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The TLR3/TICAM-1 Pathway Is Mandatory for Innate Immune Responses to Poliovirus Infection

Hiroyuki Oshiumi,*1 Masaaki Okamoto,*,1 Ken Fujii,† Takashi Kawanishi,* Misako Matsumoto,*, Satoshi Koike,† and Tsukasa Seya*

Cytoplasmic and endosomal RNA sensors recognize RNA virus infection and signals to protect host cells by inducing type I IFN. The cytoplasmic RNA sensors, retinoic acid inducible gene 1/melanoma differentiation-associated gene 5, actually play pivotal roles in sensing virus replication. IFN-β promoter stimulator-1 (IPS-1) is their common adaptor for IFN-inducing signaling. Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1), also known as TRIF, is the adaptor for TLR3 that recognizes viral dsRNA in the early endosome in dendritic cells and macrophages. Poliovirus (PV) belongs to the Picornaviridae, and melanoma differentiation-associated gene 5 reportedly detects replication of picornaviruses, leading to the induction of type I IFN. In this study, we present evidence that the TLR3/TICAM-1 pathway governs IFN induction and host protection against PV infection. Using human PVR transgenic (PVf) mice, as well as IPS-1−/− and TICAM-1−/− mice, we found that TICAM-1 is essential for antiviral responses that suppress PV infection. TICAM-1−/− mice in the PVR transgenic background became markedly susceptible to PV, and their survival rates were decreased compared with wild-type or IPS-1−/− mice. Similarly, serum and organ IFN levels were markedly reduced in TICAM-1−/−/PVf mice, particularly in the spleen and spinal cord. The sources of type 1 IFN were CD8α+ CD11c+ splenic dendritic cells and macrophages, where the TICAM-1 pathway was more crucial for PV-derived IFN induction than was the IPS-1 pathway in ex vivo and in vitro analyses. These data indicate that the TLR3/TICAM-1 pathway functions are dominant in host protection and innate immune responses against PV infection. The Journal of Immunology, 2011, 187: 5320–5327.

When RNA viruses infect mammalian cells, type I IFN is generated to suppress viral infection. IFN-inducing pathways evoked by viral dsRNA have been identified in humans and mice, and the possible involvement of these pathways in protection against viruses has been examined using gene-disrupted mice and various virus species (1). The sensing of dsRNA by the innate immune system is accomplished either by TLR3 or by cytoplasmic sensors such as dsRNA-dependent protein kinase (so-called PKR), retinoic acid inducible gene 1 (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) (2). In virus-infected cells, RIG-I and MDA5 mainly participate in type I IFN induction in conjuction with the adaptor molecule IFN-β promoter stimulator-1 (IPS-1; also known as MAVS, Cardif, or VISA) (1). The role of these molecules in host cell protection has been clearly delineated in RNA virus infection.

Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1; also called TRIF) is the adaptor of TLR3 (3–5). When TLR3 senses dsRNA on the endosomal membrane, it induces type I IFN (6, 7). The adaptor TICAM-1 plays a pivotal role in TLR3-mediated IFN-α/β induction. Once dsRNA stimulates TLR3, TICAM-1 transiently couples with TLR3 and forms a multimer, translocating to a distinct region of the cytoplasm (8). In its multimeric form, TICAM-1 recruits the kinase complex to activate IFN regulatory factors (IRF)-3 and -7, which induce type I IFN promoters (9). This concept was confirmed by the finding that MDA5−/− mice fail to induce type I IFN in response to MCMV (10, 11). In its multimeric form, TICAM-1 recruits the kinase complex to activate IRF-3 and -7, which induce type I IFN in response to MCMV (10, 11).

Many reports have mentioned the possibility that the TLR3/ TICAM-1 pathway is involved in the anti-viral IFN response (12), but no definitive evidence of the anti-viral properties of this pathway has been obtained using TICAM-1−/− mice (13). Only a DNA virus, mouse CMV (MCMV), has been shown to infect TICAM-1−/− mice, and thus mouse cells are partly protected from MCMV by the TICAM-1 pathway (5, 14).

