Published OnlineFirst March 3, 2014; DOI: 10.1158/0008-5472.CAN-13-1703

References

1. Raulet D. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 2003;3:781–90.
2. Gasser S, Raulet D. The DNA damage response, immunity and cancer. Semin Cancer Biol 2006;16:344–7.
3. Gasser S, Raulet DH. The DNA damage response arouses the immune system. Cancer Res 2006;66:3959–62.
4. Guerra N, Tan Y, Joncker N, Choy A, Gallardo F, Xiong N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 2008;28:571–80.
5. Raulet DH, Guerra N. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. Nat Rev Immunol 2009;9:568–80.
6. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature 2005;436:1186–90.
7. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. Nat Rev Cancer 2009;9:57–63.
8. Yanai H, Savitsky D, Tamura T, Taniguchi T. Regulation of the cytosolic DNA-sensing system in innate immunity: a current view. Curr Opin Immunol 2009;21:17–22.
9. Desmet CJ, Ishii KJ. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat Rev Immunol 2012;12:479–91.
10. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491–6.
11. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF family of transcription factors as regulators of host defense. Annu Rev Immunol 2001;19:623–55.
12. Packham G, Cleveland JL. c-Myc and apoptosis. Biochim Biophys Acta 1995;1242:11–28.
13. Reimann M, Loddenkemper C, Rudolph C, Schildhauer I, Teichmann B, Stein H, et al. The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. Blood 2007;110:2996–3004.
14. Wang J, Boxer LM. Regulatory elements in the immunoglobulin heavy chain gene 3′-enhancers induce c-myc deregulation and lymphomagenesis in murine B cells. J Biol Chem 2005;280:12766–73.
15. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, et al. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 1985;318:533–8.
16. Corcoran LM, Tawfilis S, Barlow LJ. Generation of B lymphoma cell lines from knockout mice by transformation in vivo with an Emu-myc transgene. J Immunol Methods 1999;228:131–8.
17. Diefenbach A, Hsia JK, Hsiung MY, Raulet D. A novel ligand for the NKG2D receptor activates NK cells and macrophages and induces tumor immunity. Eur J Immunol 2003;33:381–91.
18. Croxford JL, Tang ML, Pan MF, Huang CW, Kamran N, Phua CM, et al. ATM-dependent spontaneous regression of early Emu-myc-induced murine B-cell leukemia depends on natural killer and T cells. Blood 2013;121:2512–21.
19. Sauer M, Reiners KS, Hansen HP, Engert A, Gasser S, von Strandmann EP. Induction of the DNA damage response by IAP inhibition triggers natural immunity via upregulation of NKG2D ligands in Hodgkin lymphoma in vitro. Biol Chem 2013;394:1325–31.
20. Kim ST, Lim DS, Canman CE, Kastan MB. Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem 1999;274:37538–43.
21. Servant MJ, Grandvaux N, Hiscott J. Multiple signaling pathways leading to the activation of interferon regulatory factor 3. Biochem Pharmacol 2002;64:985–92.
22. Hiscott J, Grandvaux N, Sharma S, Tenoever BR, Servant MJ, Lin R. Convergence of the NF-kappaB and interferon signaling pathways in the regulation of antiviral defense and apoptosis. Ann N Y Acad Sci 2003;1010:237–48.
23. Stein SC, Falck-Pedersen E. Sensing adenovirus infection: activation of interferon regulatory factor 3 in RAW 264.7 cells. J Virol 2012;86:4527–37.
24. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–70.
25. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. Annu Rev Immunol 2013;31:413–41.
26. Fensterl V, Grotheer D, Berk I, Schlemminger S, Vallbracht A, Dotzauer A. Hepatitis A virus suppresses RIG-I-mediated IRF-3 activation to block induction of beta interferon. J Virol 2005;79:10968–77.
27. D'Incalci M, Covey JM, Zaharko DS, Kohn KW. DNA alkali-labile sites induced by incorporation of 5-aza-2′-deoxycytidine into DNA of mouse leukemia L1210 cells. Cancer Res 1985;45:3197–202.
28. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 2004;73:39–85.
29. Zimmermann W, Chen SM, Bolden A, Weissbach A. Mitochondrial DNA replication does not involve DNA polymerase alpha. J Biol Chem 1980;255:11847–52.
30. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, et al. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature 2008;451:725–9.
31. Adams JM, Gerondakis S, Webb E, Corcoran LM, Cory S. Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc Natl Acad Sci U S A 1983;80:1982–6.
32. Harris AW, Pinkert CA, Crawford M, Langdon WY, Brinster RL, Adams JM. The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. J Exp Med 1988;167:353–71.
33. Unni AM, Bondar T, Medzhitov R. Intrinsic sensor of oncogenic transformation induces a signal for innate immunosurveillance. Proc Natl Acad Sci U S A 2008;105:1686–91.
34. Nakajima A, Nishimura K, Nakaima Y, Oh T, Noguchi S, Taniguchi T, et al. Cell type-dependent proapoptotic role of Bcl2L12 revealed by a mutation concomitant with the disruption of the juxtaposed Irf3 gene. Proc Natl Acad Sci U S A 2009;106:12448–52.
35. Stegh AH, Kim H, Bachoo RM, Forloney KL, Zhang J, Schulze H, et al. Bcl2L12 inhibits post-mitochondrial apoptosis signaling in glioblastoma. Genes Dev 2007;21:98–111.
36. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity 2000;13:539–48.
37. Hamerman JA, Ogasawara K, Lanier LL. Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. J Immunol 2004;172:2001–5.
38. Noyce RS, Collins SE, Mossman KL. Differential modification of interferon regulatory factor 3 following virus particle entry. J Virol 2009;83:4013–22.
39. Wilkins AS. The enemy within: an epigenetic role of retrotransposons in cancer initiation. Bioessays 2010;32:856–65.
40. Takaoka A, Wang Z, Choi M, Yanai H, Negishi H, Ban T, et al. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 2007;448:501–5.

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 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).

*Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan; and †Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama 700-8558, Japan

Received for publication June 3, 2013. Accepted for publication January 13, 2014.

This work was supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture of Japan, the Ministry of Health, Labour, and Welfare of Japan, and the Kato Memorial Bioscience Foundation.

Address correspondence and reprint requests to Dr. Hiroyuki Oshiumi and Dr. Tsukasa Seya, Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku Sapporo 060-8638, Japan. E-mail addresses: oshiumi@med.hokudai.ac.jp (H.O.) and seya-tu@pop.med.hokudai.ac.jp (T.S.)

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.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301459

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

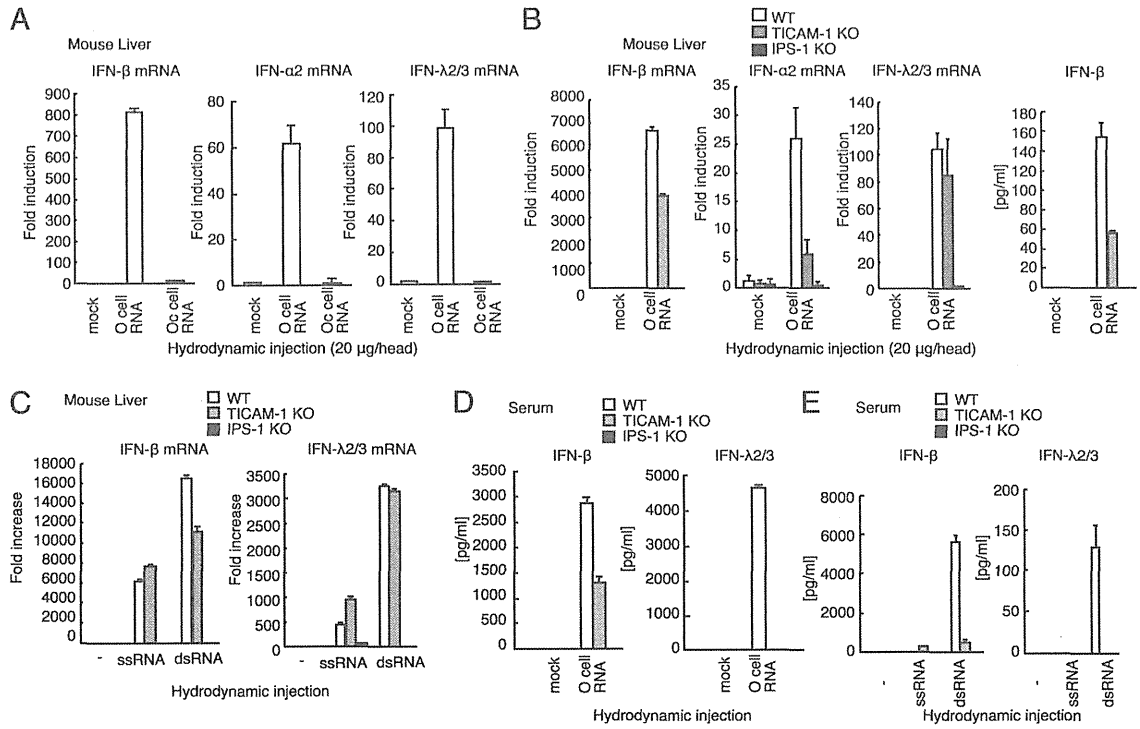

FIGURE 1. Type I and type III IFN productions in response to HCV RNA in vivo. (**A**) O cell and Oc cell RNA (20 μg) were hydrodynamically injected into wild-type mice. Six hours later, mouse livers were excised, and IFN-β, α2, and -λ2/3 mRNA levels were determined by quantitative RT-PCR. (**B**) O cell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

