Selective control of type I IFN induction by the Rac activator DOCK2 during TLR-mediated plasmacytoid dendritic cell activation

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Plasmacytoid dendritic cells (pDCs) play a key role in antiviral immunity, but also contribute to the pathogenesis of certain autoimmune diseases, by producing large amounts of type I IFNs. Although activation of pDCs is triggered by engagement of nucleotide-sensing Toll-like receptors (TLR) 7 and 9, type I IFN induction additionally requires IκB kinase (IKK) α–dependent activation of IFN regulatory factor (IRF) 7. However, the signaling pathway mediating IKK–α activation is poorly defined. We show that DOCK2, an atypical Rac activator, is essential for TLR7- and TLR9-mediated IFN-α induction in pDCs. We found that the exposure of pDCs to nucleic acid ligands induces Rac activation through a TLR-independent and DOCK2-dependent mechanism. Although this Rac activation was dispensable for induction of inflammatory cytokines, phosphorylation of IKK–α and nuclear translocation of IRF–7 were impaired in Dock2-deficient pDCs, resulting in selective loss of IFN-α induction. Similar results were obtained when a dominant-negative Rac mutant was expressed in wild-type pDCs. Thus, the DOCK2–Rac signaling pathway acts in parallel with TLR engagement to control IKK–α activation for type I IFN induction. Owing to its hematopoietic cell-specific expression, DOCK2 may serve as a therapeutic target for type I IFN–related autoimmune diseases.

Abbreviations used: DOTAP, 1,2-dioleoyloxy-3-trimethylammonium-propane; GEF, guanine nucleotide exchange factor; IKK, IκB kinase; IRAK, IL-1 receptor-associated kinase; IRF, IFN regulatory factor; MOI, multiplicity of infection; pDC, plasmacytoid DC; TRK, transferrin receptor; TLR, Toll-like receptor.

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the signaling cascades leading to type I IFN induction are not completely defined. Elucidation of this regulatory mechanism would be clinically important because type I IFNs produced by pDCs have been implicated in the pathogenesis of autoimmune diseases such as psoriasis and systemic lupus erythematosus (Blanco et al., 2001; Nestle et al., 2005; Lande et al., 2007).

Rac is a member of the Rho family of GTPases that function as molecular “switches” by cycling GDP-bound inactive states and GTP-bound active states. Once activated, Rac interacts with multiple downstream effectors to regulate various cellular functions including actin reorganization and gene expression. Stimulus-induced formation of the active GTP-bound Rac is mediated by guanine nucleotide exchange factors (GEFs). DOCK2 is a member of the CDM family of proteins (Caenorhabditis elegans CED-5, mammals DOCK180, and Drosophila melanogaster myoblast city) and is predominantly expressed in hematopoietic cells (Reif and Cyster, 2002). Although DOCK2 does not contain the Dbl homology domain and the pleckstrin homology domain that are typically found in GEFs, DOCK2 catalyzes the GTP–GDP exchange of Rac via its Docker (also known as DHR-2) domain (Brugnera et al., 2002; Côté and Vuori, 2002). There is accumulating evidence that DOCK2 functions downstream of chemokine receptors and regulates migration of certain subsets of immune cells (Fukui et al., 2001; Nombela-Arrieta et al., 2004, 2007; Kunisaki et al., 2006; Shulman et al., 2006; Nishikimi et al., 2009). Indeed, pDCs, but not myeloid DCs, from Dock2-deficient (Dock2−/−) mice exhibit a severe defect in chemokine-induced Rac activation, resulting in reduction of motility and the loss of polarity during chemotaxis (Gotoh et al., 2008). Thus, DOCK2 is a major Rac GEF that controls migration of pDCs. However, the role of DOCK2 in TLR-mediated pDC activation remains unknown.

In this paper, we present evidence that DOCK2 controls TLR7- and TLR9-mediated IFN-α induction in pDCs via Rac activation. This Rac activation occurred independently of TLR engagement but was critically required for TLR-mediated IKK-α activation and type I IFN induction. Our results thus define a novel regulatory mechanism controlling type I IFN induction in pDCs.

