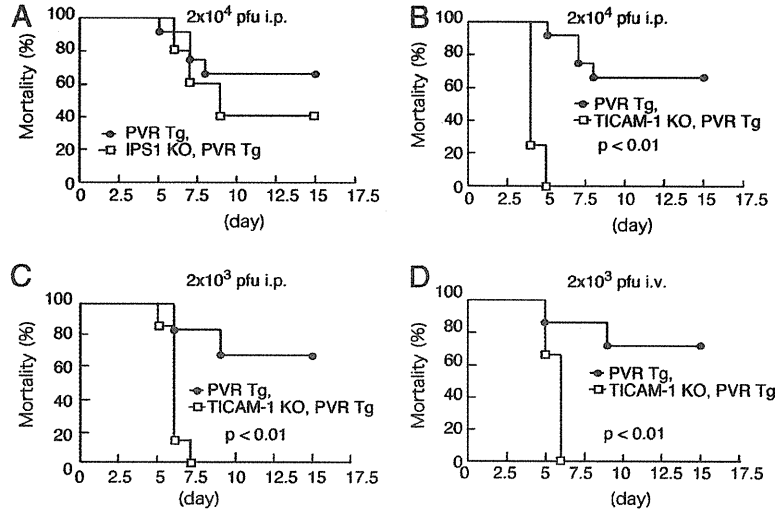

FIGURE 1. Survival of WT, TICAM-1 KO, and IPS-1 KO mice following i.p. or i.v. PV infection. *A* and *B*, PV (2×10^4 PFU) was infected via the i.p. route into WT and IPS-1 (*A*) or TICAM-1 (*B*) KO mice ($n \geq 5$), and survival was monitored for 14 d. *C* and *D*, PV (2×10^3 PFU) was infected via the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

the survival against PV infection at low dose ($<2 \times 10^4$ PFU) (Figs. 1*B*, 1*C*, 2*C*). 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.

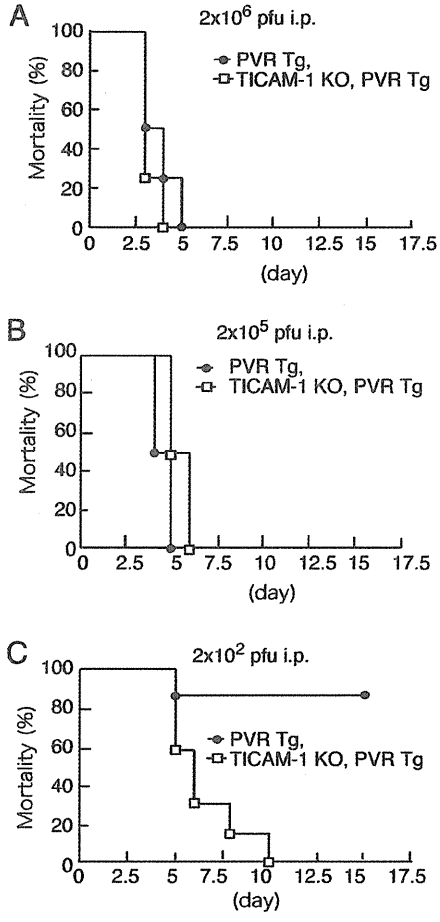

FIGURE 2. High doses of PV disable the protective effect of TICAM-1. WT and TICAM-1 KO mice ($n \geq 6$) were i.p. infected with 2×10^6 (*A*), 2×10^5 (*B*), or 2×10^2 PFU (*C*) PV and survival was monitored for 14 d.

TICAM-1–dependent type I IFN induction in PVRtg mice

PV titers in various organs were measured with WT and TICAM-$1^{-/-}$ mice i.p. injected with 2×10^4 PFU PV. In most organs, PV titers were higher in TICAM-$1^{-/-}$ mice than in WT mice at day 3 post-infection (Fig. 3*A*). The PV titer ratio in TICAM-$1^{-/-}$ versus WT mice was also high in the lung (Fig. 3*A*). In most organs except for the large intestine, high PV titers were harvested in TICAM-$1^{-/-}$ mice compared with WT mice. The difference in local PV titers between WT and TICAM-$1^{-/-}$ mice was culminated in the lung and spinal cord (Fig. 3*A*). Serum PV titers were increased within 48 h

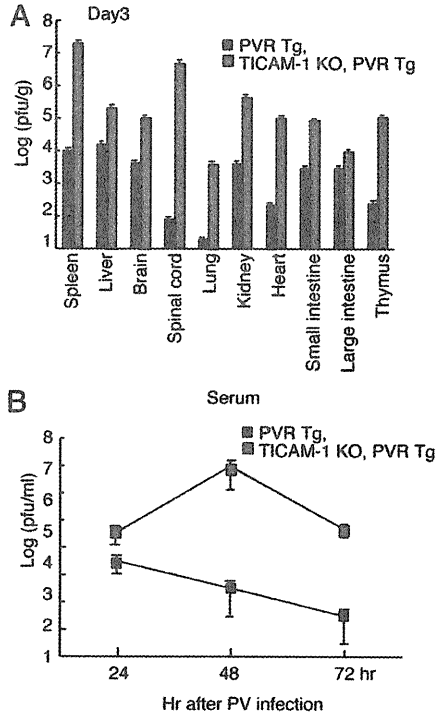

FIGURE 3. Viral titers in organs and serum following PV infection. WT and TICAM-1 KO mice were infected i.p. with 2×10^4 PFU PV. The viral titers in each organ (*A*) and sera (*B*) were measured by a plaque assay. Data are shown as means ± SD of three independent samples.

after PV i.p. injection in TICAM-1$^{-/-}$ mice compared with WT mice (Fig. 3*B*).

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. 1*B*). Only i.v. injection of high PV titers (an example shows >4 × 10^6 PFU) allowed WT mice to release type I IFN within 12 h (Supplemental Fig. 1*B*). 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. 1*C*). 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 × 10^4 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. 4*A*). In contrast, only a low increase in IFN-β mRNA was detected in the organs of TICAM-1$^{-/-}$ mice (Fig. 4*A*). IFN-α2 mRNA was upregulated in the organs of TICAM-1$^{-/-}$ and WT mice to similar extents in response to PV injection (2 × 10^4 PFU, i.p.) (Fig. 4*B*). Notable decreases in IFN-α2 mRNA were observed in the TICAM-1$^{-/-}$ spleen and spinal cord compared with WT controls (Fig. 4*B*). 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-injected TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice (Supplemental Fig. 1*D*). 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. 4*C*), with profiles similar to that of IFN-β mRNA (Fig. 4*C*). A sensor for 5′-triphosphorylated RNA, IFN-induced protein with tetratripeptide repeats 1 (IFIT-1), was also upregulated through PV infection (Fig. 4*C*). 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. 1*E*).

TICAM-1–dependent type I IFN induction by PV depends on Mf in PVRtg mice

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. 5*A*), whereas CD11c$^-$ cells barely induced type I IFN. Furthermore, IFN-β and IFN-α2 were barely induced in TICAM-1$^{-/-}$ CD11c$^+$ cells (Fig. 5*B*). Participation of IPS-1 in type I IFN induction in CD11c$^+$ myeloid cells is less compared with that of TICAM-1 (Fig. 5*B*).

