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The Specific and Essential Role of MAVS in Antiviral Innate Immune Responses

Qinmiao Sun,[2,4] Lijun Sun,[1,2,4] Hong-Hsing Liu,[2] Xiang Chen,[1,2] Rashu B. Seth,[2] James Forman,[3] and Zhijian J. Chen[1,2,*]
[1] Howard Hughes Medical Institute
[2] Department of Molecular Biology
[3] Center for Immunology
University of Texas Southwestern Medical Center
Dallas, Texas 75390

Summary

The mitochondrial antiviral signaling protein (MAVS) mediates the activation of NFκB and IRFs and the induction of interferons in response to viral infection. In vitro studies have also suggested that MAVS is required for interferon induction by cytosolic DNA, but the in vivo evidence is lacking. By generating MAVS-deficient mice, here we show that loss of MAVS abolished viral induction of interferons and prevented the activation of NFκB and IRF3 in multiple cell types, except plasmacytoid dendritic cells (pDCs). However, MAVS was not required for interferon induction by cytosolic DNA or by *Listeria monocytogenes*. Mice lacking MAVS were viable and fertile, but they failed to induce interferons in response to poly(I:C) stimulation and were severely compromised in immune defense against viral infection. These results provide the in vivo evidence that the cytosolic viral signaling pathway through MAVS is specifically required for innate immune responses against viral infection.

Introduction

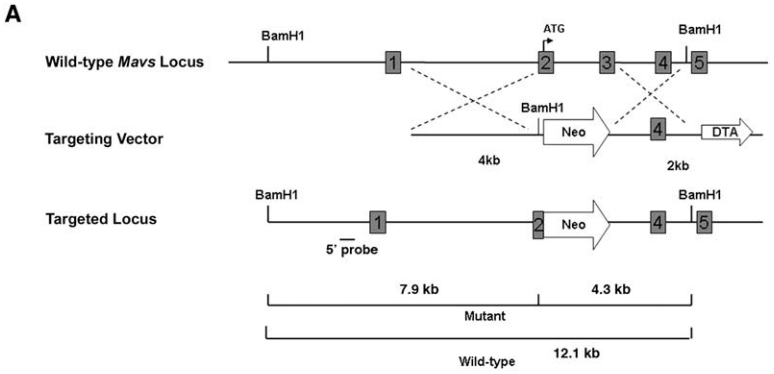
Innate immunity is the first line of defense against microbial pathogens, including viruses. Viral infection triggers the induction of type-I interferons (e.g., IFN-α and IFN-β) and other proinflammatory cytokines through two distinct signaling pathways (Honda et al., 2005; Kawai and Akira, 2006; McWhirter et al., 2005; Seth et al., 2006). One of these pathways utilizes a subfamily of Toll-like receptors (TLR3, 7, 8, and 9) to detect viral nucleic acids in the endosome after the endocytosis of viral particles. These TLRs are localized in the endosomal membranes of specialized cell types, such as pDCs (reviewed by Liu [2001]), and they recruit the adaptor protein MyD88 or TRIF to activate protein kinases, including IκB kinase complex (consisting of IKKα, IKKβ, and NEMO/IKKγ) and the IKK-related kinases (TBK1 and IKKε). The IKK complex phosphorylates the NFκB inhibitor IκB and targets IκB for degradation by the ubiquitin-proteasome pathway, thereby allowing NFκB to enter the nucleus to induce a large array of genes involved in immune and inflammatory responses (Silverman and Maniatis, 2001). TBK1 and IKKε phosphorylate another transcription factor, IRF3 or IRF7, resulting in its dimerization and nuclear translocation (Fitzgerald et al., 2003; Sharma et al., 2003). The nuclear IRFs, NFκB, and other transcription factors form an enhanceosome complex to activate the expression of interferons (Maniatis et al., 1998), which are then secreted to bind to their receptors on viral-infected as well as neighboring noninfected cells. The engagement of the interferon receptors activates the JAK-STAT signaling pathway to induce interferon-stimulated genes (ISGs), which suppress viral replication and assembly (Darnell et al., 1994).

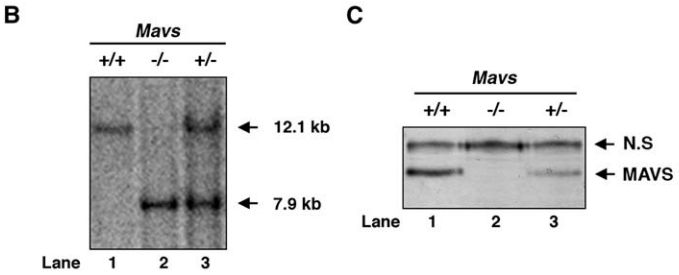
The other viral signaling pathway utilizes the retinoic acid inducible gene I (RIG-I) to detect viral double-stranded RNA (dsRNA) in the cytosol (Yoneyama et al., 2004). RIG-I binds to viral dsRNA through its C-terminal RNA helicase domain and mediates the activation of IKK and TBK1/IKKε through its N-terminal caspase activation and recruitment domains (CARD). For unknown reasons, RIG-I knockout mice are embryonic lethal, suggesting that RIG-I has unexpected functions related to animal development (Kato et al., 2005). Although the inability to obtain viable RIG-I knockout mice precludes the study of the role of RIG-I in vivo, studies using cells derived from these mice show that RIG-I is essential for innate immune responses to several RNA viruses in different cell types. However, in pDCs, loss of RIG-I had no effect on viral induction of interferons, whereas TLR7 and MyD88 are required for the immune response in these cells (Diebold et al., 2004; Heil et al., 2004; Kato et al., 2005; Lund et al., 2004).

The adaptor protein that links RIG-I to IKK and TBK1/IKKε activation is the recently identified MAVS (Seth et al., 2005), also known as IPS-1 (Kawai et al., 2005), VISA (Xu et al., 2005), or CARDIF (Meylan et al., 2005). MAVS contains an N-terminal CARD domain that interacts with the tandem CARD domains of RIG-I and a C-terminal transmembrane domain that localizes it to the mitochondrial outer membrane (Seth et al., 2005). The mitochondrial localization of MAVS is essential for its signaling function, and this property is exploited by hepatitis C virus, which deploys the NS3/4A serine protease to cleave MAVS off the mitochondria to evade the host innate immune responses (Freundt and Lenardo, 2005; Li et al., 2005; Meylan et al., 2005). Recent studies have shown that cytosolic B form DNA and the bacterium *Listeria monocytogenes* can also induce interferons (Ishii et al., 2006; Okabe et al., 2005; Perry et al., 2005; Stetson and Medzhitov, 2006). Cell culture studies have suggested that MAVS/IPS-1 is required for interferon induction by cytosolic DNA (Ishii et al., 2006); however, the in vivo evidence is lacking.