Poliovirus (PV) is a positive strand ssRNA virus that produces dsRNA intermediates during viral replication (15), modified with 5′ terminal Vpg protein (16), a characteristic feature of picornaviruses. It is generally accepted that picornaviruses are recognized by MDA5 but not RIG-I in infected cells, presumably due to the generation of this unusual dsRNA. This concept was confirmed by the finding that MDA5−/− mice fail to induce type I IFN in response to encephalomyocarditis virus (EMCV) and permit severe EMCV infection (13, 17). However, another picornavirus, coxsackie B virus (CBV) serotype 3, is recognized by TLR3 in infected cells and induces IFN-γ as an effector for suppressing CBV infection.
(18). In this study, we analyzed in vivo infection of a popular picornavirus, PV, using PVRtg transgenic (PVRtg) mice, which show a neurotropic phenotype during PV infection similar to humans (19, 20). Using this mouse model, in combination with TICAM-1−/− or IPS-1−/− mice, we present evidence that the host TICAM-1 pathway, particularly in macrophages (MF), serves as a source of type I IFN induction and protects host PVRtg mice from PV infection and paralytic death. Thus, the strategy for host protection against picornaviruses is not simply based on the MDA5-dependent dsRNA recognition, but is variable depending on picornavirus species.

Materials and Methods

Mice

All mice were backcrossed with C57BL/6 mice more than seven times before use. TICAM-1−/− (21) and IPS-1−/− mice (this study) were generated in our laboratory. TLR3−/− (4), IRF-3−/−, and IRF-7−/− mice (22) were provided by Drs. S. Akira (Osaka University, Osaka, Japan) and T. Taniguchi (University of Tokyo, Tokyo, Japan). PVRtg mice were provided as reported previously (20). All mice were maintained under specific pathogen-free conditions in the Animal Facility at Hokkaido University Graduate School of Medicine (Sapporo, Japan). Animal experiments were performed according to the guidelines set by the Animal Safety Center, Japan.

Generation of IPS-1−/−deficient mice

The IPS-1 gene was amplified by PCR using genomic DNA extracted from embryonic stem cells. The targeting vector was constructed by replacing the second and third exons with a neomycin-resistance gene cassette (Neo), and an HSV thymidine kinase driven by the PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into 129/Sv mouse-derived embryonic stem cells, G418 and ganciclovir doubly resistant colonies were selected and screened by PCR. The targeted cell line was injected into C57BL/6 blastocysts, resulting in the birth of male chimeric mice. These mice were then backcrossed with C57BL/6 mice. The disruption of the IPS-1 gene was confirmed by PCR for the long and short arms. The abolishment of IPS-1 mRNA expression was confirmed by real-time quantitative PCR (RT-qPCR).

Cells, viruses, and reagents

Wild-type (WT) and TICAM-1−/− mouse embryonic fibroblasts (MEF) were prepared from 12.5- to 13.5-d-old embryos. PV, strain Mahoney, was amplified in Vero cells, and the viral titer was determined by a plaque assay. Five- to 8-wk-old C57BL/6 female mice were used throughout this study. Mice of different genotypes were i.p. or i.v. infected with 250 μg anti-NK1.1 Ab, asialoGM1 Ab, or control PBS as described previously (21). One day later, the mice were i.p. inoculated with 105 PFU PV. One to 7 d after PV infection, depletion of peripheral NK1.1+ cells was confirmed by flow cytometry. Then, the mortality of the mice was monitored. In some experiments, the spleen cells were harvested and NK cells (DX5+ cells) were positively isolated using the MACS system (Miltenyi Biotec). The DX5+ NK cells were suspended in RPMI 1640 containing 10% FCS and mixed with 51Cr-labeled B16D8 cells at the indicated E:T ratios. After 4 h, the supernatants were harvested and [51Cr] release was measured.

Statistical analysis

Statistical significance of differences between groups was determined by the Student t test, and survival curves were analyzed by the log-rank test using Prism 4 for Macintosh software (GraphPad Software). Student t tests and χ2 goodness-of-fit tests were performed using Microsoft Excel software and a χ2 distribution table.