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. 5*C*). 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. 5*D*). Also, CD4/8α 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. 4*C*, 6*A*). However, IFN-λ and IP-10 mRNA were not detectable in PV-infected BM-Mf, the reason for which remains unclear (Fig. 6*A*). IL-12p40, a representative TICAM-1–dependent gene, was transiently upregulated

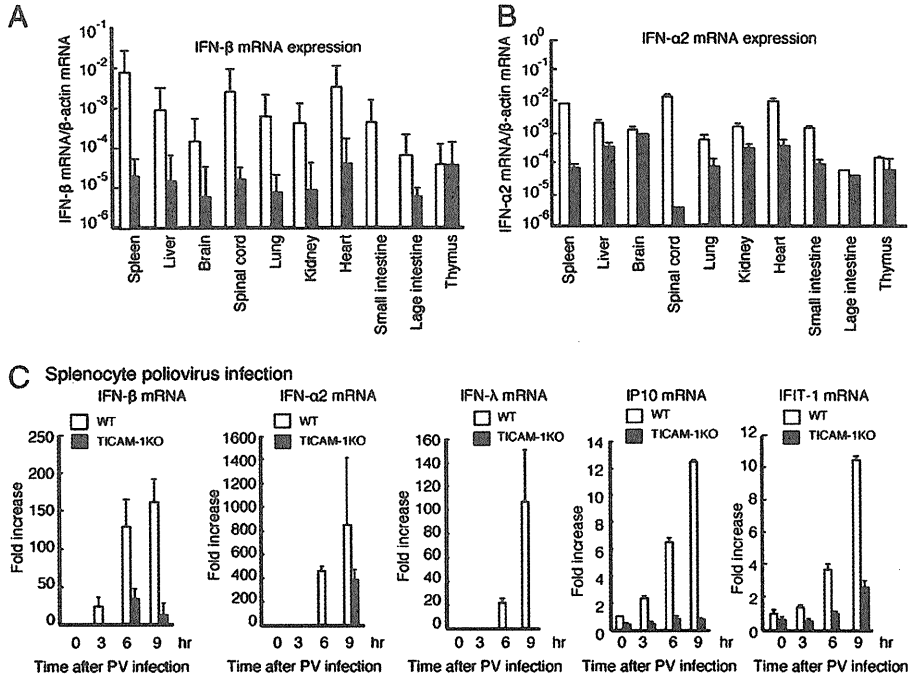

FIGURE 4. The expression of type I IFN following PV infection. *A* and *B*, WT and TICAM-1 KO mice were infected i.p. with 2 × 10^4 PFU PV. Three days postinfection, the mRNA expression levels of IFN-β (*A*) and IFN-α (*B*, *C*) were determined by RT-qPCR. *C*, Splenocytes (5 × 10^5) were infected with 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.

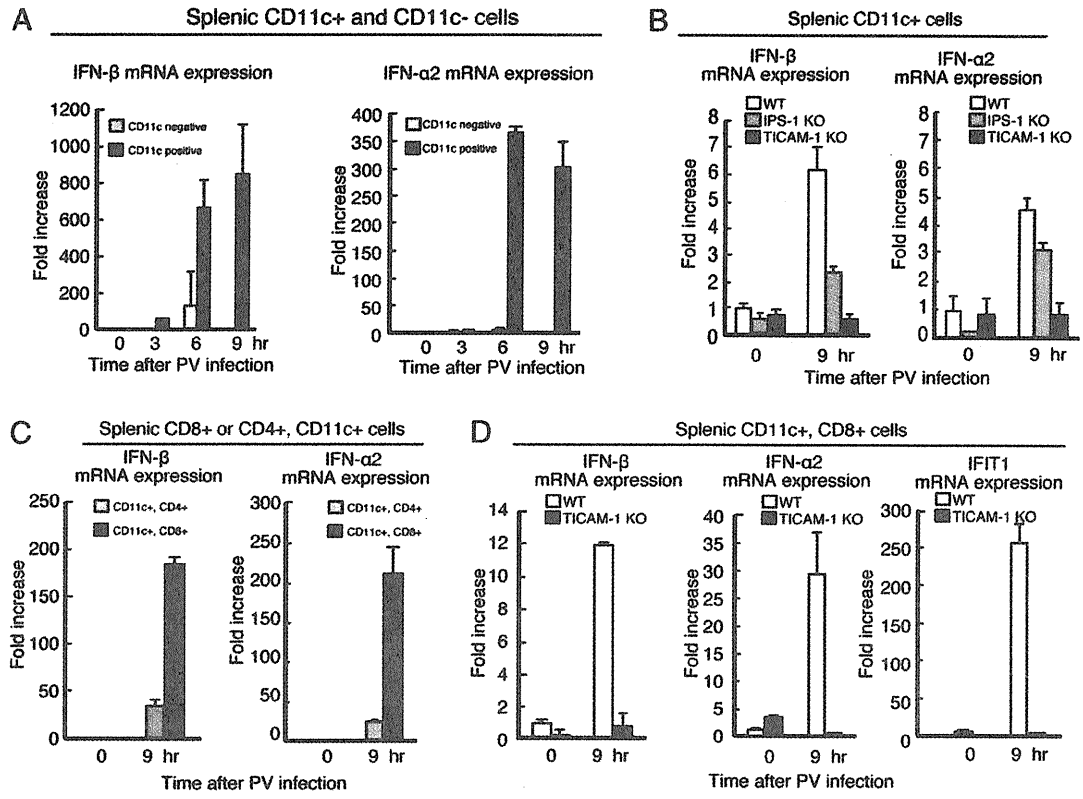

FIGURE 5. The expression of type I IFN in splenic DC. *A*, were isolated from WT spleens using the MACS system. $CD11c^+$ or $CD11c^-$ cells (5×10^5) were infected with PV (MOI of 1), and the mRNA expression of type I IFNs was measured by RT-qPCR. *B*, WT, TICAM-1, and IPS-1 knockout splenic $CD11c^+$ cells were infected with PV, and the expression of type I IFNs was measured by RT-qPCR. *C*, $CD8\alpha^+CD11c^+$ cells and $CD4^+CD11c^+$ cells were isolated from WT spleens and infected with PV (MOI of 1). The expression of type I IFNs was measured by RT-qPCR. *D*, $CD8\alpha^+CD11c^+$ splenic cells were isolated from WT and TICAM-1 KO mice and infected with PV (MOI of 1). The expressions of type I IFNs and IFIT-1 were measured by RT-qPCR. Data are shown as means ± SD and are representative of three independent experiments.

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. 6*A*, Supplemental Fig. 3) and $CD11c^+CD8^+$ splenic DC (Fig. 5*D*). 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. 6*C*). 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. 6*C*).

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. 7*A*) 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 pretreatment (Fig. 7*B*). 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. 4*A*). 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. 4*B*). 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

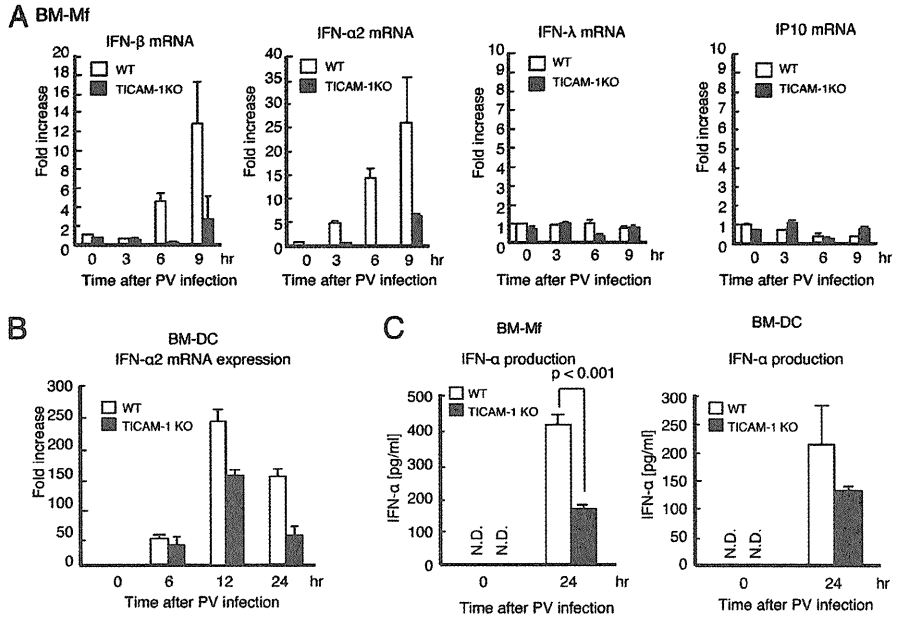

FIGURE 6. Production of type I IFN from BM-Mf and BM-DC. BM-Mf (*A*) and BM-DC (*B*) were prepared from BM cells with M-CSF and GM-CSF, respectively (32). The cells were infected with PV (MOI of 1), and the expression levels of IFN-β, IFN-α2, IFN-λ, and IP-10 were determined by RT-qPCR. *C*, IFN-α produced by PV-infected BM-Mf and BM-DC was measured by ELISA. BM-Mf and BM-DC were prepared from BM cells of WT and TICAM-1$^{-/-}$ mice as in *A* and *B*. Data are shown as means ± SD and are representative of three independent experiments.