In this report, we used MAVS-deficient cells to demonstrate that MAVS is essential for viral induction of interferons and activation of NFκB and IRF3 in multiple cell types, including fibroblasts, macrophages, and conventional DCs. However, in pDCs, MAVS is not required for viral induction of interferons and cytokines. Contrary to previous reports, we found that loss of MAVS did not affect interferon induction by cytosolic DNA or *Listeria monocytogenes*. Furthermore, we found that MAVS-deficient mice failed to induce interferons in response to

*Correspondence: zhijian.chen@utsouthwestern.edu
[4] These authors contributed equally to this work.





D

Mavs	+/+	+/-	-/-
Total #	111	223	108
%	25.1	50.5	24.4

Figure 1. Generation of *Mavs*$^{-/-}$ Mice

(A) Targeting strategy for deleting exon 2 from the ATG start codon to exon 3 of the *Mavs* locus by homologous recombination. Abbreviation: DTA, diphtheria toxin A.

(B) Southern blotting analysis of BamH1-digested genomic DNA from the mouse tails using the 5′ probe as indicated in (A).

(C) Immunoblot analysis of protein extracts from MEF cells of different genotypes using an antibody against mouse MAVS. Abbreviation: N.S., nonspecific.

(D) Offspring from the breeding of *Mavs*$^{+/-}$ mice.

poly(I:C) stimulation. Interestingly, the MAVS-deficient mice produced normal amounts of interferons in the sera when they were infected with vesicular stomatitis virus (VSV), but they were nevertheless more susceptible to viral-induced killing. Taken together, these results demonstrate the specific and essential role of MAVS in antiviral innate immunity.

Results

Generation of *Mavs*$^{-/-}$ Mice

To elucidate the role of MAVS in vivo, we generated *Mavs*-deficient (*Mavs*$^{-/-}$) mice by homologous recombination in ES cells (Figure 1A). The deletion of *Mavs* was verified by Southern and Western blotting (Figures 1B and 1C). The mutant mice were born at the Mendelian ratio (Figure 1D), and they developed and bred normally. These mice displayed no apparent abnormality at the ages of up to 8 months. We have previously shown that MAVS is localized in the mitochondrial outer membrane and that it contains a C-terminal transmembrane domain resembling those of antiapoptotic mitochondrial proteins such as Bcl-2 and Bcl-xL (Seth et al., 2005). To examine the potential role of MAVS in apoptosis, we isolated mouse embryonic fibroblasts (MEFs) from the wild-type and mutant mice and irradiated these cells with UV (Figure S1 available in the Supplemental Data with this article online). Immunoblotting experiments showed that there was no apparent difference in the UV-induced cleavage of poly (ADP-ribose) polymerase (PARP) or caspase-3 between the wild-type and *Mavs*$^{-/-}$ cells. Thus, MAVS is not essential for mouse development or survival.

Mavs$^{-/-}$ Embryonic Fibroblasts Are Defective in Antiviral Innate Immune Responses

To investigate the role of MAVS in antiviral immunity, we infected MEF cells from the wild-type and mutant mice with Sendai virus (SeV), an RNA virus of the paramyxoviridae family, and then measured interferon production by ELISA. MEF cells from *Mavs*$^{-/-}$ mice were completely defective in the production of IFN-α and IFN-β after viral infection (Figure 2A and 2B). The induction of the proinflammatory cytokine IL-6 by Sendai virus was also abolished in *Mavs*$^{-/-}$ cells, but this response was unaffected when the cells were stimulated with lipopolysaccharides (LPS) or double-stranded RNA poly(I:C), which activates TLR4 or TLR3, respectively (Figure 2C). We also examined the activation of IRF3 and NFκB by using gel shift assays, which measure the dimerization of IRF3 and DNA binding of NFκB on native gels, respectively. Viral infection led to the dimerization and nuclear translocation of IRF3 in the wild-type and heterozygous cells, but not in *Mavs*$^{-/-}$ cells (Figure 2D and data not shown). Similarly, the loss of *Mavs* abolished NFκB activation by SeV (Figure 2E), but not by LPS (Figure 2F). Because most viruses produce double-stranded RNA that is detected by the host innate immune system, we examined the role of MAVS in the cytosolic dsRNA signaling pathway. As shown in Figure 2G, transfection of poly(I:C) in MEF cells led to the dimerization of IRF3 in the wild-type cells, but not in *Mavs*$^{-/-}$ cells. Furthermore, the induction of IFN-α, IFN-β, and IL-6 by poly(I:C) was abolished in *Mavs*$^{-/-}$ cells (Figure S2). In contrast to poly(I:C) transfection, which introduced the RNA into the cytosol, addition of poly(I:C) to the media, which is known to stimulate TLR3, did not induce IFN-α or IFN-β in MEF

