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Immunity, Vol. 23, 19–28, July, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.immuni.2005.04.010

Cell Type-Specific Involvement of RIG-I in Antiviral Response

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Summary

Toll-like receptors (TLRs) play an important role in antiviral response by recognizing viral components. Recently, a RNA helicase, RIG-I, was also suggested to recognize viral double-stranded RNA. However, how these molecules contribute to viral recognition in vivo is poorly understood. We show by gene targeting that RIG-I is essential for induction of type I interferons (IFNs) after infection with RNA viruses in fibroblasts and conventional dendritic cells (DCs). RIG-I induces type I IFNs by activating IRF3 via IκB kinase-related kinases. In contrast, plasmacytoid DCs, which produce large amounts of IFN-α, use the TLR system rather than RIG-I for viral detection. Taken together, RIG-I and the TLR system exert antiviral responses in a cell type-specific manner.

Introduction

The invasion of viruses is initially sensed by the host innate immune system that provokes antiviral immune responses. Antiviral host defense is mediated by the rapid induction of type I interferons (IFNs) and proinflammatory cytokines, leading to subsequent activation of adaptive immune responses (Biron, 1999; Le Bon and Tough, 2002). Type I IFNs are produced by various types of cells after viral infection. Dendritic cells (DCs) secrete large amounts of type I IFNs in response to viruses. Among recently identified subsets of DCs, plasmacytoid DCs (pDCs) are characterized for their potent ability to secrete IFN-α (Cella et al., 1999; Colonna et al., 2002). Conventional DCs (cDCs), however, can act as specialized IFN-producing cells in a certain viral infections (Diebold et al., 2003). In addition, nonprofessional immune cells such as fibroblasts also produce IFN-β in response to viral infection.

Recent studies revealed that Toll-like receptors (TLRs) are critical for the recognition of pathogen-specific molecular patterns (PAMPs) derived from both bacterial and viral species (Akira and Takeda, 2004; Beutler, 2004; Medzhitov, 2001). Among 11 reported TLRs, TLR3, TLR4, TLR7, TLR8, and TLR9 are involved in the recognition of viral components. In mice, viral double-stranded (ds) RNA, fusion protein of respiratory syncytial virus, single-stranded (ss) RNA, and genomic DNA from herpes species and murine cytomegalovirus are recognized by TLR3, TLR4, TLR7, and TLR9, respectively (Alexopoulou et al., 2001; Kurt-Jones et al., 2000; Heil et al., 2004; Diebold et al., 2004; Lund et al., 2003, 2004; Krug et al., 2004a, 2004b). Stimulation with TLR3 and TLR4 ligands results in the activation of two IκB kinase (IKK)-related kinases, IKK-*i*, also called IKKε, and TANK binding kinase-1 (TBK1), also named as T2K, which are responsible for the phosphorylation of IFN-regulatory factor 3 (IRF3) (Fitzgerald et al., 2003; Sharma et al., 2003; McWhirter et al., 2004). Phosphorylated IRF3 dimerizes and translocates into the nucleus for the transcription of IFN-β (Yoneyama et al., 1998). In contrast, MyD88 can directly associate with IRF7, which is responsible for the induction of IFN-α after TLR9 ligand stimulation (Kawai et al., 2004). IRAK1 is also involved in IFN-α production by phosphorylating IRF-7 (Uematsu et al., 2005). Stimulation with TLR ligands also activates a transcription factor, NF-κB, which is responsible for the induction of proinflammatory cytokines.

We have previously shown that a cytoplasmic protein, retinoic-acid inducible gene-I (RIG-I), can recognize viral dsRNA to induce the type I IFN response in vitro (Yoneyama et al., 2004). RIG-I contains an N-terminal caspase recruitment domain (CARD) and a C-terminal DExD/H box RNA helicase domain (Yoneyama et al., 2004; Sun, 1997). The helicase domain is responsible for dsRNA recognition, and the CARD domain activates downstream signaling pathways. However, functional role of RIG-I in vivo has not been clarified yet. Furthermore, the relationship between RIG-I and TLRs in the recognition of viruses is unclear.

In this study, we generated RIG-I-deficient mice and showed that RIG-I, but not the TLR system, plays an essential role in antiviral responses in various cells except pDCs. Reciprocally, the TLR system, but not

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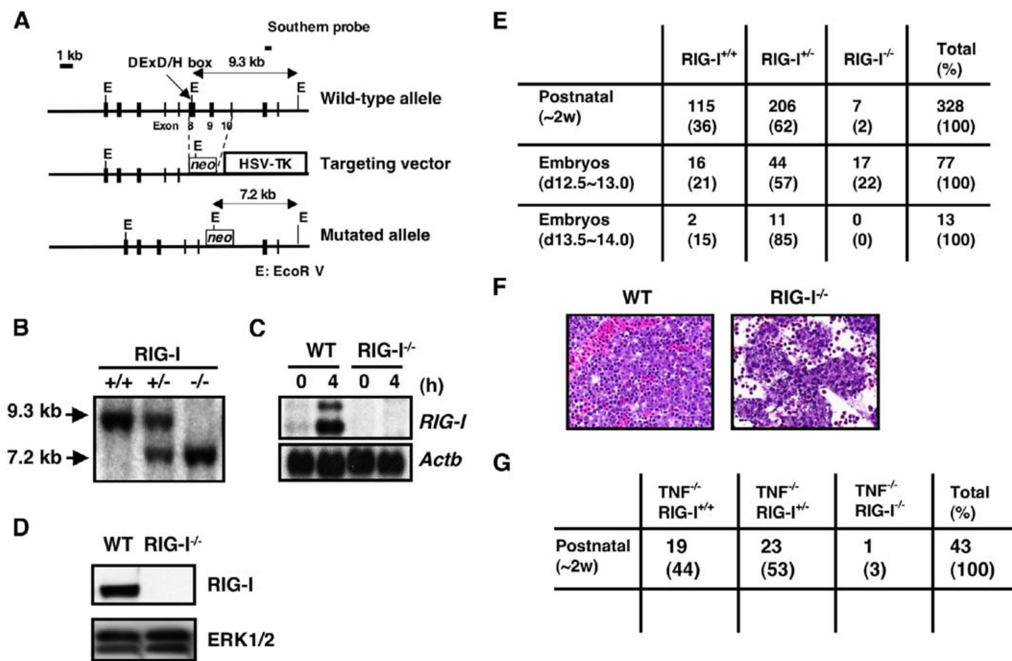

	RIG-I+/+	RIG-I+/-	RIG-I-/-	Total (%)
Postnatal (~2w)	115 (36)	206 (62)	7 (2)	328 (100)
Embryos (d12.5~13.0)	16 (21)	44 (57)	17 (22)	77 (100)
Embryos (d13.5~14.0)	2 (15)	11 (85)	0 (0)	13 (100)

	TNF-/- RIG-I+/+	TNF-/- RIG-I+/-	TNF-/- RIG-I-/-	Total (%)
Postnatal (~2w)	19 (44)	23 (53)	1 (3)	43 (100)

Figure 1. Targeted Disruption of the Murine RIG-I Gene

(A) Structure of the mouse *RIG-I* gene, the targeting vector, and the predicted disrupted gene. Closed boxes denote the coding exon. E, EcoRV; DECH, DExD/H box.

