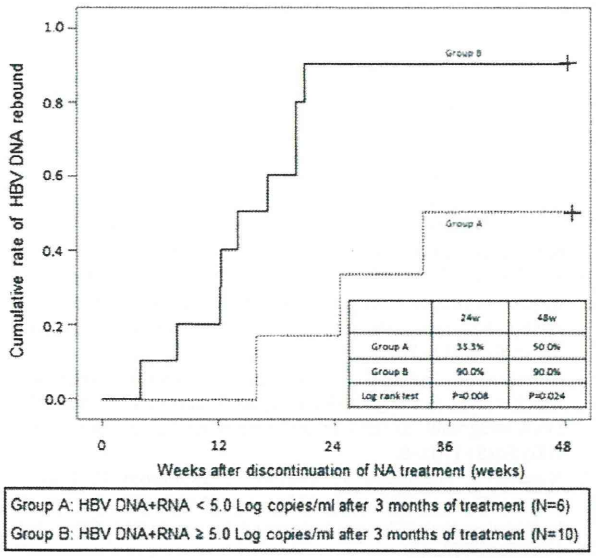

Fig. 5 Cumulative rate of ALT rebound after discontinuation of NA treatment in HBeAg-positive chronic hepatitis B patients. Six patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment were assigned to group A; the other ten patients, whose HBV DNA + RNA titers were ≥5.0 log copies/mL after 3 months of treatment, were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

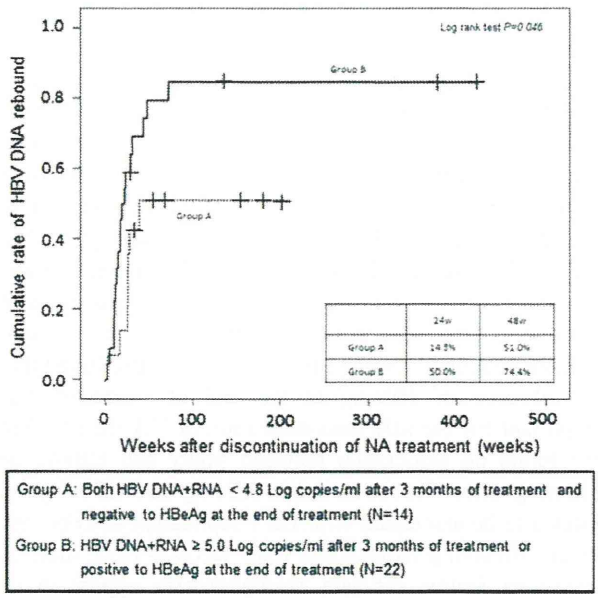

Fig. 6 Cumulative rate of ALT rebound after discontinuation of NA treatment by using combined criteria. The subjects were divided using combined criteria. Fourteen patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment and who were HBeAg negative at the end of NA treatment were assigned to group A; the other 22 patients were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

DNA + RNA titer after 3 months of treatment was found to be significantly associated with HBV DNA rebound ($P = 0.043$, OR = 9.474; Table 2). Two other factors, HBV DNA titer after 3 months of treatment and HBsAg titer at the end of treatment, were marginally associated with HBV DNA rebound ($P = 0.074$, $P = 0.070$, respectively). After 3 months of NA treatment, HBV DNA titers decreased in both the HBV DNA relapse and non-relapse groups, but HBV DNA + RNA levels in the relapse group remained high. NA therapy suppressed the production of mature HBV particles in both groups, but in the HBV DNA relapse group, high HBV replication activity was likely maintained during the treatment, and immature HBV particles associated with HBV RNA genomes were continuously produced and accumulated in hepatocytes. After discontinuation of the treatment, these accumulated immature HBV particles may have been maturated and been released from the hepatocytes. Thus, rebound of HBV DNA titers occurred rapidly after the discontinuation of NA therapy.