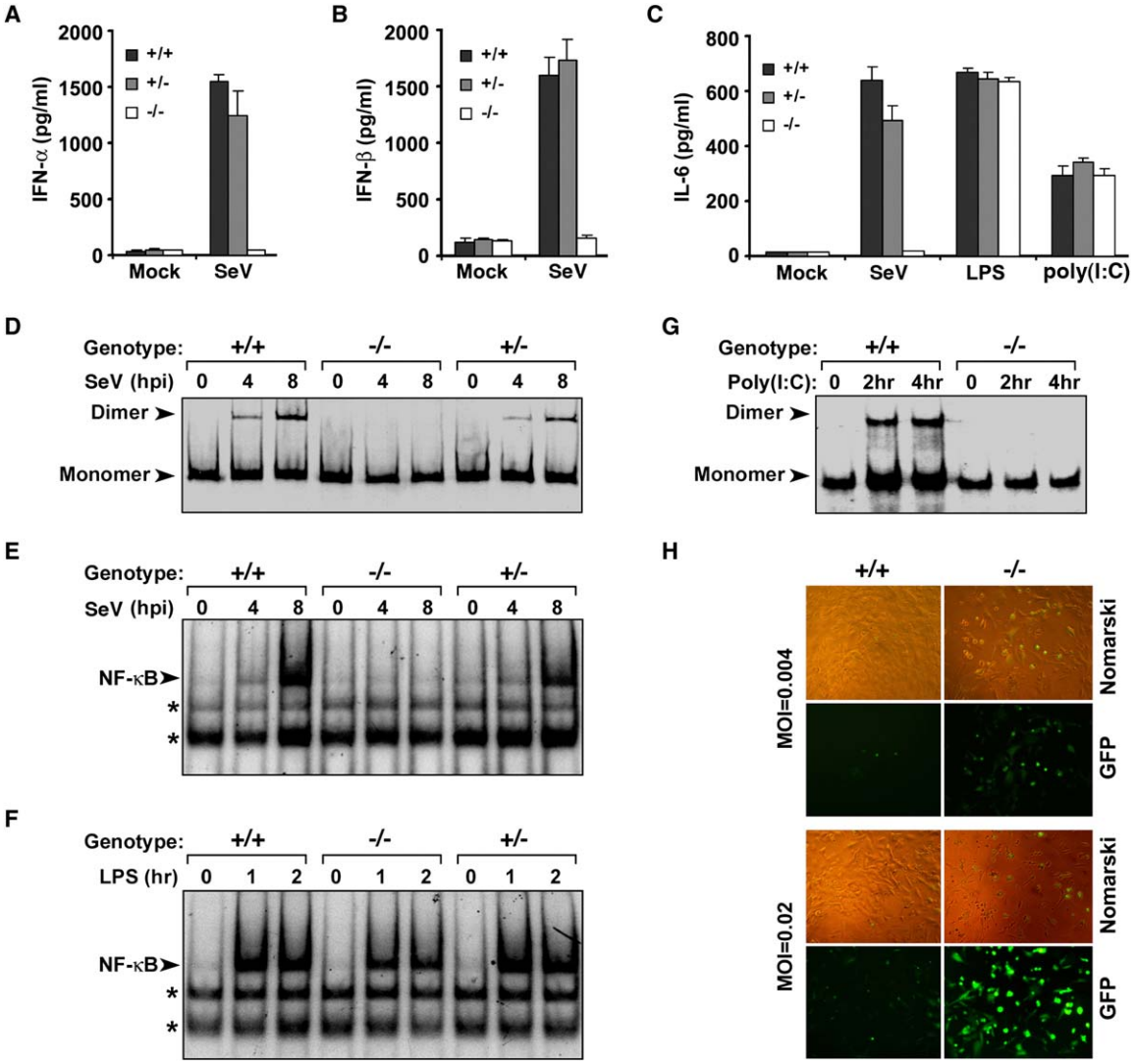


Figure 2. MAVS Is Required for Antiviral Innate Immune Responses in MEF Cells

(A–C) MEF cells were incubated with Sendai virus (SeV), LPS, or poly(I:C) for 16 hr, and the culture supernatants were harvested for ELISA analyses to measure the production of IFN-α, IFN-β, and IL-6 as indicated. Error bars represent standard deviations from the means of duplicated experiments.

(D) MEF cells were infected with SeV for the indicated times and then cell lysates were separated on 9% polyacrylamide gels under nondenaturing conditions. The IRF3 dimer and monomer were detected by immunoblotting. Abbreviation: hpi, hours postinfection.

(E and F) Electrophoretic mobility shift assays (EMSA) for NFκB DNA binding using whole-cell extracts from MEF cells infected with SeV or stimulated with LPS for the indicated times. The asterisks (*) indicate nonspecific beads.

(G) MEF cells were transfected with poly(I:C) for the indicated times and then cell extracts were prepared for analyses of IRF3 dimerization by native gel electrophoresis.

(H) MEF cells were incubated with VSV-GFP at the indicated multiplicity of infection (MOI) for 24 hr, and infection of cells was visualized by fluorescent microscopy. Nomarski microscopy showed that less *Mavs*$^{-/-}$ cells were detected after VSV-GFP infection, an indication that these cells were more susceptible to viral killing.

cells but induced IL-6 through a MAVS-independent manner (Figure S2 and Figures 2A–2C). Taken together, these results indicate that MAVS is required for signaling by Sendai virus and cytosolic dsRNA in general but is not required for signaling by TLR3 and TLR4.

To examine the role of MAVS in viral replication and survival of host cells, we infected MEF cells with vesicular stomatitis virus (VSV), an RNA virus of the rhabdoviridae family. The VSV contains a GFP fused to the cytoplasmic domain of the envelope glycoprotein (G) of the virus, allowing direct visualization of viral replication (Dalton and Rose, 2001). As shown in Figure 2H, *Mavs*$^{-/-}$ cells were much more permissible to viral replication (GFP fluorescence) and susceptible to viral killing (Normarski microscopy) as compared to wild-type cells. To quantify viral infection and killing, we used fluorescent activated cell sorting (FACS) to measure the numbers of GFP-positive cells as well as apoptotic cells that can be stained by Annexin V (Figure S3). After infection with VSV-GFP, the percentages of both GFP- and Annexin V-positive cells were significantly increased in *Mavs*$^{-/-}$ cells as compared to the wild-type cells.

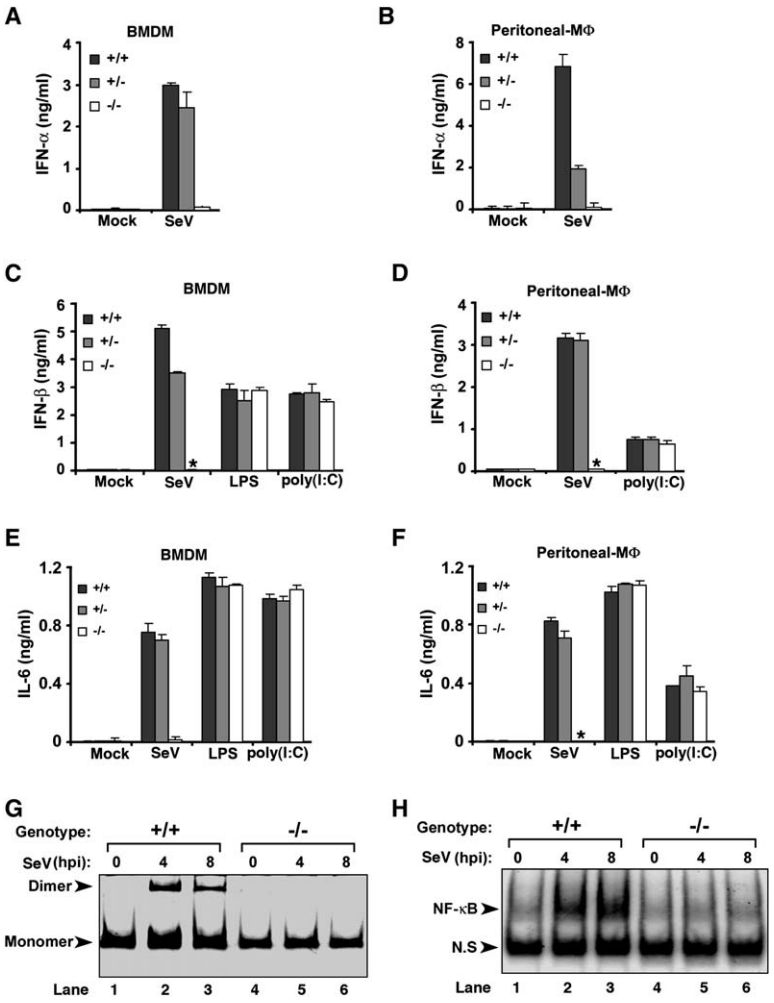


Figure 3. MAVS Is Essential for Antiviral Innate Immune Responses in Macrophages

(A–F) Bone marrow-derived macrophages (BMDMs) or peritoneal macrophages (Mϕs) were incubated with SeV, LPS, or poly(I:C) for 16 hr and then culture supernatants were collected for measurement of IFN-α, IFN-β, or IL-6 by ELISA as indicated. The asterisks (*) indicate levels that were not detectable. Error bars represent standard deviations from the means of duplicated experiments.

(G) Cell extracts from BMDMs infected with SeV for the indicated times were resolved by native gel electrophoresis and then analyzed by immunoblotting with an IRF3-specific antibody.