(B) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with EcoRV, separated by electrophoresis, and hybridized with the radiolabeled probe indicated in (A). Southern blot gave a single 9.3 kb band for wild-type (+/+), a 7.2 kb band for homozygous (–/–), and both bands for heterozygous (+/–) mice.

(C) Nothern blot analysis of MEFs. Total RNA from wild-type (WT) and RIG-I$^{-/-}$ MEFs transfected with 10 μg/ml poly (I:C) for the indicated periods was extracted and subjected to the Northern blot analysis for the expression of *RIG-I* mRNA. The same membrane was rehybridized with an *Actb* (β-actin) probe.

(D) Western blot analysis of RIG-I expression in WT and RIG-I$^{-/-}$ MEFs transfected with 10 μg/ml poly (I:C) for 8 hr. Whole-cell lysates were immunoblotted with antibody to RIG-I. The same membrane was blotted again with antibody to ERK1/2.

(E) Genotype analysis of neonatal offspring or embryos at d12.5–d13.0 or d13.5–d14.0 from the heterozygote intercrosses.

(F) Hematoxylin and eosin staining of transverse sections through the fetal liver of WT and RIG-I$^{-/-}$ embryos at d12.5.

(G) Genotype analysis of neonatal offspring from intercrossing TNF-α$^{-/-}$ RIG-I$^{+/-}$ mice.

RIG-I, is indispensable to IFN-α secretion in pDCs. Thus, induction of type I IFNs in RNA virus infection would be triggered by either RIG-I or the TLR system in a cell type-specific manner.

Results

The TLR System Is Dispensable for Viral Recognition in Fibroblasts

All known TLRs have been shown to signal through Toll/IL-1R homology (TIR) domain-containing adaptor molecules, MyD88, and/or TIR domain-containing adaptor-inducing IFN-β (TRIF). Mice deficient in both MyD88 and TRIF (MyD88$^{-/-}$TRIF$^{-/-}$) are unresponsive to stimulation with all known TLR ligands (Yamamoto et al., 2003). To examine the role of TLR signaling pathways in the recognition of RNA viruses in fibroblasts, we first infected MyD88$^{-/-}$TRIF$^{-/-}$ mouse embryonic fibroblasts (MEFs) with Newcastle disease virus (NDV) and analyzed upregulation of IFN-β and IFN-inducible genes. NDV infection induced IFN-β and IFN-inducible genes represented by IP-10 and RANTES, as well as IL-6 in MyD88$^{-/-}$TRIF$^{-/-}$ MEFs to the same extent as wild-type cells (Figure 2A). Moreover, MEFs deficient in either TLR3, TLR7, or TLR9 displayed similar results (data not shown), indicating that the antiviral response in MEFs is mediated by mechanism independent of the TLR system.

Generation of RIG-I-Deficient Mice

Recently identified RIG-I is also implicated in antiviral responses. However, the functional role of RIG-I in vivo is still unclear. To investigate the role of RIG-I in vivo, we generated *RIG-I*-deficient (RIG-I$^{-/-}$) mice by homologous recombination of ES cells (Figures 1A and 1B). Expression of RIG-I mRNA and protein was abrogated in RIG-I$^{-/-}$ cells (Figures 1C and 1D). Although most RIG-I$^{-/-}$ embryos were lethal at embryonic days 12.5 to 14.0, a few mice were born alive (Figure 1E). Nevertheless, these mice showed growth retardation and died within 3 weeks after birth. Histological analysis of embryonic day 12.5 embryos revealed massive liver degeneration (Figure 1F). A TUNEL assay revealed that the aberrant occurrence of apoptosis in the embryonic liver can explain the developmental defect of RIG-I$^{-/-}$ embryos (data not shown). Mice lacking either RelA, IKK-β, or TBK1 are also reported to be embryonic lethal because of liver apoptosis. In embryogenesis, these

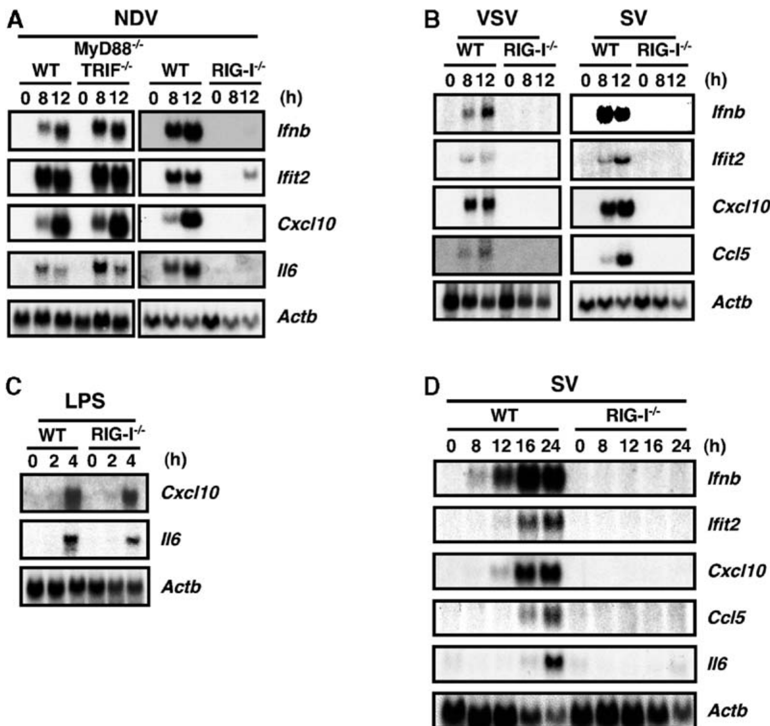

Figure 2. Abolished Responses to RNA Virus Infection in RIG-I$^{-/-}$ Fibroblasts

(A) WT, MyD88$^{-/-}$TRIF$^{-/-}$ (left), and RIG-I$^{-/-}$ (right) MEFs were infected with NDV for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *Ifnb* (IFN-β), *Cxcl10* (IP-10), *Ccl5* (RANTES), *Il6* (IL-6), and *Actb* (β-actin).

