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DOK3 Is Required for IFN-β Production by Enabling TRAF3/TBK1 Complex Formation and IRF3 Activation

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The downstream of kinase (DOK) family of adaptors is generally involved in the negative regulation of signaling pathways. DOK1, 2, and 3 were shown to attenuate TLR4 signaling by inhibiting Ras-ERK activation. In this study, we elucidated a novel role for DOK3 in IFN-β production. Macrophages lacking DOK3 were impaired in IFN-β synthesis upon influenza virus infection or polyinosinic-polycytidylic acid stimulation. In the absence of DOK3, the transcription factor IFN regulatory factor 3 was not phosphorylated and could not translocate to the nucleus to activate ifn-β gene expression. Interestingly, polyinosinic-polycytidylic acid–induced formation of the upstream TNFR-associated factor (TRAF) 3/TANK-binding kinase (TBK) 1 complex was compromised in dok3−/− macrophages. DOK3 was shown to bind TBK1 and was required for its activation. Furthermore, we demonstrated that overexpression of DOK3 and TBK1 could significantly enhance ifn-β promoter activity. DOK3 was also shown to bind TRAF3, and the binding of TRAF3 and TBK1 to DOK3 required the tyrosine-rich C-terminal domain of DOK3. We further revealed that DOK3 was phosphorylated by Bruton’s tyrosine kinase. Hence, DOK3 plays a critical role in TLR3 signaling by enabling TRAF3/TBK1 complex formation and facilitating TBK1 and IFN regulatory factor 3 activation and the induction of IFN-β production. The Journal of Immunology, 2014, 193: 840–848.

The downstream of kinase (DOK) family of adaptors comprises seven structurally related proteins with each possessing an NH2-terminal pleckstrin homology (PH), a central phosphotyrosine-binding (PTB), and a C-terminal tyrosine-rich domain (1). DOK1–3 are preferentially expressed in hematopoietic cells whereas DOK4–7 are found in neural and other cell types (2). As these proteins have no catalytic activity, they function mainly as adaptors to facilitate protein–protein interactions and possibly also as scaffolds to nucleate protein complexes in signal transduction pathways. Recently, DOK1–3 were identified as tumor suppressors for lung cancer in human and mice (3) and shown to play a role in preventing the development of aggressive histiocytic sarcoma (4).

Accumulating data suggest that DOK1–3 function to limit protein tyrosine kinase–mediated signaling in immune cells (5). In particular, DOK1 and 2 have been characterized as negative regulators of Ras-ERK signaling downstream of Ag receptors in B and T cells (6). DOK3 was also shown to have an inhibitory role as it restricts Ca2+ (7) and JNK (8) activation in B cell receptor signaling.

Unlike B cells, which express DOK1 and 3, and T cells, which express DOK1 and 2, myeloid cells express all three DOK adaptors (1). The role of DOK proteins in myeloid cells is beginning to be unraveled, and there is increasing evidence to indicate that they are involved in TLR signaling and innate immunity. TLRs are pattern recognition receptors that bind pathogen-associated molecular patterns found on microbes (9). Examples of pathogen-associated molecular patterns include the bacterial cell wall component LPS that is recognized by TLR4 and viral dsRNA that is detected by TLR3 (10). DOK1 and 2 have been shown to participate in TLR4 signaling by negatively regulating ERK activation and TNF-α production in LPS-stimulated macrophages (11). Recently, DOK3 was also demonstrated to negatively regulate LPS-induced ERK activation and the production of inflammatory cytokines in macrophages (12). Thus, it seems that DOK1–3 act similarly to inhibit TLR4 signaling in macrophages.

Whereas DOK1 and 2 were shown not to regulate TLR3 signaling (11), the role of DOK3 in TLR3 signal transduction remains to be determined. TLR3 recognition of viral dsRNA or its synthetic analog, polyinosinic-polycytidylic acid [poly(I:C)] activates the TRIF-dependent signaling pathway, resulting in IFN-β production. This signaling cascade leads to the formation of the TNFR-associated factor (TRAF) 3/TANK-binding kinase (TBK) 1 complex and activation of TBK1 that subsequently leads to the phosphorylation and activation of the transcription factor IFN regulatory factor (IRF) 3 (13). Activated IRF3 dimerizes and translocates from the cytoplasm to the nucleus to drive ifn-β mRNA synthesis (14).

Interestingly, dok3 mRNA was reported to be upregulated in virus-infected cells (15). This is in contrast to the demonstrated degradation of DOK1–3 proteins in LPS-stimulated macrophages (12), suggesting that DOK3 could have a different role in TLR3 activation in macrophages. In this study, we examined the involvement of DOK3 in TLR3 signaling and showed that the adaptor was phosphorylated upon poly(I:C) stimulation and played a critical
role in TLR3-triggered IFN-β production. More importantly, we demonstrate that DOK3 is needed for the induction of IRF3 by facilitating the formation of the upstream TRAF3/TBK1 complex and the activation of TBK1 and IRF3.