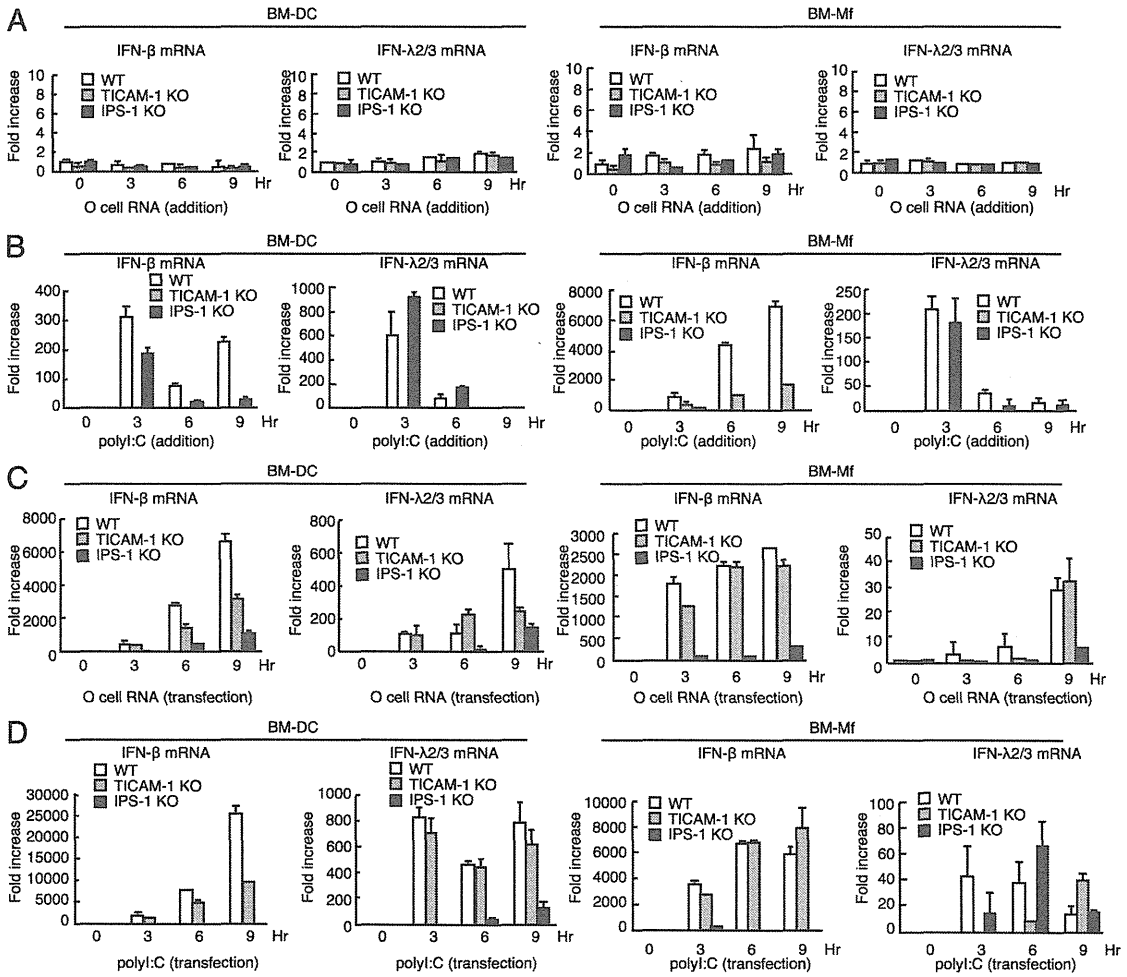

FIGURE 2. Type I and type III IFN expression in mouse DCs and Mfs in response to HCV RNA. (**A** and **B**) O cell RNA (A) or polyI:C (B) (20 μg) was added to the culture medium of BM-DCs and BM-Mfs derived from wild-type, TICAM-1 KO, and IPS-1 KO mice. IFN-β and IFN-λ2/3 mRNA levels were determined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

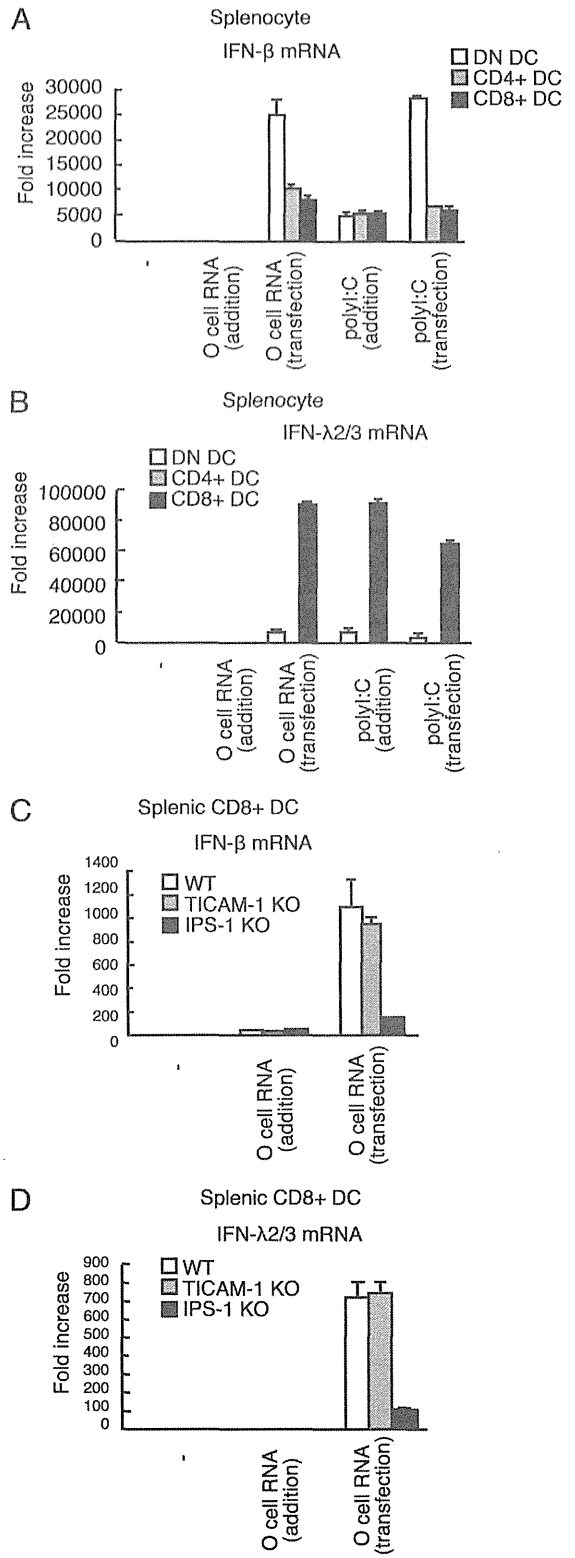

FIGURE 3. Type III IFN production by $CD8^+$ DCs. (**A** and **B**) $CD4^+$, $CD8^+$, and DN DCs were isolated from mouse spleens and stimulated with 20 μg O cell RNA without transfection or stimulated with 1 μg O cell RNA by transfection for 6 h. IFN-β (A) and -λ2/3 (B) mRNA levels were determined by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

mRNAs in response to O cell RNA and polyI:C (Fig. 2C, 2D). IPS-1 KO severely reduced IFN-λ2/3 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-λ2/3 mRNA expression in response to cytoplasmic HCV RNA.

Mice have $CD4^+$, $CD8^+$, and DN DCs. Thus, we next examined the IFN-β and -λ2/3 mRNA expression in these mouse DC subsets. As seen with BM-DCs, the mouse DCs expressed IFN-β and -λ2/3 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-λ2/3 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-λ2/3 expression in $CD8^+$ DCs in response to O cell RNA transfection (Fig. 3C, 3D). This indicated that IPS-1 was essential for IFN-λ2/3 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 -λ2/3 by $CD8^+$ DCs that were cocultured with O cells and Oc cells. Coculture with O cells but not Oc cells induced IFN-β and -λ2/3 production by $CD8^+$ DCs (Fig. 4A, 4B). Interestingly, TICAM-1 KO abolished IFN-λ2/3 mRNA expression and protein production, whereas IPS-1 KO failed to reduce IFN-λ2/3 mRNA expression and protein production in $CD8^+$ DCs (Fig. 4C, 4D). This suggested that TICAM-1 but not IPS-1 was essential for IFN-λ2/3 production by $CD8^+$ DCs when cocultured with hepatocytes with HCV replicons.

Type III IFN increases RIG-I expression in $CD8^+$ DC

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, OVA and polyI:C were i.p. injected into mice. The results showed that IFN-λ3 failed to increase OVA-specific $CD8^+$ T cells in the spleens and suggested that IFN-λ3 failed to promote cross-priming at least in our experimental condition (Fig. 5C).

Third, we examined NK cell activation by DCs. NK cells and DCs were isolated from mouse spleens and were cocultured for 24 h in the presence of IFN-α, λ3, or polyI:C. Although IFN-γ production was increased by IFN-α stimulation, IFN-λ3 failed to increase IFN-γ production (Fig. 5D). Next, we investigated a cell surface marker for NK cells when cocultured with DCs. The expression of CD69, a NK cell activation marker, was not increased by IFN-λ3 stimulation (Fig. 5E). These results indicated that, unlike IFN-α, IFN-λ3 failed to enhance the activation of NK cells by DCs.

Fourth, we investigated the expression of antiviral genes in $CD8^+$ DCs in response to IFN-λ3 stimulation. Interestingly, IFN-λ3 stimulation increased RIG-I and Mx1 but not TLR3 mRNA expression in $CD8^+$ DCs (Fig. 6A). In addition, pretreatment with IFN-λ3 augmented IFN-λ2/3 mRNA expression in $CD8^+$ DCs in response to HCV RNA (Fig. 6B). Taken together, type III IFN induced RIG-I and antiviral protein expression but failed to promote DC-mediated NK cell activation and cross-priming.

Hepatocytes express type III IFN receptors. Thus, we examined the effects of IFN-λ on mouse hepatocytes. As with IFN-α, IFN-λ3 stimulation induced both TLR3 and RIG-I mRNA expression in mouse hepatocyte (Fig. 6C). Antiviral nucleases, ISG20 and RNaseL, and an IFN-inducible gene, Mx1, were induced by IFN-λ3 or IFN-α treatment (Fig. 6C). Pretreating mouse hepatocytes with IFN-λ3 enhanced IFN-β and -λ2/3 mRNA expression in response to stimulation with HCV RNA by transfection (Fig. 6D). These results indicated that IFN-λ3 induced cytoplasmic antivirus protein expression in mouse hepatocytes. We confirmed that IFN-λ3 treatment significantly reduced HCV RNA levels in O cells with HCV replicons (Fig. 6E). A previous study also reported that IFN-λ inhibits HCV replication (13).

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

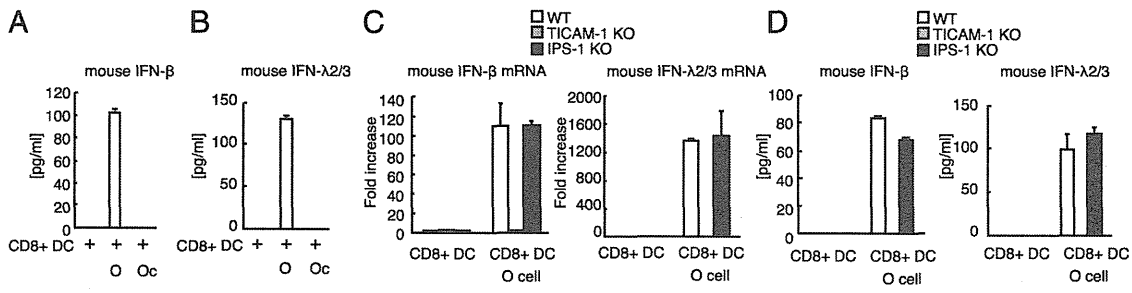

FIGURE 4. IFN-β and –λ production by $CD8^+$ DCs cocultured with hepatocytes with HCV replicons. (**A** and **B**) $CD8^+$ DCs isolated from wild-type spleens were cocultured with O cells (with HCV replicons) or Oc cells (without HCV replicons). After 24 h of coculture, IFN-β (A) and –λ2/3 (B) concentrations 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 –λ2/3 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 –λ2/3 concentrations in culture medium were determined by ELISA.