(B and C) WT and RIG-I$^{-/-}$ MEFs were infected with VSV (moi = 0.2) ([B], left), SV (moi = 20) ([B], right), or stimulated with 10 μg/ml LPS (C) for the indicated periods (*Ifit2*: ISG54). Northern blot analysis was performed using the indicated probes.

(D) Lung fibroblasts from WT and RIG-I$^{-/-}$ mice were infected with SV (moi = 20) for the indicated periods. Northern blot analysis was performed using the indicated probes.

molecules are supposed to inhibit TNF-induced cell death, as evidenced by the rescue of developmental defect under TNFR deficiency (Doi et al., 1999; Li et al., 1999; Bonnard et al., 2000). Therefore, we hypothesized that the liver apoptosis of RIG-I$^{-/-}$ mice is also due to altered TNF signaling. Therefore, we tried to generate TNF-α$^{-/-}$RIG-I$^{-/-}$ mice. However, we could obtain only 1 RIG-I$^{-/-}$TNF-α$^{-/-}$ mouse out of 43 pups born from intercrossing RIG-I$^{+/-}$TNF-α$^{-/-}$ mice (Figure 1G), indicating that TNF-α deficiency failed to rescue the embryonic lethality of RIG-I$^{-/-}$ mice. These results indicate that the developmental defect of RIG-I$^{-/-}$ mice might be caused by a mechanism(s) independent of TNF-α signaling.

Essential Role of RIG-I in Viral Recognition in Fibroblsts

RIG-I is involved in the recognition of viral dsRNA through its helicase domain. Therefore, we first examined the expression of IFN-β and IFN-inducible genes in MEFs after infection with several RNA viruses. The induction of IFN-β, IFN-inducible genes, and IL-6 in response to NDV was abrogated in RIG-I$^{-/-}$ MEFs (Figure 2A). In addition, infection with several other RNA viruses, including vesicular stomatitis virus (VSV) and Sendai virus (SV), also failed to upregulate IFN-β as well as IFN-inducible genes in RIG-I$^{-/-}$ cells (Figure 2B). In contrast, stimulation with lipopolysaccharide (LPS), a TLR4 ligand, induced the expression of IP-10 and IL-6 genes in both wild-type and RIG$^{-/-}$ cells (Figure 2C). Moreover, lung fibroblasts from RIG-I$^{-/-}$ mice did not upregulate IFN-β and IFN-inducible genes after SV infection (Figure 2D), indicating that RIG-I is essential for the induction of IFN response upon viral infection in nonprofessional immune cells like fibroblasts.

Next, we analyzed the contribution of RIG-I to antiviral effect. When MEFs were infected with VSV that expresses GFP in infected cells, RIG-I$^{-/-}$ cells have re-

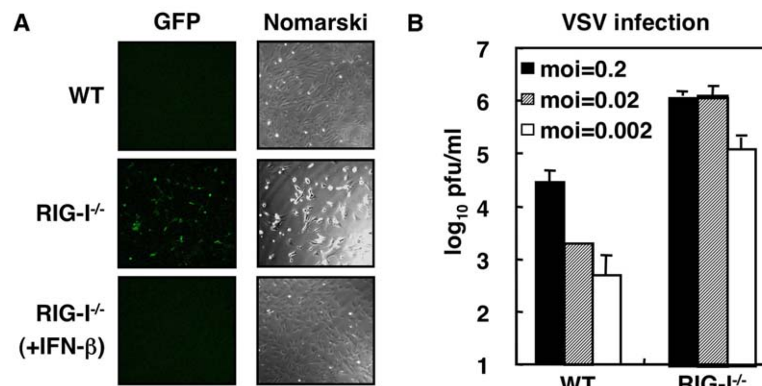

Figure 3. A Critical Role of RIG-I in Eliminating Viral Infection

(A) WT and RIG-I$^{-/-}$ MEFs pretreated with or without IFN-β were infected with VSV at moi = 0.1 for 24 hr. Cells and expression of GFP were visualized by a confocal microscopy.

(B) WT and RIG-I$^{-/-}$ MEFs were infected with the indicated moi of VSV. Virus yield in the supernatants after VSV infection was determined by plaque assay. Data are representative of three independent experiments. Indicated values are means + SD of triplicates. pfu, plaque-forming unit.

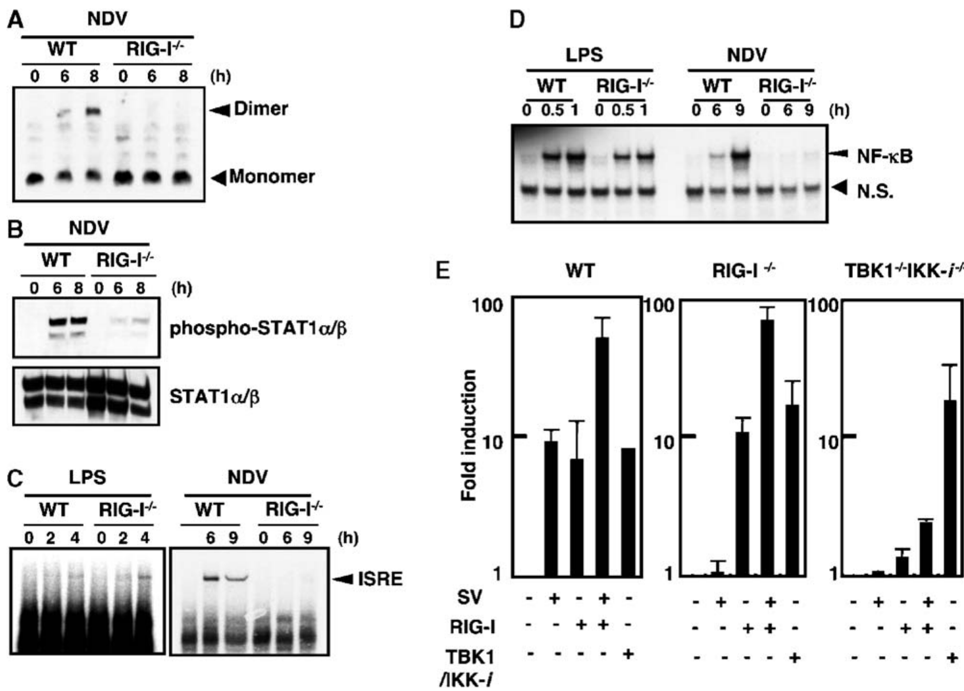

Figure 4. Activation of Signaling Cascade in RIG-I$^{-/-}$ Fibroblasts

(A) WT and RIG-I$^{-/-}$ MEFs were infected with NDV for the indicated periods. Cell lysates were prepared and subjected to native PAGE. Monomeric (arrow) and dimeric (arrowhead) forms of IRF3 were detected by Western blotting.