Materials and Methods

Mice, cells, and plasmids

Wild-type C57BL/6 and dok3−/− mice were bred in our facilities whereas Bruton’s tyrosine kinase (btk)−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). th3−/− and trf3−/− mice were provided by Osamu Takeuchi and Shizuo Akira (Osaka University, Osaka, Japan). Expression vectors with mouse were generated according to protocols from the National Advisory Committee on Laboratory Animal Research. Macrophages were differentiated from bone marrow as previously described (16). HEK293T cells were transfected with recombinant vectors encoding murine TLR3, DOK3, TRAF, TBK1, TRIF, BTK, or various truncated and site-directed mutants (Stratagene) with hemagglutinin (HA), FLAG, or GFP tag using Lipofectamine 2000 (Invitrogen, Grand Island, NY). Cells were stimulated with poly(I:C) or transfected with poly(I:C) enclosed in liposomes [poly(I:C)/LyoVec] (InvivoGen, San Diego, CA).

Immunoprecipitations and immunoblotting

Cells were lysed on ice for 30 min in phosphylosis buffer containing 1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.2 mM Na3VO4, and a mixture of protease inhibitors (Roche Applied Science). Cell homogenates were centrifuged at 13,000 rpm for 15 min at 4˚C, and supernatants were recovered for protein quantification by a BCA protein assay kit (Pierce). For immunoprecipitation studies, Abs were first coupled to Protein A/G PLUS-Agarose (SC-3003; Santa Cruz Biotechnology, Santa Cruz, CA) at 4˚C overnight. Beads were washed twice in lysis buffer and incubated with precleared cell lysates for 2 h at 4˚C and subsequently boiled in loading buffer for 5 min to release proteins. For Western blot analyses, 30 μg whole-cell lysates or 5 μg nuclear extracts isolated with NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL) were electrophoresed in 10% SDS-polyacrylamide gels and transferred onto immuno blot polyvinylidine difluoride membranes (Millipore, Billerica, MA). Membranes were subsequently probed with Abs against various signaling molecules. Abs against DOK3, ERK2, phospho-ERK2, phospho-AKT (Thr473 and Ser473), TRAF3, JNK1, p38, IRF3, IκBα, and histone deacetylase 1 (HDAC1) were from Santa Cruz Biotechnology. The anti-IRF3 Ab was generated against a synthetic peptide corresponding to amino acids 234–249 of human IRF3. The anti-phospho-p38 Abs were from Sigma-Aldrich (St. Louis, MO). Abs against phospho-p38, phospho-SAPK/JNK, phospho-AKT (Ser473), AKT, phospho-IRF3 (Ser396), phospho-TBK1 (Ser172), TBK1, and TRAF3 were from Cell Signaling Technology. The anti-phosphotyrosine (4G10) Ab was from Upstate Biotechnology.

Immunofluorescence and confocal microscopy

Cells were washed twice with cold PBS containing 1% BSA and fixed 20 min on ice with 4% paraformaldehyde in PBS. After permeabilization in 0.2% saponin/0.03 M sucrose in 1% BSA/PBS at room temperature for 10 min, cells were washed twice with cold PBS and blocked with 5% normal goat serum in 1% BSA/PBS at room temperature for 1 h before incubation with primary Abs overnight at 4˚C. The slides were washed three times with 1% BSA/PBS, incubated 1 h at room temperature with anti-HA Alexa Fluor 594 anti-mouse IgG and chicken anti-rabbit Alexa Fluor 488 Abs (Invitrogen/Molecular Probes) to reveal the respective primary Abs. Slides were washed three times with 1% BSA/PBS, mounted, and viewed with an Olympus confocal laser scanning microscope under a ×100 oil objective.

Quantitative RT-PCR

Total RNA was extracted from wild-type (WT), trif−/−, and dok3−/− macrophages using a RNeasy Mini kit (Qiagen). cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) as per the manufacturer’s protocol. Quantitative PCR was performed with an Applied Biosystems 7500 real-time PCR machine using the following primers: IFN-β, 5′-CAGCTCCAAAGAAGGACCA-3′ and 5′-GGAGCTGAATCTTCTCAGAT-3′; RANTES, 5′-GCTCTGACATGCTCTGCTC-3′; IL-12p40, 5′-CTGAGTGCAGACACGATGAC-3′ and 5′-GACTCATCCTTCTCTCCCTCG-3′; IL-12p70, 5′-CTGAGTGCAGACACGATGAC-3′ and 5′-GACTCATCCTTCTCTCCCTCG-3′; IL-6, 5′-GGTTCAGCTGCTTCGCTCTG-3′ and 5′-GTTCAGCTGCTTCGCTCTG-3′; TNF-α, 5′-GGTTCAGCTGCTTCGCTCTG-3′ and 5′-GTTCAGCTGCTTCGCTCTG-3′; IFN-γ, 5′-GGTTCAGCTGCTTCGCTCTG-3′ and 5′-GTTCAGCTGCTTCGCTCTG-3′; and 5′-AGGAGCTGCTGCTGCTGCTG-3′ and 5′-CCCATTTCATCTGCTC-3′. The concentrations of IL-12p40, TNF-α (BD Pharmingen, San Diego, CA), IFN-β (PBL InterferonSource), and RANTES (R&D Systems) were determined using commercial ELISA kits.