Results

TICAM-1 is essential for protection of PVRtg mice against PV infection

Mice lacking the mdv-5 gene abrogate the production of type I IFN in response to EMCV infection and are more susceptible to infection with EMCV (13, 17). Because EMCV is a picornavirus, it has been proposed that MDA5 is critical for sensing picornavirus infection. In infected cells, picornaviruses efficiently generate long dsRNA, which is recognized by the cytoplasmic dsRNA sensor MDA5 (23). The 5′ end of the PV genomic RNA is linked to a VPg protein (16), not to a 5′-triphosphate, a major ligand for another cytoplasmic RNA sensor, RIG-I (24, 25). Thus, we first tested, using the PVRtg mouse model (20), whether the mortality of PV-infected mice is affected by disruption of IPS-1 (Fig. 1A). Approximately 70% of WT (PVRtg) mice and ~40% of IPS-1−/− mice survived >10 d postinoculation at an i.p. dose of 2 × 104 PFU. No statistical significance between these two groups was detected (Fig. 1A). In the same experiments, TICAM-1−/− mice died within 5 d by paralysis (Fig. 1B).

We next investigated the effect of the route of PV infection on mortality in this mouse model. PV (2 × 105 PFU) was injected i.p. or i.v. into WT and TICAM-1 mice and their mortality was examined (Fig. 1C, 1D). All TICAM-1−/− mice died by paralysis within 7.5 d irrespective of the injection route. The significance of this early mortality rate of PV-infected TICAM-1−/− mice was supported by statistical analysis. The mortality rates were slightly high in WT mice compared with IPS-1−/− mice when PV loads in mice were not very high (Supplemental Fig. 1A). This tendency seemingly diminished by early death of IPS-1−/− mice with high doses of PV input. These data suggested that TICAM-1, rather than IPS-1 (or the sensors RIG-I and MDA5), is a critical factor in protecting mice from PV-mediated paralytic death. This conclusion was confirmed using RIG-I−/− and MDA5−/− mice with a PVRtg background (S. Abe, K. Fujii, and S. Koike, submitted for publication).

These results showed a discrepancy with previous indications that MDA5 is critical in picornavirus protection (13). We therefore tested the dose dependence of PV in the survival of WT versus TICAM-1−/− mice. Surprisingly, high doses of PV (2 × 104 and 2 × 105 PFU) induced paralytic death in all WT as well as TICAM-1−/− mice within 6 d (Fig. 2A, 2B). Thus, high doses of PV (>2 × 105 PFU) appear to overpower the TICAM-1 PV-protective activity in vivo, which confirmed previous findings using other picornaviruses (13). TICAM-1 was most effective in
the survival against PV infection at low dose (<2 × 10^4 PFU) (Figs. 1B, 1C, 2C). Similar results were obtained with the PV infection study (S. Abe, K. Fujii, and S. Koike, submitted for publication) when TICAM-1−/− mice were substituted with TLR3−/− or IRF-3/7 double-knockout (KO) mice. Results were confirmed using IRF-3−/− and IRF-7−/− mice (26). These results are essentially consistent with previous reports using a PVRtg/IFNAR−/− mouse model (27), in which type I IFN is critical for PV permissiveness, particularly in the intestine of PVRtg mice.

**FIGURE 1.** Survival of WT, TICAM-1 KO, and IPS-1 KO mice following i.p. or i.v. PV infection. A and R, PV (2 × 10^4 PFU) was infected via the i.p. route into WT and IPS-1 (A) or TICAM-1 (B) KO mice (n ≥ 5), and survival was monitored for 14 d. C and D, PV (2 × 10^3 PFU) was infected via the i.p. (C) or i.v. (D) route into WT and TICAM-1 KO mice (n ≥ 5), and survival was monitored for 14 d.

**FIGURE 2.** High doses of PV disable the protective effect of TICAM-1. WT and TICAM-1 KO mice (n ≥ 6) were i.p. infected with 2 × 10^6 (A), 2 × 10^5 (B), or 2 × 10^2 PFU (C) PV and survival was monitored for 14 d.