(H) Cell extracts as described in (G) were incubated with γ-^{32}P-ATP-labeled NFκB oligos and then resolved by native gel electrophoresis (EMSA). Abbreviation: N.S., nonspecific.

Thus, MAVS is essential for immune defense against viral infection and killing.

MAVS Is Required for Antiviral Innate Immune Responses in Macrophages

Next, we examined whether MAVS is required for interferon induction in macrophages. We isolated both bone marrow-derived macrophages (BMDMs) and peritoneal macrophages and infected these cells with SeV. The viral induction of IFN-α, IFN-β, and IL-6 was completely abolished in *Mavs*$^{-/-}$ macrophages (Figures 3A–3F). Furthermore, *Mavs*$^{-/-}$ macrophages failed to activate IRF3 or NFκB in response to Sendai virus (Figures 3G and 3H). In contrast, the induction of IFN-β and IL-6 by LPS or poly(I:C) was normal in *Mavs*$^{-/-}$ macrophages (Figures 3C–3F). Therefore, MAVS is specifically required for antiviral responses in macrophages.

MAVS Is Not Required for Interferon Induction by Cytosolic DNA or *Listeria monocytogenes*

Recently, it was reported that cytosolic B form DNA could elicit the induction of interferons (Ishii et al., 2006; Okabe et al., 2005; Stetson and Medzhitov, 2006) through a mechanism dependent on MAVS/IPS-1 (Ishii et al., 2006). To investigate whether MAVS is required for IFN induction by cytosolic DNA, we transfected MEF cells with double-stranded DNA poly(dA:dT) or poly(dI:dC). Both wild-type and *Mavs*$^{-/-}$ cells had a robust induction of IFN-β after DNA transfection (Figure 4A). Similarly, the induction of IFN-α and IL-6 by cytosolic DNA was intact in *Mavs*$^{-/-}$ cells (Figure S2). No cytokine induction was detected when the dsDNA was added to culture media directly without transfection, indicating that there were no contaminating TLR ligands in the DNA preparations. Native gel analyses showed that cytosolic DNA-induced activation of NFκB and dimerization of IRF3 was not affected by *Mavs* deficiency (Figures 4B and 4C). Thus, MAVS is not required for the induction of interferons or the activation of NFκB and IRF3 by cytosolic DNA.

Listeria monocytogenes is an intracellular bacterium that can induce interferon production through a pathway that depends on TBK1 and IRF3 (O'Connell et al., 2005; Stockinger et al., 2004). Recent studies have shown that *Listeria* induces interferons by releasing bacterial DNA into the cytosol (Stetson and Medzhitov, 2006), but the signaling pathway that links *Listeria* infection to IRF3 activation is not understood. To determine if MAVS is involved in the induction of interferons by *Listeria*, we infected BMDMs with *Listeria* and found that comparable amounts of IFN-β and IL-6 were produced in wild-type

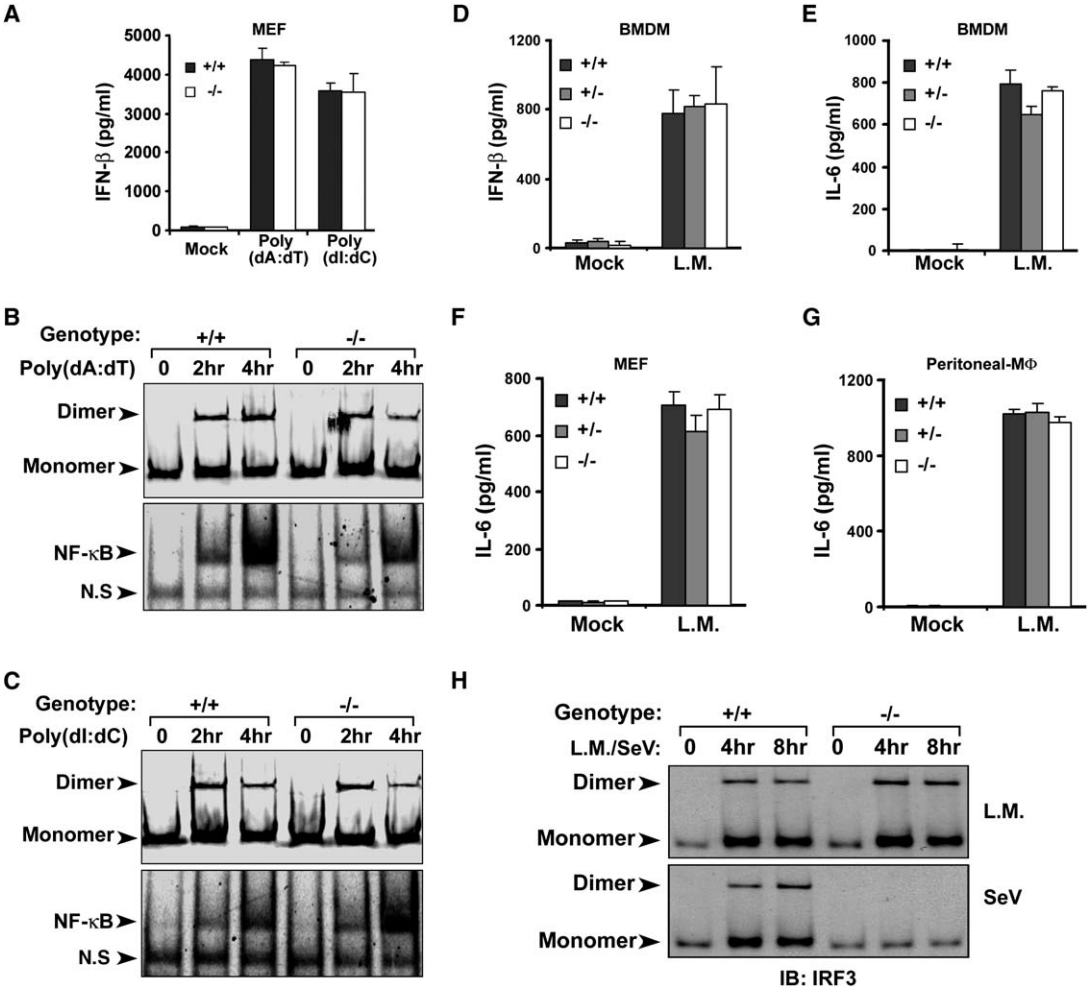


Figure 4. MAVS Is Not Required for Interferon Induction by Cytosolic DNA or *Listeria Monocytogenes*

(A) MEF cells were transfected with poly(dA:dT) or poly(dI:dC) for 16 hr and then culture supernatants were harvested for measurement of IFN-β by ELISA.

(B) MEF cells were transfected with 10 μg/ml of poly(dA:dT) DNA, and the cell lysates were resolved by native gel electrophoresis followed by immunoblotting with an IRF3 antibody (top). The same cell lysates were also analyzed for NFκB DNA binding by EMSA (bottom). Abbreviation: N.S., nonspecific.

