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IPS-1 Is Essential for Type III IFN Production by Hepatocytes and Dendritic Cells in Response to Hepatitis C Virus Infection

Masaaki Okamoto,* Hiroyuki Oshiumi,* Masahiro Azuma,* Nobuyuki Kato,† Misako Matsumoto,* and Tsukasa Seya*

Hepatitis C virus (HCV) is a major cause of chronic liver disease (1). The 3′ untranslated region (UTR) of the HCV genome is recognized by a cytoplasmic viral RNA sensor RIG-I (2), HCV RNA induces RIG-I–dependent type I IFN production to promote hepatic immune responses in vivo (2). RIG-I is a member of RIG-I–like receptors (RLRs), which include MDA5 and LGP2. RLRs trigger signal that induces type I IFN and other inflammatory cytokines through the IPS-1 adaptor molecule (3). RLRs are localized in the cytoplasm and recognize cytoplasmic dsRNAs. Another pattern recognition receptor, TLR3, recognizes dsRNAs within early endosomes or on cell surfaces (4). Human monocyte-derived dendritic cells (DCs) require TLR3 to recognize HCV RNA in vitro (5), and TLR3 induces type I IFN production through the TICAM-1 adaptor, also called Toll/IL-1R domain-containing adapter inducing IFN-β (6, 7).

IL-28B is a type III IFN (also called IFN-λ), which includes IL-28A (IFN-λ2) and IL-29 (IFN-λ1) (8). Type III IFNs interacts with heterodimeric receptors that consist of IL-10Rβ and IL-28Rα subunits (8). Polymorphisms upstream of the IL-28B (IFN-λ3) gene are significantly associated with the responses to polyethylene glycol–IFN and ribavirin in patients with chronic genotype 1 HCV infections (9–12). As seen with type I IFNs, type III IFNs have antiviral activities against HCV (13). Type I IFNs induce the expression of IFN-inducible genes, which have antiviral activities, and can promote cross-priming and NK cell activation (14). However, the roles of type III IFN in cross-priming and NK cell activation are largely unknown, and the functional differences between type I and III IFN are uncertain.

Mouse CD8+ DCs and its human counterpart BDCA3+ DCs are the major producers of type III IFNs in response to poly(I:C) (15). CD8+ DCs highly express TLR3 and have strong cross-priming capability (16). A recent study showed that TLR3 was important for type III IFN production by BDCA3+ DCs in response to cell-cultured HCV (17). RIG-I efficiently recognizes the 3′ UTR of the HCV RNA genome, and, thus, RIG-I adaptor IPS-1 is essential for type I IFN production (2). However, the role of an IPS-1–dependent pathway in type III IFN production in vivo has been underestimated. In this study, we investigated the role of an IPS-1–dependent pathway in type III IFN production in vivo and in vitro using IPS-1 knockout (KO) mice and established an essential role of IPS-1 in type III IFN production in response to HCV RNA. Our study indicated that not only TICAM-1 but also IPS-1 are essential for type III IFN production by BDCA3+ DCs and hepatocytes in response to HCV, leading to cytoplasmic antiviral protein expressions.

Materials and Methods

**Mice**

All mice were backcrossed with C57BL/6 mice more than seven times before use. The generation of TICAM-1 and IPS-1 KO mice was described...
previously (18). All mice were maintained under specific pathogen-free conditions in the Animal Facility of the Hokkaido University Graduate School of Medicine (Sapporo, Japan). Animal experiments were conducted according to the guidelines established by the Animal Safety Center, Japan.