(B) The cell extracts used in (A) were probed for phospho-STAT1 and total STAT1.

(C and D) WT and RIG-I$^{-/-}$ MEFs were stimulated with 10 μg/ml LPS or infected with NDV for the indicated periods. Nuclear extracts were prepared, and ISRE (C) and NF-κB (D) DNA binding activity was determined by EMSA. The arrowhead indicates the induced band of ISRE or NF-κB complex. N.S., nonspecific bands.

(E) WT, RIG-I$^{-/-}$, and TBK1$^{-/-}$IKK-*i*$^{-/-}$ MEFs were transiently transfected with a reporter construct containing the IFN-β promoter together with the indicated expression vectors. Transfected cells were infected with SV (moi = 20) and were subjected to the luciferase assay. Error bars show the SD of two independent experiments.

markably increased GFP-positive cells compared to wild-type cells (Figure 3A). However, priming of RIG-I$^{-/-}$ cells with IFN-β clearly decreased the number of infected cells in response to VSV infection. Given that VSV-induced IFN-β mRNA expression was abrogated in RIG-I$^{-/-}$ cells (Figure 2B), RIG-I may mediate antiviral response via induction of type I IFNs (Figure 3A). Moreover, when MEFs were infected with VSV at various multiplicities of infection (moi), viral yield of RIG-I$^{-/-}$ cells was increased about 100-fold compared with that

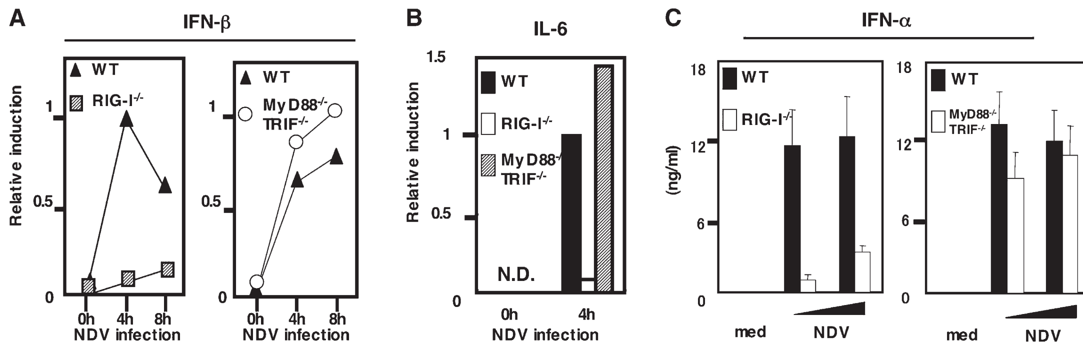

Figure 5. Abolished Responses to NDV Infection in RIG-I$^{-/-}$ cDCs

(A and B) GM-CSF-DCs used as cDCs from WT, RIG-I$^{-/-}$, and MyD88$^{-/-}$TRIF$^{-/-}$mice were infected with NDV for the indicated periods. Total RNA was extracted and supernatants were harvested. IFN-β (A) and IL-6 (B) mRNA levels were determined by Q-PCR. N.D., not detected.

(C) GM-CSF-DCs used as cDCs from WT, RIG-I$^{-/-}$, and MyD88$^{-/-}$TRIF$^{-/-}$mice were infected with increasing doses of NDV for 24 hr. IFN-α production in the supernatants was measured by ELISA. Data are shown as mean ± SD of triplicate samples of a representative from three independent experiments.

of wild-type cells (Figure 3B). These results demonstrate that RIG-I plays a critical role in eliminating viral infection through induction of IFN response.

Signaling Pathway Activated by Viral Infection

Induction of type I IFNs and proinflammatory cytokines requires activation of transcription factors IRF3 and NF-κB (Sato et al., 2000; Taniguchi and Takaoka, 2002). IRF3 is phosphorylated by TBK1 and IKK-*i*. The phosphorylated IRF3 induces the expression of the IFN-β gene. The produced of IFN-β subsequently stimulates the JAK-STAT pathway to amplify the responses (Shuai and Liu, 2003). To determine the role of RIG-I in the activation of signaling pathways, we analyzed activation of IRF3 after NDV infection in RIG-I$^{-/-}$ MEFs. NDV-induced dimerization of IRF3, as well as phosphorylation of STAT1, was abrogated in RIG$^{-/-}$ MEFs (Figures 4A and 4B). Furthermore, electrophoretic mobility shift assay (EMSA) revealed that activation of IFN stimulated a regulatory element (ISRE), and NF-κB was also abrogated in RIG-I$^{-/-}$ cells in response to NDV infection, but not to LPS stimulation (Figures 4C and 4D). These results demonstrate that RIG-I is essential for activating transcription factors initially activated after viral infection. The IFN-β-dependent reporter gene expression was induced in response to SV infection in wild-type MEFs (Figure 4E). In contrast, SV infection failed to activate the reporter gene in RIG-I$^{-/-}$ or IKK-*i*$^{-/-}$TBK1$^{-/-}$ cells. The ectopic expression of RIG-I gene restored SV-induced IFN-β promoter activity in RIG-I$^{-/-}$ cells, but not in IKK-*i*$^{-/-}$TBK1$^{-/-}$ cells. Overexpression of both TBK1 and IKK-*i* in RIG-I$^{-/-}$ cells activated the IFN-β promoter (Figure 4E), indicating that RIG-I induces type I IFNs via IKK-*i*/TBK1.

Differential Requirement of the TLR System and RIG-I in IFN-α Production in pDCs and cDCs

DCs play an important role in detecting pathogens and activating T cells by presenting antigens, producing cytokines and type I IFNs (Kaisho and Akira, 2003; Trinchieri, 2003; Malmgaard, 2004). DCs are roughly subdivided into two different subsets; conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (Cella et al., 1999; Colonna et al., 2002; Diebold et al., 2003; Malmgaard, 2004). Recent studies demonstrated that pDCs secrete large amounts of IFN-α upon viral exposure (Cella et al., 1999; Colonna et al., 2002; Diebold et al., 2003). Therefore, we examined the IFN response of RIG-I$^{-/-}$ DCs after viral infection. First, we tested cDCs induced from bone marrow (BM) in the presence of GM-CSF. The induction of CD11c^{+} DCs was not impaired in RIG-I$^{-/-}$ BM cells cultured with GM-CSF (see Figure S1A in the Supplemental Data available with this article online). Real-time PCR analysis revealed that NDV infection induced the expression of IFN-β in cDCs from wild-type mice as well as those from MyD88$^{-/-}$TRIF$^{-/-}$ mice (Figure 5A). In contrast, cDCs from RIG-I$^{-/-}$ mice showed severely impaired IFN-β induction after NDV infection. NDV-induced IL-6 production was also severely impaired in RIG-I$^{-/-}$, but not in MyD88$^{-/-}$TRIF$^{-/-}$ cDCs (Figure 5B). Next we examined the production of IFN-α in NDV-infected cDCs by ELISA. As shown in Figure 5C, production of IFN-α was severely impaired in cDCs from RIG-I$^{-/-}$, but not MyD88$^{-/-}$TRIF$^{-/-}$, mice, indicating that induction of both IFN-α and IFN-β is regulated by RIG-I in cDCs.