Although the presence of HBeAg before treatment, HBV DNA and DNA + RNA titers after 3 months of treatment, and the presence of HBeAg, HBsAg titer, and HBV DNA + RNA titer at the end of treatment were all significantly associated with ALT rebound in the univariate analysis, only the presence of HBeAg at the end of treatment was identified as an independent predictive factor for ALT rebound following multivariate analysis (Table 4). HBeAg is commonly strongly associated with the activity of HBV replication, and HBV DNA levels are high in HBeAg-positive HBV carriers. Thus, HBe seroconversion usually indicates suppression of HBV activity, and the absence of HBeAg is thought to indicate the inactivation of HBV replication.

ALT rebound following the discontinuation of NA therapy was not observed in six of the 16 patients (37.5 %) who were HBeAg-positive at the end of treatment. After examining predictive factors for ALT rebound in these HBeAg-positive patients, only the HBV DNA + RNA titer after 3 months of treatment was identified as an independent predictive factor for ALT rebound in HBeAg-positive patients (Table 6). Although the presence of HBeAg indicates high activities of HBV replication and hepatitis, it is expected to be difficult to discontinue NA therapy without ALT rebound in these patients. However, these results indicate that HBV replication activities vary greatly among individuals and suggest that it might be possible to predict future replication activity based on HBV DNA + RNA titers after 3 months of treatment.

A limitation of this study is the small sample size; as such, selection bias might have affected the internal validity of the study. As it is not common to discontinue

NA therapy in Japan, we were only able to examine 36 subjects in our study. Because HBV-related markers such as HBsAg, HBcrAg, and HBV DNA + RNA titers varied widely among individuals, HBeAg an HBV DNA + RNA titers were only marginally associated with HBV DNA or ALT rebound after the discontinuation of NA therapy. In a previous study, Matsumoto et al. [34] analyzed predictive factors for the safe discontinuation of NA therapy in 126 clinical HBeAg-negative subjects from 12 clinical centers. These authors reported that HBsAg and HBcrAg titers at the end of treatment were predictive factors for the safe discontinuation of therapy. In our study, we also found that the absence of HBeAg at the end of treatment was important for the safe discontinuation of NA therapy, but we found no association between safety and HBsAg or HBcrAg titers. However, while HBsAg and HBcrAg are known to be associated with HBV replication activity, our results involving HBeAg and HBV DNA + RNA titers as important factors for safe discontinuation appear to be consistent.

In our study, the duration of NA therapy was quite short (mean duration was 36 weeks). Similar results might be observed if the NA therapy was extended, but it might be difficult to depress the potential of infected HBV replication with long-term NA therapy. HBsAg titers represent HBV replication in human hepatocytes, and it is difficult to decrease HBsAg levels by NA therapy. Thus, HBV DNA + RNA levels might be an important factor for predicting the HBV DNA or ALT rebounds.

As it may be difficult to discontinue therapy in patients with advanced liver fibrosis, our study subjects were selected based on liver spare capacities. As shown in Fig. 1, ALT rebound is likely to occur in most patients following the discontinuation of NA therapy, and severe hepatitis could occur in some patients. Thus, if the liver spare capacity were low, NA therapy would not be discontinued; the patients in this study were selected solely based on clinical aspects, which may have influenced our interpretation of the results.

In conclusion, HBV replication activity was found to be an important predictor of safe discontinuation of NA therapy. These findings suggest that monitoring of serum HBV DNA + RNA levels would be a useful method for predicting the re-activation of chronic hepatitis B following discontinuation of NA therapy.

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Conflict of interest None to declare.

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LETTER TO THE EDITOR

Availability of monitoring serum HBV DNA plus RNA during nucleot(s)ide analogue therapy

Masataka Tsuge · Kazuaki Chayama

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We appreciate the comments by Kurosaki et al. on the article entitled "Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleot(s)ide analogue (NUC) treatments in chronic hepatitis B patients" [1]. They raised three important questions: (1) whether HBV DNA levels measured by transcription-mediated amplification and hybridization (TMA-HPA) can be used as an alternative to HBV DNA plus RNA levels measured by RT-PCR; (2) whether post-treatment monitoring of serum HBV DNA plus RNA might serve as a predictor of safe discontinuation after long term NUC; and (3) whether serum HBV DNA plus RNA titer is a predictor of favorable response to sequential interferon therapy.