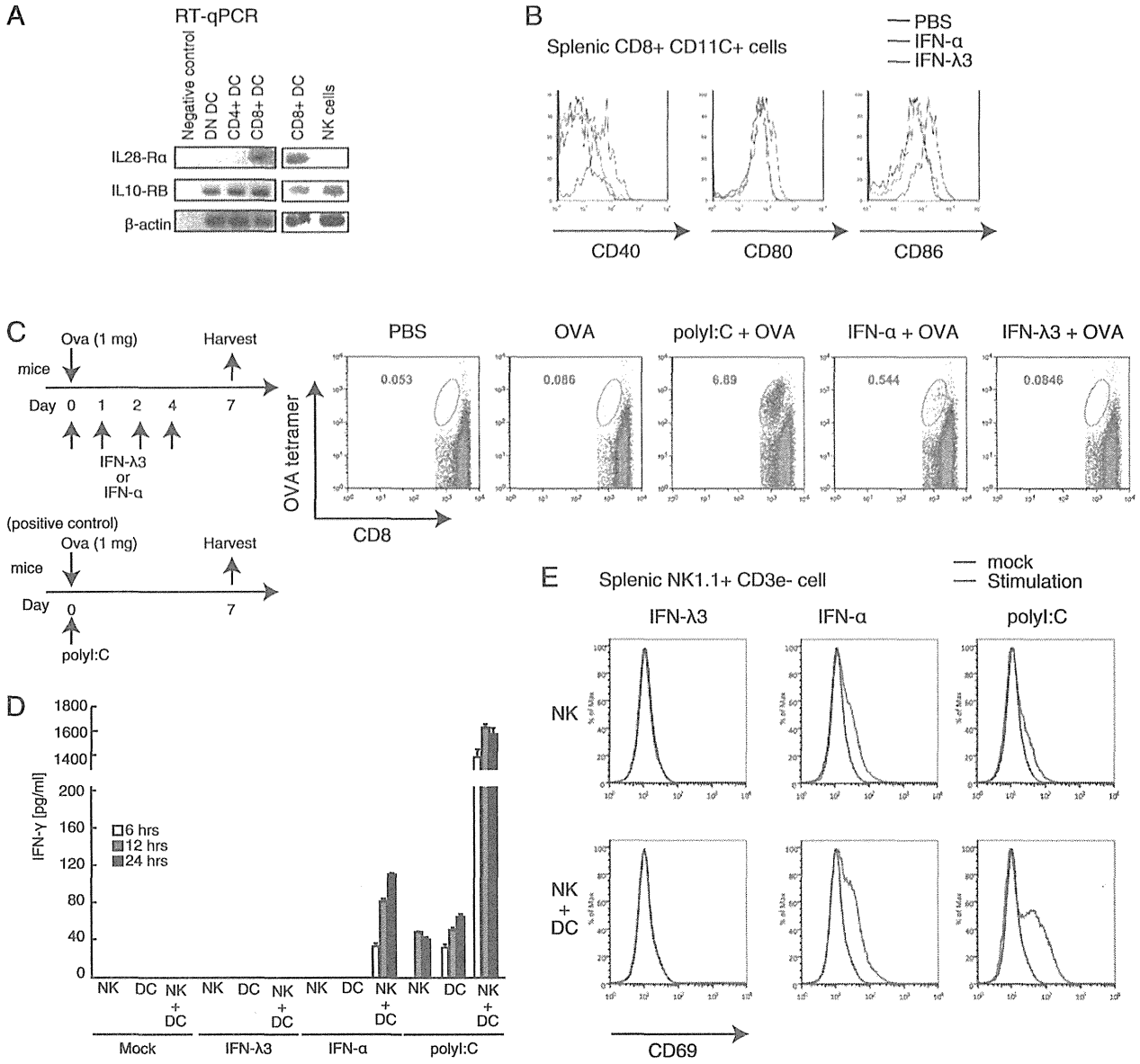

FIGURE 5. IFN-λ effects on DC functions. (**A**) DN, $CD4^+$, $CD8^+$ DCs, and NK cells were isolated from wild-type mouse spleens. IL-28Rα and IL-10RB mRNA were determined by RT-PCR. (**B**) A total of 0.5 μg IFN-λ3 or 1×10^5 IU IFN-α was i.p. injected into mice. Six hours after injection, spleen $CD8^+$ DCs were isolated, and cell surface expressions of CD40, 80, and 86 were determined by FACS analysis. (**C**) OVA and IFN-λ or IFN-α were i.p. injected into mice on day 0, and then IFN-λ or IFN-α was injected into mice on days 1, 2, and 4. Spleens were excised on day 7, and OVA (SL8)-specific $CD8^+$ T cells were determined by a tetramer assay. For a negative control, PBS in place of IFN was injected on days 0, 1, 2, and 4. For a positive control, polyI:C and OVA were injected into mice on day 0. (**D**) NK cells and $CD11c^+$ DCs were isolated from mouse spleens and then stimulated with 1000 U/ml IFN-α, 100 ng/ml IFN-λ3, or 100 μg/ml polyI:C. IFN-γ concentrations in the culture medium at the indicated times were determined by ELISA. (**E**) NK cells were isolated from mouse spleens and then cultured with or without spleen $CD11c^+$ DCs. Cells were stimulated with 1000 U/ml IFN-α, 100 ng/ml IFN-λ3, or 20 μg polyI:C. CD69 expression on NK cells was determined by FACS analysis.

cytoplasmic HCV RNA. Moreover, our data showed that IFN-λ 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-λ in response to stimulation with cytoplasmic HCV RNA. Furthermore, IFN-λ enhanced the mRNA expression of IFN-λ itself in $CD8^+$ DCs, which suggested a positive feedback loop for IFN-λ mRNA expression in $CD8^+$ DCs. IFN-λ 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-λ stimulation in hepatocytes and $CD8^+$ DCs. These results established a novel role of IPS-1 in innate immune response against HCV via IFN-λ production. IFN-λ pretreatment markedly increased IFN-β 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-λ.

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. It

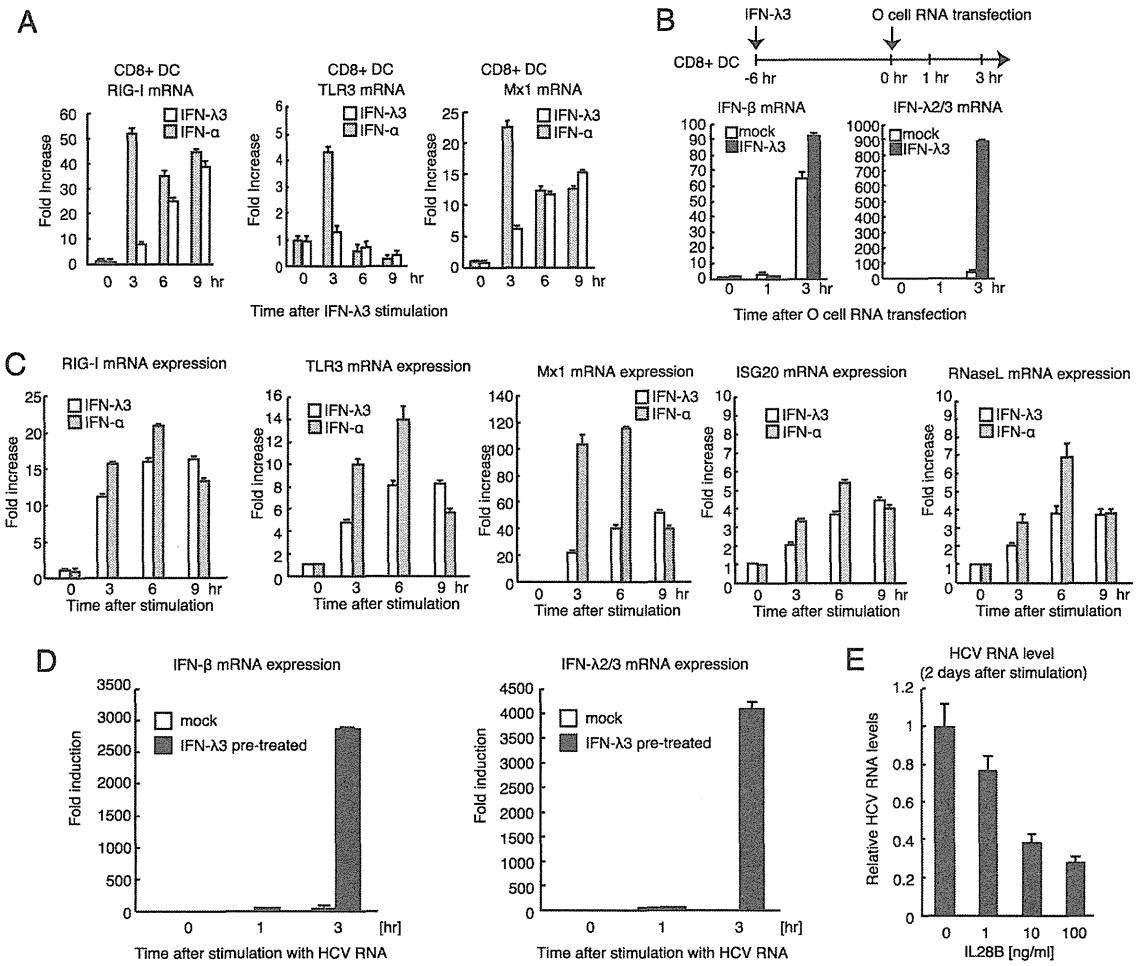

FIGURE 6. Antiviral responses induced by IFN-λ. (**A**) Mouse spleen CD8$^+$ DCs were stimulated with 100 ng/ml IFN-λ3 or 1000 IU/ml IFN-α, after which RIG-I, TLR3, and Mx1 mRNA levels were determined by quantitative RT-PCR. (**B**) Mouse spleen CD8$^+$ DCs were treated with 100 ng/ml IFN-λ3 for 6 h. O cell RNA was transfected into CD8$^+$ DCs, and IFN-β and -λ2/3 mRNA levels were determined by quantitative RT-PCR at the indicated times. (**C**) Mouse hepatocyte cell line cells were stimulated with 1000 U/ml IFN-α or 100 ng/ml IFN-λ3. RIG-I, TLR3, Mx1, ISG20, and RNaseL mRNA levels were determined by quantitative RT-PCR. (**D**) Mouse hepatocyte cell line cells were treated with 100 ng/ml IFN-λ3 for 6 h, and then O cell RNA was transfected into these cells. IFN-β and -λ2/3 mRNA levels were measured by quantitative RT-PCR at the indicated times. (**E**) O cells that contain HCV 1b full-length replicons were treated with human IL-28B at indicated concentration for 2 d. HCV RNA levels were determined by quantitative RT-PCR. HCV RNA levels were normalized to GAPDH mRNA expression.

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 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.

IFN-α augmented DC-mediated NK cell activation and cross-priming, whereas IFN-λ failed to augment DC-mediated NK cell activation and cross-priming in our experimental conditions. However, as seen with IFN-α, IFN-λ could induce RNaseL and ISG20 mRNA expression. These data indicated that IFN-λ induces cytoplasmic antiviral proteins to eliminate infected virus. A previous study showed that IPS-1 is required for initial antiviral response but dispensable for the protective adaptive immune response to influenza A virus (34). Thus, it is expected that IPS-1–mediated IFN-λ production would be required for initial antiviral response to 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.