**Cell lines and reagents**

Human hepatocyte cell lines O cells and O cured (Oc) cells that contained HCV 1b replicons were provided by N. Kato (Okayama University). Mouse hepatocyte cell line was described previously (19). PolyIC was purchased from GE Healthcare and dissolved in saline. An OVA (HEK 293 SLS) tetramer was purchased from MBL. PE-CD80, -CD86, -NK1.1, FITC-CD8, and allophyocyaconin-CD3e Abs were purchased from BioLegend, and PE-CD40, FITC-CD69, and allophyocyaconin-CD11c Abs were from eBioscience. An ELISA kit for IFN-β was purchased from PBL Biological Laboratories, and ELISA kits for mouse IL-28 (IFN-λ2/3) (Invitrogen) were purchased from Abcam and eBioscience. An ELISA kit for mouse IFN-γ was purchased from eBioscience. ELISA was performed according to the manufacturer’s instructions. Mouse IFN-α and IFN-λ3 (IL-28B) were purchased from Miltenyi Biotec and R&D Systems, respectively.

**Cell preparation**

Spleen CD8+ and CD4+ DCs were isolated using CD8+ DC isolation kit and CD4+positive isolation kit, according to manufacturer’s instruction (Miltenyi Biotec). Spleen CD11c+ DCs were isolated using CD11c microbeads. To obtain splenic double-negative (DN) DCs, CD4+ and CD8+ cells were depleted from mouse spleen cells using CD4 and CD8 MicroBeads (Miltenyi Biotec), and then CD11c+ DCs were positively selected using CD11c MicroBeads (Miltenyi Biotec). We confirmed that 90% of isolated cells were CD4- , CD8- , and CD11c+ DCs. Splenic NK cells were isolated using mouse DX5 Microbeads (Miltenyi Biotec). The cells were analyzed by flow cytometry on a FACSCalibur instrument (BD Biosciences), followed by data analysis using FlowJo software.

**Generation of bone marrow–derived DCs and bone marrow–derived macrophages**

Bone marrow cells were prepared from the femur and tibia. The cells were cultured in RPMI 1640 medium with 10% FCS, 100 μM 2-ME, and 10 ng/ml murine GM-CSF or culture supernatant of L929 expressing M-CSF. Medium was changed every 2 d. Six days after isolation, cells were collected.

**Hydrodynamic injection**

Total RNA from the human hepatocyte cell lines O cells and Oc cells was extracted using TRizol reagent (Invitrogen). HCV genotype 1b 3’ UTR RNA, including the polyU/UC region, was synthesized using T7 and SP6 RNA polymerase and purified with TRizol, as described previously (20). RNA was i.v. injected into a mouse by a hydrodynamic method using a Transit Hydrodynamic Gene Delivery System (Takara), according to the manufacturer’s instruction.

**Quantitative PCR**

For quantitative PCR, total RNA was extracted using TRizol reagent (Invitrogen), after which 0.1–1 μg RNA was reverse transcribed using a high-capacity cDNA transcription kit with an RNase inhibitor kit (Applied Biosystems), according to the manufacturer’s instructions. Quantitative PCR was performed using a Step One real-time PCR system (Applied Biosystems). The expression of cytokine mRNA was normalized to that of β-actin mRNA, and the fold increase was determined by dividing the expressions in each sample by that of wild type at 0 h. PCR primers for mouse IFN-λ amplified both IFN-λ2 and λ3 mRNA. The primer sequences are described in Supplemental Table 1.

**Activation of NK cells in vitro**

NK cells and CD11c+ DCs were isolated from spleens using DX5 and CD11c MicroBeads (Miltenyi Biotec), respectively. A total of 2 x 10^6 NK cells and CD11c+ DCs were activated in vitro with a mixture of CD80, CD86, CD40, NK1.1, and CD11c antibodies. HCV ssRNA or HCV dsRNA (5 μg) was hydrodynamically injected into wild-type, TICAM-1 KO, and IPS-1 KO mice. Six hours after injection, serum IFN-β and -λ2/3 concentrations were determined by ELISA.