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 Mf/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 epithelical 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).

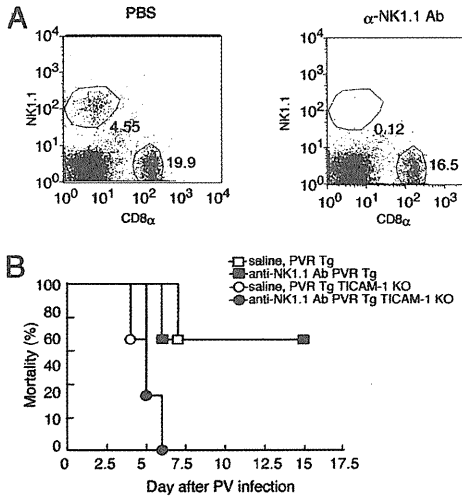

FIGURE 7. Effect of NK cells on mortality of PV-infected TICAM-1$^{-/-}$ PVRtg mice. *A*, To block the NK cell activity in mice, NK1.1 Ab or PBS (control) was i.p. injected into WT mice ($n \geq 6$). After 24 h, spleen cells were isolated from the mice and the fraction of NK1.1$^+$ cells was measured by FACS analysis. *B*, NK1.1 Ab or PBS was i.p. injected into WT and TICAM-1 KO mice. After 24 h, the mice were infected i.p. with PV, and survival was monitored for 15 d.

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 eIF4G (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 Mf/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.

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Figure S1

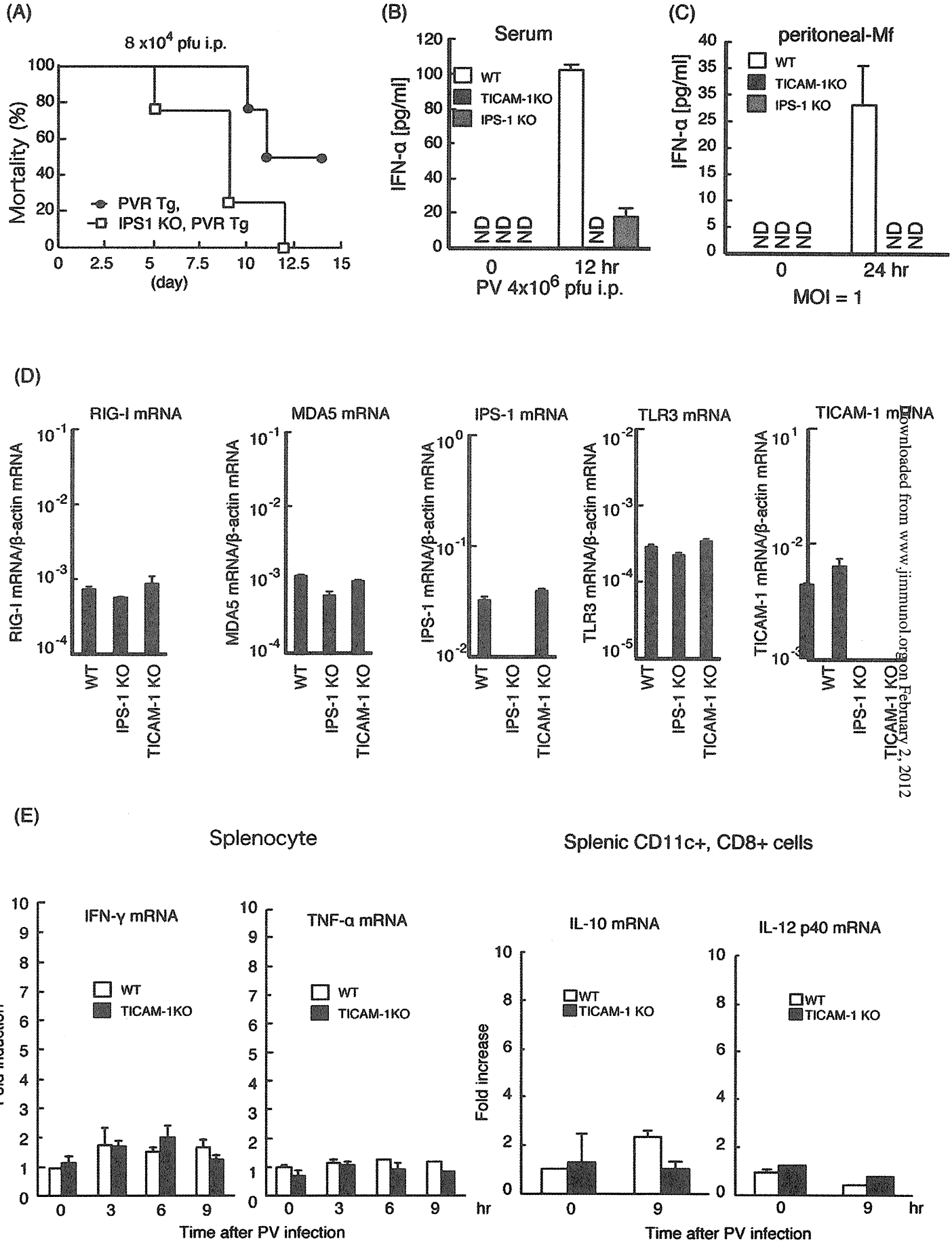
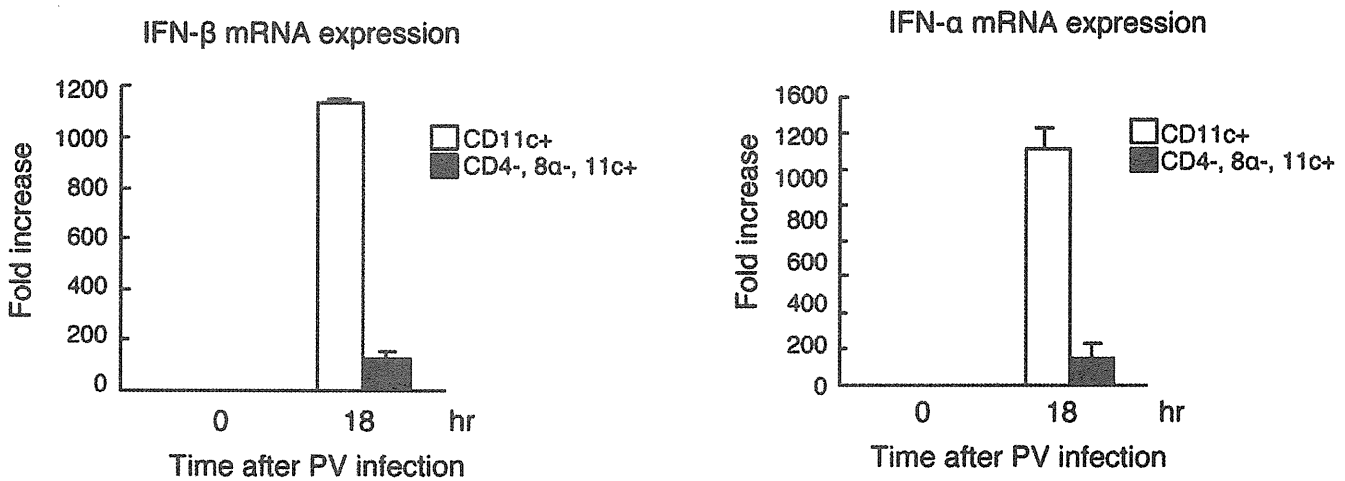