The presence of HBV RNA in serum is an indicator of ongoing transcription of the HBV pregenome from cccDNA in hepatocytes and may occur even when production of mature HBV particles is effectively suppressed by inhibition of reverse transcription by NUC. As we previously reported, lamivudine resistant strains emerge more easily under such conditions [2], but HBV RNA gradually decreases under continued suppression of reverse transcription and generally becomes undetectable in patients following a year of NUC treatment.

The first question Kurosaki et al. was whether HBV DNA titers measured by TMA-HPA assay, which actually represent HBV DNA plus RNA titers, can be used as an alternative to HBV DNA plus RNA measured by RT-PCR. As we showed in our previous report [2], levels obtained by TMA-PHA assay correlated well with those obtained by RT-PCR during NUC therapy ($r = 0.955$, $P < 0.0001$) [2]. Therefore, measurement of TMA-PHA is a reasonable alternative to RT-PCR. Although the sensitivity of HBV nucleic acids by TMA-HPA assay is lower than RT-PCR, measurement of HBV nucleic acids may provide useful information, especially for those patients who started NUC therapy with high pretreatment HBV DNA levels. RT-PCR is more useful in patients who had relatively lower HBV levels at the beginning of NUC therapy.

The second question was whether monitoring of serum HBV DNA plus RNA at the end of treatment serves as a predictor of safe discontinuation after long term NUC. We found that HBV RNA can be detected in patients who became negative for HBV DNA after long term NUC therapy, and measurement of HBV RNA in patients receiving long term NUC therapy may yield important insight into the risk of reactivation of HBV if NUC therapy is discontinued. However, we have not analyzed enough such patients, and a prospective study is necessary to evaluate the predictive value of HBV RNA plus RNA measurement.

The third question was whether serum HBV DNA plus RNA titer is a predictor of favorable response to sequential NUC and interferon therapy. The mechanisms of these drugs is different, and interferon is not associated with serum HBV RNA because it does not disturb reverse

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transcription but instead suppresses HBV transcription in hepatocytes. In our previous study [3], HBV RNA was negative before administration of NUC and became positive soon after the beginning of NUC therapy, peaking at weeks two to four and then gradually decreasing. We assumed that, after HBV RNA levels have been reduced during long term NUC therapy, HBV RNA should become undetectable during interferon therapy [3]. We tried to assess the predictive effect of HBV RNA titer immediately prior to interferon administration in patients who received sequential therapy, but, incidentally, HBV RNA was undetectable in all patients just before interferon treatment [3]. As we did not show results for sequential therapy in our study in Journal of Gastroenterology [1], results of the 26 patients (20 males, 6 females) who underwent sequential therapy patients in that study are described below. Ten patients were positive for HBeAg at the end of NUC therapy. HBV DNA rebound was observed in 13 patients within 24 weeks after discontinuation of NUC therapy, and ALT rebound occurred in 9 patients. HBV DNA rebound was significantly associated with serum HBV DNA plus RNA titer following 3 months of NUC treatment ($P = 0.029$, Mann–Whitney U test), and ALT rebound was significantly associated with serum HBV DNA titer and DNA plus RNA titer following 3 months of NUC treatment ($P = 0.041$, $P = 0.016$, respectively, Mann–Whitney U test) and the existence of HBeAg at the end of NUC treatment ($P = 0.009$, Fisher's exact test). Although it is necessary to confirm these results in a large, prospective study, we conclude from these results that HBV RNA plus DNA is a predictor for sequential therapy.

Due to the complicated nature of chronic HBV infection and immunological reaction of the host, it is difficult to completely predict the effect of any type of therapy. Further study should be done to identify conditions for safe discontinuation of NUC because otherwise patients must continue lifelong NUC therapy. We thank Kurosaki et al. for their helpful comments and appreciate the opportunity to respond to their questions.