**FIGURE 1.** Type I and type III IFN productions in response to HCV RNA in vivo. (A) O cell and Oc cell RNA (20 μg) were hydrodynamically injected into wild-type mice. Six hours later, mouse livers were excised, and IFN-β, α2, and -λ2/3 mRNA levels were determined by quantitative RT-PCR. (B) O cell RNA (20 μg) with HCV replicons was hydrodynamically injected into wild-type, TICAM-1 KO, and IPS-1 KO mice. Six hours after injection, IFN-β, α2, and -λ2/3 mRNA levels in liver were determined by quantitative RT-PCR. IFN-β protein levels in mouse livers were determined by ELISA. (C) HCV ssRNA or HCV dsRNA (5 μg) was hydrodynamically injected into wild-type, TICAM-1 KO, and IPS-1 KO mice. Six hours after injection, serum IFN-β and -λ2/3 mRNA levels were determined by quantitative RT-PCR. (D) O cell RNA (20 μg) with HCV replicons was hydrodynamically injected into wild-type, TICAM-1 KO, and IPS-1 KO mice. Six hours after injection, serum IFN-β and -λ2/3 concentrations were determined by ELISA.
Next, we examined cytokine mRNA expression in mouse tissues in response to i.p. injected polyI:C. IFN-β, IFN-α2, and IFN-α4 mRNA expression was detectable in both wild-type and TICAM-1 KO mice livers, whereas IFN-λ2/3 mRNA expression was not detected in TICAM-1 KO mouse liver (Supplemental Fig. 1B–1E). A recent study showed that TLR3 KO abolished IFN-λ serum levels in response to i.v. polyI:C injection (15). Our results and those in the previous study confirmed that TICAM-1 is essential for type III IFN expression in response to polyI:C.

**IPS-1 plays a crucial role in type III IFN production in response to HCV in vivo**

IPS-1 is essential for type I IFN production in response to HCV RNA and polyI:C in vivo (2, 3). We investigated whether IPS-1 could induce type III IFN production. An ectopic expression study using IPS-1 and TICAM-1 expression vectors showed that both TICAM-1 and IPS-1 activated the IFN-l1 promoter (Supplemental Fig. 2A, 2B), which suggested that IPS-1 has the ability to induce IFN-l1 expression. A deletion analysis showed that a 150- to 556-aa region of TICAM-1 and the transmembrane region of IPS-1 were essential for IFN-l1, -l2, and -l3 promoter activations (Supplemental Fig. 2C, 2D).

Hydrodynamic injection is a highly efficient procedure to deliver nucleic acids to the mouse liver (21), and Gale Jr. and colleagues...
(2) previously used a hydrodynamic assay to assess the role of RIG-I in type I IFN production in response to HCV RNA in vivo. Thus, to investigate the response to HCV RNA in vivo, we also used a hydrodynamic assay. We used RNA extracted from hepatocyte cell lines, O cells and Oc cells. O cells are derived from HuH-7 cells and contain HCV 1b full-length replicons (22). Oc cells were obtained by eliminating these replicons using IFN-α treatment (22). RNAs extracted from O cells (with HCV RNA) and Oc cells (without HCV RNA) were hydrodynamically injected into mouse livers, after which the cytokine expressions in mouse livers were determined. In wild-type mouse liver, O cell but not Oc cell RNA induced IFN-α2, β, and λ mRNA expression (Fig. 1A), which indicated that these cytokines were expressed in response to HCV RNAs within O cells that contained the HCV genome and replication intermediates in hepatocyte. Knockout of IPS-1 severely reduced IFN-α2 and α2 mRNA expressions in mouse liver in response to hydrodynamically injected O cell RNA (Fig. 1B). IFN-λ2/3 protein level in mouse liver was also reduced by IPS-1 knockout (Fig. 1B). Although TICAM-1 was essential for IFN-α2/3 mRNA expression in liver in response to i.p. injected polyI:C (Supplemental Fig. 1), TICAM-1 was dispensable for IFN-α2/3 mRNA expression in response to hydrodynamically injected O cell RNA (Fig. 1B). In contrast, IPS-1 was essential for IFN-λ2/3 mRNA expression in response to hydrodynamically injected O cell RNA (Fig. 1B). A requirement for IPS-1 for IFN-λ2/3 mRNA expression in the liver was also found when in vitro synthesized HCV dsRNAs and ssRNAs were used for the hydrodynamic assay (Fig. 1C). These results suggested that IPS-1 plays a crucial role in type III IFN production in response to HCV RNA in vivo.