Acknowledgments

IFN-λ1 and 2/3 reporter plasmids and O cells with HCV replicons were gifted from T. Imamichi (National Institutes of Health) and N. Kato (Okayama University), respectively.

Disclosures

The authors have no financial conflicts of interest.

References

1. Lauer, G. M., and B. D. Walker. 2001. Hepatitis C virus infection. *N. Engl. J. Med.* 345: 41–52.
2. Saito, T., D. M. Owen, F. Jiang, J. Marcotrigiano, and M. Gale, Jr. 2008. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 454: 523–527.
3. Kumar, H., T. Kawai, H. Kato, S. Sato, K. Takahashi, C. Coban, M. Yamamoto, S. Uematsu, K. J. Ishii, O. Takeuchi, and S. Akira. 2006. Essential role of IPS-1 in innate immune responses against RNA viruses. *J. Exp. Med.* 203: 1795–1803.
4. Matsumoto, M., and T. Seya. 2008. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv. Drug Deliv. Rev.* 60: 805–812.
5. Ebihara, T., M. Shingai, M. Matsumoto, T. Wakita, and T. Seya. 2008. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. *Hepatology* 48: 48–58.
6. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 301: 640–643.
7. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 4: 161–167.
8. Sheppard, P., W. Kindsvogel, W. Xu, K. Henderson, S. Schlutsmeyer, T. E. Whitmore, R. Kuestner, U. Garrigues, C. Birks, J. Roraback, et al. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat. Immunol.* 4: 63–68.
9. Thomas, D. L., C. L. Thio, M. P. Martin, Y. Qi, D. Ge, C. O'Huigin, J. Kidd, K. Kidd, S. I. Khakoo, G. Alexander, et al. 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 461: 798–801.
10. Tanaka, Y., N. Nishida, M. Sugiyama, M. Kurosaki, K. Matsuura, N. Sakamoto, M. Nakagawa, M. Korenaga, K. Hino, S. Hige, et al. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* 41: 1105–1109.
11. Suppiah, V., M. Moldovan, G. Ahlenstiel, T. Berg, M. Weltman, M. L. Abate, M. Bassendine, U. Spengler, G. J. Dore, E. Powell, et al. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.* 41: 1100–1104.
12. Ge, D., J. Fellay, A. J. Thompson, J. S. Simon, K. V. Shianna, T. J. Urban, E. L. Heinzen, P. Qiu, A. H. Bertelsen, A. J. Muir, et al. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461: 399–401.
13. Marcello, T., A. Grakoui, G. Barba-Spaeth, E. S. Machlin, S. V. Kotenko, M. R. MacDonald, and C. M. Rice. 2006. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* 131: 1887–1898.
14. Le Bon, A., N. Etchart, C. Rossmann, M. Ashton, S. Hou, D. Gewert, P. Borrow, and D. F. Tough. 2003. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat. Immunol.* 4: 1009–1015.
15. Lauterbach, H., B. Bathke, S. Gilles, C. Traidl-Hoffmann, C. A. Luber, G. Fejer, M. A. Freudenberg, G. M. Davey, D. Vremec, A. Kallies, et al. 2010. Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *J. Exp. Med.* 207: 2703–2717.
16. Schulz, O., S. S. Diebold, M. Chen, T. I. Näslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljeström, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433: 887–892.
17. Yoshio, S., T. Kanto, S. Kuroda, T. Matsubara, K. Higashitani, N. Kakita, H. Ishida, N. Hiramatsu, H. Nagano, M. Sugiyama, et al. 2013. Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-λ in response to hepatitis C virus. *Hepatology* 57: 1705–1715.
18. Oshiumi, H., M. Okamoto, K. Fujii, T. Kawanishi, M. Matsumoto, S. Koike, and T. Seya. 2011. The TLR3/TICAM-1 pathway is mandatory for innate immune responses to poliovirus infection. *J. Immunol.* 187: 5320–5327.
19. Aly, H. H., H. Oshiumi, H. Shime, M. Matsumoto, T. Wakita, K. Shimotohno, and T. Seya. 2011. Development of mouse hepatocyte lines permissive for hepatitis C virus (HCV). *PLoS One* 6: e21284.
20. Oshiumi, H., M. Ikeda, M. Matsumoto, A. Watanabe, O. Takeuchi, S. Akira, N. Kato, K. Shimotohno, and T. Seya. 2010. Hepatitis C virus core protein abrogates the DDX3 function that enhances IPS-1-mediated IFN-beta induction. *PLoS One* 5: e14258.
21. Liu, F., Y. Song, and D. Liu. 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 6: 1258–1266.
22. Ikeda, M., K. Abe, H. Dansako, T. Nakamura, K. Naka, and N. Kato. 2005. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem. Biophys. Res. Commun.* 329: 1350–1359.
23. Goutagny, N., A. Fatmi, V. De Ledinghen, F. Penin, P. Couzigou, G. Inchauspé, and C. Bain. 2003. Evidence of viral replication in circulating dendritic cells during hepatitis C virus infection. *J. Infect. Dis.* 187: 1951–1958.
24. Sansonno, D., A. R. Iacobelli, V. Cornacchiulo, G. Iodice, and F. Dammacco. 1996. Detection of hepatitis C virus (HCV) proteins by immunofluorescence and HCV RNA genomic sequences by non-isotopic in situ hybridization in bone marrow and peripheral blood mononuclear cells of chronically HCV-infected patients. *Clin. Exp. Immunol.* 103: 414–421.
25. Stone, A. E., S. Giugliano, G. Schnell, L. Cheng, K. F. Leahy, L. Golden-Mason, M. Gale, Jr., and H. R. Rosen. 2013. Hepatitis C virus pathogen associated molecular pattern (PAMP) triggers production of lambda-interferons by human plasmacytoid dendritic cells. *PLoS Pathog.* 9: e1003316.
26. Itoh, K., A. Watanabe, K. Funami, T. Seya, and M. Matsumoto. 2008. The clathrin-mediated endocytic pathway participates in dsRNA-induced IFN-beta production. *J. Immunol.* 181: 5522–5529.
27. Dreux, M., U. Garaigorta, B. Boyd, E. Décembre, J. Chung, C. Whitten-Bauer, S. Wieland, and F. V. Chisari. 2012. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. *Cell Host Microbe* 12: 558–570.
28. Morelli, A. E., A. T. Larregina, W. J. Shufesky, M. L. Sullivan, D. B. Stolz, G. D. Papworth, A. F. Zahorchak, A. J. Logar, Z. Wang, S. C. Watkins, et al. 2004. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* 104: 3257–3266.
29. Matsumoto, M., K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, and T. Seya. 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* 171: 3154–3162.
30. Pham, T. N., S. A. MacParland, P. M. Mulrooney, H. Cooksley, N. V. Naoumov, and T. I. Michalak. 2004. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. *J. Virol.* 78: 5867–5874.
31. Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437: 1167–1172.
32. Li, X. D., L. Sun, R. B. Seth, G. Pineda, and Z. J. Chen. 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc. Natl. Acad. Sci. USA* 102: 17717–17722.
33. Saito, T., R. Hirai, Y. M. Loo, D. Owen, C. L. Johnson, S. C. Sinha, S. Akira, T. Fujita, and M. Gale, Jr. 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc. Natl. Acad. Sci. USA* 104: 582–587.
34. Koyama, S., K. J. Ishii, H. Kumar, T. Tanimoto, C. Coban, S. Uematsu, T. Kawai, and S. Akira. 2007. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. *J. Immunol.* 179: 4711–4720.

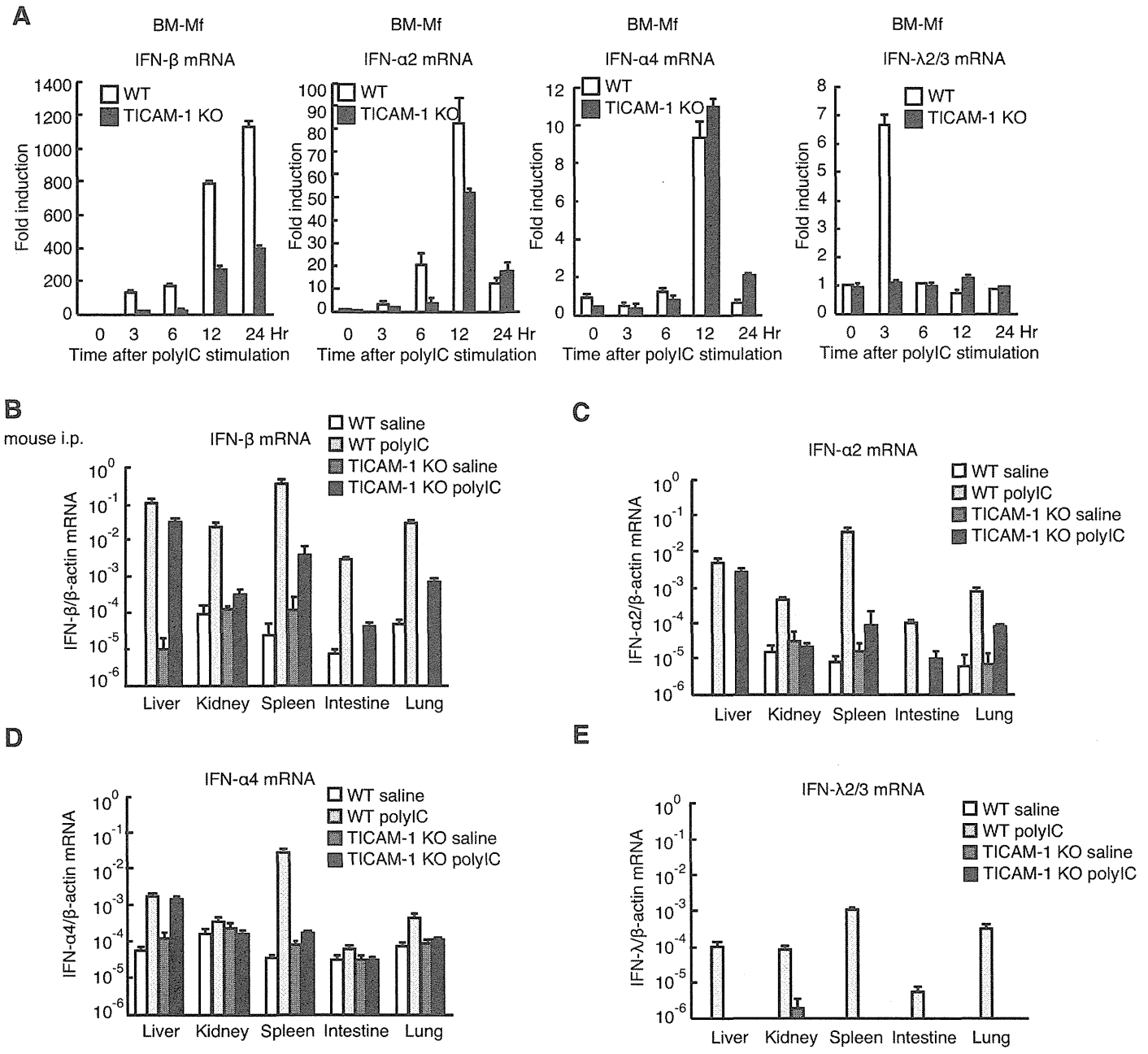

Figure S1

Type I IFN production in response to polyI:C stimulation.