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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

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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

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Hepatitis C virus (HCV) is a major cause of liver disease. The innate immune system is essential for controlling HCV replication, and HCV is recognized by RIG-I and TLR3, which evoke innate immune responses through IPS-1 and TICAM-1 adaptor molecules, respectively. IL-28B is a type III IFN, and genetic polymorphisms upstream of its gene are strongly associated with the efficacy of polyethylene glycol–IFN and ribavirin therapy. As seen with type I IFNs, type III IFNs induce antiviral responses to HCV. Recent studies established the essential role of TLR3–TICAM-1 pathway in type III IFN production in response to HCV infection. Contrary to previous studies, we revealed an essential role of IPS-1 in type III IFN production in response to HCV. First, using IPS-1 knockout mice, we revealed that IPS-1 was essential for type III IFN production by mouse hepatocytes and $CD8^+$ dendritic cells (DCs) in response to cytoplasmic HCV RNA. Second, we demonstrated that type III IFN induced RIG-I but not TLR3 expression in $CD8^+$ DCs and augmented type III IFN production in response to cytoplasmic HCV RNA. Moreover, we showed that type III IFN induced cytoplasmic antiviral protein expression in DCs and hepatocytes but failed to promote DC-mediated NK cell activation or cross-priming. Our study indicated that IPS-1–dependent pathway plays a crucial role in type III IFN production by $CD8^+$ DCs and hepatocytes in response to HCV, leading to cytoplasmic antiviral protein expressions. *The Journal of Immunology*, **2014, 192: 2770–2777.**

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).

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM-DC, bone marrow–derived dendritic cell; BM-Mf, bone marrow–derived macrophage; DC, dendritic cell; HCV, hepatitis C virus; KO, knockout; Mf, macrophage; Oc, O cured; RLR, RIG-I–like receptor; UTR, untranslated region.

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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 polyI: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 in response to HCV.

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). PolyI:C was purchased from GE Healthcare and dissolved in saline. An OVA ($H2K^b$-SL8) tetramer was purchased from MBL. PE-CD80, -CD86, -NK1.1, FITC-CD8, and allophycocyanin-CD3e Abs were purchased from BioLegend, and PE-CD40, FITC-CD69, and allophycocyanin-CD11c Abs were from eBioscience. An ELISA kit for IFN-β was purchased from PBL Biomedical Laboratories, and ELISA kits for mouse IL-28 (IFN-λ2/3) 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 2×10^5 NK