RESULTS AND DISCUSSION

DOCK2 is required for TLR7/9-mediated type I IFN induction in pDCs

To examine the role of DOCK2 in TLR-mediated cytokine production, we first measured serum IFN-α and IL-12p40 after intravenously injecting TLR ligands into WT and Dock2−/− mice. When A-type CpG DNA (D19), a TLR9 ligand (Vertehelyi et al., 2001), was administered, both WT and Dock2−/− mice comparably produced IL-12p40 (Fig. 1 A). Similar results were obtained with a TLR7 ligand R848 (Fig. 1 A; Hemmi et al., 2002), indicating that DOCK2 deficiency does not affect TLR7/9-mediated inflammatory cytokine production. Surprisingly, however, both CpG-A and R848 failed to induce IFN-α production in Dock2−/− mice (Fig. 1 A).

Although pDCs are normally generated in the BM in the absence of DOCK2, Dock2−/− mice exhibit a severe reduction of pDCs in the spleen and lymph nodes (Gotoh et al., 2008). Therefore, this mislocalization of pDCs may have affected TLR7/9-mediated IFN-α production in vivo. To determine more precisely the role of DOCK2 in type I IFN induction, we purified pDCs from Flt3 ligand–induced BM-derived DCs and stimulated them in vitro with CpG-A, R848, and another TLR9 ligand CpG-B (1668; Krieg et al., 1995). Although WT pDCs produced considerable amounts of IFN-α and IFN-β in response to these TLR ligands, TLR7/9-mediated type I IFN induction was severely impaired in Dock2−/− pDCs (Fig. 1, B–D). Similar results were obtained when naive BM pDCs were stimulated with CpG-A (Fig. 1 E). In contrast, induction of IL-12p40 and IL-6 and up-regulation of costimulatory molecules occurred normally in Dock2−/− pDCs (Fig. 1 F and not depicted). These results indicate that DOCK2 selectively controls type I IFN induction pathway during TLR7/9-mediated pDC activation.

Influenza A virus and HSV type 2 (HSV-2) are known to activate the TLR7 and TLR9 signaling pathways, respectively (Lund et al., 2003; Diebold et al., 2004). To examine whether DOCK2 is also important for virus-induced IFN-α production, WT and Dock2−/− pDCs were stimulated with these viruses in vitro. Although WT pDCs produced large amounts of IFN-α depending on a multiplicity of infection (MOI), production of IFN-α by Dock2−/− pDCs was markedly suppressed at any MOIs tested (Fig. 1 G). Similar results were obtained when heat- or UV-inactivated virus and purified HSV-2 DNA were used (Fig. 1, H and I), indicating that DOCK2 controls IFN-α induction independently of virus replication. In contrast, such a drastic difference was not found in IL-12p40 production, although Dock2−/− pDCs produced less IL-12p40 than WT pDCs at a certain MOI (Fig. 1 G).

DOCK2 controls TLR7/9-mediated type I IFN induction via Rac activation

To explore the mechanism by which DOCK2 controls type I IFN induction, we first compared ligand uptake between WT and Dock2−/− pDCs. The Cy5-labeled CpG-A was comparably incorporated into both types of pDCs (Fig. 2 A), indicating that DOCK2 deficiency does not affect CpG-A uptake. However, although WT pDCs exhibited a localized accumulation of F-actin in response to CpG-A, F-actin polymerization, as previously suggested in macrophages (Sanjuan et al., 2006), appeared to be triggered at the cell surface, independently of TLR9 engagement. Indeed, after CpG-A stimulation, Thr9−/− pDCs also exhibited polarized morphology with focused distribution of F-actin (Fig. 2 B). In human pDCs, it has been reported that CXCL16, a scavenger receptor, recognizes CpG-A at the cell surface and mediates its uptake (Gursel et al., 2006). However, CXCL16 deficiency did not affect CpG-A uptake, actin polymerization,
and IFN-α induction in mouse pDCs (Fig. S1, A–C). In addition, CpG-A uptake and actin polymerization were insensitive to pertussis toxin and the Src family kinase inhibitor PP2 (Fig. S1, D and E), ruling out the possible involvement of G protein-coupled receptors and tyrosine kinase signaling pathways in these cellular functions, although treatment with PP2, but not pertussis toxin, inhibited induction of both IFN-α and IL-12p40 in WT pDCs (Fig. S1 F).