Then we generated Flt3L-induced BM-derived DCs (Flt3L-DCs), which contained pDCs, stimulated them with NDV, and measured IFN-α production. The population of pDCs obtained by culture with Flt3L from wild-type and RIG-I$^{-/-}$ mice was not altered (Figure S1B). Unexpectedly, Flt3L-DCs from RIG-I$^{-/-}$ mice produced similar amounts of IFN-α and IL-6 as those from wild-type mice (Figures 6A and 6B). On the other hand, IFN-α and IL-6 production from MyD88$^{-/-}$TRIF$^{-/-}$ Flt3L-DCs as well as MyD88$^{-/-}$ Flt3L-DCs were severely impaired compared to those from wild-type Flt3L-DCs (Figures 6A and 6B). Since cells obtained by Flt3L cultures contained both cDCs (CD11c^{+}B220^{-}) and pDCs (CD11c^{+}B220^{+}), we costained RIG-I$^{-/-}$ or MyD88$^{-/-}$ FltL-DCs for IFN-α, CD11c, and B220 and analyzed them by flow cytometry. As shown in Figure 6C, the production of IFN-α in CD11c^{+}B220^{+} pDCs was comparable between wild-type and RIG-I$^{-/-}$ Flt3L-DCs, whereas that from CD11c^{+}B220^{-} cells was severely impaired in RIG-I$^{-/-}$ Flt3L DCs. In contrast, production of IFN-α in pDCs was severely impaired in MyD88$^{-/-}$ mice (Figure 6D). IFN-α production in MyD88$^{-/-}$ cDCs was normal, albeit the frequency of IFN-α-positive cells was lower than that of pDCs. On the other hand, neither pDCs nor cDCs from MyD88$^{-/-}$ mice responded to CpG-ODN stimulation. To examine whether IFN-β is also regulated by the TLR system, we purified pDCs from Flt3L-DCs, infected them with NDV, and assessed the induction of mRNA for IFNs and IL-6 by real-time PCR. As shown in Figure 6C, the induction of mRNA for IFN-β, IFN-α4, IFN-α6/8, and IL-6 was severely impaired in MyD88$^{-/-}$ pDCs, indicating that induction of both IFN-α and IFN-β is mainly regulated by MyD88 in pDCs. These results indicate that the TLR system rather than RIG-I is a prerequisite for the recognition of viruses to induce type I IFNs in pDCs. Since pDCs highly express TLR7 (Heil et al., 2004; Diebold et al., 2004) and TLR9 (Lund et al., 2003; Krug et al., 2004a), we analyzed Flt3L-DCs from either TLR7$^{-/-}$ or TLR9$^{-/-}$ mice for NDV responsiveness. Wild-type Flt3L-DCs produced IFN-α in a dose- and time-dependent manner (Figures 6A and 6B). In contrast, TLR7$^{-/-}$ Flt3L-DCs showed impaired IFN-α production in response to NDV infection (Figures 6A and 6B). TLR9$^{-/-}$ Flt3L-pDCs also showed impaired IFN-α production to a lesser extent compared to TLR7$^{-/-}$ cells (Figures 6A and 6B). These results indicate that TLR7 and TLR9 are involved in NDV recognition in pDCs.

We further investigate the role of RIG-I in cDCs and pDCs directly purified from mouse spleens. The composition of splenic cDCs and pDCs was not altered between wild-type and RIG-I$^{-/-}$ mice (Figure S1C). We purified CD11c^{high}mPDCA1^{-} cDCs and CD11c^{+}mPDCA1^{+} pDCs from splenocytes from wild-type, MyD88$^{-/-}$, or RIG-I$^{-/-}$ mice by FACS sorting. Infection with NDV induced the production of IFN-α in both pDCs and cDCs from wild-type mice (Figure 7). In contrast, pDCs, but not cDCs, from MyD88$^{-/-}$ mice showed severely impaired production of IFN-α in response to NDV infection (Figure 7A). Reciprocally, the IFN-α response was defective in RIG-I$^{-/-}$ splenic cDCs (Figure 7B), although pDCs from wild-type and RIG-I$^{-/-}$ mice secreted com-