Determination of IRF3 activation by ELISA

Activated mouse IRF3 was measured in cell nuclear extracts using ELISA according to the manufacturer’s instructions (TransAM; Active Motif). Briefly, a 96-well plate of which oligonucleotides containing the IRF3 consensus binding sites were immobilized were used to measure DNA binding activity of activated IRF3. The activated forms of IRF3 in nuclear cell extracts bind to the oligonucleotides, and the amount bound is subsequently measured using Abs specific to IRF3. Protein concentrations determined by a BCA protein assay kit (Pierce) were normalized across all samples prior to the ELISA and measurement was taken at 450 nm.

Virus infection

Influenza A virus, IVR-116 (A/New Caledonia/20/99 [H1N1] × IRV-6 [H3N2], NIH/NS2, NIBSC code 06/108), was obtained from the National Institute for Biological Standards and Control (Potters Bar, U.K.). The virus was propagated three times in Vero cells (ATCC CCL-81) in OptiPro serum-free medium (Invitrogen, catalog no. 12309-019) supplemented with 5 μg/ml porcine trypsin (Sigma-Aldrich, catalog no. T5266, 1500 N-benzoyl-l-arginine ethyl ester units/mg) and subsequently stored at −80˚C. The virus was thawed at room temperature and used to infect WT and dok3−/− primary macrophage cultures in OptiMEM (Invitrogen) at a multiplicity of infection of 10 per cell for 3, 6, 12, or 24 h. The experiment was performed in triplicates. Total RNA was extracted from virus-infected cells using TRizol (Invitrogen), and viral DNA was detected with semiquantitative RT-PCR using the following primers: forward, 5′-AAGGGCTTTCCAGCAGAAGG-3′; reverse, 5′-CCCCATTTCATCTGCTC-3′. Densitometric analysis of bands obtained in semiquantitative RT-PCR analyses was carried out using a Bio-Rad imaging densitometer and multianalysis software (Bio-Rad Laboratories), and the pixel intensity (absorbance units/mm2) of the specific bands were normalized to that corresponding to β-ACTIN, which acted as loading control, and this ratio was expressed as fold increase in intensity over the control non-stimulated sample.

Expression of recombinant proteins in bacteria and kinase activity assays

The coding region of DOK3, BTK, and kinase-dead BTK (K430R) were cloned into pDEST41 (Pharmacia Biotech) and transformed into Escherichia coli BL21 (DE3) cells (Invitrogen). The cells were then cultured at 37˚C until the OD600 value reached 0.6. Subsequently, isopropyl β-D-thiogalactoside (Invitrogen) was added to the culture media to a final concentration of 0.5 mM to induce protein expression for 2 h at 27˚C. Cells were harvested by centrifugation and lysed by sonication in PBS. GST-tagged proteins were then purified by affinity chromatography with glutathione-Sepharose beads (Amersham Biosciences) under native conditions. The activity of the purified kinases and substrates was determined using the ADP-Glo kinase assay (Promega) as per the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using an unpaired t test (Prism; GraphPad Software, San Diego, CA). A p value <0.05 was considered statistically significant.
Results
DOK3 is required for influenza virus and poly(I:C)-triggered IFN-β production

It was shown previously by deep sequencing that dok3 mRNA was upregulated within 2 h of vaccinia virus infection of HeLa cells (15). However, the relevance of this observation for host antiviral response remains unclear. To determine the significance of this finding, we first examined whether WT macrophages were also able to upregulate dok3 mRNA upon influenza virus infection. As shown in Fig. 1A, dok3 mRNA, as measured by standard RT-PCR, was significantly increased in virus-infected cells. Next we infected WT and dok3−/− macrophages with influenza virus and monitored their production of IFN-β over time. As depicted in Fig. 1B, WT macrophages rapidly synthesized ifn-β mRNA within 6 h of virus infection. However, dok3−/− macrophages had severe impairment of ifn-β mRNA synthesis at 6 h and remained so up to 24 h of infection. As a result, they could not secrete any substantial amount of IFN-β when assayed by ELISA at the 24 h time point after infection compared with WT infected controls (Fig. 1C). Concomitant with the defect in ifn-β gene induction, RT-PCR analysis of influenza virus NS protein mRNA at 12 h postinfection revealed more pronounced presence of the viruses in mutant compared with WT cells (Fig. 1D).