(A) Wild-type and TICAM-1 KO BM-Mf were stimulated with 50 μg of polyI:C (without transfection), and IFN-β, α2, α4, and λ2/3 expressions were determined by RT-qPCR.

(B-E) 50 μg of PolyI:C was i.p. injected into wild-type and TICAM-1 KO mice. Six hours after injection, the tissues were isolated, and total RNA was extracted. The expression of IFN-β (B), α2 (C), α4 (D), and λ2/3 (E) in indicated tissues were measured by RT-qPCR.

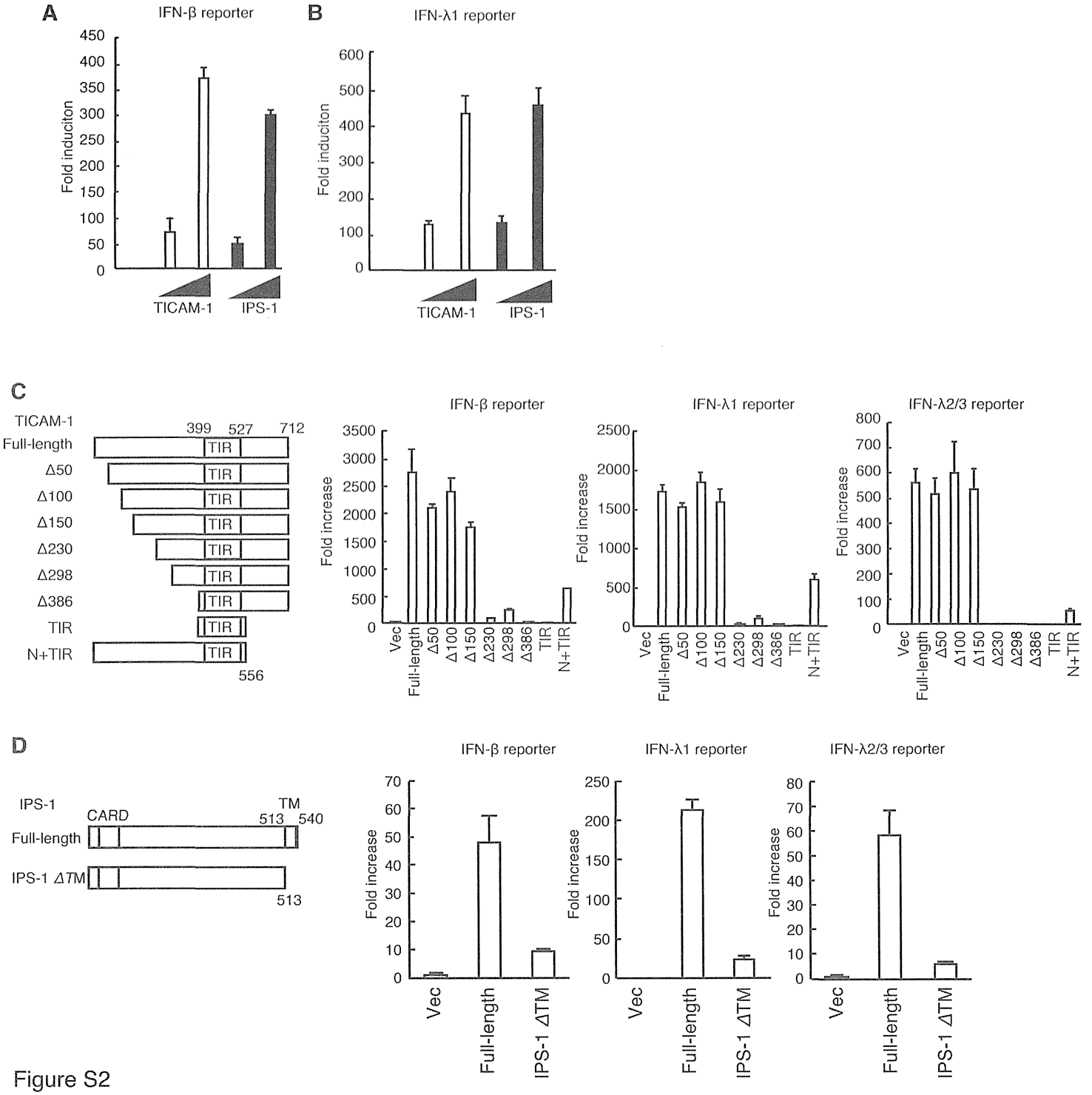

Figure S2

The ability of IPS-1 to induce type I and III IFNs.

TICAM-1 and IPS-1 expression vectors were transfected into HEK293 with p125luc (IFN-β) (A) or pIFN-λ1 luc (human IFN-λ) (B) reporter plasmids. 24 hours after transfection, the luciferase activity was measured.

(C) TICAM-1 fragment expression vectors were transfected into HEK293 cells with p125luc, pIFN-λ1, or λ2/3 luc reporter plasmids. 24 hours after transfection, the luciferase activity was measured.

(D) IPS-1 fragment expression vectors were transfected into HEK293 cells with p125luc, pIFN-λ1, or λ2/3 luc reporter plasmids. 24 hours after transfection, the reporter activities were measured.

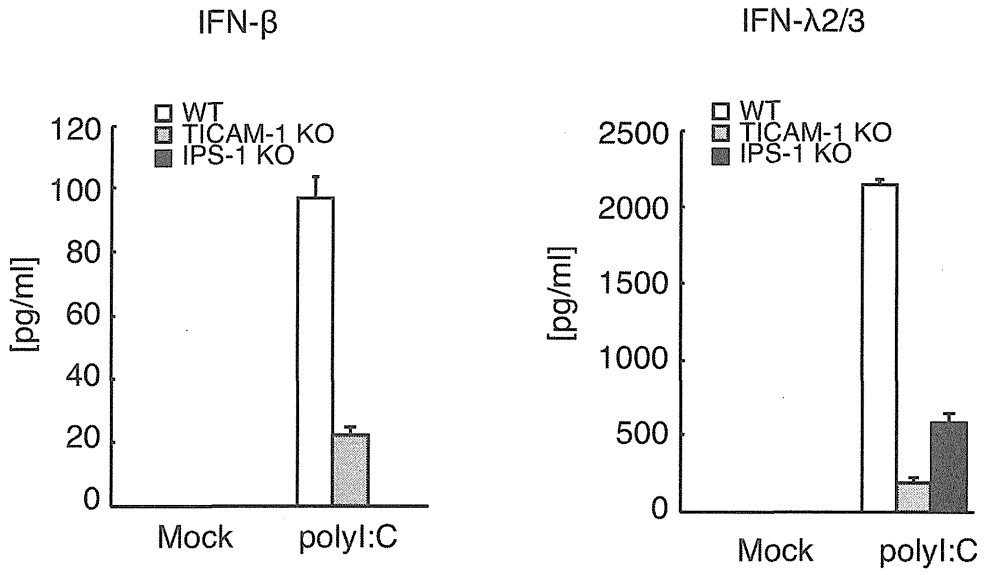

Figure S3

polyI:C (20 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.

Supplemental Table S1 Primer sequences for RT-qPCR

mouse β-actin	F	TTTGCAGCTCCTTCGTTGC
	R	TCGTCATCCATGGCGAACT
mouse IFN-β	F	CCAGCTCCAAGAAAGGACGA
	R	CGCCCTGTAGGTGAGGTTAT
mouse IFN-α2	F	TACTCAGCAGACCTTGAACC
	R	GGTACACAGTGATCCTGTGG
mouse IFN-α4	F	CTGCTGGCTGTGAGGACATACT
	R	AGGCACAGAGGCTGTGTTTCTT
mouse IFN-λ2/3	F	TCCCAGTGGAAGCAAAGGATTG
	R	TCAAGCACCTCTTCTCGATGG
mouse TLR3	F	TTGCGTTGCGAAGTGAAGAA
	R	ACTTGCCAATTGTCTGGAAACA
mouse RIG-I	F	GCCCTGTACCATGCAGGTTAC
	R	AGTCCCAACTTTCGATGGCTT
mouse Mx1	F	TCAAGCCTGGAGTGTCAAGTGCC
	R	AATCTGGAAGCCTTGCCACTGGG
mouse RNaseL	F	GCGAACACATCAATGAGGAAAA
	R	CTGCCTCTGGAACGCTGAG
mouse ISG20	F	CAATGCCCTGAAGGAGGATA
	R	TGTAGCAGGCGCTTACACAG
mouse IL10Rb	F	GTCGTGCTGTGGCTCATTTA
	R	AGCAGAAACGTGCTGTGATG
mouse IL28Ra	F	CAGGTGGAATTCTGGAAGGA
	R	CCTTGCTGGAGAGGAATCTG
HCV NS5B	F	CCGAGGCCAGACAGGTCATA
	R	GGCTGCAGAGGCCTTCAAGT
Human GAPDH	F	GAGTCAACGGATTTGCTCGT
	R	TTGATTTTGCAGGGATCTCG

The International Journal of Biochemistry & Cell Biology 53 (2014) 329–333

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Cells in focus

Dendritic cell subsets involved in type I IFN induction in mouse measles virus infection models

Hiromi Takaki*, Hiroyuki Oshiumi, Misako Matsumoto, Tsukasa Seya*

Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita-ku, Sapporo 060-8638, Japan

ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form 28 April 2014
Accepted 1 May 2014
Available online 4 June 2014

Keywords:
Dendritic cells
Measles virus
Mouse model
Type I interferon (IFN)
Immune suppression

ABSTRACT

Measles caused by measles virus (MV) infection remains important in child mortality. Although the natural host of MV is human, mouse models expressing MV entry receptors (human CD46, CD150) and disrupting the interferon (IFN) pathways work for investigating immune responses during early MV infection *in vivo*. Dendritic cells (DCs) are primary targets for MV in the mouse models and are efficiently infected with several MV strains in the respiratory tract *in vivo*. However, questions remain about what kind of DC in a variety of DC subsets is involved in initial MV infection and how the RNA sensors evoke circumventing signals against MV in infected DCs. Since type I IFN-inducing pathways are a pivotal defense system that leads to the restriction of systemic viral infection, we have generated CD150-transgenic mice with disrupting each of the IFN-inducing pathway, and clarified that DC subsets had subset-specific IFN-inducing systems, which critically determined the DC's differential susceptibility to MV.

1. Introduction

The pathogenic measles virus (MV) causes measles in infants. The MV genome is a nonsegmented negative single-stranded RNA consisting of six genes that encode the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The P gene encodes P protein and the nonstructural V and C proteins. Although the nonstructural V and C proteins of wild type (WT) strains of MV are important in suppressing the host interferon (IFN) response in human cells (Gerlier and Valentin, 2009), WT strains of MV are less able to suppress type I IFN production in murine cells than in human cells (Shingai et al., 2005), suggesting that V and C proteins are relatively ineffective suppressors for IFN response in murine cells.