Having found that CpG-A induces actin polymerization in TLR9-independent and DOCK2-dependent mechanisms, we compared Rac activation among WT, Th9<sup>−/−</sup>, and Dock2<sup>−/−</sup> pDCs. In response to CpG-A, Rac was activated in Th9<sup>−/−</sup> pDCs to the same extent and at the same kinetics as in WT pDCs (Fig. 2 D). However, CpG-A–induced Rac activation was almost totally abolished in Dock2<sup>−/−</sup> pDCs (Fig. 2 D). This defect in Rac activation was also observed when Dock2<sup>−/−</sup> pDCs were stimulated with other TLR ligands such as R848, influenza A virus, HSV-2, and purified HSV-2 DNA (Fig. 2, E–G). In contrast, Rac activation and actin polymerization occurred normally in Dock2<sup>−/−</sup> myeloid DCs stimulated with CpG-A (Fig. S2), indicating that the effect of DOCK2 deficiency on TLR ligand–induced Rac activation is limited to pDCs.

To directly examine whether Rac activation is involved in type I IFN induction in pDCs, we retrovirally transduced WT pDCs to express a dominant-negative Rac mutant and IFN-α induction in mouse pDCs (Fig. S1, A–C). In addition, CpG-A uptake and actin polymerization were insensitive to pertussis toxin and the Src family kinase inhibitor PP2 (Fig. S1, D and E), ruling out the possible involvement of G protein-coupled receptors and tyrosine kinase signaling pathways in these cellular functions, although treatment with PP2, but not pertussis toxin, inhibited induction of both IFN-α and IL-12p40 in WT pDCs (Fig. S1 F).

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(T17N-Rac). Compared with the cells expressing the WT Rac control, the expression of T17N-Rac markedly suppressed CpG-A–induced IFN-α production without affecting IL-12p40 levels (Fig. 2 H). Similarly, the expression of T17N-Rac abrogated influenza A virus–mediated induction of IFN-α, but not IL-12p40, in WT pDCs (Fig. 2 I). These

Figure 2. DOCK2 controls TLR7/9-mediated type I IFN induction in pDCs via Rac activation. (A) The uptake of CpG-A was compared between WT and Dock2−/− pDCs after cells were incubated with 3 µM CpG-A–Cy5 at 37°C (filled area) or 4°C (open area) for 1 h. Before assay, cell surface fluorescence was quenched with 0.2% trypan blue. Representative profiles of three independent experiments are shown. (B and C) BM-derived pDCs from WT, Dock2−/−, and Tlr9−/− mice were stimulated with 3 µM CpG-A for the indicated times (B) or CpG-A–coated polystyrene beads for 1 min (C). Cells were then fixed and stained with phalloidin. Representative images of two independent experiments are shown. DIC, differential interference contrast. Bars, 10 µm. (D–G) Activation of Rac was analyzed for BM-derived pDCs from WT (+/+), Dock2−/−, and Tlr9−/− mice after stimulation with 3 µM CpG-A (D), 100 nM R848 (E), influenza A virus, or HSV-2 at a MOI of 0.5 (F) or 2.18 µg/ml HSV-2 DNA (G) for the indicated times. Data are representative of three (D and F) or two (E and G) independent experiments. (H and I) BM-derived WT pDCs were retrovirally transduced to express either T17N-Rac–IRES–GFP or WT Rac–IRES–GFP. After fluorescence-activated cell sorting, GFP-positive cells (H, 4 × 10⁴ cells per well; I, 6 × 10⁴ cells per well) were stimulated with 3 µM CpG-A (H) or influenza A virus at an MOI of 0.5 (I) for 24 h. Data indicate the levels of IFN-α and IL-12p40 in cell culture supernatants (mean ± SD of triplicate wells) and are representative of five (H) and two (I) independent experiments. **, P < 0.001; *, P < 0.01. ND indicates below the detectable limit.
results indicate that DOCK2 controls TLR7/9-mediated type I IFN induction in pDCs through Rac activation.