Figure 1. DOK3-deficient macrophages are impaired in IFN-β production and influenza virus clearance. (A) Semiquantitative RT-PCR analyses of dok3 mRNA in WT macrophages at 24 h postinfection with influenza virus. (B) Real-time RT-PCR analyses of ifn-β mRNA synthesis in WT and dok3−/− macrophages infected with influenza virus for various times. (C) ELISA measurement of IFN-β secretion by WT and dok3−/− macrophages after 24 h of influenza virus infection. (D) Semiquantitative RT-PCR analysis of influenza virus NS RNA in WT and dok3−/− macrophages at 12 h postinfection. Densitometry was performed on the band corresponding to the influenza NS gene and normalized to that of β-ACTIN and this was set as 1 for the respective uninfected samples. The numerical values after comparison with control samples are provided below each corresponding lane. (E) Quantitative real-time PCR analysis of influenza virus NS mRNA in WT and dok3−/− macrophages at 24 h postinfection. (F) Quantitative real-time PCR analyses of ifn-β mRNA synthesis in WT, dok3−/−, and trif−/− macrophages stimulated with naked poly(I:C) for 2 h. (G) ELISA measurement of IFN-β secretion by poly(I:C)-stimulated WT and dok3−/− macrophages at 6 h postinfection. Quantitative real-time PCR analyses of IFN-β mRNA synthesis in WT and dok3−/− macrophages stimulated with (H) liposome-enclosed poly(I:C), (I) poly(deoxyadenylc-thymidylic) acid [p(dA:dT)], and (J) CpG-ODN are shown. Control experiments represented uninfected or non-stimulated cells. Expression of ifn-β mRNA was normalized to that of β-actin mRNA. Triplicate experiments were performed. *p < 0.005.
This was further confirmed by real-time PCR quantification of influenza virus NS mRNA at 24 h postinfection, which showed the presence of greater amounts of viral NS mRNA in virus-infected dok3−/− macrophages compared with WT cells (Fig. 1E). Taken together, these data suggest that DOK3 plays a positive role in mediating IFN-β production, as its absence impairs ifn-β mRNA synthesis and IFN-β protein secretion during host antiviral response, thereby allowing the accumulation of a greater amount of viruses inside the mutant cells.

Influenza virus replicates via a dsRNA intermediate, and this is recognized by TLR3 and RIG-I (17). To address whether DOK3 could play a role in the signaling of these two innate immune receptors, we stimulated WT and dok3−/− cells with naked poly(I:C) to engage only TLR3 or transfected them with liposome-enclosed poly(I:C) to engage RIG-I. Our results indicated that WT macrophages readily synthesize ifn-β mRNA when treated with naked poly(I:C). Alternatively, dok3−/− macrophages behaved similar to trif−/− macrophages in that they could not respond to naked poly(I:C) stimulation (Fig. 1F). To corroborate this finding, we also measured IFN-β protein production and showed that dok3−/− macrophages were impaired in IFN-β secretion when stimulated with naked poly(I:C) for 6 h (Fig. 1G). As it was possible that DOK3 deficiency could affect other cytokine responses during TLR3 signaling, we also examined IL-12 (Supplemental Fig. 1A) and TNF-α (Supplemental Fig. 1B) production in naked poly(I:C)-stimulated WT and dok3−/− macrophages and found them to be unaffected in the absence of DOK3. Next we examined the response of WT and dok3−/− macrophages to RIG-I engagement by treating them with liposome-enclosed poly(I:C) or poly(I:C)/LyoVec. Mutant macrophages were also impaired in ifn-β mRNA synthesis when poly(I:C) was transfected into the cytosol of these cells using LyoVec (Fig. 1H). We further stimulated WT and dok3−/− macrophages with dsDNA/poly(deoxyadenylic-thymidylic) acid or CpG ODN, both of which were also known to induce IFN-β production in macrophages, and found that ifn-β mRNA synthesis was unaffected by these stimuli in mutant cells (Fig. 1I, 1J). Hence, the data indicate that DOK3 is likely to be specifically involved in macrophage responses to RNA ligands through the engagement of TLR3 and RIG-I and leads to IFN-β production.

DOK3 is phosphorylated in macrophages upon poly(I:C) stimulation

DOK3 is phosphorylated upon B cell Ag receptor engagement (6). Hence, we examined whether DOK3 would also be phosphorylated when WT macrophages were treated with naked poly(I:C) or transfected with poly(I:C)/LyoVec as a further indication that the adaptor could be involved in TLR3 and RIG-I signaling. Western blot analysis of immunoprecipitated DOK3 revealed that the protein was indeed tyrosine phosphorylated in naked poly(I:C)-stimulated WT macrophages (Fig. 2A). This phosphorylation was dependent on TRIF signaling, as it was abrogated in naked poly(I:C)-stimulated trif−/− macrophages (Fig. 2B). As a further indication that DOK3 was involved in TRIF signaling, gene induction of the TRIF-dependent chemokines Rantes (Fig. 2C) and IP10 (Fig. 2D) were also defective in naked poly(I:C)-stimulated dok3−/− macrophages. We also measured the secretion of RANTES protein and confirmed that it was indeed defective in naked poly(I:C)-stimulated dok3−/− macrophages (Fig. 2E). Furthermore, we showed that DOK3 was phosphorylated in poly(I:C)/LyoVec-transfected WT macrophages, which signal via RIG-I (Fig. 2F). Taken together, the data indicate that DOK3 is phosphorylated upon TLR3 and RIG-I engagement and is required for these receptors to signal IFN-β production. For subsequent experiments, we mainly used naked poly(I:C) stimulation of macrophages, which we showed previously to engage only TLR3 (16), to study the role of DOK3 in the induction of the IFN response.