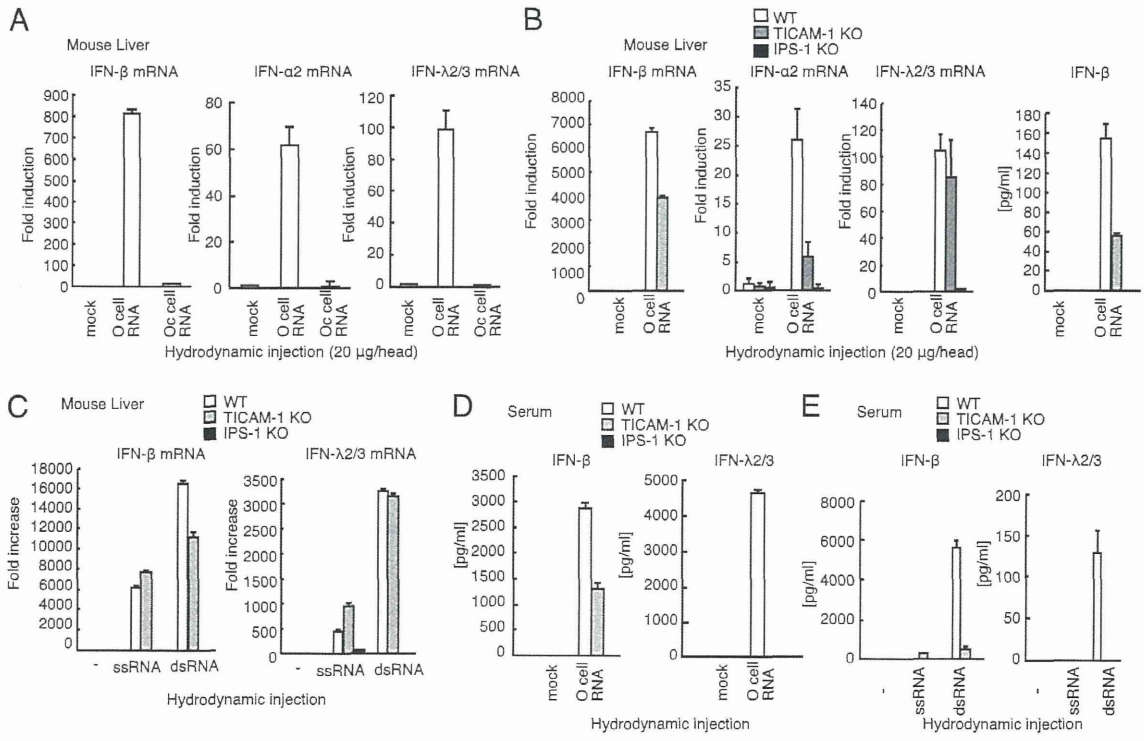

FIGURE 1. Type I and type III IFN productions in response to HCV RNA in vivo. (**A**) O cell and Oc cell RNA (20 μg) were hydrodynamically injected into wild-type mice. Six hours later, mouse livers were excised, and IFN-β, α2, and -λ2/3 mRNA levels were determined by quantitative RT-PCR. (**B**) O cell 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, IFN-β and -λ2/3 mRNA levels in liver 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. (**E**) 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.

cells and 1×10^5 DCs was cocultured with IFN-λ, IFN-α, or polyI:C. After 6, 12, and 24 h, IFN-γ concentrations in the supernatants were determined by ELISA. To determine CD69 expression, $NK1.1^+$ and $CD3e^+$ cells in 24-h sample were gated.

Ag-specific T cell expansion in vivo

OVA (1 mg) and IFN-λ (0.5 μg) or 1×10^5 IU IFN-α were i.p. injected into mice on day 0, and then 0.5 μg IFN-λ or 1×10^5 IU of IFN-α was injected into mice on days 1, 2, and 4. On day 7, spleens were homogenized and stained with FITC CD8α Ab and PE-OVA tetramer for detecting OVA (SL8)-specific $CD8^+$ T cell population. For a negative control, PBS in place of IFN was injected on days 0, 1, 2, and 4. For a positive control, 100 μg polyI:C and OVA were injected into mice on day 0.

Results

TICAM-1 is essential for type III IFN production in response to polyI:C

DCs require the TLR3 adaptor TICAM-1 to produce type III IFN in response to polyI:C (15). Adding polyI:C to culture medium for mouse bone marrow–derived macrophages (BM-Mf) induced IFN-β, IFN-α2, IFN-α4, and IFN-λ2/3 mRNA expression, and TICAM-1 KO abolished IFN-λ2/3 mRNA expression (Supplemental Fig. 1A). These results suggested an essential role for TICAM-1 in type III IFN expression by BM-Mf.

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-λ1 promoter (Supplemental Fig. 2A, 2B), which suggested that IPS-1 has the ability to induce IFN-λ1 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-β, -λ1, and 2/3 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