CD46 (also called MCP) was first identified as an MV entry receptor for laboratory-adapted and vaccine strains of MV. CD46 is expressed in all human nucleated cells including epithelial cells (Gerlier and Valentin, 2009). In 2000, human CD150, a signaling lymphocyte activation molecule (SLAM), was identified as the second MV entry receptor for all MV strains including WT (Tatsuo et al., 2000). Expression of CD150 is restricted to activated lymphocytes, dendritic cells (DCs), and macrophages (Delpeut et al., 2012), consistent with the lymphotropism of MV. However, the expression pattern of CD150 does not explain why WT strains of MV infect epithelial cells that do not express CD150. Recently, human nectin-4 (also called poliovirus receptor-related 4, PVRL4) was identified as the third entry receptor for WT strains of MV (Mühlebach et al., 2011; Noyce et al., 2011). Expression of nectin-4 is restricted to the basolateral surface of epithelial cells (Delpeut et al., 2012). Thus, laboratory-adapted and vaccine strains of MV use CD46 and CD150 as entry receptors, and WT strains of MV use CD150 and nectin-4. Initial infection with WT stains of MV *via* CD150 occurs in DCs and alveolar macrophages (AMs) and secondary spreading of MV infection is established in lymphocytes through infected DCs and AMs. Ultimately, MV-infected lymphocytes systemically spread to distal sites including the respiratory tract and then MV infects epithelial cells *via* nectin-4, resulting in release of MV into the airway lumen of the infected lung (Delpeut et al., 2012). C-type lectin DC-SIGN (also called CD209) has an important role for infection of DCs by laboratory-adapted and WT strains of MV (de Witte et al., 2006), although DC-SIGN is dispensable for MV entry. Both attachment and infection of immature DCs with MV are blocked by DC-SIGN inhibitors, suggesting that DC-SIGN is critical for enhancement of CD46/CD150-mediated infection of DCs (de Witte et al., 2006).

Human CD150 transgenic (Tg) and CD150 knock-in mice were generated as MV infection models to study receptor tropism and the immune dynamics of MV (Hahm et al., 2003, 2004; Ohno et al., 2007; Sellin et al., 2006; Shingai et al., 2005; Welstead et al., 2005) and these mice were somehow permissive to MV *in vivo*.

* Corresponding authors at: Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan. Tel.: +81 11 706 7866; fax: +81 11 706 7866.
E-mail addresses: tahiromi@sci.hokudai.ac.jp (H. Takaki), seya-tu@pop.med.hokudai.ac.jp (T. Seya).

http://dx.doi.org/10.1016/j.biocel.2014.05.001
1357-2725/

Systemic infection by WT strains of MV *in vivo* was observed in CD150Tg/*Ifnar*$^{-/-}$ mice, generated by crossing CD150Tg mice with mice having the disrupted IFN receptor 1 (*Ifnar*) gene; the other is CD150Tg/*Stat1*$^{-/-}$ mice, generated by crossing CD150Tg mice with mice knocked out for the signal transduction and activator of transcription 1 (*Stat1*) gene, which is a major signaling molecule for the IFN receptor (Shingai et al., 2005; Welstead et al., 2005). Both models indicate the importance of the IFNAR pathway for restricting MV *in vivo* infection in mice. DCs and AMs are primary targets for MV intranasally inoculated into CD150Tg models (Ferreira et al., 2010), since these cells express CD150 and are located in the lung where host cells firstly encounter MV. Results from mouse models for MV *in vivo* infection reflect *in vitro* high susceptibility of human monocyte-derived DCs (moDCs) to MV. DCs and AMs are the first target cells during early MV infection in monkeys (de Swart et al., 2007; Lemon et al., 2011). All these data indicate that type I IFN produced by DCs and AMs primarily protects hosts from systemic MV infection.

In this review, we summarized the mouse model studies on the host antiviral response to MV infection, which involves both toll-like receptors (TLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) in specific DC subsets.

2. Type I IFN-inducing pathways respond to viral RNA

The IFN response, which is the induction of type I IFN-α/β is a major antiviral defense pathway that confers virus resistance to neighboring cells. Previous reports showed that viral RNA is detected by cytoplasmic pattern recognition receptors (PRRs) such as RIG-I and the melanoma differentiation-associated gene 5 (MDA5) (Kawai and Akira, 2009). MDA5 and RIG-I detect long and short dsRNA, respectively (Kato et al., 2008). TLR3 recognizes extracellular double-stranded RNA (dsRNA) in the endosome whereas RIG-I and MDA5 sense cytoplasmic dsRNA (Fig. 1). TLR3 recruits the adaptor, Toll/interleukin-1 receptor (TIR) homology domain-containing adaptor molecule 1 (TICAM-1, also called TRIF) in response to dsRNA and induces type I IFN production. Activation of RLRs is regulated by multiple consecutive processes including dephosphorylation, ubiquitination and oligomerization of RLR (Gack et al., 2007; Wies et al., 2013). The CARD domain of RLRs is phosphorylated by unknown kinases in steady state, prohibiting RLR activation (Wies et al., 2013). Viral infection activates RLRs *via* dephosphorylation by serine–threonine phosphatases PP1α and PP1γ (Wies et al., 2013). The dephosphorylated RLRs provide signals through the mitochondrial antiviral signaling protein (MAVS; also called VISA, Cardif, or IPS-1) to induce type I IFN. Disrupting these adaptor genes results in failure to activate IFN regulatory factor (IRF)-3 and IRF-7, abrogating type I IFN production and antiviral host defense. Virus-derived single-stranded RNA (ssRNA) is recognized by TLR7 and TLR8 which are in the endosome. MyD88-dependent signaling is activated upon viral RNA recognition by TLR7 to induce type I IFNs (Kawai and Akira, 2009). Unlike ubiquitous RLRs, TLR expression is restricted to particular cell types with a different set of TLRs (Table 1) (Edwards et al., 2003). This differential expression pattern of TLRs directs specific sets of cells to respond to particular TLR ligands, which enhance a variety of immune responses.

3. Type I IFN induction in MV-infected murine DCs

Studies in mice with targeted gene deletions provide insight into the mechanisms of type I IFN induction in response to MV infection *in vivo* and *in vitro*. Bone marrow-derived DCs (BMDCs) were used to study MV permissiveness of DCs, initially in CD150Tg mice (Ohno et al., 2007; Shingai et al., 2005; Welstead et al., 2005). Studies using BMDCs from CD150Tg mice in combination with *Mavs*$^{-/-}$, *Irf3*$^{-/-}$/*Irf7*$^{-/-}$, *Ticam1*$^{-/-}$ and *Myd88*$^{-/-}$ mice showed that type I IFN expression in BMDCs completely relied on MAVS but not TICAM-1 and MyD88 (Takaki et al., 2014). Surprisingly, BMDCs derived from CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ mice produce a detectable IFN-β in response to MV infection, which confers nonpermissiveness to CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs (Takaki et al., 2014). A pharmacological study indicated that MV-derived IFN-β expression partially depended on NF-κB (Takaki et al., 2014). A recent study using West Nile virus showed that IRF3/IRF7 and IRF5 coordinately regulate the type I IFN response in DCs (Lazear et al., 2013). For MV, IRF5 might be a transcription factor for MAVS-dependent and IRF3/IRF7-independent type I IFN induction in BMDCs (Fig. 2).

An *in vivo* MV infection study using a CD150Tg mouse model revealed that MAVS disruption scarcely led MV permissiveness or type I IFN gene expression in the spleen compared to CD150Tg mice (Takaki et al., 2013). *In vitro* infection assays showed that isolated cell subsets of CD11c^{+} DCs, but not T or B cells, mainly produced type I IFN in response to MV infection through a MAVS-independent pathway. Various types of DCs have been identified in mouse secondary lymphoid tissues, including three CD11c^{high} subsets of conventional DCs (cDCs): CD8α^{+}, CD4^{+} and CD4^{-} CD8α^{-} double negative (DN) DCs (Vremec et al., 2000), and one subset of CD11c^{low} plasmacytoid DCs (pDCs) (Asselin-Paturel et al., 2001). These DC subsets express different sets of TLR genes and have distinct functions (Table 1) (Edwards et al., 2003; Luber et al., 2010). Mouse pDCs express most TLRs except TLR3 and therefore respond to a wide range of pathogen-associated molecular patterns including TLR7 ligand (Boonstra et al., 2003; Edwards et al., 2003). CD8α^{+} DCs express high amounts of TLR3, but not TLR7 (Edwards et al., 2003) and mainly participate in poly I:C-induced cross-presentation. Although a CD4^{+} and DN DCs have a similar TLR expression pattern (Edwards et al., 2003), CD4^{+} DCs but not DN DCs express TLR7 protein at low levels (Takaki et al., 2013). Type I IFN expression is induced in CD4^{+} DCs and pDCs, but not CD8α^{+} and DN DCs that are isolated from MAVS-disrupted mice during *in vitro* MV infection (Takaki et al., 2013). This result indicates that type I IFN-inducing pathways in pDC and CD4^{+} DCs are independent of the MAVS pathway. A pharmacological study showed that the MyD88 pathway is involved in a MAVS-independent type I IFN-inducing pathway (Takaki et al., 2013). This result was confirmed using CD150Tg/*Myd88*$^{-/-}$ pDCs, suggesting that TLR7 is responsible for recognition of MV RNA in CD4^{+} and pDCs. Since the RLR-MAVS pathway usually senses endogenous viral RNA in CD4^{+} DCs (Luber et al., 2010), MAVS disruption highlights that the MyD88 pathway participates in initial type I IFN induction in CD4^{+} DCs in MV infection (Fig. 2). However, CD150Tg/*Myd88*$^{-/-}$ mice are not permissive to MV infection *in vivo*, both MyD88 in pDCs and CD4^{+} DCs and MAVS in other cells contribute to protection against systemic MV infection.

Since TLR7 is in the endosome, viral RNA transport to the endosome is required to activate the TLR7/MyD88 pathway. Autophagy is required for the recognition of vesicular stomatitis virus by TLR7 to transport cytosolic viral replication intermediates into the lysosome, leading to type I IFN production in pDCs (Lee et al., 2007) IFN-β mRNA expression is induced in UV-irradiated MV-infected CD150Tg/*Mavs*$^{-/-}$ DCs; however, treatment with an autophagy inhibitor prevented this IFN-β induction (unpublished data). These data suggest that autophagy but not viral replication is required for MV-mediated type I IFN induction *via* TLR7 in MAVS-disrupted murine DCs.