DOK3 deficiency impairs the activation of IRF3

Expression of ifn-β mRNA requires the coordinated actions of three major transcription factors, those of AP-1, NF-κB, and IRF3 (18). It remains to be determined whether the absence of DOK3 would affect any of these signaling pathways downstream of poly(I:C) stimulation in macrophages. The induction of AP-1 is dependent on MAPK signaling (19). Because DOK1 and 2 are
known to participate in Ras-ERK signaling downstream of TLR4 (11) and growth receptors (20), and DOK3 is involved in JNK signaling downstream of immune receptors (21), we examined whether ERK, JNK, and p38 MAPK signaling would be affected in poly(I:C)-stimulated \( \text{dok3}^{-/-} \) macrophages. Surprisingly, the induction of these MAPKs was largely normal in the mutant cells (Supplemental Fig. 2A). We next examined the activation of NF-κB and again found this signaling pathway to be intact, as the kinetics of degradation of the inhibitory IκB subunit was comparable between poly(I:C)-stimulated WT and \( \text{dok3}^{-/-} \) macrophages (Supplemental Fig. 2B). Thus, DOK3 deficiency did not affect NF-κB or MAPK signaling in naked poly(I:C)-stimulated macrophages. Finally, we investigated the activation of IRF3 in poly(I:C)-stimulated \( \text{dok3}^{-/-} \) macrophages. This transcription factor is activated by serine phosphorylation and translocates from the cytoplasm to the nucleus of cells to bind the \( \text{ifn-} \) gene promoter (22).

Other than nuclear translocation, we also examined IRF3 activation by phosphorylation. Indeed, IRF3 activation as indicated by Ser396 phosphorylation was also defective in poly(I:C)-stimulated \( \text{dok3}^{-/-} \) macrophages (Fig. 3C). Finally, our ELISA measuring IRF3 promoter/DNA binding activity demonstrated that IRF3 was indeed activated in poly(I:C)-stimulated WT but not \( \text{dok3}^{-/-} \) macrophages (Fig. 3D). Taken together, our data indicate

**FIGURE 3.** Defective IRF3 phosphorylation and nuclear localization in poly(I:C)-stimulated macrophages lacking DOK3. (A) Confocal microscopy examining IRF3 nuclear localization in nontreated (media) and poly(I:C)-stimulated WT, \( \text{dok3}^{-/-} \), \( \text{trif}^{-/-} \), and \( \text{tlr3}^{-/-} \) macrophages. Cells were stained with FITC-labeled anti-IRF3 Ab (green). Nuclei of cells were stained with DAPI (blue). Original magnification \( \times 100 \). (B) Western blot analysis examining nuclear IRF3 in poly(I:C)-stimulated WT and \( \text{dok3}^{-/-} \) macrophages. Nuclear extracts from nontreated and stimulated cells were first probed with anti-IRF3 Ab and subsequently with anti-HDAC1 Ab to serve as loading control. (C) Western blot analysis examining IRF3 phosphorylation in poly(I:C)-treated WT and \( \text{dok3}^{-/-} \) macrophages. Cells were stimulated for various times and whole-cell lysates were probed with an Ab that recognized phospho-Ser\(^{396}\) residue of IRF3. The IRF3, tubulin, and \( \beta \)-actin blots were included as loading controls. Data shown are representative of more than three independent experiments. (D) IRF3 promoter/DNA-binding activity was measured using poly(I:C)-stimulated nuclear extract proteins obtained at 2 h time point from WT and \( \text{dok3}^{-/-} \) macrophages and assayed using an IRF3 TransAM (Active Motif) ELISA kit. Data shown are representative of two independent experiments.
that DOK3 deficiency specifically impairs IFR3 but not NF-κB or MAPK signaling and that it likely affects IFR3 activation farther upstream in the signaling cascade.

**DOK3 is required for TBK1 activation and synergizes with TBK1 to induce IFN-β gene expression**

The lack of IFR3 phosphorylation in poly(I:C)-stimulated dok3−/− macrophages prompted us to examine whether the activation of kinases required for IFR3 phosphorylation would be compromised. Two kinases are important for the activation of IFR3, namely TBK1 (23) and AKT (24), and the activation of AKT is further dependent on TBK1 during TLR3 signaling (25). Our Western blot analyses indicated that the activation of AKT, as indicated by phosphorylation of its Thr308 and Ser473 residues, was defective in poly(I:C)-stimulated dok3−/− macrophages (Fig. 4A). More importantly, the activation of TBK1, as determined by examining the phosphorylation of its Ser172 residue, was also impaired in poly(I:C)-stimulated dok3−/− macrophages (Fig. 4B). These data together indicated that the impairment in IFR3 activation was mainly due to defective TBK1 activation in poly(I:C)-stimulated dok3−/− macrophages. To better understand the relationship between DOK3 and TBK1, we overexpressed DOK3 or TBK1 or both in HEK293T cells and examined their effect in inducing Ifn-β promoter activity using a luciferase-based reporter assay (Fig. 4C). DOK3 alone had no effect on the induction of Ifn-β promoter activity whereas TBK1 alone could induce a certain level of activity. Interestingly, when DOK3 was expressed in increasing concentrations with TBK1 in HEK293T cells, there was substantial induction of Ifn-β promoter activity that was significantly higher than that achieved with TBK1 alone. Hence, DOK3 acts in concert with TBK1 to further induce Ifn-β promoter activation.