Type I IFN signaling and TLR engagement are unaffected in Dock2<sup>−/−</sup> pDCs

Although activation of basally expressed IRF-7 induces type I IFN gene expression, robust type I IFN production requires IFN signal-dependent IRF-7 expression. This positive-feedback regulation involves a complex called IFN-stimulated gene factor 3, which is composed of STAT-1, STAT-2, and IRF-9 (Honda et al., 2006). As CpG-A–mediated If7 induction was significantly reduced in Dock2<sup>−/−</sup> pDCs at 3 h after stimulation (Fig. 3 A), we examined whether DOCK2 functions downstream of type I IFN receptors. Although CpG-A–induced STAT-1 phosphorylation was impaired in Dock2<sup>−/−</sup> pDCs (Fig. 3 B), STAT-1 was normally phosphorylated in these cells in response to IFN-α (Fig. 3 C). Consistent with this finding, the expression of If7 and Cxcl10, another IFN-inducible gene, were comparably up-regulated in WT and Dock2<sup>−/−</sup> pDCs after stimulation with IFN-α (Fig. 3 D). These results suggest that DOCK2 controls the initial IFN induction pathway rather than the IFN signal–dependent positive-feedback loop.

Both TLR7 and TLR9 reside in the ER and are recruited to endolysosomes upon stimulation to detect internalized ligands (Latz et al., 2004; Leifer et al., 2004). As TLR9 engagement by CpG-A in early endosomes is suggested to be important for robust type I IFN induction (Honda et al., 2005a; Guiducci et al., 2006), we examined whether DOCK2 deficiency alters subcellular localization of TLR9 and CpG-A. Although the majority of YFP-tagged TLR9 was localized in the ER under unstimulated conditions, this proportion was markedly reduced upon stimulation, irrespective of DOCK2 expression (Fig. 3 E). More importantly, in WT and Dock2<sup>−/−</sup> pDCs, TLR9-YFP and CpG-A-Cy5 comparably accumulated in the transferrin receptor (TfR)–positive endosomal compartments at 1.5 and 6 h after stimulation (Fig. 3 F), indicating that DOCK2 deficiency does not interfere with TLR engagement in early endosomes. When LAMP-1–positive late endosomes/lysosomes were analyzed, the proportion of TLR9 and CpG-A colocalized with LAMP-1 somewhat decreased in Dock2<sup>−/−</sup> pDCs at 6 h, compared with that in WT pDCs (Fig. 3 G). Although the precise meaning of this difference remains unknown, it seems unlikely that this difference directly affects the ability of IFN-α induction because such a difference was not found when WT and Dock2<sup>−/−</sup> pDCs were stimulated with CpG-B (Fig. S3).

DOCK2-mediated Rac activation is critical for IKK-α activation in pDCs

Having found that TLR engagement in early endosomes is unaffected in the absence of DOCK2, we next examined the nuclear translocation of IRF-7 by staining pDCs with anti–IRF-7 antibody and DAPI. Although IRF-7 was expressed in the cytoplasm of unstimulated pDCs, stimulation with CpG-A induced nuclear accumulation of IRF-7 in WT, but not Dock2<sup>−/−</sup>, pDCs (Fig. 4 A). Recent evidence indicates that PI3Ks (phosphatidylinositol-3 kinases) control the nuclear translocation of IRF-7 (Guiducci et al., 2008). However, phosphorylation of Akt, a downstream effector of PI3Ks, as well as that of ERK, JNK, and p38 occurred normally in Dock2<sup>−/−</sup> pDCs stimulated with CpG-A (Fig. 4 B). Similarly, DOCK2 deficiency did not affect CpG-A–induced degradation and autophosphorylation of IRF-3, which is also essential for IRF-7 activation (Fig. 4, C and D; Uematsu et al., 2005).