In contrast to BMDCs, type I IFN gene expression is observed in DCs and splenocytes derived from MV-infected CD150Tg/*Mavs*$^{-/-}$ mice, which prevents DCs from MV infection *in vivo* in these mice (Takaki et al., 2013, 2014). RIG-I/MAVS but not TLR7/MyD88 mediates the antiviral response to RNA virus in conventional DCs. The

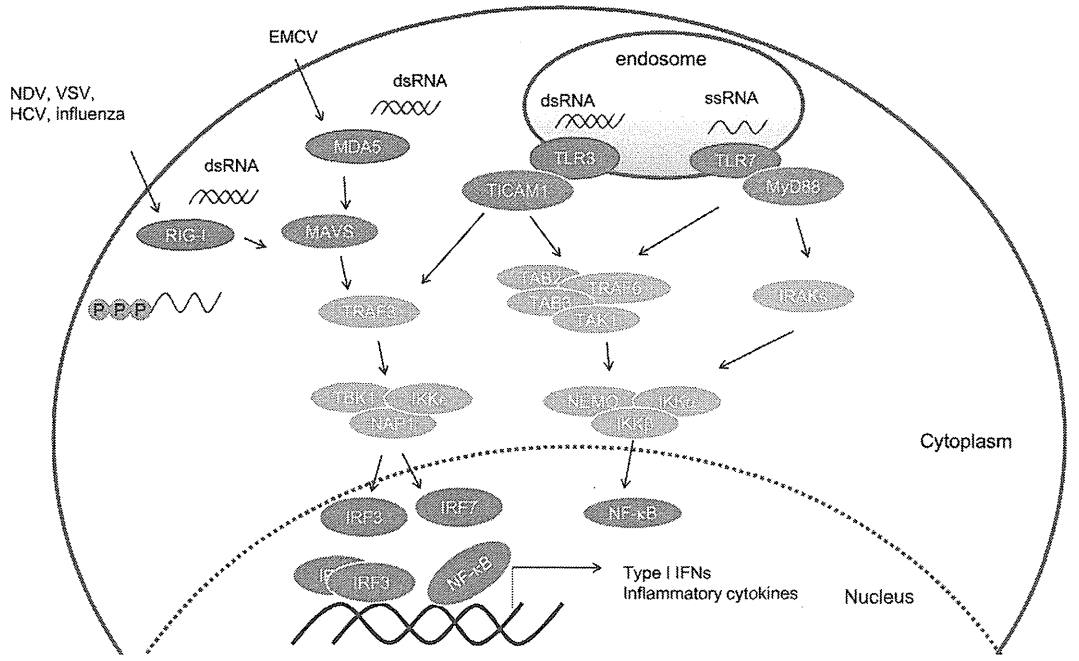

Fig. 1. Recognition of RNA by RLRs and TLRs. Double-stranded RNA (dsRNA) synthesized by RNA virus replication in infected cells is recognized by endosomal TLR3 and cytosolic RIG-I like receptors (RLRs), RIG-I and MDA5. They differentially recognize viral dsRNA products such that long dsRNA chains fit in MDA5, 5′-triphosphates short dsRNA couple with RIG-I and structured RNA activate TLR3 (Tatematsu et al., 2013). The outline of their signaling cascades that lead to the activation of IRF3 and NF-κB is overviewed (Kawai and Akira, 2009). Single-stranded RNA (ssRNA) is recognized by endosomal TLR7, leading to the activation of NF-κB and IKK α/β *via* adaptor protein MyD88. Transcription factor activation resuls in expression of type I IFN and inflammatory cytokines. NDV, newcastle disease virus; SeV, sendai virus; HCV, hepatitis C; EMCV, encephalomyocarditis virus

Table 1
Expression of TLRs in murine and human DC subset.

			TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
Mouse	Conventional DCs ($CD11c^{high}$ $B220^{-}$)	$CD4^{+}$	+	+	–	+	+	+	+	–	+	–
		$CD4^{-}CD8\alpha^{-}$	+	+	+/–	+	+	+	+/–	–	+	–
		$CD4^{-}$	+	+	+	+	–	+	–	–	+	–
	Plasmacytoid DCs ($CD11c^{low}$ $B220^{+}$ $PDCA{-}1^{+}$)		+	+	–	+	+/–	+	+	–	+	–
Human	Myeloid DCs ($CD11c^{+}$)		+	+	+	+	+	+	+	+/–	–	+
	Monocyte-derived DCs (moDCs)		+	+	+	+	+	+/–	+/–	+	–	–
	Plasmacytoid DCs ($CD11c^{-}$ $BDCA2^{+}$ $BDCA4^{+}$)		+/–	–	–	–	–	–	+	–	+	+

TLR expression in murine and human DC subset is described in refs (Jarrossay et al., 2001; Kadowaki et al., 2001; Edwards et al., 2003; Luber et al., 2010).

studies using reporter mouse that expresses green fluorescence protein (GFP) under the control of the *Ifn-α6* promoter show that intranasal infection with newcastle disease virus (NDV) induces GFP expression in AMs and cDCs in lung as an initial defense *via* the RLR pathway (Kumagai et al., 2007). Although systemic NDV infection leads to GFP expression in not only pDCs but also cDCs and AMs, the frequency of GFP positive cells is higher in pDCs than in other cells. Thus, the activation of different subsets of DCs would be important to produce type I IFNs in systemic and local RNA virus infection.

Similar to murine DCs, PRRs expression differs with subsets of human DCs (Table 1) (Jarrossay et al., 2001; Kadowaki et al., 2001). In cDCs, MV transcription is required to activate type I IFN response, since UV-irradiated MV is unable to promote IFN-β production (Duhen et al., 2010). Type I IFN induction by pDCs depends on the recognition of MV RNA *via* the endosomal pathway, since UV-irradiated MV infection induces IFN-α production and this induction is cancelled by an endosomal acidification inhibitor in pDCs (Duhen et al., 2010). Although MV can inhibit TLR7 and TLR9-mediated type I IFN induction by MV-V and MV-C proteins in human pDCs (Pfaller and Conzelmann, 2008; Schlender et al., 2005; Yamaguchi et al., 2014), it remains unknown whether MV proteins act as suppressors in murine DCs. Moreover, MV interacts with human DC-SIGN to enhance infection of human DCs (de Witte et al., 2006). However, how MV-H protein binds murine CIRE/DC-SIGN is unknown. The findings in murine DCs may differ from those in human DCs when infected with MV.

4. Type I IFN and cytokines in the context of MV immunosuppression

DCs contribute to MV-induced immunosuppression, including downregulation of costimulatory molecules and inhibition of IL-12 production following lipopolysaccharide stimulation (Coughlin et al., 2013; Hahm et al., 2004, 2007). MV infection suppressed BMDCs development *via* type I IFN that acts through STAT2-dependent signaling but independent of the STAT1 signaling (Hahm et al., 2005). Furthermore, *in vivo* MV infection induces a T helper type 2 response, enhances apoptosis, and induces regulatory T cells (Koga et al., 2010; Sellin et al., 2009). Blocking IL-10 signaling prevents MV-induced immunosuppression in CD150 knock-in mice, indicating that IL-10 participates in immunosuppression (Koga et al., 2010). In addition, high amounts of IL-10 are produced in $CD4^{+}$ T cells obtained from MV-infected CD150Tg mice (Takaki

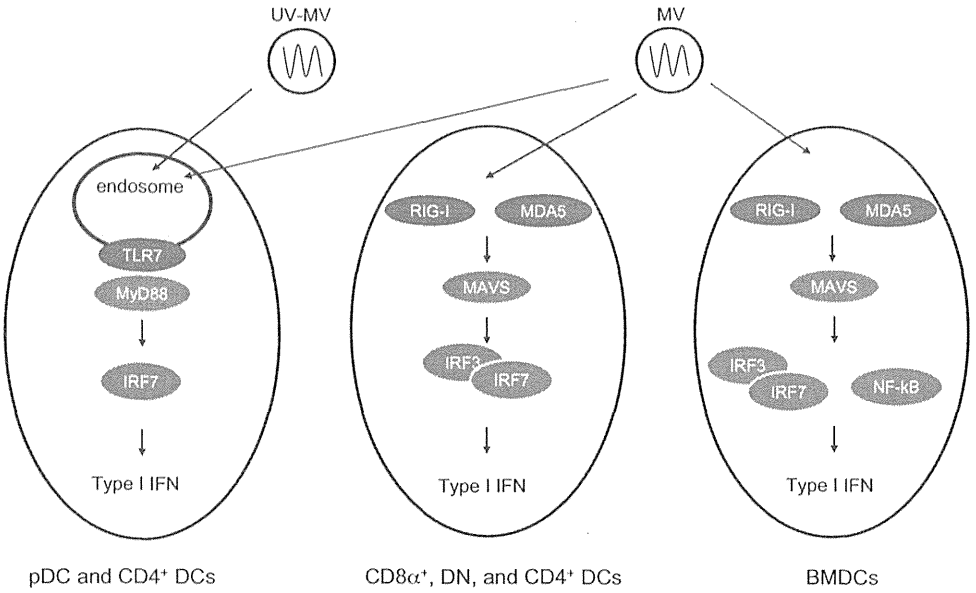

Fig. 2. Recognition of MV RNA in mouse DC subsets. DC subsets have their own viral RNA sensors to induce type I IFN. MV specifically infects these DC subsets. The ways for IFN induction in each DC subset are shown schematically. UV-MV; UV-irradiated MV

et al., 2014). In early infection by lymphocytic choriomeningitis virus (LCMV), type I IFN is produced *via* the TLR7/MyD88 pathway in pDCs. MDA5/MAVS-mediated type I IFN induction in other cells is required for sustained type I IFN responses to acute and chronic LCMV infection (Wang et al., 2012). Thus, different sources of type I IFN and signaling pathways affect immune responses to viral infection. Besides IL-10, IL-12 and type I IFN, other cytokines and signaling molecules affect MV-mediated immunomodulation. Further analysis is needed to clarify the function of DCs that modulate MV-induced immunosuppression.

Acknowledgements

We are grateful to Dr. M. Takeda (National Institute of Infectious Diseases, Japan) for fruitful discussions. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for 'Carcinogenic Spiral') and the Ministry of Health, Labor, and Welfare of Japan, and by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (Host factors determining tropism and pathogenicity of zoonoses in association with innate immune system) and Japan Society for the promotion of Science Fellows (21-1368).

References

Asselin-Paturel C, Boonstra A, Dalod M, Durand I, Yessaad N, Dezutter-Dambuyant C, et al. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat Immunol 2001;2:1144–50.

Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, et al. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. J Exp Med 2003;197:101–9.

Coughlin MM, Bellini WJ, Rota PA. Contribution of dendritic cells to measles virus induced immunosuppression. Rev Med Virol 2013;23:126–38.

de Swart RL, Ludlow M, de Witte L, Yanagi Y, van Amerongen G, McQuaid S, et al. Predominant infection of CD150+ lymphocytes and dendritic cells during measles virus infection of macaques. PLoS Pathog 2007;3:e178.

de Witte L, Abt M, Schneider-Schaulies S, van Kooyk Y, Geijtenbeek TB. Measles virus targets DC-SIGN to enhance dendritic cell infection. J Virol 2006;80:3477–86.

Delpeut S, Noyce RS, Siu RW, Richardson CD. Host factors and measles virus replication. Curr Opin Virol 2012;2:773–83.

Duhen T, Herschke F, Azocar O, Druelle J, Plumet S, Delprat C, et al. Cellular receptors, differentiation and endocytosis requirements are key factors for type I IFN response by human epithelial, conventional and plasmacytoid dendritic infected cells by measles virus. Virus Res 2010;152:115–25.