(C) Similar to (B), except that poly(dI:dC) was used to stimulate cells.

(D and E) BMDMs were infected with *Listeria monocytogenes* (L.M.) for 16 hr, and the culture supernatants were harvested for measurement of IFN-β and IL-6 by ELISA.

(F and G) MEF cells or peritoneal macrophages were infected with L.M. for 16 hr, and the induction of IL-6 was measured by ELISA.

(H) Cell lysates from MEF cells infected with L.M. or SeV were resolved by native gel electrophoresis and then immunoblotted with an antibody against IRF3.

and *Mavs*$^{-/-}$ macrophages (Figures 4D and 4E). The loss of MAVS also did not affect IL-6 induction by *Listeria* in MEFs and peritoneal macrophages (Figures 4F and 4G) nor did it affect the dimerization of IRF3 (Figure 4H). Taken together, these results indicate that MAVS is dispensable for interferon induction and IRF3 activation by *Listeria*, further reinforcing the conclusion that MAVS is not required for interferon induction by cytosolic DNA.

MAVS Is Required for Interferon Induction in cDCs, but Not pDCs

DCs play a pivotal role in bridging innate and adaptive responses, and these cells can be classified into conventional (cDCs) and plasmacytoid dendritic cells (pDCs), the latter being high producers of IFN-α/β (Liu, 2001). We isolated cDCs and pDCs from the bone marrow cultured with GM-CSF and Flt-3 ligand, respectively, and purified them by FACS sorting (the purities of cDCs and pDCs were 90%–95%; Figure S4). These cells were stimulated with Sendai virus to measure cytokine production by ELISA. Although cDCs derived from the wild-type and heterozygous mice were fully capable of producing IFN-α, IFN-β, and IL-6, cDCs from *Mavs*$^{-/-}$ mice were severely defective in producing these cytokines (Figures 5A–5C). When cDCs from *Mavs*$^{-/-}$ mice were stimulated with LPS, poly(I:C), or CpG DNA (a TLR9 ligand), normal production of IL-6 was detected (Figure 5C). In sharp contrast to cDCs and other cell types, pDCs from *Mavs*$^{-/-}$ mice produced comparable levels of IFN-α and IFN-β to those in wild-type mice in

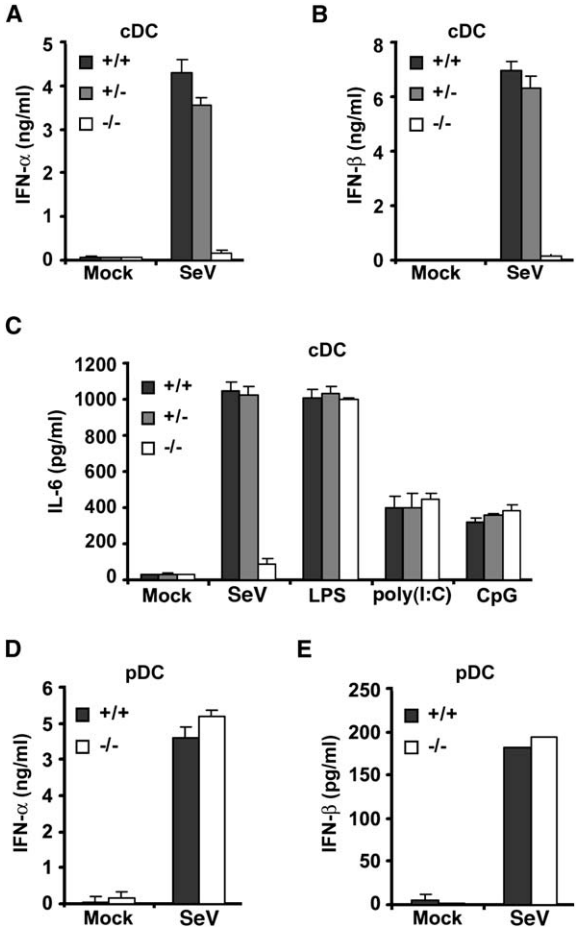


Figure 5. MAVS Is Essential for Interferon Induction in cDCs, but Not pDCs

(A–C) cDCs were isolated from bone marrow cells after stimulation with GM-CSF. These cells were incubated with SeV (A–C), LPS, poly(I:C), or CpG DNA (C), and the production of IFN-α, IFN-β, and IL-6 was measured by ELISA.

(D and E) pDCs were isolated from Flt-3L-stimulated bone marrow cells and purified by FACS. These cells were stimulated with SeV for 16 hr and then culture supernatants were harvested for measurement of IFN-α and IFN-β by ELISA.

Error bars represent standard deviations from the means of duplicated experiments.

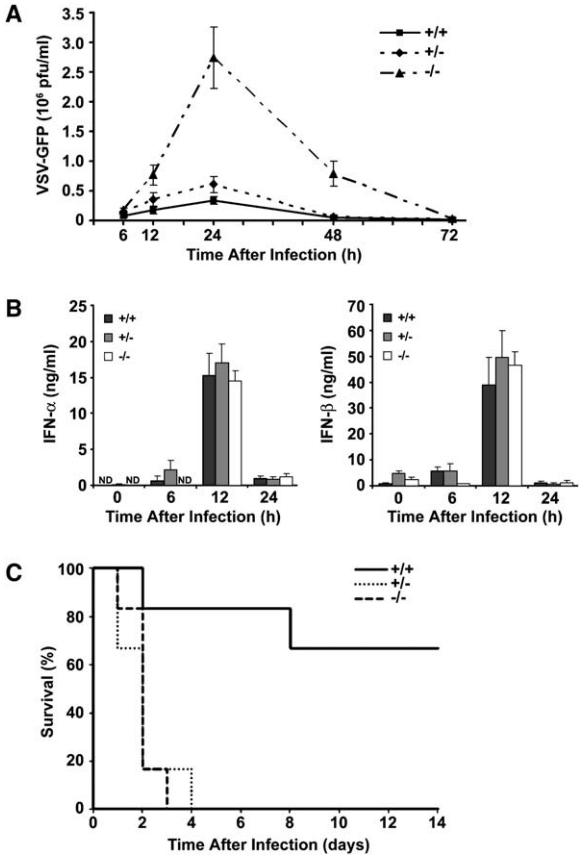


Figure 6. MAVS Is Required for Antiviral Immune Defense In Vivo

(A) Wild-type (n = 7), *Mavs*$^{+/-}$ (n = 5), and *Mavs*$^{-/-}$ (n = 5) mice were infected with VSV-GFP (2×10^8 pfu) via tail vein injection. The sera were collected from the mice at different time points as indicated and used to measure viral titers by plaque assays. The error bars indicate the standard error of the mean (SEM).

(B) Sera collected as in (A) were used for measurement of IFN-α and IFN-β by ELISA. The error bars indicate SEM.