**DOK3 binds TBK1 and TRAF3 and is required for TRAF3/TBK1 complex formation**

Our data so far suggested that DOK3 acted at the level of TBK1 activation in poly(I:C)-stimulated macrophages (Fig. 4). TBK1 is known to form a complex with TRAF3 downstream of TLR3, RIG-I, and other innate immune receptor signaling to activate IFR3 (26). Furthermore, in TLR3 signaling, the TRAF3/TBK1 complex is recruited to the TRIF adaptor following activation (27). Hence, we first examined whether TRAF3/TBK1 complex formation would be compromised in the absence of DOK3. As shown in Fig. 5A, we were able to coimmunoprecipitate both TRAF3 with TBK1 (top panel) and TBK1 with TRAF3 (bottom panel) in poly(I:C)-stimulated WT macrophages but were unable to do so in the mutant cells, indicating that TRAF3 and TBK1 interaction did not occur when DOK3 was absent. Because DOK3 is an adaptor protein, we directly assessed whether it could bind either TBK1 or TRAF3 or both. Our overexpression studies indicated that TBK1 could coinmuno precipitate with DOK3 (Fig. 5B), suggesting that DOK3 physically binds TBK1. We also performed endogenous coimmunoprecipitation experiments using WT macrophages and surprisingly found DOK3 and TBK1 to be constitutively bound to each other (Fig. 5C, left and right panels). We also examined the interaction between TRAF3 and DOK3 and demonstrated that the two molecules bind each other (Fig. 5B). Furthermore, the binding of TRAF3 to DOK3 appeared to be more intense than the binding of TRAF2 or TRAF6 to DOK3, suggesting that the binding of TRAF3 to DOK3 is specific (Supplemental Fig. 3A). We also tested the binding of DOK3 to TRIF and showed that the two molecules did not bind each other, unlike the interaction of TRAF3 with TRIF, which were known to interact with each other (28) and served as controls for our studies (Supplemental Fig. 3B). These data together suggest that DOK3 likely acts as a bridge to facilitate the formation of the TRAF3/TBK1 complex that is necessary for TBK1 and IFR3 activation, leading to IFN-β production.

**DOK3 is a multidomain adaptor containing a PH, PTB, and C-terminal domain**

To better understand the interaction between TBK1 and DOK3, we expressed various mutant forms of DOK3 bearing specific or combinations of different domains (as illustrated in Fig. 5D) and examined their abilities to further enhance TBK1 activation of Ifn-β promoter activity. It was apparent from these studies that the C-terminal domain of DOK3 was required to act in concert with TBK1 to induce Ifn-β promoter activity, as its deletion alone (as in the mutant form D2) substantially impaired the induction of the reporter luciferase gene expression (Fig. 5E). This finding together with the data obtained in Fig. 5B also implied that the C-terminal domain of DOK3 might be responsible for binding TBK1. To test this possibility, we overexpressed the various mutant forms of DOK3 and TBK1 in HEK293T cells and examined their physical associations via coimmunoprecipitation experiments (Fig. 5F). Indeed, TBK1 could coimmunoprecipitate with full-length DOK3 as well as the mutant forms bearing the PTB and C-terminal domains (D4) or the mutant form bearing only the C-terminal domain (D5), indicating that the C-terminal domain of DOK3 binds TBK1. Similar experiments also indicated that the C-terminal domain of DOK3 binds TRAF3 (Fig. 5G). Hence, DOK3 binds both TBK1 and TRAF3 via its C-terminal domain.

**DOK3 is phosphorylated by BTK in poly(I:C)-induced signaling pathway**

Because DOK3 is phosphorylated upon poly(I:C) stimulation (Fig. 2A), we were also interested to identify the kinase responsible for this event. We recently demonstrated a role for BTK in TLR3 signaling (16) and wondered whether BTK could phosphorylate DOK3. To examine this possibility, we immunoprecipitated DOK3 from poly(I:C)-stimulated WT and btk−/− macrophages and showed that the adaptor protein was not phosphorylated in the mutant cells (Fig. 6A). To definitively demonstrate that TLR3-induced DOK3 phosphorylation is BTK-dependent, we also immunoprecipitated DOK3 from poly(I:C)-stimulated but
BTK-inhibited (LFM-A13) or control DMSO-treated macrophages and showed that the adaptor protein was also not phosphorylated in LFM-A13–treated and therefore BTK-inhibited cells (Fig. 6B). Furthermore, coexpression of DOK3 with BTK or with a constitutively active form of BTK (E41K) led to the phosphorylation of DOK3 in HEK293T cells and coexpressed with anti-FLAG Ab and immunoblotted (IB) with anti-HA Ab to examine possible association of DOK3 with TBK1 and TRAF3. The FLAG immunoblot was included to show equivalent immunoprecipitation of FLAG-tagged DOK3 whereas whole-cell lysates (WCL) were immunoblotted to demonstrate expression of HA-tagged TBK1 or TRAF3 proteins. (C) Western blot analyses of endogenous TBK1/DOK3 interaction in primary macrophages that were nontreated (0 min) or stimulated for 60 min with naked poly(I:C). Western blots were performed as in (A). (D) Pictogram depicting FLAG-tagged full-length DOK3 protein or mutants harboring various combinations of PH, PTB, or tyrosine-rich carboxyl terminal domains. (E) The C-terminal domain of DOK3 acts in concert with TBK1 to further induce ifn-β promoter activity. HEK293T cells were transfected with empty vectors (control) or vectors carrying TBK1 and/or DOK3 or various mutant forms of DOK3 and assayed for the induction of luciferase activity as in Fig. 4C. Data shown are representative of three independent experiments. *p < 0.001. (F and G) The C-terminal domain of DOK3 binds TBK1 and TRAF3. HEK293T cells were transfected with either HA-tagged TBK1 (F) or HA-tagged TRAF3 (G) together with FLAG-tagged full-length DOK3 or various DOK3 mutants. Cell lysates were immunoprecipitated with anti-HA Ab and immunoblotted with anti-FLAG Abs to examine for association and with anti-HA for immunoprecipitation control. Whole-cell lysates were also included to ensure expression of the various transfected constructs. *NS, nonspecific band. Data shown are representative of three independent experiments.