**FIGURE 3.** Viral titers in organs and serum following PV infection. WT and TICAM-1 KO mice were infected i.p. with 2 × 10^4 PFU PV. The viral titers in each organ (A) and sera (B) were measured by a plaque assay. Data are shown as means ± SD of three independent samples.
after PV i.p. injection in TICAM-1−/− mice compared with WT mice (Fig. 3B).

IFN-α/β levels were measured with sera from WT, IPS-1−/−, and TICAM-1−/− mice, but they were barely detected in these PV-infected mice (Supplemental Fig. 1B). Only i.v. injection of high PV titers (an example shows >4 × 10^4 PFU) allowed WT mice to release type I IFN within 12 h (Supplemental Fig. 1B). No IFN was detected in blood in TICAM-1−/− and IPS-1−/− mice even in this high-dose setting. However, IFN-α production was reproduced in a cell type level (peritoneal Mf) in vitro (Supplemental Fig. 1C). PV infection-mediated cell death (28) and degradation of MDA5 protein (29) may be major causes for this undetectable type I IFN production during in vivo PV infection.

**TICAM-1 pathway contributes to IFN-β induction in WT mice with low PV titers**

We next determined the mRNA levels of type I IFN in each organ extracted from PV (2 × 10^6 PFU)-infected WT and TICAM-1−/− mice. IFN-β mRNA was upregulated in all of the organs tested in WT mice within 12 h in response to PV injection (i.p.) (Fig. 4A). In contrast, only a low increase in IFN-β mRNA was detected in the organs of TICAM-1−/− mice (Fig. 4A). IFN-α2 mRNA was upregulated in the organs of TICAM-1−/− and WT mice to similar extents in response to PV injection (2 × 10^6 PFU, i.p.) (Fig. 4B). Notable decreases in IFN-α2 mRNA were observed in the TICAM-1−/− spleen and spinal cord compared with WT controls (Fig. 4B). The mRNA levels of genes associated with type I IFN induction were evaluated by qPCR, and no unique differences were observed between the splenocytes from PV-infected TICAM-1−/− and IPS-1−/− mice (Supplemental Fig. 1D). Hence, type I IFN mRNA is generally upregulated via TICAM-1 in the local organs of PVRtg WT mice during PV infection.

The mRNA levels of IFN-inducible genes and other cytokines were determined in spleen cells after PV infection. IFN-λ and IFN-γ-induced protein 10 (IP-10) mRNA were upregulated in the spleen cells of WT, but not TICAM-1−/− mice, after PV infection (multiplicity of infection [MOI] of 1) (Fig. 4C), with profiles similar to that of IFN-β mRNA (Fig. 4C). A sensor for 5′-triphosphorylated RNA, IFN-induced protein with tetratripeptide repeats 1 (IFIT-1), was also upregulated through PV infection (Fig. 4C). TNF-α, IL-10, IL-12p40, and IFN-γ, which may be associated with infectious cell death, were barely upregulated in spleen cells in response to PV infection (Supplemental Fig. 1E).

**FIGURE 4.** The expression of type I IFN following PV infection. A and B, WT and TICAM-1 KO mice were infected i.p. with 2 × 10^4 PFU PV. Three days postinfection, the mRNA expression levels of IFN-β (A) and IFN-α (B, C) were determined by RT-qPCR. C, Splenocytes (5 × 10^5) were infected with PV (MOI of 1) and the mRNA expression levels of IFN-β, IFN-α2, IFN-λ, IP-10, and IFIT-1 were measured by RT-qPCR. Data are shown as means ± SD and are representative of three independent experiments.

The types of cells that participate in type I IFN induction in the spleen were examined by sorting spleen cells. IFN-β and IFN-α2 were found to be induced in WT CD11c^+ DC (Fig. 5A), whereas CD11c^- cells barely induced type I IFN. Furthermore, IFN-β and IFN-α2 were barely induced in TICAM-1−/− CD11c^- cells (Fig. 5B). Participation of IPS-1 in type I IFN induction in CD11c^- myeloid cells is less compared with that of TICAM-1 (Fig. 5B).