(C) *Mavs*$^{+/+}$, *Mavs*$^{+/-}$, and *Mavs*$^{-/-}$ mice (n = 6 for each genotype) were infected with wild-type VSV (Indiana strain; 5×10^7 pfu) via tail vein injection, and the survival of the mice was monitored for 5 weeks. The mice that survived the viral infections at 2 weeks remained alive after 5 weeks.

response to SeV infection (Figures 5D and 5E). Thus, as shown for RIG-I (Kato et al., 2005), the role of MAVS in interferon induction is cell type dependent (see Discussion).

MAVS Is Essential for Antiviral Immune Defense In Vivo

To investigate the role of MAVS in antiviral responses in vivo, we injected wild-type and mutant *Mavs* mice with VSV-GFP through the tail vein and then collected sera to measure viral titers and interferon production. At 12–48 hr after viral infection, the viral titers in the *Mavs*$^{-/-}$ mice were significantly higher than those in the wild-type and heterozygous mice (Figure 6A). Nevertheless, the virus was largely cleared in both wild-type and mutant mice at 72 hr postinfection, suggesting that the immune system was still effective in clearing the virus in the absence of MAVS. Consistent with this notion, the sera of the *Mavs*$^{-/-}$ mice contained similar amounts of IFN-α and IFN-β to those of wild-type mice (Figure 6B), indicating that some cells in mice, likely pDCs, could still produce sufficient amounts of interferons when the RIG-I-MAVS pathway was crippled.

To determine if MAVS deficiency affects the survival of mice after viral infection, we infected mice with the wild-type VSV (Indiana strain) and monitored their survival. As shown in Figure 6C, whereas the majority of wild-type mice (4/6) survived VSV infection, all of the *Mavs*$^{+/-}$ and *Mavs*$^{-/-}$ mice died within 4 days after the viral infection. The high mortality rate of *Mavs*$^{+/-}$ mice was surprising, as cell culture studies showed that *Mavs*$^{+/-}$ cells were capable of inducing interferons. To determine if there is a quantitative difference in the viability of mice carrying different copies of *Mavs*, we infected these mice with VSV-GFP, which is less virulent (Figure S5). When 2×10^8 pfu of the virus was used to infect each

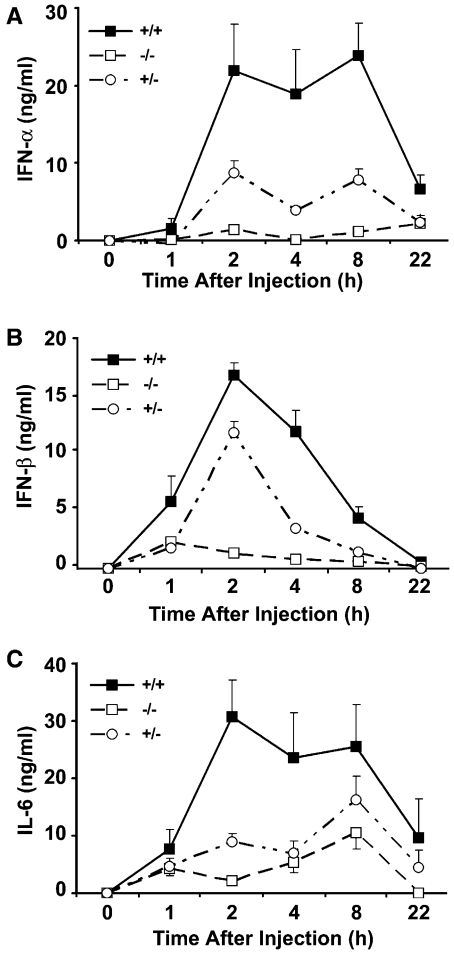


Figure 7. MAVS Is Required for Interferon Induction By Poly(I:C) In Vivo

Wild-type (n = 5), *Mavs*$^{+/-}$ (n = 5), and *Mavs*$^{-/-}$ (n = 6) mice were injected intravenously with 200 μg of poly(I:C) and then sera were collected at indicated times for ELISA to measure the concentrations of IFN-α (A), IFN-β (B), and IL-6 (C). Littermates of 10- to 12-week-old mice were used for this experiment. The error bars indicate SEM.

mouse, all *Mavs*$^{-/-}$ mice died within 8 days (n = 5), whereas two out of five *Mavs*$^{+/-}$ and five out of seven wild-type mice survived at 12 days. At 1 × 10^8 pfu per mouse, 50% of *Mavs*$^{-/-}$ mice (n = 6) succumbed to viral infection at day 12, whereas three out of four *Mavs*$^{+/-}$ and all of wild-type (n = 4) mice remained alive. When the viral titer was further reduced to 5 × 10^7 pfu per mouse, none of the mice died of viral infection within 20 days, although the *Mavs*$^{-/-}$ mice appeared sick and later recovered (data not shown). Thus, MAVS protects the mice from VSV-induced mortality in a manner that depends on its gene dosage as well as the viral titer. These results suggest that interferons produced by pDCs and other cells through the TLR pathway are not sufficient to protect mice from viral killing and that the RIG-I-MAVS pathway may provide innate immunity through local production of interferons and/or other antiviral molecules. In the absence of MAVS, the initial high viral load might have caused irreversible damages to the mice so that they failed to recover even after the virus was cleared. The observation that *Mavs*$^{+/-}$ mice were vulnerable to VSV killing is particularly interesting, because it suggests that the expression level of MAVS is critical to antiviral immune defense (see below).

MAVS Is Required for Interferon Induction by Poly(I:C) In Vivo

As pDCs appear to be the major source of interferons elicited by VSV infection in vivo and this may mask the contribution of interferon production by other cell types, we sought to examine the role of MAVS in interferon induction by poly(I:C), which activates the cytosolic RNA sensing pathway in most cell types. Intravenous injection of poly(I:C) led to a rapid and robust induction of IFN-α, IFN-β, and IL-6 in the sera of wild-type mice (Figures 7A–7C). In contrast, the *Mavs*$^{-/-}$ mice failed to produce IFN-α and IFN-β, and the induction of IL-6 within the first 4 hr of poly(I:C) injection was also impaired. However, there was a late induction of IL-6 in *Mavs*$^{-/-}$ mice, albeit at a much reduced level as compared to the wild-type (Figure 7C; 8 hr time point). This may be due to the induction of IL-6 through TLR3, but not MAVS (see also Figure 3C). Interestingly, *Mavs*$^{+/-}$ mice produced intermediate levels of IFN-α, IFN-β, and IL-6, indicating that the gene dosage of *Mavs* is important for the cytosolic RNA signaling pathway. As the amount of MAVS protein in *Mavs*$^{+/-}$ cells is about half of that in the wild-type cells (Figure 1C), these results indicate that the steady-state level of MAVS protein is a limiting factor in mounting an effective antiviral response in vivo.