FIGURE 5. DOK3 is required for TRAF3/TBK1 complex formation and binds TRAF3 and TBK1 via its C-terminal domain. (A) Defective formation of TRAF3/TBK1 complex in poly(I:C)-stimulated dok3−/− macrophages. WT and dok3−/− macrophages were nontreated or stimulated with poly(I:C) for 60 min and TRAF3 was immunoprecipitated (IP) to examine TBK1 association (top panel) and vice versa with TBK1 immunoprecipitated to examine TRAF3 association (bottom panel) via Western blot analyses. (B) DOK3 binds TRAF3 and TBK1. HEK293T cells were cotransfected with FLAG-tagged DOK3 and HA-tagged TBK1 or HA-tagged TRAF3 and cell lysates were immunoprecipitated with anti-FLAG Ab and immunoblotted (IB) to examine possible association of DOK3 with TBK1 and TRAF3. The FLAG immunoblot was included to show equivalent immunoprecipitation of FLAG-tagged DOK3 whereas whole-cell lysates (WCL) were immunoblotted to demonstrate expression of HA-tagged TBK1 or TRAF3 proteins. (C) Western blot analyses of endogenous TBK1/DOK3 interaction in primary macrophages that were nontreated (0 min) or stimulated for 60 min with naked poly(I:C). Western blots were performed as in (A). (D) Pictogram depicting FLAG-tagged full-length DOK3 protein or mutants harboring various combinations of PH, PTB, or tyrosine-rich carboxyl terminal domains. (E) The C-terminal domain of DOK3 acts in concert with TBK1 to further induce ifn-β promoter activity. HEK293T cells were transfected with empty vectors (control) or vectors carrying TBK1 and/or DOK3 or various mutant forms of DOK3 and assayed for the induction of luciferase activity as in Fig. 4C. Data shown are representative of three independent experiments. *p < 0.001. (F and G) The C-terminal domain of DOK3 binds TBK1 and TRAF3. HEK293T cells were transfected with either HA-tagged TBK1 (F) or HA-tagged TRAF3 (G) together with FLAG-tagged full-length DOK3 or various DOK3 mutants. Cell lysates were immunoprecipitated with anti-HA Ab and immunoblotted with anti-FLAG Abs to examine for association and with anti-HA for immunoprecipitation control. Whole-cell lysates were also included to ensure expression of the various transfected constructs. *NS, nonspecific band. Data shown are representative of three independent experiments.
assayed at 24 h after transfection for luciferase activity. Data shown are promoter luciferase (IFN-$\beta$-luc) reporter and Renilla luciferase and assayed at 24 h after transfection for luciferase activity. Data shown are representative of two independent experiments. *$p < 0.05$.