Splenic CD8α^-CD11c^- and CD4^-CD11c^- cells were separated by MACS beads and their response to PV (MOI of 1) was analyzed by determining the mRNA levels of type I IFN (Fig. 5C). CD8α^-CD11c^- cells, but not the CD4^-CD11c^- cells, of WT mice were responsible for type I IFN induction by PV. There was a CD4^-CD8α^- population of DC in the spleen and this type of cells did not induce type I IFN in response to PV (Supplemental Fig. 2). The generation of the mRNA of type I IFN and IFIT-1 by PV infection was abrogated in the TICAM-1−/− CD8α^-CD11c^- splenic DC (Fig. 5D). Also, CD4^-CD8^- double-negative DC failed to express type I IFNs (Supplemental Fig. 2). Thus, CD8α^-CD11c^- DC, which reportedly express TLR3 (30), are the source of type I IFN in PV-infected PVRtg mice.

We finally confirmed that type I IFN is locally induced in TLR3^- myeloid cells during PV infection. BM-Mf and BM-DC were prepared from mouse BM and challenged with PV (MOI of 1). These cells express TLR3 in the endosome as previously reported about mouse BM-DC (30) and human monocyte-derived DC (31). BM-Mf showed similar profiles of type I IFN mRNA to those of PV-infected splenocytes (Figs. 4C, 6A). However, IFN-λ and IP-10 mRNA were not detectable in PV-infected BM-Mf, the reason for which remains unclear (Fig. 6A). IL-12p40, a representative TICAM-1-dependent gene, was transiently upregulated...
in BM-Mf ~4 h after PV infection (Supplemental Fig. 3). Similarly, but less prominently, the profiles of type I IFN and IL-12p40 were observed in BM-DC (Fig. 6A, Supplemental Fig. 3) and CD11c+CD8+ splenic DC (Fig. 5D). Therefore, taken together, these results indicate that IL-12 and IFN-α/β are only minimally upregulated in splenic DC in a PV-dependent manner.

The production of IFN-α was determined by ELISA in the supernatant of PV-infected BM-Mf and BM-DC (Fig. 6C). BM-Mf prepared from WT mice generated higher amounts of IFN-α than did those from TICAM-1−/− mice. Although similar results were obtained with BM-DC, the effect of TICAM-1 depletion was not statistically significant (Fig. 6C).

NK cells and MEF do not play major roles in protection against PV infection

Using NK1.1-depleted mice, we tested the possible participation of NK cells in the protection of PVRtg mice from PV infection (Fig. 7). NK1.1+ cells were depleted from mouse blood 1 d after injection (i.p.) of NK1.1 Ab into WT (Fig. 7A) and TICAM-1−/− mice. After PV challenge, WT mice inoculated with control saline and NK1.1 Ab survived similarly, whereas TICAM-1−/− mice were all killed by PV within 7.5 d irrespective of NK1.1 pre-treatment (Fig. 7B). Hence, NK cell activation does not affect PV-derived death. The lack of TICAM-1 was also found to have no effect on the NK cell-mediated rescue of PV-infected mice.

Mouse fibroblasts are known to be a potential source of type I IFN (13). We therefore checked whether MEF induce type I IFN and protection against PV (Supplemental Fig. 4). MEF from WT PVRtg mice were susceptible to PV, with cell death being observed at an MOI of 1. MEF from TICAM-1−/− PVRtg mice were 1 log more susceptible to PV, with cell death occurring at an MOI of 0.1 (Supplemental Fig. 4A). IFN-β was upregulated in PV-infected MEF to only a slightly higher level in PVRtg MEF than in TICAM-1−/− PVRtg MEF (Supplemental Fig. 4B). These results suggested that the large difference in the PV survival rate between WT and TICAM-1−/− mice is not caused by NK cells or type I IFN induction by fibroblasts. The TICAM-1 pathway plays a key role for producing IFN-α/β in Mf/DC, but not in fibroblasts, during PV infection in PVRtg mice.