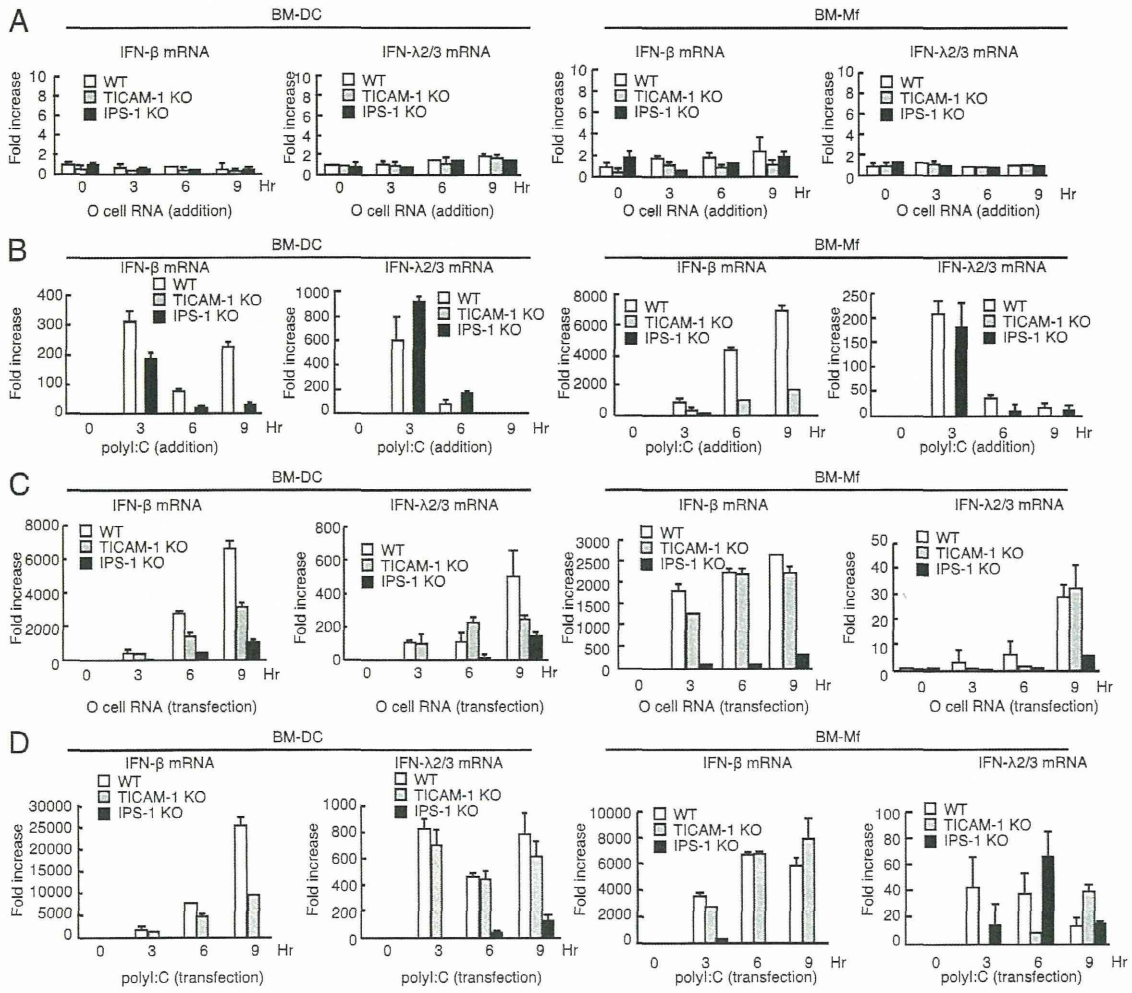

FIGURE 2. Type I and type III IFN expression in mouse DCs and Mfs in response to HCV RNA. (**A** and **B**) O cell RNA (A) or polyI:C (B) (20 μg) was added to the culture medium of BM-DCs and BM-Mfs derived from wild-type, TICAM-1 KO, and IPS-1 KO mice. IFN-β and IFN-λ2/3 mRNA levels were determined by quantitative RT-PCR at indicated hours. (**C** and **D**) O cell RNA (C) or polyI:C (D) (1 μg) was transfected into BM-DCs and BM-Mfs derived from wild-type, TICAM-1 KO, or IPS-1 KO mice. IFN-β (C) and -λ2/3 (D) mRNA levels were determined by quantitative RT-PCR.