To corroborate the role of IPS-1 in type III IFN production, we next measured serum IFN-α and -β levels in response to hydrodynamic injection of O cell RNA, HCV ssRNA, and HCV dsRNA. Interestingly, IPS-1 KO markedly reduced serum IFN-α2/3 levels (Fig. 1D, 1E). Unexpectedly, TICAM-1 KO also reduced serum IFN-α levels (Fig. 1D, 1E). Because TICAM-1 was dispensable for IFN-α mRNA expression in the liver, it is possible that serum IFN-α was produced from DCs in other tissues in a TICAM-1–dependent manner, as described below. Our data indicated that both TICAM-1 and IPS-1 are essential for type III IFN in response to HCV RNA in vivo. When polyI:C was hydrodynamically injected, knockout of TICAM-1 or IPS-1 moderately reduced IFN-α2/3 levels in sera (Supplemental Fig. 3).

DCs produce type III IFN through an IPS-1–dependent pathway in response to cytoplasmic HCV RNA

HCV proteins and minus strands of its genome are detected in DCs and macrophages (Mfs) of chronically HCV-infected patients (23, 24), and recent study showed that DCs produce type I and III IFNs in response to HCV (17, 25). Thus, we assessed the role of IPS-1 in type III IFN production by DCs and Mfs in response to HCV RNA. Surprisingly, adding O cell RNA into the culture medium did not induce any IFN-β and -α2/3 mRNA expression (Fig. 2A), whereas adding polyI:C into culture medium efficiently induced IFN-β and -α2/3 mRNA expression (Fig. 2B), and TICAM-1 KO abolished the IFN-α2/3 mRNA expression in bone marrow–derived DCs (BM-DCs) and BM-Mfs (Fig. 2B). It has been shown that polyI:C is preferentially internalized and activates TLR3 in human monocyte-derived DCs, whereas in vitro transcribed viral dsRNA hardly induced IFN-β production in monocyte-derived DCs (26). Thus, there is a possibility that, unlike polyI:C, TLR3 ligand in O cell RNA was not delivered to endosome where TLR3 is localized. Next, cells were stimulated with O cell RNA or polyI:C by transfection. BM-DCs and BM-Mfs expressed IFN-β and -α2/3

![FIGURE 3. Type III IFN production by CD8+ DCs. (A and B) CD4+, CD8+, and DN DCs were isolated from mouse spleens and stimulated with 20 μg O cell RNA without transfection or stimulated with 1 μg O cell RNA by transfection for 6 h. IFN-β (A) and -α2/3 (B) mRNA levels were determined by quantitative RT-PCR. (C and D) CD8+ DCs were isolated from wild-type, TICAM-1 KO, or IPS-1 KO mouse spleens. O cell RNA (20 μg) was added to the culture medium, or 1 μg O cell RNA was transfected into CD8+ DCs. Six hours after transfection, IFN-β (C) and -α2/3 (D) mRNA levels were determined by quantitative RT-PCR.](http://www.jimmunol.org/)

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mRNAs in response to O cell RNA and polyI:C (Fig. 2C, 2D). IPS-1 KO severely reduced IFN-α/2/3 mRNA expression in BM-DCs and BM-Mfs in response to O cell RNA (Fig. 2C). These results indicated that IPS-1 in BM-DCs and BM-Mfs plays a crucial role in IFN-α/2/3 mRNA expression in response to cytoplasmic HCV RNA.