Discussion

In this study, we demonstrated that PV infection is exacerbated in TICAM-1−/− PVRtg mice. There are a number of RNA-sensing molecules that serve as anti-virus agents and function in a cell type-specific manner. Based on trials using gene-disrupted mice and human viruses, RIG-I has been reported to be essential for sensing infection by rhabdoviruses, influenza viruses, paramyxoviruses, and flaviviruses, whereas MDA5 is important for sensing picornavirus infection (13, 33). In previous studies on picornaviruses, however, only EMCV and several species of picornaviruses have been employed for the KO mice analyses (13). The essential role of type I IFN in PV tropism has been well characterized in PVRtg mice (34). To our knowledge, this study is the first to investigate the sensor that detects PV infection in PVRtg PV-sensitive mice. Because RIG-I and MDA5 use the adaptor IPS-1, we constructed an IPS-1−/− mouse strain for this
study. Unexpectedly, however, IPS-1 was dispensable for protection against PV infection in vivo. This study, taken together with other reports (33, 35, 36), suggests that each virus species has its own strategy to evade host immune attack. This is true even in picornavirus subspecies. Although the IPS-1 pathway involving RIG-I and MDA5 is important for sensing and preventing cytoplasmic virus replication, other steps also participate in critical regulation of virus replication. PV infection is the case where MDA5 is not absolutely critical, but TICAM-1 is essential, for virus protection.

The TICAM-1 pathway participates in driving NK/CTL activation in DC/Mf (21, 37). This pathway is involved in type I IFN induction, as in the IPS-1 pathway, but cells expressing TLR3 are limited. The TLR3 distribution profile by flow cytometry confirms its expression in myeloid cells in mice (30). The TICAM-1 pathway converges with the IPS-1 pathway via the molecular complex of IRF-3–activating kinases (38), and therefore activation of the TICAM-1 pathway induces type I IFN and other IFN-inducible genes (39). Nevertheless, gene induction profiles differ between the TICAM-1 and IPS-1 pathways (40), which may explain the functional distinction between the sensor that is triggered in the virus-infected cells (MDA5/IPS-1) and the sensor that is required for DC/Mf to mount immune responses. Studying these gene functions will be an important issue for functional discrimination between the intrinsic versus extrinsic sensors.

RIG-1/MDA5 are distributed over almost all organs, including MI/DC. An interesting point concerns what the function is of the IPS-1 pathway in Mf/DC. Without conditional KO mice, we have an experimental limit to discriminate between their intrinsic function that is triggered in PV-infected cells and the extrinsic function leading Mf/DC to driving the innate immune response. Because the TLR3/TICAM-1 pathway is conserved in Mf/DC, the CNS, fibroblasts, and epithelial cells, it is reasonable that their functions are rather specified in Mf/DC and the neuronal system in PV infection.

However, except several examples such as rhabdovirus (41) and hepatitis C virus (HCV) (32), no definitive evidence has been reported supporting the role of TLR3/TICAM-1 in anti-RNA virus function using KO mice, unlike IPS-1 (35, 36). In previous studies, we used RNA viruses and their mouse models of measles virus, respiratory syncitial virus, vesicular stomatitis virus, influenza virus, and rotavirus infection (12), but we were unable to demonstrate solid antiviral function of the TLR3/TICAM-1 pathway in these models (12). Accordingly, which type I IFN, IFN-inducible gene, NK cell, or CTL is an effector for antagonizing viral replication still remains uncharacterized. To our knowledge, the results of our present study first demonstrated that the TLR3/TICAM-1 pathway is indispensable for induction of the type I IFN effector, but not NK cell activation, which is a critical event in the elimination of virus-infected cells and host protection against PV. IL-12 and IFN-γ are not upregulated in splenic DC in a PV-dependent manner. Furthermore, CTL are unlikely to be involved in our present model, since they would not function within the time scale of several days after initial infection (42).
How PV circumvents host-inducible type I IFN is an intriguing point. Three lines of evidence have supported the presence of unique mechanisms by which PV infection abrogates MDA5-mediated type I IFN production by infected cells and accelerates TLR3-mediated DC maturation through phagocytosis of PV-infected cell debris. First, proteases encoded in the PV genome process the PV polyprotein to produce functional viral proteins (43). PV 2A and 3C proteases also contribute to the degradation of elF4G (44) and TATA-binding protein (45), respectively, the cleavage of which induces the translational and transcriptional “shutoff” of host protein synthesis (28). Thus, blocking the synthesis of host cell proteins by PV involves stopping IFN production. Second, MDA5 is degraded in PV-infected cells in a proteasome- and caspase-dependent manner, resulting in the lack of type I IFN production (29). Third, PV-mediated apoptosis occurs in a caspase-dependent manner to disable infected cells from inducing an IFN response (46), with the MDA5-dependent innate response to PV infection becoming minimal within 3 h postinfection. Additionally, RIG-I is also cleaved by the viral protease 3C (47), and additional RIG-I functions are subsequently disrupted. Hence, the RIG-I/MDA5 functional time frames should be narrow and ineffective in PV-infected cells.