IKK-α is involved in phosphorylation and nuclear translocation of IRF-7 (Hoshino et al., 2006). IKK-α carries two serine residues at positions 176 and 180 in the activation loop, which are phosphorylated during cellular stimulation (Häcker and Karin, 2006). In WT pDCs, IKK-α was phosphorylated at these sites in response to CpG-A (Fig. 4 E). However, such phosphorylation was hardly detected in both Dock2<sup>−/−</sup> and Th9<sup>−/−</sup> pDCs (Fig. 4, E and F). Moreover, IKK-α phosphorylation was inhibited in WT pDCs by treating them with a cell-permeable dominant-negative Rac mutant (Tat–T17N-Rac; Fig. 4 G). A critical role of this IKK-α phosphorylation in type I IFN induction was examined by luciferase assays. Overexpression of MyD88 augmented IRF-7–induced Ifna promoter activation (Fig. S4). This augmentation was inhibited by coexpression of an IKK-α mutant encoding alanines instead of serines at positions 176 and 180 (IKK-α SSAA) as well as a kinase-inactive IKK-α mutant (Fig. S4). Conversely, Ifna promoter activation was further enhanced by coexpression of an IKK-α mutant with glutamates at these sites (IKK-α SSEE), which mimics the effect of phosphorylated serines (Fig. S4). Collectively, these results suggest that DOCK2-mediated Rac activation controls type I IFN induction, at least in part, through phosphorylation and activation of IKK-α.

In this paper, we have provided evidence that DOCK2 couples ligand stimulation to type I IFN induction in pDCs via Rac activation. We found that the exposure of pDCs to nucleic acid ligands induces Rac activation through a TLR-independent and DOCK2-dependent mechanism. This Rac activation was not required for TLR engagement in early endosomes and IFN signal–dependent positive feedback regulation. However, TLR-mediated IKK-α activation was severely impaired in pDCs lacking DOCK2 and those expressing a dominant-negative Rac mutant, resulting in selective loss of type I IFN induction in these cells. Our results thus indicate that the DOCK2–Rac signaling pathway acts in parallel with TLR engagement to control IKK-α activation for type I IFN induction. Although the precise mechanism by which Rac controls IKK-α activation is currently unknown, it has been reported that activation of IKK complex is regulated by a redox-dependent mechanism in other receptor systems (Oakley et al., 2009). As Rac is a cytosolic component of the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, Rac may therefore control IKK-α activation through generation of reactive oxygen species. Alternatively, PAK (p21–activated kinase), a downstream
Although pDCs play a key role in antiviral immunity, there is growing evidence that pDCs also contribute to the pathogenesis of autoimmune diseases by producing large amounts of type I IFNs. Thus far, several molecules, including IKK-α and IRF-7, have been shown to control type I IFN production. However, the precise mechanisms by which these molecules regulate IFN production remain unclear. The study by Gotoh et al. aimed to investigate the role of DOCK2, a Rac effector, in IFN production by pDCs.

The authors used BM-derived pDCs to study the effects of DOCK2 on IFN production. They showed that the absence of DOCK2 did not affect the expression of type I IFNs in response to CpG-A stimulation. Furthermore, they demonstrated that the phosphorylation of STAT-1 was not altered in DOCK2−/− pDCs, indicating that DOCK2 is not involved in the activation of STAT-1 in response to type I IFN stimulation.

The authors also analyzed the localization of TLR9 in pDCs and showed that the absence of DOCK2 did not affect the endosomal localization of TLR9. These findings suggest that DOCK2 is not involved in the regulation of TLR9-mediated IFN production.

In conclusion, the study by Gotoh et al. provides new insights into the mechanisms by which DOCK2 regulates IFN production in pDCs. The findings suggest that DOCK2 is not directly involved in the regulation of IFN production and that other molecules may be required to control IFN production in pDCs.
IFN induction in pDCs. However, their ubiquitous expression precludes the use as therapeutic targets. Unlike these molecules, DOCK2 expression is limited to hematopoietic cells (Fukui et al., 2001). In addition, we have demonstrated in this paper that DOCK2 selectively controls type I IFN induction during TLR7/9-mediated pDC activation. Therefore, DOCK2 may be a novel therapeutic target for controlling type I IFN–related autoimmune diseases.

MATERIALS AND METHODS

Mice. Dock2−/−, Tlr9−/−, and Cxcl16−/− mice have been described elsewhere (Hemmi et al., 2000; Fukui et al., 2001; Shimaoka et al., 2007). Dock2−/− mice were backcrossed with C57BL/6 mice for more than eight generations before use. Tlr9−/− mice and Cxcl16−/− BM cells were provided by S. Akira (Osaka University, Osaka, Japan) and S. Ueha and K. Matsushima (both from the University of Tokyo, Tokyo, Japan), respectively. Age-matched and sex-matched C57BL/6 mice were used as WT controls. Mice were kept under specific pathogen-free conditions in the animal facility of Kyushu University. Animal protocols were approved by the committee of Ethics on Animal Experiment, Faculty of Medical Sciences, Kyushu University.