(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-β and α2 mRNA expressions in mouse liver in response to hydrodynamically injected O cell RNA (Fig. 1B). IFN-β protein level in mouse liver was also reduced by IPS-1 knockout (Fig. 1B). Although TICAM-1 was essential for IFN-λ2/3 mRNA expression in liver in response to i.p. injected polyI:C (Supplemental Fig. 1), TICAM-1 was dispensable for IFN-λ2/3 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-λ 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-λ2/3 levels (Fig. 1D, 1E). Unexpectedly, TICAM-1 KO also reduced serum IFN-λ levels (Fig. 1D, 1E). Because TICAM-1 was dispensable for IFN-λ mRNA expression in the liver, it is possible that serum IFN-λ 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-λ2/3 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 -λ2/3 mRNA expression (Fig. 2A), whereas adding polyI:C into culture medium efficiently induced IFN-β and -λ2/3 mRNA expression (Fig. 2B), and TICAM-1 KO abolished the IFN-λ2/3 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 -λ2/3

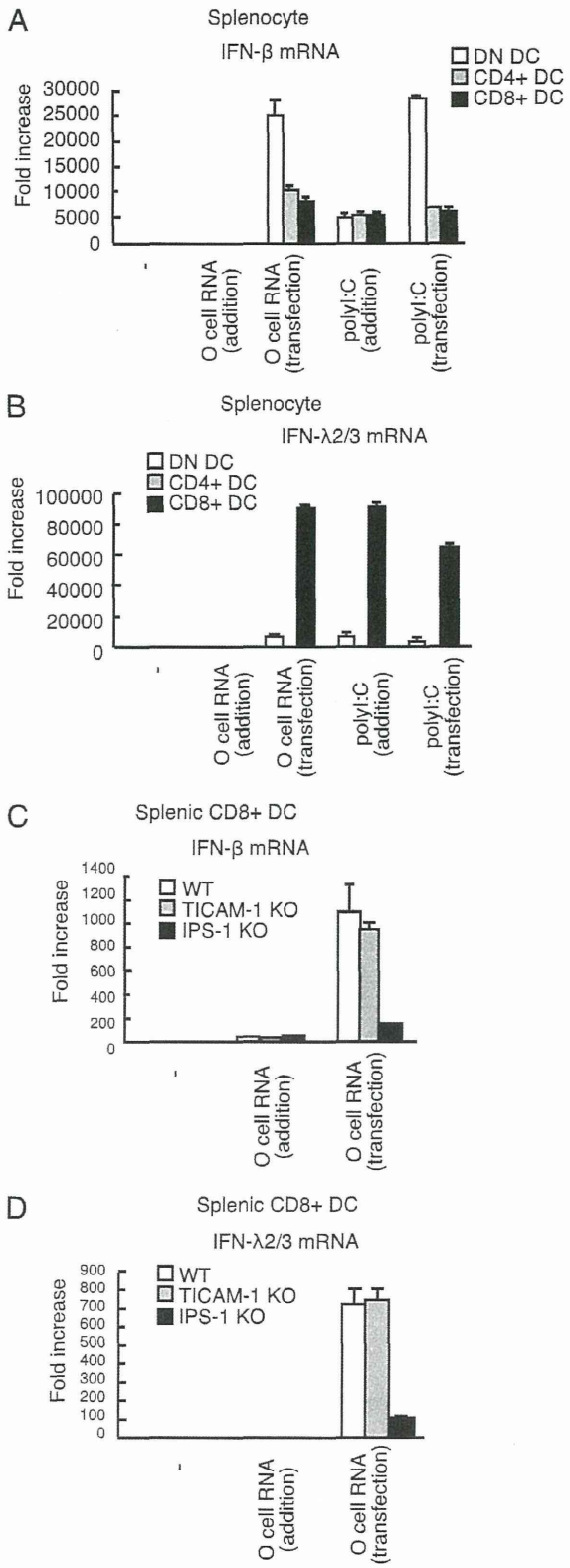

FIGURE 3. Type III IFN production by $CD8^+$ DCs. (**A** and **B**) $CD4^+$, $CD8^+$, and DN DCs were isolated from mouse spleens and stimulated with 20 μg O cell RNA without transfection or stimulated with 1 μg O cell RNA by transfection for 6 h. IFN-β (A) and -λ2/3 (B) mRNA levels were determined by 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 -λ2/3 (D) mRNA levels were determined by quantitative RT-PCR.