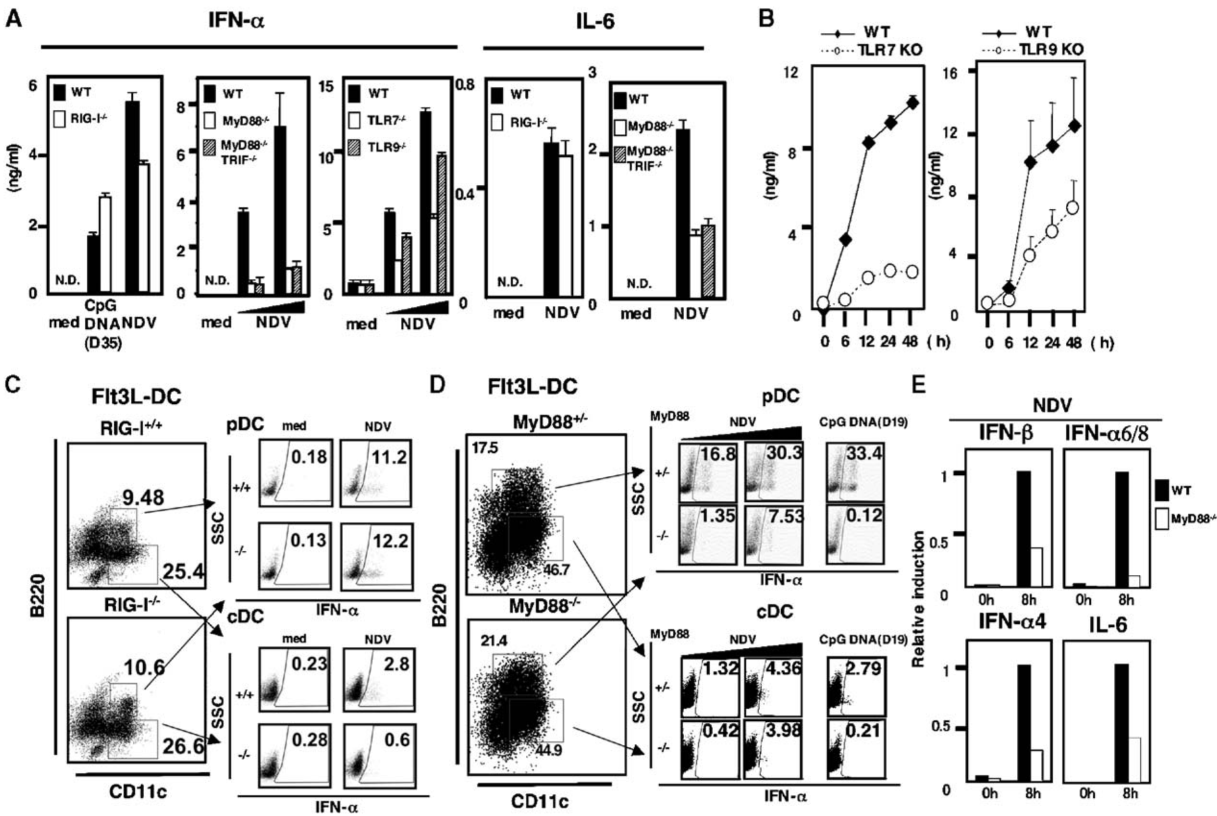

Figure 6. The TLR System, Rather than RIG-I, Is a Prerequisite for the Recognition of NDV to Induce IFN-α and IL-6 in pDCs

(A) Flt3L-DCs from WT, MyD88$^{-/-}$, TLR7$^{-/-}$, TLR9$^{-/-}$, MyD88$^{-/-}$TRIF$^{-/-}$, and RIG-I$^{-/-}$ mice were infected with NDV or stimulated with 3 μM CpG DNA (D35). Production of IFN-α (left) and IL-6 (right) was measured by ELISA. Data are representative of three independent experiments. Indicated values are means + SD of triplicates. N.D., not detected.

(B) Flt3L-DCs from WT, TLR7$^{-/-}$, and TLR9$^{-/-}$ mice were infected with NDV for the indicated time, and IFN-α production was measured. Data are shown as mean ± SD of triplicate samples of a representative from three independent experiments.

(C and D) Flt3L-DCs from RIG-I$^{+/+}$ and RIG-I$^{-/-}$(C) or MyD88$^{+/-}$ and MyD88$^{-/-}$ mice (D) were infected with NDV or stimulated with 2 μM CpG DNA (D19). Cells were fixed and subjected to intracellar IFN-α staining as described in Experimental Procedures. Induction of IFN-α in pDCs and cDCs was separately analyzed based on the gates indicated.

(E) Purified CD11c$^+$B220$^+$ DCs were infected with NDV. Total RNA was harvested and IFN-β, IFN-α6/8, IFN-α4 and IL-6 mRNA levels were measured by Q-PCR.

parable amounts of IFN-α. Thus, these observations indicate that cDCs and pDCs mainly exploit RIG-I and the TLR system, respectively, to recognize RNA viruses.

Discussion

We showed here that RIG-I was critical for RNA virus-mediated IFN response in fibroblasts and cDCs by generating RIG-I$^{-/-}$ mice. Most RIG-I$^{-/-}$ mice were embryonic lethal at embryonic days 12.5 to 14.0 because of liver degeneration, although a few mice overcame the developmental defect and were born alive. Genes related to NF-κB activation in TNF-α signaling and TBK1, a kinase responsible for IRF-3 phosphorylation, are known to regulate the development of the liver. Although the mice deficient in either RelA, IKK-β, or TBK1 are embryonic lethal due to liver apoptosis, disruption of TNF-α signaling rescues the developmental defect. Therefore, we hypothesized that TNF signaling is also involved in the liver degeneration of RIG-I$^{-/-}$ embryos. However, TNF-α deficiency failed to rescue the embryonic lethality of RIG-I$^{-/-}$ mice. Thus, a mechanism(s) independent of TNF-α signaling is responsible for the liver degeneration of RIG-I$^{-/-}$ mice. Further studies are required to determine the cause of the developmental defect in RIG-I$^{-/-}$ mice.

The responses to several single-stranded RNA viruses, NDV, VSV, and SV, were abrogated in RIG-I$^{-/-}$ MEFs. Furthermore, increased viral yield and the restored resistance to VSV infection by the IFN-β pretreatment in RIG-I$^{-/-}$ MEFs demonstrate that RIG-I exerts its antiviral activity via inducing type I IFNs. In addition to RIG-I, TLR3, TLR4, TLR7, and TLR9 have been implicated in the recognition of viruses. TLR3 and TLR4 activate TRIF to induce type I IFNs (Yamamoto et al., 2003), and TLR7 and TLR9 utilize MyD88 to activate type I IFNs (Hemmi et al., 2002; Hoshino et al., 2002; Kawai et al., 2004). Nevertheless, MEFs and cDCs from MyD88$^{-/-}$TRIF$^{-/-}$ mice displayed normal type I IFN induction upon NDV infection, indicating that the TLR system is not required in these cells for the induction of type I IFNs after viral infection. TLRs are transmembrane proteins that localize either on plasma membrane or in intracellular vesi-

cles, indicating that TLRs can detect PAMPs in extracellular or luminal spaces. On the other hand, RIG-I is a cytoplasmic protein without a transmembrane domain. These observations suggest that cytoplasmic detection of infected viruses plays a main role in antiviral responses in MEFs and cDCs.

Protein kinase R (PKR) is also one of the sensors of viral invasion (Williams, 2002). PKR is a cytosolic protein composed of dsRNA binding motifs and a serine/threonine kinase domain. Binding of PKR with viral dsRNA results in the phosphorylation of the translation initiation factor, eIF2α, and translational inhibition (Williams, 2002). In addition, production of type I IFNs by dsRNA is possibly regulated by PKR (Diebold et al., 2003; Yang et al., 1995). However, a report showed that the amount of type I IFN induced by NDV was not altered in PKR$^{-/-}$ MEFs (Smith et al., 2001). Since induction of IFN-β after NDV, VSV, and SV is solely dependent on RIG-I in MEFs, it is possible that RIG-I, but not PKR, is a bona fide cytoplasmic receptor for the recognition of RNA viruses.