The hijacked cells release virions and die irrespective of blocking of the IPS-1 pathway. These infected cells are degrading into apoptotic debris containing virus dsRNA when RIG-I/MDA5 is ineffective at inducing IFN (48). Phagocytic internalization of this infected debris containing viral dsRNA into endosomes in Mi/DC is a critical event for TLR3 stimulation (37). If this is the case in PV-infected PVRtg mice, dsRNA-containing debris produced by apoptosis of PV-infected cells may play a major role in the activation of the TICAM-1 pathway in myeloid cells, as is the case for another positive-stranded RNA virus, HCV (32). In HCV studies, dead cells act as carriers of viral dsRNA to the endosomes of DC (32). HCV induces cellular immunity including NK activation driven by the DC TICAM-1 pathway. PV, however, barely induces NK cell activation.

The results of the present study were obtained using the PVRtg mouse model for human PV infection. Possible limitations of this model may include the fact that PV natural infection in humans occurs postinfection of the intestine by a low dose of PV and the PV mouse model is unable to reproduce this infectious route (27). The difference in PV infection between human and the PVrtg mouse might reflect the difference of the IFN-inducing system in humans and mice. However, the response to neurovirulence and death by PV infection occurs similarly in mice and humans. PVRtg mice are susceptible to neuronal infection and the IFNAR−/− phenotype further enhances systemic PV infection (27, 34). The G (Sabin vaccine) and A forms (WT) of PV, which harbor G or A residues in their stem-loop V structures, respectively, show different levels of toxicity or neurovirulence (49). The lower toxicity of the vaccine strain is due to suppression of PTB-mediated protein synthesis in the G form. These results are essentially reproducible in the PVRtg mouse model (50). Our findings further indicate the essential role of the TICAM-1 pathway in the PVrtg model system for the PV-mediated induction of type I IFN in vivo. How this finding is associated with PV-mediated paralytic death and aberrance in the neuronal system is an open question for further understanding the PV neurovirulence and host defense.

In studies on virus infection in neurons, there was no difference between TLR3−/− and WT mice in the brain of reovirus infection (51). TLR3−/− mice have less severe neuroinvasiveness and survive longer than do WT mice in rabies virus infection (41). Further extensive studies have been performed with West Nile virus (WNV). TLR3−/− or TICAM-1−/− mice became more resistant to WNV infection than did WT mice (52). Compared to these earlier results, a recent report showed that lack of TLR3 enhances WNV mortality and increases viral burden in the brain (53). TNF-α and IL-6 are induced for inflammation, and high IL-10 production causes an increase of mortality in WNV-infected mice (54). TICAM-1 signaling is undoubtedly involved in the modulation of these cytokine productions and WNV replication in the nervous system (53, 54). In patients with herpes simplex encephalitis, functional deficiency of TLR3 or TICAM-1 is a critical factor for disease progression (55). The TLR3 responses in the CNS may differ from those in the immune system we examined (54, 56). How PV infection modulates IFN/cytokine-inducing signaling in the nervous system is an interesting issue. The possibility remains that cytokines, such as TNF-α, IL-10, IL-12p40, and IFN-γ, might be associated with the removal of infectious cells as in other virus infections, and the antiviral function of TLR3 ligands in PV-infected mice requires further elucidation.