Figure S2



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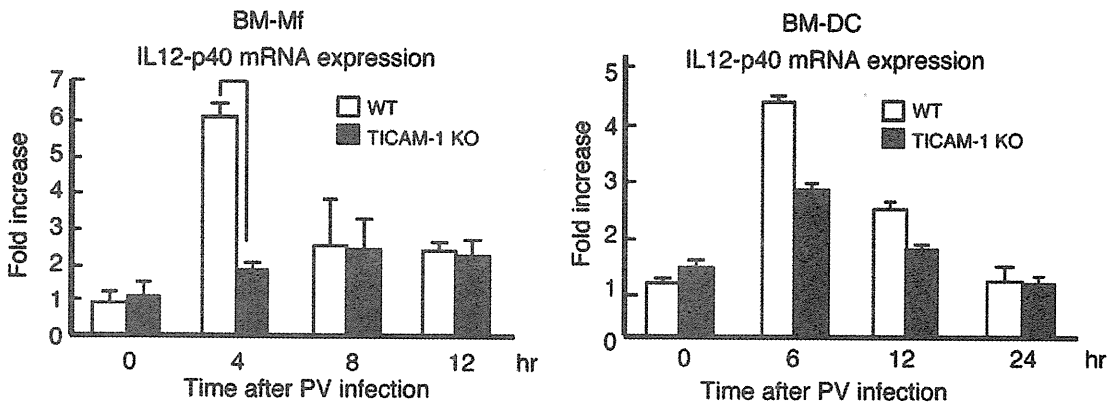
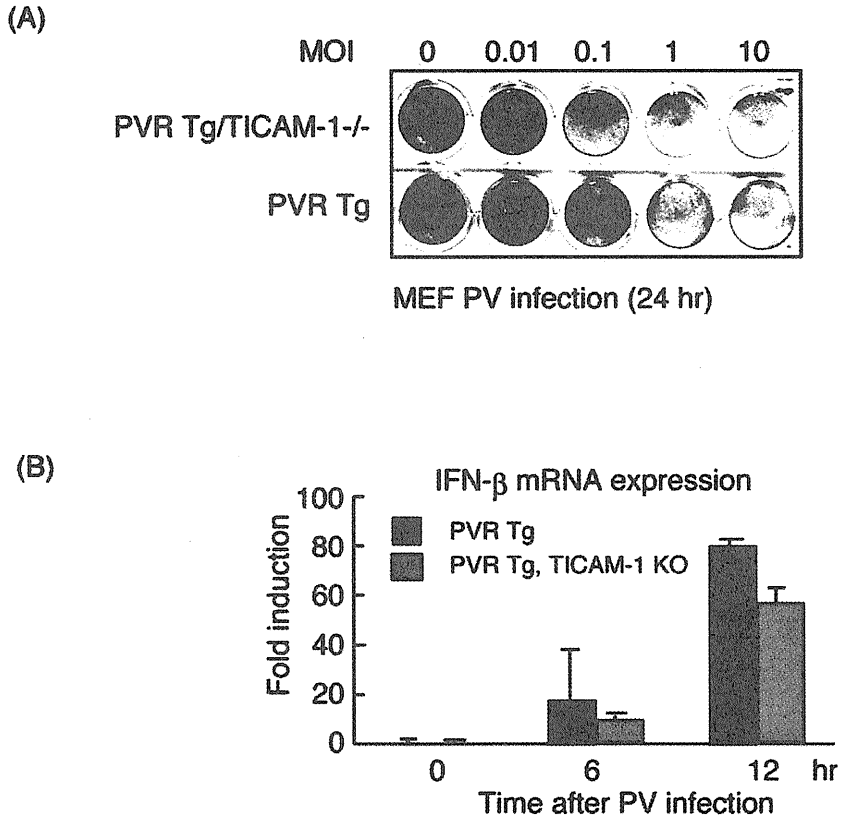


Figure S4



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.

Will There Be an HCV Meeting in 2020? Summary of the 17th International Meeting on Hepatitis C Virus and Related Viruses

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Hepatitis C virus (HCV), which was discovered in 1989, is a major etiologic agent in human liver disease. Approximately 130 million people, or 2% of the population, worldwide are infected. The 17th International Meeting on Hepatitis C Virus and Related Viruses was held September 10–14, 2010, in Yokohama, Japan. The meeting was attended by almost 700 scientists from all over the world who are interested in the fundamental aspects of the molecular virology, immunology, pathogenesis, prevention, and treatment of HCV infection. Two special opening lectures given by Masaaki Komatsu and Takashi Gojobori focused attention on the related research fields of autophagy and genome biology, respectively. In the subsequent sessions, the latest research, original studies, and controversies were presented in 9 keynote lectures, 82 oral presentations, and 329 poster presentations.

Viral Entry

The opening scientific session of this meeting focused on the viral host cell entry processes. Thomas Baumert presented the keynote lecture, which included an overview of the HCV cell entry process and recent advances at his laboratory. These included the finding that HCV variants that reinfect the liver after transplantation demonstrate more efficient cell entry and are less susceptible to neutralization by host antibodies. He also described the isolation of monoclonal antibodies against claudin-1 that do not inhibit either extracellular or direct cell-to-cell HCV transfer.

Alexander Ploss described the establishment of a mouse model for studying HCV cell entry. They utilized an HCV cell culture virus (HCVcc) expressing recombinase and transgenic mice bearing a recombinase-activatable fluorescent protein. Bioluminescent imaging indicated that only mice transduced with CD81 and occludin supported HCVcc entry. The presence of an intact immune system in these animals makes it particularly important for the testing of HCV vaccine candidates. Danyelle N. Martin described a role for transferrin receptor 1 (TfR1) in mediating HCV cell entry. The inhibition of HCV entry with TfR1 antibodies and silencing, suggest this factor should be added to the growing list of cellular proteins required for HCV cell entry. Joachim Lupberger presented results from a study showing an essential role for the epidermal growth factor receptor (EGFR) in HCV cell entry. He found that EGFR is required for both mediating the interactions between two other entry factors, CD81 and CLDN1, and catalyzing the fusion activity of viral glycoproteins.

Translation/Replication

Volker Lohmann began the session by describing what is known of the functions of viral nonstructural proteins and their associated host cellular factors in viral translation and replication. He included an overview of viral isolates and model systems currently used, and presented data addressing the mechanisms for efficient replication of the JFH-1 isolate.

Several reports have focused on the molecular basis of the architecture and composition of membrane-associated sites for HCV replication, which often induce membrane alterations, such as the so-called membranous web. Brenno Wolk demonstrated that NS4B is sufficient to direct all nonstructural proteins into the viral replication complex compartment, and that intragenotype-specific interactions are required for NS4B-dependent recruitment of NS5A. Ines Romero-Brey showed that the membranous web predominantly contains double-membrane vesicles with various diameters. These vesicle structures were connected to the endoplasmic reticulum (ER) through funnel-like structures.