**FIGURE 6.** BTK phosphorylates DOK3. (A) DOK3 is not phosphorylated in poly(I:C)-stimulated hbk$^{-/-}$ macrophages. Western blot analysis of DOK3 phosphorylation in WT and hbk$^{-/-}$ macrophages upon poly(I:C) stimulation is shown. Cells were stimulated with poly(I:C) and cell lysates were immunoprecipitated (IP) with an anti-DOK3 Ab or control IgG Abs and immunoblotted (IB) with an anti-phosphotyrosine Ab (4G10). Membranes were subsequently stripped and immunoblotted with DOK3 Ab for loading control. (B) DOK3 is phosphorylated in poly(I:C)-stimulated but not BTK-inhibited macrophages. Cells were treated with DMSO as control or with the BTK-inhibitor LFM-A13, and DOK3 was immunoprecipitated and probed with anti-phosphotyrosine Ab and reprobed with anti-DOK3 Ab as control. (C) BTK kinase activity is required for DOK3 phosphorylation. HEK293T cells were nontransfected or transfected with FLAG-tagged DOK3 together with HA-tagged BTK (K430R). Cell lysates were immunoprecipitated with anti-FLAG Ab and immunoblotted with anti-phosphotyrosine (4G10) or anti-FLAG Abs. Whole-cell lysates (WCL) were included to show expression of the transfected gene constructs. (D) BTK physically binds DOK3. HEK293T cells were transfected with FLAG-tagged DOK3 and with or without HA-tagged BTK. Cell lysates were immunoprecipitated with anti-FLAG Ab and immunoblotted with anti-HA Ab. Whole-cell lysates were included to indicate expression of the transfected gene constructs. (E) In vitro kinase phosphorylation assay examining the activities of purified WT and kinase-dead BTK (K430R) kinases on DOK3 as substrate as measured with the ADP-Glo kinase assay. Data are plotted as nanomoles phosphate transferred from ATP to a specific substrate. (F) BTK acts in concert with DOK3 and TBK1 to further increase $ifn-\beta$ gene expression. HEK293T cells were transfected with various combinations of vectors bearing TBK1, DOK3, and BTK together with plasmids encoding IFN-$\beta$ promoter luciferase (IFN-$\beta$-luc) reporter and Renilla luciferase and assayed at 24 h after transfection for luciferase activity. Data shown are representative of two independent experiments. $p < 0.05$.

**Discussion**

Our present study arises as a follow-up of a published observation indicating that dok3 mRNA was induced during virus infection (15). Thus, we explored the role of DOK3 in innate immunity by employing poly(I:C)-stimulation and influenza virus infection of dok3$^{-/-}$ macrophages and demonstrated that the adaptor protein was required for the induction of IRF3 and production of IFN-$\beta$. Further biochemical characterizations indicated that DOK3 could bind both TRAF3 and TBK1 but not TRIF, and that in the absence of DOK3, the formation of the TRAF3/TBK1 complex and the activation of TBK1, as indicated by its Ser$^{172}$ phosphorylation, was defective. These data suggest that DOK3 acts as a bridge to bring TRAF3 and TBK1 together and is also needed for TBK1 activation that is required for the subsequent induction of IRF3 and IFN-$\beta$ production.

It is currently not known how TBK1 is activated by phosphorylation. A recent protein crystallization study suggested that TBK1 was activated by transautophosphorylation (30). According to this model, TBK1 is first maintained in an inactive state until recruited to a signaling platform where it can undergo autoactivation owing to greater concentration of the kinase present or activation by another effector kinase that is recruited to the same scaffold. Our current findings fit into this model in that we show DOK3 binds TBK1 and that DOK3 is needed for TBK1 activation by phosphorylation and TBK1 complex formation with TRAF3. Thus, DOK3 could be part of the signaling scaffold needed for the recruitment and activation of TBK1. We further identified the SH2-target motif of DOK3 to be important for its interaction with TBK1, and consistent with this finding, coexpression of DOK3 with TBK1 synergizes IFN-$\beta$-promoter activity whereas a truncated DOK3 lacking the C terminus did not manifest this synergy. Hence, these data strongly supported the hypothetical model in which DOK3 is the scaffold that serves to concentrate TBK1 for activation, either through autophosphorylation or by a different effector kinase. Additionally, given that the TRAF3/TBK1 complex is formed downstream of many immune signaling pathways (31), our data further suggest that DOK3 could be involved in the signaling of innate immune receptors other than TLR3 and RIG-I. This notion could be explored in future experiments. Alternatively, our stimulation with dsDNA and CpG suggested that DOK3 might be specific for the innate sensing of RNA ligands.

We recently demonstrated a role for BTK in phosphorylating TLR3 during an antiviral response (16). In the present study, we identify another target of BTK in TLR3 signaling by showing that it could also phosphorylate DOK3 upon poly(I:C) stimulation. Hence, there seems to be a broader and more coordinated role for BTK in the antiviral response. A global proteome study of BTK’s binding partners could help to identify new targets of BTK in the host antiviral response.

Myeloid cells express DOK1–3 (1). DOK1 and 2 had been shown to play a role in TLR4 signaling where they negatively regulate ERK activation and TNF-$\alpha$ production in macrophages (11). Previously, DOK3 has largely been characterized in the context of B cell receptor (32) and Fc$\gamma$RIIb (33) signaling where it serves as a negative regulator by interacting with Grb2 and the inhibitor SHIP to restrict the intensity of Ca$^{2+}$ mobilization (7) and limit JNK activation (8). While this manuscript was being prepared, a report was published describing DOK3 as a negative regulator of TLR4 signaling in the same manner as DOK1 and 2 by inhibiting ERK as it was degraded upon LPS but not poly(I:C) stimulation (12). In contrast, we demonstrate here a positive role for DOK3 in the induction of IFN-$\beta$ production during virus infection and poly(I:C) stimulation of macrophages. We observed that DOK3 did not undergo degradation upon poly(I:C) stimulation, consistent with previous findings (12). Its presence facilitates TBK1 and TRAF3 interaction during virus infection and likely enables maximal transduction of downstream signaling for optimal IFN-$\beta$ production.

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Disclosures
The authors have no financial conflicts of interest.

References