Mice have CD4+, CD8+, and DN DCs. Thus, we next examined the IFN-β and -α/2/3 mRNA expression in these mouse DC subsets. As seen with BM-DCs, the mouse DCs expressed IFN-β and -α/2/3 mRNA in response to polyI:C but not O cell RNA in the culture medium, whereas stimulation with polyI:C or O cell RNA by transfection strongly induced their expression (Fig. 3A, 3B). Interestingly, CD8+ DCs highly expressed IFN-α/2/3 mRNA in response to stimulation with polyI:C or O cell RNA by transfection compared with CD4+ and DN DCs (Fig. 3A, 3B), and IPS-1 KO but not TICAM-1 KO severely reduced IFN-α/2/3 expression in CD8+ DCs in response to O cell RNA transfection (Fig. 3C, 3D). This indicated that IPS-1 was essential for IFN-α/2/3 mRNA expression in CD8+ DCs in response to cytoplasmic HCV RNA.

It was recently reported that exosomes mediate cell-to-cell transfer of HCV RNA from infected cells to cocultured DCs (27). We examined the production of IFN-β and -α/2/3 by CD8+ DCs that were cocultured with O cells and Oc cells. Coculture with O cells but not Oc cells induced IFN-β and -α/2/3 production by CD8+ DCs (Fig. 4A, 4B). Interestingly, TICAM-1 KO abolished IFN-α/2/3 mRNA expression and protein production, whereas IPS-1 KO failed to reduce IFN-α/2/3 mRNA expression and protein production in CD8+ DCs (Fig. 4C, 4D). This suggested that TICAM-1 but not IPS-1 was essential for IFN-α/2/3 production by CD8+ DCs when cocultured with hepatocytes with HCV replicons.

Type III IFN increases RIG-I expression in CD8+ DCs

The receptor for type III IFN consists of IL-10RB and IL-28Rα subunits (8). DN and CD4+ DCs and NK cells did not express IL-28Rα mRNA, whereas CD8+ DCs expressed both IL-10RB and IL-28Rα mRNAs (Fig. 5A). Thus, we investigated the effects of IFN-λ on DC function.

First, we examined DC cell surface markers. Unlike IFN-α, IFN-λ3 hardly increased CD40, 80, and 86 surface marker expressions on CD8+ DCs (Fig. 5B). Second, we examined the effects of IFN-λ3 on cross-priming because CD8+ DCs have high cross-priming capability. OVA, IFN-α-, and/or IFN-λ3 were i.p. injected into mice according to the indicated schedules (Fig. 5C). Seven days after injection, OVA (SL8)-specific CD8+ T cells in spleens were quantified by tetramer staining. For a positive control, OVAg-specific CD8+ T cells in spleens were cocultured with O cells (with HCV replicons) or Oc cells (without HCV replicons). After 24 h of coculture, IFN-β (A) and -α/2/3 (B) concentrations in culture medium were determined by ELISA. (C) CD8+ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons for six hours, and then IFN-β and -α/2/3 mRNA expression was determined by RT-qPCR. (D) CD8+ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons. IFN-β and -α/2/3 concentrations in culture medium were determined by ELISA.

**FIGURE 4.** IFN-β and -λ production by CD8+ DCs cocultured with hepatocytes with HCV replicons. (A and B) CD8+ DCs isolated from wild-type spleens were cocultured with O cells (with HCV replicons) or Oc cells (without HCV replicons). After 24 h of coculture, IFN-β (A) and -α/2/3 (B) concentrations in culture medium were determined by ELISA. (C) CD8+ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons for six hours, and then IFN-β and -α/2/3 mRNA expression was determined by RT-qPCR. (D) CD8+ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons. IFN-β and -α/2/3 concentrations in culture medium were determined by ELISA.

**Discussion**

Previous studies have established the importance of the TLR3 pathway for type III IFN production in response to polyI:C (15) or HCV (17). In this study, we established the importance of IPS-1–dependent pathway for type III IFN production in response to cytoplasmic HCV RNA in vivo and in vitro using a mouse model. These data indicated that there are at least two main pathways for type III IFN production in vivo, as follows: one is TICAM-1–dependent, and the other is IPS-1–dependent.