Several DDX DEAD-box RNA helicases were identified as host factors associated with HCV replication. Yasuo Ariumi presented the cross-talk of HCV with DDX proteins and the role of distinct DDX proteins in viral replication. Tetsuro Shimakami and Selena M. Sagan reported the importance of miR-122 to not only enhance IRES-mediated translation, but stabilize positive-strand HCV RNA by binding to its 5′ extremity. Enzymatic activity of host phosphatidyl-inositol-4 kinase III alpha was shown to be critically involved in HCV replication and the activity is regulated by HCV NS5A (Simon Reiss). Nam-Joon Cho reconstituted a functionally active full-

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length HCV polymerase on a biomimetic membrane platform. Deborah Harrus found that guanosine triphosphate specifically stimulates the initial step of de novo initiation by stimulating transition of newly formed linker primer.

Assembly and Release

In the keynote lecture, Guangxiang G. Luo presented an overview of particle assembly and release, and the impact of apolipoprotein (Apo) E in the entry and assembly of HCV. He demonstrated the inhibition of HCVcc entry by treatment with anti-ApoE antibody and the direct interaction of ApoE with NS5A.

Ann L. Wozniak showed an important role for p7 in the production of infectious particles. Their data suggest that p7 stimulates virus production through the alkalization of intracellular vesicles. Ophelia Granio showed that both p7 and NS2 are required for the recruitment of core from lipid droplets (LDs) to ER. Costin-Ioan I. Popescu showed that NS2 accumulated in dotted structures in the ER in juxtaposition with Core and LDs. They concluded that cross-talk among Core, E1, E2, p7, and NS2 was essential for virion assembly. Vlastimil Jirasko demonstrated point mutations in the transmembrane regions of NS2 impaired the particle production and suggested that NS2 serves as a platform of viral and cellular proteins that coordinates HCV assembly. Qisheng Li identified the proviral function of IKKα by genome wide siRNA screening. IKKα regulates lipid metabolism and biogenesis of LDs and may enhance production of virus particles. The very low-density lipoproteins are secreted via a Golgi-dependent pathway. Bryan R. Bishe demonstrated the important role of phosphatidylinositol-4-phosphate and its interacting protein GOLPH3 in HCV secretion in the trans-Golgi network. Roland Remenyi showed 3-dimensional visualization of the HCV life cycle in cultured cells by electron tomography. They detected virus-like particles at various cytoplasmic locations. Viral particles in the proximity of LDs and within sponge-like inclusion were observed. These results provide ultrastructural visualization of putative assembly sites close to LDs.

Host Factors

In the invited lecture, Sara Cherry presented an overview of high-throughput screening toward the identification of host factors required for viral infection.

The contribution of autophagy to the HCV life cycle was also presented in this section, most notably, host factors linked with lipids. Tsubasa Munakata showed that the fatty acid synthase is required for efficient HCV replication. They also suggested the importance of palmitate for HCV replication. Samantha L. Blackham presented both the thioredoxin-interacting protein and the peroxisome proliferator activated receptor-α have significant effects on HCV replication. The host factors functioning on infectious HCV particle production were also reported. Takayuki Hishiki demonstrated the isoform dependent binding affinities of ApoE for low-density lipoprotein receptors and they affect infectivity of HCV. Laurent Chatel-Chaix found that Y-box binding protein interacted with HCV NS3 protein and viral RNA and was relocalized from nucleocytoplasmics site to the core-containing surface of LDs. Mohsan Saeed reported that the ER-associated degradation pathway was activated by HCV infection in a viral envelope protein-dependent manner. Po-Yuan Ke showed that HCV infection induces the unfolded protein response and activates the autophagic pathway. They proposed that autophagy contributes to the suppression of HCV in an autolysosome formation-dependent manner. Hiroto Kambara did not find any effects on HCV replication by inhibition of autophagosome formation in replicon cells. They proposed a role for autophagy induced by HCV infection to avoid the generation of vacuolation harmful to cell survival. Qisheng Li reported the network map of cellular pathways and machineries that are associated with HCV life cycle.

Very low-density lipoprotein is now considered to be one of a component of HCV particles. LDs are composed of fatty acid, triglyceride, and cholesterol, surrounded by several types of lipoproteins. In addition, Daniel J. Felmlee reported that chylomicron-associated viruses may be generated by virion association while in the vascular compartment. Francois Jean showed that the serine protease inhibitor protein Spn4A was modified to be directed to Site-1 protease specifically and was introduced into adenovirus vector to inhibit cholesterol and fatty acid syntheses for down-regulation of HCV propagation. The modified serpin could suppress Site-1 protease activity, reduce the LD, and block HCVcc infection. Nicolas Menzel tried to identify novel cellular factors involved in HCV assembly and release and found ERK inhibitor and cytosolic phospholipase A2 (cPLA2) inhibitor reduce viral production. cPLA2 inhibitor also reduced the amount of LD-associated core and supernatant ApoB/E. cPLA2 may be crucial for assembly of infectious HCV particles, possibly through participating in the formation of lipoproteins. Kohji Moriishi reported that the proteasome activator PA28γ participates in HCV propagation. PA28γ may participate in the propagation of HCV by regulating the degradation of Core in both ubiquitin-dependent and -independent manners. NS5A is regulated by phosphorylation of several host protein kinases. Takahiro Masaki identified 79 serine threonine protein kinases that were tightly associated with NS5A. Two of these may regulate the production of viral particles and/or viral replication.

Innate Immunity

The early phase of host defense against viral infection has largely been delineated based on recent advances in innate immunity. In the invited lecture, Manoj N. Krishnan introduced his comprehensive study on the Toll-like receptor 3–TRIF (TICAM-1) pathway. Using RNAi and polyI:C, he screened the genes specifically upregulated via the TRIF (TICAM-1) pathway. He expected that some viral infections are selectively blocked by the IPS-1 pathway, while others are blocked by the TRIF pathway.

Michael Gale, Jr., identified IFITM1 inhibits HCV infection. IFITM1 assembles with CD81 and tranlocates to the tight junction. This translocation of CD81 hampers the receptor function of CD81. They also discovered a novel pathway for ISGF3 activation. A non-receptor type tyrosine kinase-1 triggers activation of ISGF3 independent from the classical IFNAR pathway. IP-10 is a chemokine and is a negative predictor for pegylated interferon (IFN)/ribavirin therapy. Matthew L. Albert indicated that there is a 2-amino-acid–deleted form of IP-10 that serves as an antagonist for intact IP-10, and this form abrogates an early virologic response. As this IP-10 truncation is mediated by dipeptidylpeptidase IV, they believed that dipeptidylpeptidase IV is a novel therapeutic target for HCV patients during IFN therapy. Joo Chun Yoon suggested that activation of natural killer cells is inhibited by HCV-infected hepatocytes. They claimed that the early phases of HCV infection may be established through the failure of virus-inducible natural killer cell activation. Shin-ichiro Nakagawa reported that polyI:C induces both type I IFN and IFN-λ in human hepatocytes. The antiviral effect appears to parallel the induction of IFN-λ. This, together with the report by Emmanuel Thomas, suggests that the IFN-λ system is activated in HCV infected hepatocytes.

Adaptive Immunity

In a keynote lecture, Robert Thimme summarized the mechanisms of HCV-induced T-cell dysfunction. Multifaceted factors contribute to the hyporesponsiveness of T cells, including viral mutations, primary T-cell failure, lack of support from dendritic cells, expression of inhibitory molecules on T cells, and abundance of regulatory T cells (Tregs). Whether the ability of HCV-specific CTLs is comparable with that of CTLs having other specificities remains controversial. Bianca Seigel showed that HCV-specific CTLs are functionally impaired when compared with other CTLs, irrespective of their expression of inhibitory receptors or differentiation stages. CD161 is a C-type lectin that is expressed in HCV-specific $CD8^+$ T cells with tissue homing phenotype. Vicki M. Fleming found that $CD4^+CD161^+$ T cells produce large amounts of inflammatory cytokines and accumulate in the liver, where they are thought to exert pro-inflammatory roles. Naruyasu Kakita reported that certain adaptive Tregs, known as interleukin (IL)-10–producing type 1 Tregs, are increased in HCV-positive hepatocellular carcinoma patients, and their significance in hepatocellular carcinoma was greater than that of natural Tregs. Even in patients who have attained a sustained virologic response, trace amounts of HCV RNA are sporadically detectable in plasma. Barbara Rehermann reported the inoculation studies of such plasma. Residual HCV RNA in patients was able to infect chimpanzees and induced broad, HCV-specific T-cell responses. HCV RNA levels continued to be high when T-cell responses declined, suggesting that such HCV remains transmissible as hepatotropic pathogens.