Analysis of signaling pathways revealed that RIG-I is indispensable for the activation of both IRF3 and NF-κB in response to viral infection and that TBK1 and IKK-*i* are activated downstream of RIG-I to trigger IFN response. TBK1 and IKK-*i* are essential for the induction of type I IFNs in TLR4 signaling, indicating that both the RIG-I and TLR signaling pathways converge to TBK1/IKK-*i* to activate IRF3. Given that TBK1 and IKK-*i* are dispensable for NF-κB activation, RIG-I should control a signaling pathway leading to NF-κB in addition to the TBK1/IKK-*i*/IRF3 pathway (Fitzgerald et al., 2003; Sharma et al., 2003; McWhirter et al., 2004; Hemmi et al., 2004). It is interesting to explore the mechanism of how RIG-I signals to activate NF-κB.

Although many cell types produce type I IFNs, DCs are known to be a potent inducer of type I IFNs after viral infection. Among subsets of DCs, pDCs are identified as the major source of type I IFNs in vivo after viral infection. However, depletion experiments revealed that pDCs are not essential for type I IFN response to lymphocytic choriomeningitis virus, and conventional DCs are still a candidate for a type I IFN inducer (Dalod et al., 2002). First, analysis of cDCs from RIG-I$^{-/-}$ mice revealed that RIG-I plays an essential role in type I IFNs and IL-6 production in the cells. However, pDCs from RIG-I$^{-/-}$ mice showed normal production of IFN-α in response to NDV infection. By contrast, the TLR system plays an important role in pDCs, but not in cDCs, suggesting that pDCs have a specific and elaborate mechanism to produce IFN-α. Of note, although the amount of IFN-α mRNA induced was severely impaired, the induction of IFN-α in Flt3L-cultured pDCs from MyD88$^{-/-}$ mice was not completely abrogated. These suggest that a pathway(s) other than TLRs also contributes to virus-induced IFN response to some extent. It is possible that RIG-I marginally plays a role in type I IFN production even in pDCs.

Analysis of pDCs deficient in each TLR revealed that TLR7 mainly plays an important role in NDV recognition, although TLR9 also partially participates in this process. Since TLR7 recognizes viral single-stranded RNA and is expressed in endosomal membranes (Lee et al., 2003; Heil et al., 2003), pDCs may sense the presence of ss RNA of NDV by endosomal uptake of its virions. In contrast, cDCs and fibroblasts may recognize viruses only after the invasion into the cytoplasm. Further study will also be needed to clarify the functional difference between the two types of type I IFN induction systems in in vivo viral infection.

Experimental Procedures

Generation of RIG-I-Deficient Mice

The RIG-I gene was isolated from genomic DNA extracted from ES cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 3.6 kb fragment encoding the RIG-I ORF (exons 8–10) with a neomycin-resistance gene cassette (*neo*), and a herpes simplex virus thymidine kinase (HSV-TK) driven by PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into ES cells, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and further confirmed by Southern blotting. Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed in order to obtain RIG-I$^{-/-}$ mice.

Mice, Cells, Virus, and Reagents

MyD88$^{-/-}$ (Adachi et al., 1998), TLR7$^{-/-}$ (Hemmi et al., 2002), TLR9$^{-/-}$ (Hemmi et al., 2000), and MyD88$^{-/-}$TRIF$^{-/-}$ (Yamamoto et al., 2003) mice were as described. TNF-α $^{-/-}$ mice were kindly provided by K. Sekikawa (Tagawa et al., 1997). TBK1$^{-/-}$IKK-*i*$^{-/-}$ MEFs were as described (Hemmi et al., 2004). RIG-I$^{-/-}$ MEFs were prepared from day 12.5–13.5 embryos. Lung fibroblasts and bone marrow cells were prepared from 1- to 2-week-old RIG-I$^{-/-}$ mice. Recombinant vesicular stomatitis virus (VSV) was a gift from T. Abe and Y. Matsuura (Osaka University). Sendai virus (SV) Z strain was provided by T. Shioda (Osaka University). LPS from *Salmonella minnesota* Re-

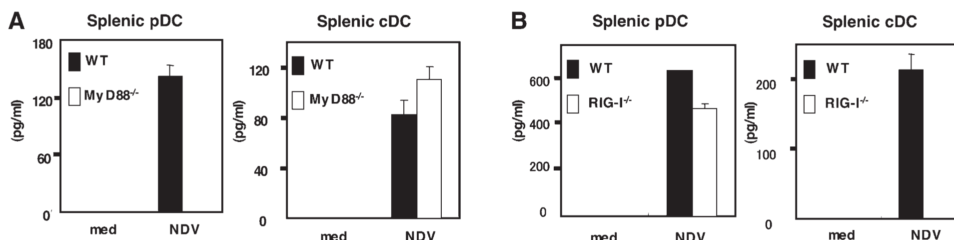

Figure 7. Splenic pDCs Utilize the TLR System, and RIG-I Is Essential in Splenic cDCs for the Recognition of NDV to Induce IFN-α

(A) Splenic CD11c^{+}mPDCA-1^{+} pDCs and CD11c^{high}mPDCA-1^{-} cDCs were purified from WT and MyD88$^{-/-}$ mice by FACS sorting. pDCs and cDCs were infected with NDV for 24 hr, and IFN-α production from pDCs (1 × 10^{4}) or cDCs (1 × 10^{4}) was measured by ELISA.

(B) Splenic CD11c^{+}mPDCA-1^{+} pDCs and CD11c^{high}mPDCA-1^{-} cDCs were purified from WT and RIG-I$^{-/-}$ mice by FACS sorting. pDCs and cDCs were infected with NDV for 24 hr. IFN-α production from pDCs (2 × 10^{4}) or cDCs (2 × 10^{4}) was measured by ELISA.

Data are shown as mean ± SD of triplicate samples of a representative from two independent experiments.

595 was purchased from Sigma-Aldrich. Poly (I:C) was purchased from Amersham Biosciences. Polyclonal anti-IRF3 antibody was raised as described (Yamamoto et al., 2003). Polyclonal anti-RIG-I antibody was raised against amino acids 907–920 of mouse RIG-I. As IFN-β pretreatment, 1000 U/ml IFN-β was used 15 hr before VSV infection. Synthesized oligodeoxynucleotides were purchased from Hokkaido System Science. The sequences and backbones of oligodeoxynucleotides (ODN) are as described (Hemmi et al., 2003); D19, ggTGCATCGATGCAgggggG; D35, ggTGCATCGATGCAGG GggG.

Nothern Blot Analysis

Total RNA from MEFs was extracted using TRIzol reagent (Invitrogen), electrophoresed, transferred to nylon membranes, and then hybridized with an indicated cDNA probe. To detect the expression of RIG-I mRNA, a 400 bp fragment (exons 5–8) containing DExD/H box domain was used as a probe. The same membrane was rehybridized with a β-actin probe.