Reagents. The following CpG oligonucleotides were synthesized at Hokkaido System Science: CpG-A (D19), ggTGCATCGATGCAgggggG; and CpG-B (1668), tccatgacgttcctgatgct (uppercase and lowercase letters indicate bases with phosphodiester- and phosphorothioate-modified backbones, respectively). The Cy5-labeled CpG-A and CpG-B were obtained from Sigma-Aldrich. R848 was purchased from Enzo Life Sciences, Inc., and pertussis toxin and PP2 were obtained from EMD. For viral infection, HSV-2 (186 strain) and influenza A/New Caledonia/20/99 (H1N1) were used.

Figure 4. DOCK2-mediated Rac activation is critical for IKK-α activation in pDCs. (A) Subcellular localization of IRF-7 was compared between WT and Dock2−/− pDCs after stimulation with CpG-A. DAPI was used to stain nuclei. Representative images of three independent experiments are shown. Bar, 5 µm. (B) BM-derived pDCs from WT and Dock2−/− mice were stimulated with 3 µM CpG-A for the indicated times and analyzed for phosphorylation of Akt, ERK, JNK, or p38. Data are representative of at least two independent experiments. (C and D) BM-derived WT and Dock2−/− pDCs were stimulated with 3 µM CpG-A for the indicated times and analyzed for the expression (C) and autophosphorylation (D) of IRAK-1. Data are representative of two independent experiments. (E–G) BM-derived pDCs from WT, Dock2−/−, and Tlr9−/− mice were stimulated with 3 µM CpG-A for the indicated times and analyzed for serine phosphorylation of IKK-α at positions 176 and 180. (G) Before stimulation, cells were treated with bacterially expressed GFP-tagged Tat-T17N-Rac or Tat-WT Rac (500 nM each) for 30 min at 37°C. Data are representative of three (E and G) or two (F) independent experiments.

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In some experiments, the virus was used for stimulation after inactivation by exposure to 1 J/cm² UV light with a UV cross-linker or incubation at 35°C for 30 min. HSV-2 186 viral DNA was purified on 5–20% potassium acetate gradient. Cytokine ELISA kits were purchased from R&D Systems (IFN-α and IFN-β) and Thermo Fisher Scientific (IL-12p40 and IL-6).

**In vivo treatment.** Mice were anesthetized and intravenously injected with 200 μl R848 (50 nmol) or CpG-A complexed with the cationic lipid 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP). For preparation of CpG-A/DOTAP mixture, 5 μg CpG-A resolved in 50 μl PBS was incubated with 30 μl (30 μg) of DOTAP Liposomal Transfection Reagent (Roche) in a polystyrene tube containing 70 μl PBS for 15 min at room temperature. The sample was then diluted with 50 μl PBS to make 200 μl of CpG-A/DOTAP mixture.

**Cell preparation and retrovirus-mediated gene transfer.** Fcβ ligand-induced BM-derived pDCs, GM-CSF–induced BM-derived myeloid DCs, and naive BM pDCs were prepared as described elsewhere (Gotoh et al., 2008). The retroviral vector pMX (provided by T. Kitamura, University of Tokyo, Tokyo, Japan) was used to generate the plasmid encoding TLR9–YFP, T17N-Rac–IRES–GFP, or WT Rac–IRES–GFP. These plasmids were transfected into Platinum-E packaging cells using FuGENE 6 transfection reagent (Roche). The cell culture supernatants were harvested 48 h after transfection, supplemented with 5 μg/ml polybrene and 10 ng/ml Flk3 ligand, and used to infect BM-derived pDCs. After centrifugation at 2,000 rpm for 1 h, plates were incubated for 8 h at 32°C and for 16 h at 37°C. Before assay, two additional retroviral infections were performed at daily intervals.