Pathogenesis

In the invited lecture, Michael Diamond presented new mechanisms for West Nile virus immune evasion via 2'O methylation of viral RNA to subvert host innate immunity.

Genome-wide analysis of quantitative data (transcriptomics, proteomics, and metabolomics) facilitates systems biology analysis of HCV infection. Deborah L. Diamond analyzed the pathways involved in the progression of chronic hepatitis, namely, fibrosis and carcinogenesis, and found that molecules relating to cell metabolism including fatty acid oxidation enzymes and antioxidant systems may be master regulators of liver disease progression in HCV infection. HCV core protein has been shown to play a key role in the development of steatosis in HCV infected liver, especially in patients with genotype 3a HCV infection. Sophie Clement-Leboube showed that PTEN expression was down-regulated in the HCV infected liver. Analysis of lipo-viral-particle from hepatitis C patients by Olivier Diaz revealed that empty lipo-viral-particle lacking HCV RNA outnumbers those with RNA. The presence of virus-modified lipoproteins in HCV-infected patients may play a role in the pathogenesis of hepatitis C. Massimiliano Pagani used serum miRNA signatures to monitor liver disease in HCV infection and found miRNome candidates that are specific for HCV disease progression. Shuhei Taguwa showed that Con1 replicon induces incomplete autophagy through the dysfunction of autolysosomal acidification, which results in the secretion of immature cathepsin B in cells. Because the secretion of the protein is enhanced in many types of tumors, this observation may be associated with the pathogenesis of liver tumorigenesis in HCV infection.

The existence of extrahepatic manifestations is another issue of interest. Essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis, and Sjögren syndrome are conditions that have been shown to correlate

with HCV infection. Nicola A. Fletcher reported that brain microvascular endothelial cells express all the recognized entry factors for HCV, and brain microvascular endothelial cells actually support infection by HCVpp and HCVcc. This suggests potential disorders of the central nervous system in HCV infection.

Treatment

In the keynote lecture, Masashi Mizokami presented "Genome-wide association study and its application for HCV treatment." He emphasized that the functional relevance of IL-28B single nucleotide polymorphisms should be elucidated to further advance the progress of research on the mechanisms of chronic HCV infection and treatment.

Yasuhiro Asahina presented that genetic variation in IL-28B is associated with gene expression involving innate immunity. Minor alleles of IL-28B, as well as a higher RIG-I/IPS-1 ratio are associated with null viral response. Martin Laggins correlated IL-28B genetic variation with pretreatment levels of IP-10 and HCV RNA throughout therapy. The favorable genetic variation of IL-28B single nucleotide polymorphisms (major allele) was significantly associated with lower baseline IP-10. Masao Honda revealed that hepatic IFN-stimulated genes (ISGs) are associated with genetic variation in IL-28B and the outcome of IFN therapy for chronic hepatitis C using microarray gene expression profiling of the biopsied liver samples. Multivariate logistic regression analysis showed that ISGs, fibrosis stage, and ISDR mutations were strongly associated with viral response. Hepatic ISGs were associated with the IL-28B polymorphism and expression was significantly higher in patients with the minor genotype than in those with the major genotype. Takashi Motomura also analyzed ISG expression using liver transplantation samples. Expression of ISGs in recipients' liver carrying the minor allele of IL-28B was significantly up-regulated when compared with the major allele. Surprisingly, IFN sensitivity for recurrent hepatitis C after liver transplantation is influenced by IL-28B genetic variation not only in recipients, but also in donors.

Drug Development

This session opened with a keynote lecture by Raffaele De Francesco describing the current state of drug development for patients with chronic hepatitis C. Because of the rapid development of NS3/4A, NS5A, and NS5B inhibitors, he finally presented the hopeful message "Will there be an HCV meeting in 2020?".

Lotte Coelmont characterized an NS5A D320E variant showing low-level resistance to DEB025, a cyclophilin (Cyp)-binding molecule. This study suggests that DEB025 presents a high barrier to resistance, and that D320E confers low-level resistance to DEB025 by reducing the need for CypA-dependent isomerization of NS5A. Paul Targett-Adams reported that NS5A inhibitors stimulated redistribution of NS5A from the ER to ring-like structures in the cytoplasm, and disrupted colocalization with NS5B. This study suggests that NS5A inhibitors perturb formation of new replication complexes rather than acting on preformed complexes. Luis M. Schang developed a family of small synthetic rigid amphiphiles with large hydrophilic heads and small, planar and rigid hydrophobic tails, called RAFIs (rigid amphipathic fusion inhibitors), which inhibit the infectivity of enveloped virions including HCV. Emmanuel Thomas screened host genes involving the anti-HCV activity of ribavirin. Among 64 host genes, several candidate genes were identified as host factors involving ribavirin's anti-HCV activity. Interestingly, silencing of the *ITPA* gene increased the anti-HCV activity of ribavirin. Pablo Gastaminza identified a novel family of 1,2-diamines as an anti-HCV reagent from a chemical library. The analysis of ~300 derivatives identified several compounds with enhanced potency and low cytotoxicity.

Vaccines/Epidemiology

HCV therapeutic vaccines are aimed to induce effective T-cell responses. Marianne Mikkelsen reported that vaccination of mice with recombinant adenovirus expressing HCV NS3 fused to the MHC class II chaperon protein invariant chain significantly enhanced NS3 specific $CD8^+$ T-cell responses, and protected mice against NS3-expressing vaccinia virus challenge. This vaccination induced polyfunctional $CD8^+$ memory T cells. Lars Frelin aimed to restore immunologic function through vaccination in a transgenic mouse model with impaired HCV-specific T-cell responses owing to a persistent presence of hepatic HCV NS3/4A antigens. They found that heterologous sequences improved activation and expansion of NS3/4A-specific T cells in a wild-type host, as well as in a tolerant NS3/4A-transgenic mouse model. The authors also suggested an important role for Tregs in the impaired HCV-specific T-cell responses.

Livia M.G. Rossi examined antibody cross-immunoreactivity against different HVR1 variants to identify antigens with a possible application of HCV vaccine development. The authors identified a small set of HVR1 variants that cross-immunoreacted with a large number of HVR1 peptides, thus suggesting their potential use in the development of HCV vaccine candidates.

Conclusion

HCV2010 in Yokohama was successful and contributed to the progress of research in the field. HCV infection remains one of the most serious worldwide health problems. The goals of this symposium were to

increase the scientific understanding of this virus and gain insights applicable to future efforts to control its infection. From this point of view, we gained further fundamental understanding about HCV at the meeting. The discovery of IL-28B as a new host factor involved in HCV treatment and pathogenesis had a major impact on HCV research. New treatment advances have been made in recent years and will continue in the near future. We would like to conclude that this meeting was successful in providing opportunities for exchanging up-to-date information and international collaboration. The next meeting will take place in Seattle, Washington, from September 8–12, 2011 (http://www.hcv2011.org/).

Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Development of Mouse Hepatocyte Lines Permissive for Hepatitis C Virus (HCV)

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Abstract

The lack of a suitable small animal model for the analysis of hepatitis C virus (HCV) infection has hampered elucidation of the HCV life cycle and the development of both protective and therapeutic strategies against HCV infection. Human and mouse harbor a comparable system for antiviral type I interferon (IFN) induction and amplification, which regulates viral infection and replication. Using hepatocytes from knockout (ko) mice, we determined the critical step of the IFN-inducing/amplification pathways regulating HCV replication in mouse. The results infer that interferon-beta promoter stimulator (IPS-1) or interferon A receptor (IFNAR) were a crucial barrier to HCV replication in mouse hepatocytes. Although both IFNARko and IPS-1ko hepatocytes showed a reduced induction of type I interferons in response to viral infection, only IPS-1-/- cells circumvented cell death from HCV cytopathic effect and significantly improved J6JFH1 replication, suggesting IPS-1 to be a key player regulating HCV replication in mouse hepatocytes. We then established mouse hepatocyte lines lacking IPS-1 or IFNAR through immortalization with SV40T antigen. Expression of human (h)CD81 on these hepatocyte lines rendered both lines HCVcc-permissive. We also found that the chimeric J6JFH1 construct, having the structure region from J6 isolate enhanced HCV replication in mouse hepatocytes rather than the full length original JFH1 construct, a new finding that suggests the possible role of the HCV structural region in HCV replication. This is the first report on the entry and replication of HCV infectious particles in mouse hepatocytes. These mouse hepatocyte lines will facilitate establishing a mouse HCV infection model with multifarious applications.

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Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of mortality and morbidity throughout the world infecting around 3.1% of the world's population [1]. The development of much needed specific antiviral therapies and an effective vaccine has been hampered by the lack of a suitable small animal model. The determinants restricting HCV tropism to human and chimpanzee hosts are unknown. Replication of HCV strain JFH1 has been demonstrated in mouse cells only upon antibody selection [2], highlighting the very limited replication efficiency. Human CD81 and occludin have been implicated as important entry receptors for retrovirus particles bearing HCV glycoproteins, HCV pseudoparticles (HCVpp), into NIH3T3 murine cells [3]. However, HCV infection, spontaneous replication and particle production by mouse cells have not yet been reported.

In mammalian cells, the host detects and responds to infection by RNA-viruses, including HCV, by primarily recognizing viral RNA through several distinct pathogen recognition receptors (PRRs), including the cell surface and endosomal RNA sensors Toll-like receptors 3 and 7 (TLR3 and TLR7), and the cytoplasmic RNA sensors retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) [4]. The detection of virus infection by these receptors leads to the induction of interferons (IFNs) and their downstream IFN-inducible anti-viral genes through distinct signaling pathways [5]. Type I IFN is an important regulator of viral infections in the innate immune system [6]. Another type of IFN, IFN-lambda, affects the prognosis of HCV infection, and its response to antiviral therapy [7,8].

Mutations impairing the function of the RIG-I gene and the induction of IFN were essential in establishing HCV infectivity in human HuH7.5 cells [9]. Similarly, the HCV-NS3/4a protease is known to cleave IPS-1 adaptor molecule, inducing further downstream blocking of the IFN-inducing signaling pathway [10]. These data clearly demonstrate that the host RIG-I pathway is crucial for suppressing HCV proliferation in human hepatocytes. Using a similar strategy, we investigated whether suppressing the antiviral host innate immune system conferred any advantage on HCV proliferation in mouse hepatocytes. We examined the possibility of HCV replication in mice lacking the expression of key factors that modulate the type I IFN-inducing pathways. Only gene silencing of the IFN receptor (IFNAR) or IPS-1 was sufficient to establish spontaneous HCV replication in

mouse hepatocytes. To establish a cell line permissive for HCV replication, which is required for further *in vitro* studies of the HCV life cycle in mouse hepatocytes, we immortalized IFNAR- and IPS-1-knockout (ko) mice hepatocytes with SV40 T antigen. Upon expression of the human (h)CD81 gene, these newly established cell lines were able to support HCV infection for the first time in mouse hepatocytes. Viral factors required for HCV replication in mouse hepatocytes were also analyzed.

Results

IPS-1-mediated IFN signaling is important for HCV replication in mouse hepatocytes

As a first step in establishing HCV infection in mice, we tested the susceptibility of mouse hepatocytes to persistent expression of HCV proteins after RNA transfection. *In vitro* transcribed chimeric J6JFH1 RNA, in which the HCV structural and non-structural regions were from J6 and JFH1 isolates respectively, was transfected into hepatocytes from wild-type mice. We used a highly sensitive polyclonal antibody derived from HCV-patient serum for the detection of HCV proteins. No HCV proteins were detected five days after transfection (Fig. 1 A), suggesting that wild-type mouse hepatocytes were unable to maintain HCV replication. We then tried to find and block the pathway used by mouse hepatocytes for the detection of viral-RNA and the induction of IFN response. Mouse hepatocytes did not show the expression of either TLR3 or TLR7 as detected by RT-PCR, unlike IPS-1 and RIG-I which was fairly detected (Fig. S1), suggesting that the cytoplasmic RIG-I/IPS-1 pathway is the main pathway utilized by mouse hepatocytes for the detection of RNA viruses. We then checked the susceptibility of hepatocytes from TICAM-1ko, IPS-1ko and IFNARko mice to the prolonged expression of HCV proteins (Fig. 1B–D). Only IPS-1- and IFNARko mouse hepatocytes showed expression of J6JFH1 proteins five days after transfection (Fig. 1), indicating the importance of impaired IPS-1 and/or IFNAR receptors for HCV persistence. Similarly, the detection of the J6JFH1-RNA in transfected hepatocyte lines from various knockout mice showed higher levels in IPS-1 or IFNAR knockout cells compared to TICAM-1knockout cells in which a rapid decline of J6JFH1-RNA levels was noticed similar to the non-replicating control JFH1GND construct (Fig. S2). These data clearly suggest that the RIG-I/IPS-1 but not TLR3/TICAM-1 is the main pathway utilized for the detection of HCV-RNA and the induction of anti-viral immune response in mouse hepatocytes. Its suppression significantly improves HCV replication in mouse hepatocytes.

Establishment and characterization of immortalized mouse hepatocyte cell lines lacking expression of the IFNAR or IPS-1 gene

We further established mouse hepatocyte lines with disrupted IFNAR or IPS-1 genes through immortalization with SV40T antigen, and used these cell lines to study factors required for the HCV life cycle. Hepatocytes were transduced with SV40T-expressing lentivirus vectors. Six weeks after transduction, hepatocytes transduced with SV40T showed continuous proliferation and clonally proliferating hepatocyte lines were selected. SV40T-immortalized IFNARko and IPS-1ko clones were designated IRK (Fig. 2 A) and IPK (Fig. 2 B), respectively. 20 IRK and 19 IPK clones were picked up, of which IRK clones 2 and 4 (IRK2 and IRK4) and IPK clones 10 and 17 (IPK10 and IPK17) were most closely related to primary mouse hepatocytes in term of differentiation (Fig. 2 C) and were used in the following experiments. Expression of SV40T was confirmed by RT-PCR analysis (data not shown). IRK2, IRK4, IPK10 and IPK17, but not the non-hepatocytic NIH3T3 cells, displayed albumin and hepatocyte nuclear factor 4 (HNF4) expression similar to that observed in liver tissue, but did not express the bile duct marker, cytokeratin. IRK and IPK cells did not show expression of IFNAR and IPS-1 respectively (Fig. 2 C).

