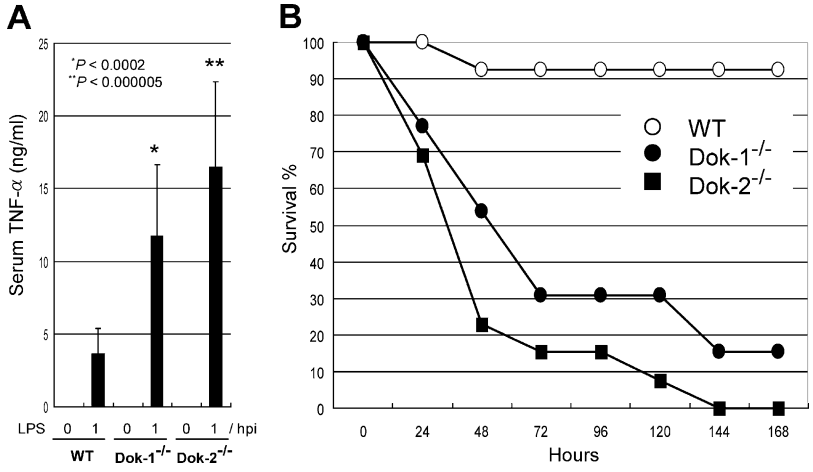

Figure 4. Mice lacking Dok-1 or Dok-2 are hypersensitive to LPS. (A) Serum concentration of TNF-α of 8-wk-old mice at 1 h after injection (1 hpi) with LPS to the peritoneal cavity or before it (0 hpi) was examined with ELISA and shown with SD ($n = 7–13$). The maximal p-value compared with the wild-type is indicated. (B) Mice at 8 wk of age ($n = 13$ for each) were injected with LPS as in A and monitored up to 7 d. Data representative of duplicate experiments are shown.

ated signaling, our findings, at the very least, provide a novel target for controlling the innate immune response.

MATERIALS AND METHODS

Reagents, mice, and cells. LPS purified from *Escherichia coli* 0111:B4 (Sigma-Aldrich), poly(I:C) (Amersham Biosciences), CpG ODN (5′-TCCATGACGTTCCTGATGCT-3′; QIAGEN), and Pam_3CSK_4 (EMC microcollections) were purchased. The generation of Dok-1 or Dok-2 KO mice was described (14, 15), and these mice were backcrossed to C57BL/6 for at least eight generations. Mice were kept under specific pathogen-free conditions and subjected to experiments at 8–12 wk of age. Experiments and animal care were performed according to institutional guidelines. Peritoneal exudate cells (PECs) were collected with 2 mM EDTA/PBS 3 d after an i.p. injection of 0.5 ml of 3% thioglycollate (Nissui). Resident PECs were obtained by the same procedure without a thioglycollate injection. These cells were washed and resuspended in DMEM containing 15% FCS. After several hours of incubation in culture plates, adherent PECs and resident PECs were used as peritoneal macrophages and peritoneal resident macrophages, respectively. BM cells were cultured in DMEM containing 10 ng/ml of murine M-CSF (PeproTech) and 15% FCS. After 7 d of culture, adherent cells were maintained in the absence of M-CSF for 24 h and used as BM-derived macrophages. RAW 264.7 cells were cultured in DMEM containing 15% FCS.

Flow cytometry. A single cell suspension of peritoneal resident or RAW 264.7 macrophages was treated with 10 or 1.0 μg/ml LPS, respectively, and 2.0 μg/ml brefeldin A (Sigma-Aldrich) for 16 h, and then the former cells were stained with PE-conjugated mAbs to CD11b (BD Biosciences). Intracellular TNF-α was stained with a CytoStain kit (BD Biosciences), and flow cytometry was performed with a FACSCalibur (Becton Dickinson). Data representative of quintuplicate experiments are shown (refer to Fig. 1 A).

NO production assay. To evaluate NO production, cells were cultured for 24 h and the NO_2^- concentration in the medium was measured with a NO_2/NO_3 Assay kit-CII (Dojindo).

Immunoprecipitation and immunoblotting. Cells treated with 1.0 or 2.0 μg/ml LPS, 10 μM CpG ODN, 100 μg/ml poly(I:C), or 100 ng/ml Pam_3CSK_4 were solubilized in 1.0% NP-40–based TNN buffer (18). For immunoprecipitation, cell lysates were cleared and incubated with antibodies to mouse Dok-1 (A3) or Dok-2 (M20; Santa Cruz Biotechnology, Inc.) followed by incubation with protein G–Sepharose (Amersham Biosciences). The immune complex was washed and collected as immunoprecipitates. For immunoblotting, immunoprecipitates or cleared cell lysates were separated by SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories), which was then incubated with antibodies to phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho–IκB-α (Cell Signaling), phospho-JNK (Thr183/Tyr185), ERK, IκB-α, Dok-2 (H192), p120 rasGAP (Santa Cruz Biotechnology, Inc.), or phosphotyrosine (4G10; Upstate Biotechnology), followed by incubation with secondary horse radish peroxidase–labeled (Amersham Biosciences) or AP-labeled (Santa Cruz Biotechnology, Inc.) antibodies. The blots were visualized with the ECL system (Amersham Biosciences) or BCIP/NBT system (Promega). Data representative of triplicate experiments are shown.

Gel mobility shift assay. The nuclear extracts of cells treated with 1.0 μg/ml LPS were incubated with a specific probe for the NF-κB DNA binding site, electrophoresed, and visualized by autoradiography as described previously (29). Data representative of triplicate experiments are shown.

Forced expression of Dok-1, Dok-1 YF, or Dok-2 in RAW 264.7 cells. cDNA for mouse Dok-1, Dok-1 YF, or Dok-2 fused with the flag tag at the COOH terminus was generated by PCR. Each cDNA and the IRES-GFP fragment were appropriately inserted into the mammalian expression vector pA-puro (30). The expression plasmid was confirmed by sequencing and transfected into RAW 264.7 cells with FuGENE 6 (Roche). The puromycin-resistant clones were further selected for Dok-1 or Dok-2 expression.

ELISA. The serum TNF-α concentration of mice at 1 h after injection with LPS (25 mg per weight kg) to the peritoneal cavity or before it, was measured with an ELISA kit (Biosource International).

Statistical analysis. Statistical analysis was performed with Student's *t* test and analyzed using Microsoft Excel Software.

Online Supplemental Material. Fig. S1 shows normal expression of LPS receptors on macrophages from mice lacking Dok-1 or Dok-2. Fig. S2 shows normal TNF-α production upon stimulation of TLR9, TLR3, or TLR2 of these macrophages. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20041817/DC1.

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Note added in proof: After acceptance of this manuscript, Niki et al. (Niki, M., A. Di Cristofano, M. Zhao, H. Honda, H. Hirai, L. Van Aelst, C. Cordon-Cardo, and P.P. Pandolfi. 2004. *J. Exp. Med.* 200:1689–1695) reported a role of Dok-1 and Dok-2 in leukemia suppression.

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