Western Blot Analysis

MEFs were transfected with 10 μg/ml poly (I:C) for 8 hr. The cells were then lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, and protease inhibitor cocktail (Roche). The cell lysates were dissolved by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blotted with the specific antibody to the indicated protein, and visualized with an enhanced chemiluminescence system (NEN Life Science Product).

Histological Analysis

For histological analysis, fetuses derived at 12.5 days postcoitum were fixed with 3.7% formaldehyde. Transverse sections through the fetal liver (5 μm) were cut and stained with hematoxylin and eosin.

Plaque Assay

Culture supernatants were collected from MEFs infected with VSV. Virus yield in culture supernatants was determined by standard plaque assay. Prepared BHK cells were infected with serial dilutions of the recovered viruses. The cells were overlaid with DMEM containing 1% low melting agarose and incubated for 24 hr. Then plaques were counted.

Electrophoretic Mobility Shift Assay

MEFs (1×10^6) were stimulated with 10 μg/ml LPS or infected with NDV for the indicated periods. Nuclear extracts were purified from cells using lysis buffer (10 mM HEPES-KOH [pH 7.8], 10 mM KCl, 10 mM EDTA [pH 8.0]), and then incubated with a specific probe for ISRE or NF-κB DNA binding sites, electrophoresed, and visualized by autoradiography (Yamamoto et al., 2003).

Native PAGE Assay

MEFs (1×10^6) were infected with NDV for the indicated periods and then lysed. The cell lysates in native PAGE sample buffer (62.5 mM Tris-Cl [pH 6.8], 15% glycerol, and 1% deoxycholate) were separated on a native PAGE and then immunoblotted with anti-IRF3 antibody (Yamamoto et al., 2003).

Luciferase Assay

Mammalian expression plasmid for mouse RIG-I was constructed in the pEF-BOS. The cDNA fragment encoding the ORF of RIG-I was amplified by RT-PCR from a total RNA prepared from mouse lung fibroblasts. MEFs obtained from wild-type, RIG-I$^{-/-}$, or TBK1$^{-/-}$ IKK-*i*$^{-/-}$ mice were transiently transfected with reporter constructs containing the IFN-β promoter together with the empty vector (control), RIG-I, or TBK-I and IKK-*i* expression vector. As an internal, a Renilla luciferase construct was transfected. Transfected cells were mock treated (mock) or infected with SV (moi = 20) for 24 hr, lysed, and subjected to the luciferase assay using a dual-luciferase reporter assay system (Promega)

Preparation of Dendritic Cells

To prepare in vitro bone marrow DCs, bone marrow cells were prepared from femora and tibia. Then cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 μM 2-ME, and 100 ng/ml human Flt3 ligand (PeproTech) or 10 ng/ml murine GM-CSF (PeproTech) (Hemmi et al., 2003). After 6–8 days, the cells were collected and used as Flt3L-induced BMDCs (Flt3L-DCs) or GM-CSF-induced BMDCs (GM-CSF-DCs), respectively. In the case of culturing GM-CSF-DCs, medium was changed every 2 days. To prepare splenocytes containing DCs, spleens were cut unto small fragments and incubated with RPMI 1640 medium containing 400 U/ml collagenase (Wako) and 15 μg/ml DNase (Sigma) at 37°C for 20 min. For the last 5 min, 5 mM EDTA was added. Single cell suspensions were prepared after RBC lysis and subjected to the negative selection of T and B cells using Thy1.2 and anti-CD19 microbeads (Miltenyi).

Cells purified from negative selection were used for splenic pDCs and cDCs. To prepare splenic pDCs and cDCs, CD11c^{+}mPDCA-1^{+} cells and CD11c^{high}mPDCA-1^{-} cells were sorted by FACS Vantage (BD bioscience) and used as splenic pDCs and cDCs, respectively. The purity of sorted cells was 97%–99% (data not shown).

Quantitative Real-Time PCR

For Q-PCR, RNA from GM-CSF-DCs infected with NDV was quantitated, and 1 μg of RNA was reverse-transcribed using Superscript2 (Invitrogen) according to the manufacturer's instructions, with random hexamers as primers. Q-PCR analysis was done using the 7700 Sequence Detector (Applied Biosystem). Q-PCR was conducted in a final volume of 25 μl containing cDNA amplified as described before, 2× PCR Master Mix (Applied Biosystem), and the following primers: 18s rRNA (Applied Biosystem) as an internal control, IFN-α4, IFN-α6/8, IFN-β, and IL-6 (Assay on Demand). Amplification conditions were: 95°C (10 min), 35 cycles of 95°C (15 s), 60°C (60 s), 50°C (120 s).

Measurement of IFN-α and IL-6 Production

Flt3L-DCs (2×10^5) were infected NDV or stimulated with 3 μM synthesized oligodeoxynucleotides, D35 for 24 hr. Culture supernatants were collected and analyzed for IFN-α or IL-6 production with enzyme-linked immunosorbent assay (ELISA). ELISA kits for mouse IFN-α and IL-6 were purchased from PBL Biomedical Laboratories and R&D Systems, respectively.

Flow Cytometry

For intracellular IFN-α staining, Flt3L-DCs were treated with NDV or 2 μM D19 for 4.5 hr. Golgi stop (BD PharMingen) was added for an additional 3.5 hr, and cells were collected and fixed in paraformaldehyde. Staining was performed in saponin-containing buffer using a mixture of rat anti-mouse IFN-α antibodies (clone F18, Hycult Biotechnology b.v. and clone RMMA-1, PBL Biomedical Laboratories), followed by biotinylated mouse anti-rat IgG (Jackson ImmunoResearch), and Streptavidin-APC (PharMingen). Cells were subsequently stained with CD11c-FITC (clone HL3) and B220-PE (clone RA3-6B2) and analyzed on a FACS Calibur (BD Bioscience).

Supplemental Data

Supplemental Data are available for this article at http://www.immunity.com/cgi/content/full/23/1/19/DC1/.

Acknowledgments

We thank Drs. T. Abe and Y. Matsuura for providing VSV, Dr. T. Shioda for providing SV, and Dr. K. Sekikawa for providing TNF-α$^{-/-}$ mice. We also thank T. Kawai and K. Ishii for helpful discussions, M. Hashimoto for excellent secretarial assistance, and N. Iwami, N. Okita, Y. Fujiwara, M. Shiokawa, A. Shibano, and N. Kitagaki for technical assistance.

Received: October 4, 2004
Revised: April 15, 2005
Accepted: April 20, 2005
Published: July 26, 2005

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