Replication of the HCV genome in IRK and IPK cells

To assess the permissiveness of the established cell lines to HCV replication, we transduced IRK4 and IPK17 cells with J6JFH1 RNA and monitored the HCV protein and RNA levels by IF (Fig. 3 A) and real time RT-PCR (Fig. 3 B). The number of cells expressing HCV proteins, as detected by IF, increased over time, indicating the continuous proliferation of J6JFH1 in these cells. However, the ratio between infected and non-infected cells did not significantly change over time for 7 days after transfection. Similarly, the amount of total J6JFH1 RNA in 1 μg of total cellular RNA was reasonably constant. By contrast, the level of

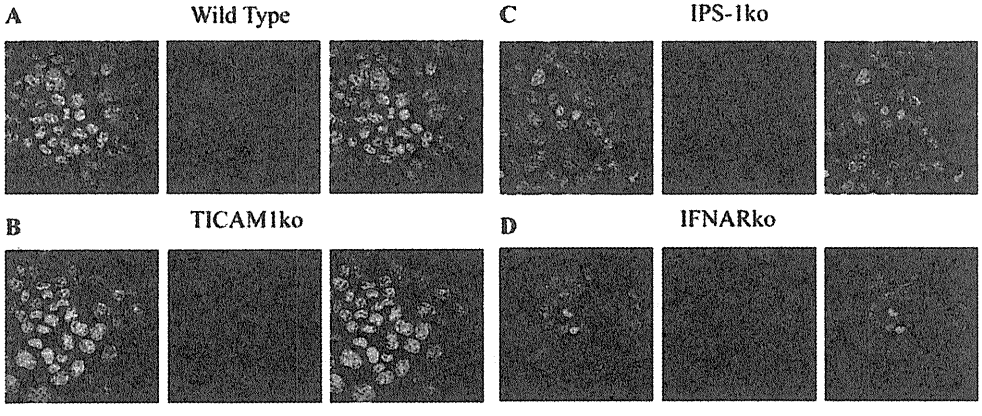

Figure 1. IF detection of of J6JFH1 proteins' expression 5 days after transfection of J6JFH1-RNA through electroporation into wild type (A), TICAM-1ko (B), IPS-1ko (C), and IFNARko (D), freshly isolated primary hepatocytes. A highly sensitive polyclonal antibody extracted from HCV-patient serum (AbS3) was used for the detection. Staining of the uninfected hepatocytes from different Ko mice was also performed and they showed negative for HCV proteins (data not shown).
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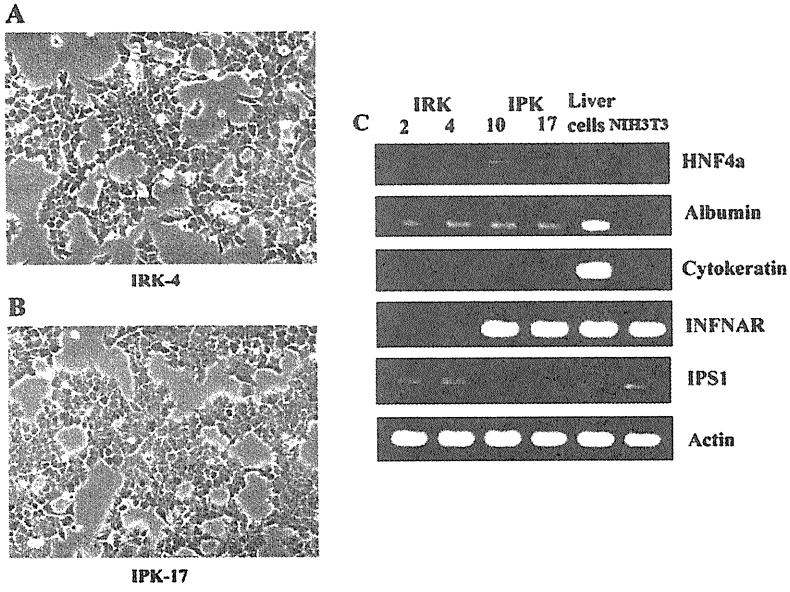

Figure 2. Morphological characteristics of IRK-4 (A) and IPK-17 (B) cells. (C) RT analysis for the expression of albumin, HNF4, cytokeratin, interferon A receptor, and IPS-1 in 2 IFNAR-KO cell lines (IRK2 and 4), 2 IPS-1-KO cell lines (IPK-10 and 17), total liver, and NIH3T3 cells.
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JFH1GND RNA carrying a mutation in NS5B hampering HCV replication, rapidly declined, indicating the requirement of continuous HCV replication for the maintenance of HCV positivity in the transfected mouse hepatocytes. Similar data were obtained from IRK2 and IPK10 cells (data not shown).

IPS-1-dependent/Interferon-independent pathway is responsible for HCV's cytopathic effect

In comparison to IPS-1ko hepatocytes, J6JFH1-RNA in IFNARko were lower and decreased further after its transfection, while higher stable levels of J6JFH1-RNA were maintained in IPS-1ko cells (Fig. 3 B and Fig. S2). Similarly, larger numbers of HCV-positive cells were detected in IPS-1ko hepatocytes compared with their IFNARko counterparts (Fig. 3 A), suggesting that the IPS-1 disruption benefits HCV replication in a distinct manner from IFNAR disruption. To measure the interferon induction after RNA virus infection in those cells, we used a highly infectious RNA-Virus (VSV) and measured the induction of interferon after its infection. All the interferons measured showed similar suppression of induction in IFNARko and IPS-1ko hepatocytes (Fig. 4). Surprisingly, cellular cytopathic effect that was monitored after transfection of J6JFH1-RNA was markedly reduced in IPS-1ko but not in IFNARko hepatocytes after transfection (Fig. 5A). This suppression was accompanied by an increase of J6JFH1-RNA levels in IPS-1ko cells, suggesting that minimal cellular damage induced by HCV replication in IPS-1-/- cells led to the improvement of HCV proliferation in mouse hepatocytes (Fig. 5B).Reduction of HCV-induced cellular cytotoxicity (Fig.5C), and improvement of HCV replication (Fig.5D) in wild type, and IFNAR-KO cells were found when we cultured the cells with a pan-caspase inhibitor, zVAD-fmk, 2 days before and after HCV-RNA transfection. We reasoned that the IPS-1 pathway rather than the IFNAR pathway capacitates hepatocytes to induce HCV-derived apoptotic cell death and its disruption resulted in the circumvention of cell death.

Human CD81 is required for HCV infection of mouse hepatocytes

Similar to the primary mouse hepatocytes, immortalized mouse hepatocytes showed the expression of all the mouse counterparts of human HCV entry receptors (Fig. S3). Human CD81 and hOccludin, but not other human HCV receptors such as SR-B1 or claudin1, have previously been reported to be essential for HCVpp entry into NIH3T3 mouse cells [3]. We then expressed hCD81 and/or hOccludin in IRK2 and IRK4 cells using lentivirus vectors. Using a MOI of 10, 95% transfection efficiency was achieved (Fig. S4) with lentivirus vector. We next tested the effect of these proteins on HCV particle (HCVcc) infection. Human CD81 alone was found to be required for J6JFH1 infection into all IRK and IPK cells tested (Fig. S5 and Fig. 6 A, and B). For the first time in mouse hepatocytes, HCV proteins were detected in nearly 1% of the cells used for infection. These data demonstrated the importance of hCD81 in establishing HCVcc infection in mouse hepatocytes.

Viral factors affecting HCV replication in mouse hepatocytes

After successfully establishing J6JFH1 infection in mouse hepatocytes, we attempted to infect these cells with other strains of HCV. Human CD81-expressing IPK17 cells were infected with full-length JFH1FL, however, no infection was detected (data not shown). This might be due to a problem in infection and/or replication. We further examined the replication efficiency of JFH1FL, the subgenomic JFH1 replicon and the J6JFH1 chimera in two different mouse hepatocyte lines and the HuH7.5.1 cell line. The persistent expression of HCV proteins was detected seven days after RNA transfection. Although HCV proteins were detected in HuH7.5.1 cells in all cases (Fig. 7 C), only J6JFH1 proteins were detected in the mouse hepatocyte lines, suggesting for the first time the importance of the J6 structural region for the replication of HCV in mouse hepatocytes (Fig. 7 A, and B).