Discussion

We have presented genetic evidence that MAVS is essential for antiviral innate immune responses in fibroblasts, macrophages, and cDCs, but not in pDCs. The cell type-specific requirement of MAVS in antiviral immunity is similar to that of RIG-I (Kato et al., 2005). However, RIG-I knockout mice are not viable, suggesting that RIG-I might be involved in other processes currently not understood. In contrast, *Mavs*$^{-/-}$ mice displayed no apparent developmental abnormality, but they were severely compromised in immune responses against viral infection. Thus, our results provide the genetic evidence that the cytosolic viral sensing and signaling pathway through MAVS is essential for antiviral immune defense in vivo. Interestingly, normal levels of interferons are still detected in the sera of *Mavs*$^{-/-}$ mice, suggesting that other cell types such as pDCs can still produce interferons in the absence of MAVS. Nevertheless, *Mavs*$^{-/-}$ mice have higher initial viral loads and are much more vulnerable to viral killing. Thus, although the host immune system is endowed with different cell types that provide two partially redundant antiviral pathways, TLR and RIG-I/MDA-5, each of these pathways is important for effective antiviral responses in vivo.

Previous studies have presented conflicting data concerning the role of MAVS in TLR signaling (Seth et al., 2006). Although two reports showed that RNAi of MAVS/IPS-1 did not affect interferon induction by TRIF or poly(I:C) (Kawai et al., 2005; Seth et al., 2005), another report found that MAVS/VISA interacts with TRIF and is required for TLR3 signaling (Xu et al., 2005). Using MAVS-deficient cells, we have now provided the genetic evidence that MAVS is not required for interferon

induction by TLRs in multiple cell types. Therefore, TLRs and RIG-I are parallel antiviral signaling pathways that detect viral RNA in topologically different locations. The TLRs involved in viral sensing, including TLR3, 7, 8, and 9, are localized on the endosomal membrane, with the ligand sensing domain facing the lumen of the endosome (O'Neill, 2004). These TLRs detect viral RNA after the endocytosis and disassembly of the viral particles and then transduce signals through the cytosolic Toll-IL1 receptor (TIR) domain, which recruits MyD88 or TRIF to activate NFκB and IRFs. In contrast to TLRs, RIG-I is a cytosolic receptor that detects double-stranded RNA generated during viral replication in the cytosol. It has been proposed that some RNA viruses such as respiratory syncytial virus (RSV) and SeV, which enter host cells through fusion with the plasma membrane, induce type-I interferons through cytosolic RNA sensing pathway instead of TLR pathways in most cells, including pDCs (Hornung et al., 2004). Indeed, SeV has been shown to induce interferons in pDCs derived from *Myd88*$^{-/-}$ and *Pkr*$^{-/-}$ mice (Hornung et al., 2004). Because signaling by TLR7 and TLR9 in pDCs is strictly dependent on MyD88, our finding that *Mavs*$^{-/-}$ pDCs are still fully capable of inducing interferons (Figures 5D and 5E) raises the interesting possibilities that either MAVS and MyD88 function redundantly or there is a third pathway (MAVS and MyD88 independent) that mediates the induction of type-I interferons in pDCs in response to infection by some viruses such as SeV.

The TLR and RIG-I pathways utilize distinct adaptors to activate IKK and TBK1/IKKε. The use of MAVS as an essential adaptor in the RIG-I pathway is particularly interesting, as the function of MAVS depends on its localization on the mitochondrial membrane. The importance of the mitochondrial localization of MAVS is underscored by the recent discovery that hepatitis C virus (HCV) employs a serine protease, NS3/4A, to cleave MAVS off the mitochondrial membrane, thereby blocking interferon induction by the viral RNA (Li et al., 2005; Meylan et al., 2005). However, the in vivo situations appear to be more complicated, as microarray analyses have revealed abundant intrahepatic expression of ISGs in HCV-infected chimpanzees, suggesting that IFNs are produced even though MAVS might have been cleaved in these animals (Bigger et al., 2004; Wieland and Chisari, 2005). This conundrum is reminiscent of our observation that *Mavs*$^{-/-}$ mice remain capable of producing high levels of interferons after VSV infection. Thus, it is possible that both VSV and HCV can induce interferons through the TLR pathway in some cell types such as pDCs. Although HCV is a hepatotropic virus, it can also infect and replicate in extrahepatic tissues such as B cells (Machida et al., 2006). In addition, a recent study has shown that the hepatoma cell line Huh7 expresses TLR7 (Lee et al., 2006), which could induce interferons in response to viral infection. Although the source of interferons in HCV-infected host remains to be investigated, the cleavage of MAVS by the HCV protease likely contributes to the pathogenesis of this widespread virus, as suggested by our finding that *Mavs*$^{-/-}$ mice were highly susceptible to viral killing. The importance of MAVS in antiviral defense may partially explain why the HCV protease inhibitors are highly effective in rapidly reducing HCV viral loads in early-stage clinical trials (Lamarre et al., 2003); these inhibitors are expected to not only inhibit viral replication and assembly but also restore the host antiviral innate immunity by blocking the cleavage of MAVS.

Recent studies have uncovered a signaling pathway that induces interferons in response to cytosolic DNA, which can be introduced to the host cells through bacterial or viral infection or under certain pathological conditions that fail to degrade self-DNA during apoptosis (Ishii et al., 2006; Okabe et al., 2005; Stetson and Medzhitov, 2006). It has also been shown that *Listeria monocytogenes* induces interferon by releasing the bacterial DNA into the host cytoplasm (Stetson and Medzhitov, 2006). In addition, it was reported that RNAi of MAVS/IPS-1 partially inhibited the induction of an interferon reporter in cultured cells, leading to the proposal that MAVS/IPS-1 is required for signaling by cytosolic DNA (Ishii et al., 2006). However, our experiments using MAVS-deficient cells have shown clearly that MAVS is not required for interferon induction by cytosolic DNA or *Listeria monocytogenes*. Previous studies have also shown that RIG-I is not required for interferon induction by cytosolic DNA (Ishii et al., 2006) and that TLR, NOD1, NOD2, and RIP2 are dispensable for interferon induction by *Listeria monocytogenes* (O'Connell et al., 2005; Stockinger et al., 2004). Thus, further studies are required to identify the sensor(s) of cytosolic DNA and the adaptor(s) that transduce the DNA signals to the activation of IKK and TBK1/IKKε.

Our in vivo studies of *Mavs* mutant mice showed that both *Mavs*$^{-/-}$ and *Mavs*$^{+/-}$ mice were highly vulnerable to killing by VSV, suggesting that the dosage of MAVS is critical for antiviral immune defense. Because the *Mavs* mutant mice had normal levels of interferons in the circulation, the sensitivity of these mice to viral killing is likely due to the defect in the local production of antiviral molecules (Levy, 2002). Interferons and cytokines may be such molecules, as the production of IFN-α, INF-β, and IL-6 was abolished in *Mavs*$^{-/-}$ mice that were stimulated with poly(I:C). A recent study has shown that poly(I:C) induces interferons through Mda5, but not RIG-I (Kato et al., 2006). Thus, our results provide the in vivo evidence that MAVS is essential for signaling downstream of Mda5, and it serves as a convergent point for both Mda5 and RIG-I pathways. Interestingly, the induction of interferons and IL-6 by poly(I:C) was also severely compromised in *Mavs*$^{+/-}$ mice, suggesting that one copy of *Mavs* is not sufficient to mediate full antiviral responses. That *Mavs*$^{+/-}$ mice are severely immune compromised may have important implications in human immunogenetics. It will be of great interest to analyze the *Mavs* gene locus in human populations to determine whether individuals homozygous and heterozygous for *Mavs* mutations have increased susceptibility to viral diseases.