**F-actin localization.** After fixation with 4% paraformaldehyde, cells were treated with 0.1% saponin in PBS containing 0.1% BSA and stained with Alexa Fluor 488–conjugated phalloidin (Invitrogen). For preparation of immobilized CpG-A microsphere, 10 μl Fluospheres polystyrene microsphere beads (10 μm; Invitrogen) were coated with 100 μM CpG-A overnight and washed five times with PBS containing 0.1% BSA. All images were taken with a laser-scanning confocal microscope (LSM 510 META; Carl Zeiss, Inc.).

**Pulldown assay, immunoblotting, and in vitro kinase assay.** To assess Rac activation, aliquots of the cell extracts were kept for total lysate controls, and the remaining extracts were incubated with glutathione-S-transferase fusion Rac-binding domain of PAK1 at 4°C for 60 min. The bound proteins and the same amounts of total lysates were analyzed by SDS-PAGE, and blots were probed with anti-Rac antibody (23A8; Millipore). Activation of STAT-1, Akt, ERK, JNK, p38, or IKK-α/β was analyzed by immunoblot with the phosphospecific antibody for STAT-1 (Tyr701; 9171; Cell Signaling Technology), Akt (Ser473; 9271; Cell Signaling Technology), ERK (Tyr204; sc-7383; Santa Cruz Biotechnology, Inc.), JNK (Thr183/Tyr185; 9252; Cell Signaling Technology), p38 (Thr180/Tyr182; 9211; Cell Signaling Technology), or IKK-α/β (Ser176/180; 2697; Cell Signaling Technology), respectively. In some experiments, cells were treated before stimulation with bacterially produced Tat–T7N-Rac or Tat–WT Rac. For in vitro kinase assay, cell lysates were immunoprecipitated with anti-IRAK1 antibody (06–872; Millipore). The immunoprecipitates were incubated for 30 min at 30°C in kinase buffer (20 mM Hepes, pH 7.6, 10 mM MgCl₂, 2 mM MnCl₂, 50 mM NaCl, 0.5 μM ATP, 10 μM PTP 32P-ATP, 0.1 mM Na₃Vo₄, 10 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate, 1 mM DTT, 10 mM NaF, and complete protease inhibitor). Samples were washed with lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 20 μg/ml aprotinin, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na₃Vo₄, and 1% NP-40) and then subjected to SDS-PAGE and autoradiography.

**Immunofluorescence microscopy.** To assess subcellular localization of TLR9, fixed cells were made permeable for 5 min with 0.2% (vol/vol) Triton X-100 and were stained with anti-TIR antibody (C2; BD) or anti–LAMP-1 antibody (1D4B; BD), followed by Alexa Fluor 546–conjugated anti–rat IgG antibody (Invitrogen). In some experiments, cells were treated with ER-Tracker Red (Invitrogen). The DeltaVision SoftWorX Restoration Microscopy system (Applied Precision) with a 63× objective attached to a cooled charge-coupled device camera was used for microscopy. Images were acquired with the DeltaVision SoftWorX Resolve 3D capture program and were collected as a stack of 0.2-μm increments. After deconvolution, images were viewed as a single section on the z axis. The area of TLR9 or CpG DNA localized together with TIR, LAMP-1, or ER–Tracker was calculated in each cell using the MetaMorph imaging system (Universal Imaging). The percentage of colocalization was determined by dividing the area of TLR9 or CpG DNA localized together with TIR, LAMP-1, or ER–Tracker by the total area of TLR9 or CpG DNA, respectively.

To examine the nuclear translocation of IRF-7, fixed cells were permeabilized for 5 min with 0.5% (wt/vol) saponin (Sigma-Aldrich). After being blocked with 20% goat serum for 30 min, cells were stained with anti–IRF-7 antibody (Invitrogen) at 4°C overnight and incubated with biotin-conjugated anti–rabbit IgG (Jackson Immunoresearch Laboratories) followed by Alexa Fluor 488–conjugated streptavidin (Molecular Probes). The cells were washed and mounted using fluorescent mounting medium (Dako) with DAPI (Wako Chemicals USA, Inc.). All images were taken with a laser-scanning confocal microscope (LSM 510 META).

**Real-time PCR.** After treatment with RNase-free DNase I (Invitrogen), RNA samples were reverse transcribed with random primers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR analysis was performed using specific primers (TaqMan Gene Expression Assays; Applied Biosystems).