We revealed that IFN-λ was efficiently produced by CD8+ DCs, the mouse counterpart of human BDCA3+ DCs, in response to...
cytoplasmic HCV RNA. Moreover, our data showed that IFN-\(\alpha\) stimulation increased the mRNA expression of RIG-I but not that of TLR3 in CD8\(^+\) DCs, and CD8\(^+\) DCs required IPS-1 to produce IFN-\(\alpha\) in response to stimulation with cytoplasmic HCV RNA. Furthermore, IFN-\(\alpha\) enhanced the mRNA expression of IFN-\(\alpha\) itself in CD8\(^+\) DCs, which suggested a positive feedback loop for IFN-\(\alpha\) mRNA expression in CD8\(^+\) DCs. IFN-\(\alpha\) failed to promote DC-mediated NK activation or cross-priming at least in our experimental conditions, whereas antiviral proteins, such as ISG20 and RNaseL, were efficiently induced by IFN-\(\alpha\) stimulation in hepatocytes and CD8\(^+\) DCs. These results established a novel role of IPS-1 in innate immune response against HCV via IFN-\(\alpha\) production. IFN-\(\lambda\) pretreatment markedly increased IFN-\(\beta\) mRNA expression in response to HCV RNAs in mouse hepatocyte but not in CD8\(^+\) DCs (Fig. 6B, 6D). Although the underlying mechanism is unclear, it is possible that there is a cell-type–specific role of IFN-\(\lambda\).

It was recently reported that BDCA3\(^+\) DCs require TLR3 for type III IFN production in response to cell-cultured HCV (17). They used a HCV 2a JFH1 strain that cannot infect human DCs in vitro (5). We also showed that the TLR3 adaptor TICAM-1 was essential for type III IFN production by CD8\(^+\) DCs when cocultured with O cells with HCV replicons. Thus, TLR3 appears to be essential for type III IFN production by DCs that are not infected with HCV.
has been shown that exosomes are internalized efficiently by DCs and sorted into early endosomes, where TLR3 is localized (28, 29). Unlike the transfected HCV RNA, exosome-enclosed HCV RNA might be efficiently sorted and released within early endosomes of CD8+ DC, where TLR3 is localized, leading to TLR3-dependent IFN-λ2/3 production. Although HCV JFH1 infection particles fail to infect DCs in vitro, previous studies indicated that HCV infects DCs in chronically infected patients (23, 24, 30). In human patient DCs and hepatocytes infected with HCV, the IPS-1 pathway could play a pivotal role in type III IFN production.

Knockout of TICAM-1 failed to reduce IFN-λ2/3 mRNA expression in mouse liver after HCV RNA hydrodynamic injection, whereas knockout of TICAM-1 abolished IFN-λ2/3 mRNA levels in sera after HCV RNA hydrodynamic injection (Fig. 1B, 1D). Considering that there is a positive feedback loop for IFN-λ production, it is possible that TICAM-1 and IPS-1 pathways augment IFN-λ production each other in vivo; however, we do not exclude a possibility that TICAM-1 is involved in posttranscriptional step of IFN-λ production.

HCV NS3-4A protease cleaves IPS-1 to suppress host innate immune responses (31, 32). However, it is notable that a mutation within the RIG-I gene in HuH7.5 cells increases cellular permissiveness to HCV infection (33). This indicates that the RIG-I pathway is functional at least during the early phase of HCV infection before NS3-4A cleaves IPS-1. Thus, we propose that IPS-1 is important for type III IFN production during the early phase of HCV infection.

In summary, our results provide insights into type III IFN production mechanism in response to HCV RNA in vivo and identify IPS-1 as a molecule crucial for producing type III IFN from hepatocyte and CD8+ DCs in response to cytoplasmic HCV RNA.
Disclosures
The authors have no financial conflicts of interest.

References