Experimental Procedures

Generation of *Mavs*-Deficient Mice

Genomic DNA containing the *Mavs* gene was isolated from 129/Sv mouse ES cell genomic DNA by PCR. The targeting vector was constructed by replacing a 1.7 kb fragment spanning from the ATG start codon of exon 2 to exon 3 of *Mavs* with a PGK-*Neo* positive selection cassette, which also contained two *loxP* sites flanking PGK-*Neo*.

After electroporation of the *Mavs* targeting vector, three independently targeted ES cell clones were injected into C57BL/6 blastocysts to produce chimeric mice. Chimeric mice obtained from two targeted ES clones were bred to C57BL/6 mice to obtain germline transmission. The heterozygous F1 progenies were intercrossed to obtain $Mavs^{-/-}$ mice. Mice from these independent clones displayed indistinguishable phenotypes. The mice used in this study were 129/Sv/C57BL/6 hybrids. However, only littermates from the crossing of heterozygous mice were used in the same experiments. All mice described in this report were engineered and housed in animal facilities at the University of Texas Southwestern Medical Center in compliance with guidelines set by Institutional Animal Care and Use Committee.

Antibodies

To generate polyclonal antibodies against mouse MAVS, a recombinant protein containing residues 127–276 of mouse MAVS was expressed in *E. coli* as a His_6-tagged protein and affinity purified. This protein fragment was used to immunize rabbits, and the sera were further purified by using the antigen column to obtain the MAVS antibodies. The antibody for mouse IRF3 was purchased from Zymed Inc, and antibodies for PARP and caspase 3 were from Cell Signaling Inc. FITC-conjugated antibodies against CD11c and CD11b, PE-conjugated antibody against B220, and APC-Annexin V were purchased from BD Pharmingen.

Cells

Embryonic fibroblasts from wild-type and mutant mice were prepared from day 13.5 embryos and cultured in DMEM supplemented with 10% FBS. Bone marrow cells were prepared from the femurs and tibiae of mice. These cells were cultured in RPMI 1640 containing 10% FBS, 10 mM HEPES (pH 7.4), 50 μM β-mercaptoethanol, and 100 ng/ml human Flt3 ligand (peproTech) or 10 ng/ml murine GM-CSF (peproTech). After 6–8 days, the cells were collected and used as Flt3L-induced BMDCs or GM-CSF-induced BMDCs, respectively. Flt3L-induced BMDCs were stained with antibodies against CD11c and B220 and sorted by FACS. FACS sorting was carried out with FACSVantage SE (with DIVA upgrade) after CD11c and B220 staining. $CD11c^+$ $B220^+$ cells and $CD11c^+$ $B220^-$ cells were used as bone marrow pDCs and cDCs, respectively. The purity of pDCs and cDCs was greater than 90% based on FACS analysis.

To isolate BMDMs, 1×10^7 bone marrow cells were cultured in DMEM containing 10% FBS and 10 ng/ml CSF-1 (Sigma). Twenty-four hours later, nonadherent cells were transferred to a new flask and cultured for 3 days before 10 ml fresh media containing CSF-1 were added and cells were cultured for another 4 days. Mature macrophages were harvested by collagenase (Roche) digestion and cultured on 96-well or 12-well plates for experiments. For peritoneal macrophages, cells were obtained by lavage of the peritoneal cavity with DMEM. Blood cells were lysed in ammonium chloride, and macrophages were collected and resuspended in DMEM containing 10% FBS for further experiments.

Stimulation of Cells and Functional Assays

To stimulate MEF cells or macrophages with cytosolic DNA, poly (dA:dT) or poly(dI:dC) (10 μg/ml; GE Biosciences) was incubated with lipofectamine 2000 (Invitrogen; 1 μl LF2000/μg DNA) at room temperature for 20 min and then added to cultured cells at the final concentration of 10 μg/ml of DNA. To stimulate TLR3, TLR4, or TLR9, cells were incubated with poly(I:C) (10 μg/ml; GE Biosciences), LPS (10 μg/ml; Sigma), or CpG-2084 DNA (5′-TCCTGACGTTGAAGT-3′; 5 μg/ml) (Lund et al., 2004), respectively. After incubation for indicated time periods, cell extracts were prepared to measure NFκB activation by electrophoretic mobility shift assays (EMSA) or IRF3 activation by native gel dimerization assays (Seth et al., 2005). For EMSA, whole-cell extracts were incubated with ^{32}P end-labeled NFκB oligos (5′-AGTTGAGGGGACTTTCCCAGG-3′), and the protein-DNA complex was resolved by native gel electrophoresis. To measure the production of cytokines, culture supernatants were collected for ELISA. The ELISA kits for mouse IFN-α and IFN-β were purchased from PBL Biomedical Laboratories (Piscataway, NJ), and the IL-6 ELISA kit was from BD Biosciences (San Diego, CA).

Viral and Bacterial Infection of Cells

SeV (Cantell strain) and VSV (Indiana strain) have been described previously (Seth et al., 2005). VSV-GFP virus (kindly provided by Dr. Genhong Cheng; UCLA) was propagated in BHK21 cells (Oganesyan et al., 2006). *Listeria monocytogenes* (10403 serotype) was cultured in 3.7% Brain-Heart Infusion overnight (Berg et al., 2003). The bacteria were washed three times in PBS before being used to infect cells.

For viral infection, cells grown in media containing 1% FBS were incubated with viruses at the indicated MOIs for 1 hr before replacement with the complete media containing 10% FBS. For *Listeria* infection, cells were incubated with the bacteria at MOI of 10 or 100 in antibiotic-free DMEM containing 10% FBS. After infection for 1 hr, excess bacteria were washed away and cells were incubated in complete media containing 50 μg/ml gentamycin.

Viral Infection and Poly(I:C) Injection in Mice and Measurement of Viral Titer

Mice of different genotypes were infected with VSV (5×10^7 pfu per mouse) or VSV-GFP (1×10^8 or 2×10^8 pfu per mouse) via tail vein injection. The viability of the infected mice was monitored for 2–5 weeks. Sera were collected at different time points to measure interferon induction by ELISA, as described above, and viral titers by plaque assays. For plaque assays, BHK21 cells were incubated with viral samples at serial dilutions for 1 hr and then overlaid with 1.5% methylcellulose in MEM containing 1% FBS. Forty-eight hours later, cells were fixed in methanol and stained with 0.1% crystal violet. Plaques were counted to calculate viral titer. For poly(I:C) injection, 200 μg of poly(I:C) was injected into each mouse intravenously, and the sera were collected for ELISA as described above.

Supplemental Data

Supplemental Data include five figure and can be found with this article online at http://www.immunity.com/cgi/content/full/24/5/633/DC1/.

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