**Reporter assay.** Reporter assay was performed as described elsewhere (Hoshino et al., 2006). In brief, 293T cells were seeded on a 24-well plate (7 × 10⁴ cells per well). The next day, these cells were transfected with 56 ng of luciferase reporter plasmid alone or together with a combination of the expression plasmids for MyD88 (65.6 ng), IRF-7 (216.8 ng), or IKK-α and its mutants (228 or 456 ng). Cell lysates were prepared 24 h after transfection and luciferase activity was measured by the Dual-luciferase reporter assay system (Promega).

**Statistical analysis.** P-values were calculated using the two-tailed Student’s t test.

**Online supplemental material.** Fig. S1 shows the effect of CXCL16 deficiency, pertussis toxin, or PP2 on CpG-A uptake, actin polymerization, and cytokine production in pDCs. Fig. S2 shows that DOCK2 deficiency does not affect CpG-A–induced actin polymerization and Rac activation in myeloid DCs. Fig. S3 shows that DOCK2 deficiency does not alter subcellular localization of TLR9 and CpG-B in pDCs. Fig. S4 shows that the serine phosphorylation of IKK-α at position 176/180 is critical for IRF-7–mediated type I IFN induction. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091776/DC1.

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Figure S1. The effect of CXCL16 deficiency, pertussis toxin, or PP2 on CpG-A uptake, actin polymerization, and cytokine production in pDCs. (D–F) Before assay, WT pDCs were pretreated for 1 h with 500 ng/ml pertussis toxin (PTx), 4 µM PP2, or vehicle (DMSO; Mock). (A and D) The uptake of CpG-A was compared between WT and Cxcl16−/− pDCs (A) or among WT pDCs treated with Mock, PTx, or PP2 (D). Cells were incubated with 3 µM CpG-A–Cy5 at 37°C (filled area) or 4°C (open area) for 1 h in the presence or absence of inhibitors. (B and E) CpG-A–induced actin polymerization was compared between WT and Cxcl16−/− pDCs (B) or among WT pDCs treated with Mock, PTx, or PP2 (E). After being stimulated with 3 µM CpG-A in the presence or absence of inhibitors, cells were fixed and stained with phalloidin. Bars, 10 µm. (C and F) Cytokine production was compared between WT and Cxcl16−/− pDCs (C) or among WT pDCs treated with Mock, pertussis toxin, or PP2 (F). Cells were stimulated with 3 µM CpG-A for 24 h in the presence or absence of inhibitors. Data indicate the levels of IFN-α and IL-12p40 in cell culture supernatants (mean ± SD of triplicate wells). All data are representative of two independent experiments.
Figure S2. DOCK2 deficiency does not affect CpG-A–induced actin polymerization and Rac activation in myeloid DCs. BM-derived WT and Dock2−/− myeloid DCs were left untreated or stimulated with 3 µM CpG-A for the indicated times. (A) Cells were then fixed and stained with phalloidin. Bar, 10 µm. (B) Activation of Rac was analyzed. Data are representative of two independent experiments.
Figure S3. DOCK2 deficiency does not alter subcellular localization of TLR9 and CpG-B in pDCs. BM-derived WT and Dock2−/− pDCs were retrovirally transduced with TLR9–YFP. Cells were left untreated or stimulated with 1 μM CpG-B–Cy5 for the indicated times. After fixation with 4% paraformaldehyde, cells were stained for TfR (A) or LAMP-1 (B), and the area of TLR9–YFP or CpG-B–Cy5 merged with TfR (A) or LAMP-1 (B) was calculated in each cell. Data are pooled from two independent experiments and are expressed as the percentage of colocalization (mean ± SD) for 20 cells analyzed per group. Bars, 5 μm.
Figure S4. The serine phosphorylation of IKK-α at position 176/180 is critical for IRF-7–mediated type I IFN induction. 293T cells were transiently transfected with an Ifna4 promoter–driven reporter plasmid alone or together with a combination of the expression vector for MyD88, IRF-7, IKK-α (WT), IKK-α (Ser176/180Ala, SSAA), IKK-α (Lys44Ala, KA), or IKK-α (Ser176/180Glu, SSEE). After 24 h, cell lysates were prepared and promoter activity was measured by luciferase assay. Data are representative of three independent experiments.