A picornavirus CBV activates the TLR3/TICAM-1–IFN-γ axis in host-infected cells to induce type II IFN (18). It is possible that CBV promotes TLR3-dependent IFN-γ induction in lymphocytes rather than the type I IFN-inducing pathway. In the model of PV infection, however, the TICAM-1 pathway does not contribute to type II IFN induction. These findings indicate that picornaviruses, that is, EMCV, CBV, and PV, have independently evolved to adapt to the host innate immune system and cope with the IFN-inducing system. If this is the case, host responses against picornaviruses may not be unimodally raised by MDA5 but may provide differentially adapted strategies. EMCV tropism reported previously (13) is clearly distinct from those of other picornaviruses. In this article, we present evidence that PV infection is protected by the TICAM-1 pathway that extrinsically induces type I IFN. Virus-produced dsRNA may differentially act on host cells depending on each virus species and accomplish circumvention from host innate sensing systems, maintaining virus tropism.

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Disclosures
The authors have no financial conflicts of interest.

References
adaptor molecule I-mediated NF-κB and interferon regulatory factor-3 activation.


Figure S2

IFN-β mRNA expression

Fold increase

Time after PV infection

IFN-α mRNA expression

Fold increase

Time after PV infection
Figure S4

(A)

Initial MOI: 0, 0.01, 0.1, 1, 10

PVR Tg/TICAM-1/-

PVR Tg

MEF PV infection (24 hr)

(B)

IFN-β mRNA expression

Fold induction

Time after PV infection

0 6 12 hr

PVR Tg

PVR Tg, TICAM-1 KO
Supplemental figure legends

Figure S1. Production of IFN-α following PV infection. (A) 8×10^4 pfu of PV were intraperitoneally injected into wild-type and IPS-1 knockout (KO) mice, and the survival was monitored for 14 days. n=4. (B) 4×10^6 pfu of PV was intravenously injected into wild-type (WT), TICAM-1 knockout (KO) mice, and IPS-1 knockout mice (KO), and the cytokine levels in sera were measured by ELISA. (C) Peritoneal macrophages (peritoneal-Mf) (41) were induced from WT, TICAM-1 KO, and IPS-1 KO mice, and infected with PV (MOI=1) in a 24-well plate. The concentrations of IFN-α in the culture supernatants were measured by ELISA. (D) Splenocytes were isolated from WT, IPS-KO and TICAM-1 KO mice, and the expression of RIG-I, MDA5, TLR3, TICAM-1 and IPS-1 was measured by RT–qPCR. Data are shown as means ± SD and are representative of three independent experiments. (E) Splenocytes and CD8α+/CD11c+ cells were isolated from WT and TICAM-1 KO mice, and the expression levels of IFN-γ, IP-10, TNF-α, and IL-12 p40 were measured by RT–qPCR. Data are shown as means ± SD and are representative of three independent experiments.

Figure S2. No induction of type I IFN by CD4−/CD8α−/CD11C+ splenic dendritic cells (DC) was observed in response to PV. Splenocytes were harvested from PVRtg wild-type (WT) mice 18 h post PV intraperitoneal infection. Total CD11c+ cells and CD4−/CD8α−/CD11C+ cells were separated by flow cytometry. Fold increases in IFN mRNA were determined by RT–qPCR. Data are shown as means ± SD and are representative of three independent experiments.

Figure S3. IL-12p40 induced by PV infection in Mf. Bone marrow (BM)-macrophages (Mf) and BM-dendritic cells (DC) were prepared from the BM cells of wild-type and TICAM-1 knockout mice, and were infected with PV (MOI=1) in a 24-well plate. The expression of IL-12 p40 was measured by RT–qPCR. Data are shown as means ± SD and are representative of three independent experiments.

Figure S4. TICAM-1 was not a strong inducer for IFN-β in mouse embryonic fibroblasts (MEF). (A) Wild-type (WT) and TICAM-1 knockout (KO) MEF were infected with PV at the indicated MOI for 24 h. The cells were fixed and stained with
crystal violet. WT and TICAM-1 KO MEF were infected with PV (MOI=1), and the expression of IFN-β was measured by RT–qPCR. Data are shown as means ± SD and are representative of three independent experiments.