Edwards AD, Diebold SS, Slack EM, Tomizawa H, Hemmi H, Kaisho T, et al. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur J Immunol 2003;33:827–33.

Ferreira CS, Frenzke M, Leonard VH, Welstead GG, Richardson CD, Cattaneo R. Measles virus infection of alveolar macrophages and dendritic cells precedes spread to lymphatic organs in transgenic mice expressing human signaling lymphocytic activation molecule (SLAM, CD150). J Virol 2010;84:3033–42.

Gack MU, Shin YC, Joo CH, Urano T, Liang C, Sun L, et al. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature 2007;446:916–20.

Gerlier D, Valentin H. Measles virus interaction with host cells and impact on innate immunity. Curr Top Microbiol Immunol 2009;329:163–91.

Hahm B, Arbour N, Naniche D, Homann D, Manchester M, Oldstone MB. Measles virus infects and suppresses proliferation of T lymphocytes from transgenic mice bearing human signaling lymphocytic activation molecule. J Virol 2003;77:3505–15.

Hahm B, Arbour N, Oldstone MB. Measles virus interacts with human SLAM receptor on dendritic cells to cause immunosuppression. Virology 2004;323:292–302.

Hahm B, Cho JH, Oldstone MB. Measles virus-dendritic cell interaction via SLAM inhibits innate immunity: selective signaling through TLR4 but not other TLRs mediates suppression of IL-12 synthesis. Virology 2007;358:251–7.

Hahm B, Trifilo MJ, Zuniga EI, Oldstone MB. Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. Immunity 2005;22:247–57.

Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 2001;31:3388–93.

Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 2001;194:863–9.

Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, et al. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med 2008;1601–10, J205.

Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 2009;21:317–37.

Koga R, Ohno S, Ikegame S, Yanagi Y. Measles virus-induced immunosuppression in SLAM knock-in mice. J Virol 2010;84:5360–7.

Kumagai Y, Takeuchi O, Kato H, Kumar H, Matsui K, Morii E, et al. Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. Immunity 2007;27:240–52.

Lazear HM, Lancaster A, Wilkins C, Suthar MS, Huang A, Vick SC, et al. IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling. PLoS Pathog 2013;9:e1003118.

Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 2007;315:1398–401.

Lemon K, de Vries RD, Mesman AW, McQuaid S, van Amerongen G, Yüksel S, et al. Early target cells of measles virus after aerosol infection of non-human primates. PLoS Pathog 2011;7:e1001263.

Luber CA, Cox J, Lauterbach H, Fancke B, Selbach M, Tschopp J, et al. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. Immunity 2010;32:279–89.

Mühlebach MD, Mateo M, Sinn PL, Prüfer S, Uhlig KM, Leonard VH, et al. Adherens junction protein nectin-4 is the epithelial receptor for measles virus. Nature 2011;480:530–3.

Noyce RS, Bondre DG, Ha MN, Lin LT, Sisson G, Tsao MS, et al. Tumor cell marker PVRL4 (nectin 4) is an epithelial cell receptor for measles virus. PLoS Pathog 2011;7:e1002240.

Ohno S, Ono N, Seki F, Takeda M, Kura S, Tsuzuki T, et al. Measles virus infection of SLAM (CD150) knockin mice reproduces tropism and immunosuppression in human infection. J Virol 2007;81:1650–9.

Pfaller CK, Conzelmann KK. Measles virus V protein is a decoy substrate for IkappaB kinase alpha and prevents Toll-like receptor 7/9-mediated interferon induction. J Virol 2008;82:12365–73.

Schlender J, Hornung V, Finke S, Günthner-Biller M, Marozin S, Brzózka K, et al. Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. J Virol 2005;79:5507–15.

Sellin CI, Davoust N, Guillaume V, Baas D, Belin MF, Buckland R, et al. High pathogenicity of wild-type measles virus infection in CD150 (SLAM) transgenic mice. J Virol 2006;80:6420–9.

Sellin CI, Jégou JF, Renneson J, Druelle J, Wild TF, Marie JC, et al. Interplay between virus-specific effector response and Foxp3 regulatory T cells in measles virus immunopathogenesis. PLoS ONE 2009;4:e4948.

Shingai M, Inoue N, Okuno T, Okabe M, Akazawa T, Miyamoto Y, et al. Wild-type measles virus infection in human CD46/CD150-transgenic mice: CD11c-positive dendritic cells establish systemic viral infection. J Immunol 2005;175:3252–61.

Takaki H, Honda K, Atarashi K, Kobayashi F, Ebihara T, Oshiumi H, et al. MAVS-dependent IRF3/7 bypass of interferon β-induction restricts the response to measles infection in CD150Tg mouse bone marrow-derived dendritic cells. Mol Immunol 2014;57:100–10.

Takaki H, Takeda M, Tahara M, Shingai M, Oshiumi H, Matsumoto M, et al. The MyD88 pathway in plasmacytoid and CD4+ dendritic cells primarily triggers Type I IFN production against measles virus in a mouse infection model. J Immunol 2013;191:4740–7.

Tatematsu M, Nishikawa F, Seya T, Matsumoto M. Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA. Nat Commun 2013;4:1833.

Tatsuo H, Ono N, Tanaka K, Yanagi YSLAM. (CDw150) is a cellular receptor for measles virus. Nature 2000;406:893–7.

Vremec D, Pooley J, Hochrein H, Wu L, Shortman K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J Immunol 2000;164:2978–86.

Wang Y, Swiecki M, Cella M, Alber G, Schreiber RD, Gilfillan S, et al. Timing and magnitude of type I interferon responses by distinct sensors impact CD8T cell exhaustion and chronic viral infection. Cell Host Microbe 2012;11:631–42.

Welstead GG, Iorio C, Draker R, Bayani J, Squire J, Vongpunsawad S, et al. Measles virus replication in lymphatic cells and organs of CD150 (SLAM) transgenic mice. Proc Natl Acad Sci U S A 2005;102:16415–20.

Wies E, Wang MK, Maharaj NP, Chen K, Zhou S, Finberg RW, et al. Dephosphorylation of the RNA sensors RIG-I and MDA5 by the phosphatase PP1 is essential for innate immune signaling. Immunity 2013;38:437–49.

Yamaguchi M, Kitagawa Y, Zhou M, Itoh M, Gotoh B. An anti-interferon activity shared by paramyxovirus C proteins: inhibition of toll-like receptor 7/9-dependent alpha interferon induction. FEBS Lett 2014;588:28–34.

Research Article

Journal of Innate Immunity

J Innate Immun 2015;7:47–58
DOI: 10.1159/000365113

Received: March 14, 2014
Accepted after revision: June 9, 2014
Published online: August 8, 2014

A MAVS/TICAM-1-Independent Interferon-Inducing Pathway Contributes to Regulation of Hepatitis B Virus Replication in the Mouse Hydrodynamic Injection Model

Chean Ring Leong[a] Hiroyuki Oshiumi[a] Masaaki Okamoto[a] Masahiro Azuma[a]
Hiromi Takaki[a] Misako Matsumoto[a] Kazuaki Chayama[b] Tsukasa Seya[a]

[a]Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Sapporo, and
[b]Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

Key Words
Type I interferon · Hepatitis B virus regulation · Toll/IL-1R homology domain-containing adaptor molecule 1 · Mitochondrial antiviral signaling protein · Pathogen-associated molecular patterns

Abstract
Toll-like receptors (TLRs) and cytoplasmic RNA sensors have been reported to be involved in the regulation of hepatitis B virus (HBV) replication, but remain controversial due to the lack of a natural infectious model. Our current study sets out to characterize aspects of the role of the innate immune system in eliminating HBV using hydrodynamic-based injection of HBV replicative plasmid and knockout mice deficient in specific pathways of the innate system. The evidence indicated that viral replication was not affected by MAVS or TICAM-1 knockout, but absence of interferon regulatory factor 3 (IRF-3) and IRF-7 transcription factors, as well as the interferon (IFN) receptor, had an adverse effect on the inhibition of HBV replication, demonstrating the dispensability of MAVS and TICAM-1 pathways in the early innate response against HBV. *Myd88*$^{-/-}$ mice did not have a significant increase in the initial viremia, but substantial viral antigen persisted in the mice sera, a response similar to *Rag2*$^{-/-}$ mice, suggesting that the MyD88-dependent pathway participated in evoking an adaptive immune response against the clearance of intrahepatic HBV. Taken together, we show that the RNA-sensing pathways do not participate in the regulation of HBV replication in a mouse model; meanwhile MyD88 is implicated in the HBV clearance.

Introduction

Hepatitis B virus (HBV) is a noncytopathic human DNA (hepadna) virus that infects hepatocytes causing acute and chronic hepatitis [1]. More than 360 million people are chronically infected with HBV worldwide. Although less than 5% of HBV-infected patients develop persistent infections that progress to liver cirrhosis and hepatocellular carcinoma, HBV causes about 20% of hepatocellular carcinoma deaths [2]. The adaptive immune response is widely acknowledged as pivotal in the defense against HBV. However, the role of innate immunity during HBV infection remains controversial since analysis in patients at the early stage of infection is unfeasible. In ad-

1662–811X/14/0071–0047$39.50/0

E-Mail karger@karger.com
www.karger.com/jin

Dr. Tsukasa Seya
Department of Microbiology and Immunology
Graduate School of Medicine, Hokkaido University
Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638 (Japan)
E-Mail seya-tu@pop.med.hokudai.ac.jp

dition, no reliable cell-based in vitro infection system or convenient animal model is available.

During HBV infection, the HBV genome is delivered into the nucleus. Infection is defined by the formation of covalently closed circular DNA. Following formation of covalently closed circular DNA, viral mRNA and pregenomic RNA are transcribed [3, 4]. The pregenomic RNA is subsequently converted to a partially double-stranded genome by the viral DNA polymerase. Unlike other DNA viruses, HBV uses an RNA proviral intermediate that must be copied back into DNA for replication. Although these replication steps are sequestered in the nucleus of infected cells, cytoplasmic DNA/RNA sensors are reported to affect the efficacy of HBV replication [5, 6]. The association between cytoplasmic pattern recognition receptors and the dynamics of the HBV life cycle in HBV-infected cells needs to be clarified.

Viral RNA is sensed by the innate immune system by either Toll-like receptor 3 (TLR3) or cytoplasmic sensors such as retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). RIG-I and MDA5 mainly participate in type I interferon (IFN) induction in conjunction with the adaptor molecule, mitochondrial antiviral signaling protein (MAVS; also called IPS-1, Cardif, or VISA) [7–9]. The Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1; also called TRIF) is the adaptor of TLR3, which senses viral RNA on the endosomal membrane [8–10]. Several DNA sensors, most of which signal through STING for type I IFN induction, have been reported in recent years [11]. A few reports have also mentioned that MAVS participates in DNA sensing in certain human cells whereby poly-dA/dT DNA is found to signal via RIG-I. Later, it was also shown that poly-dA/dT serves as a template for RNA polymerase III to make RIG-I ligands [12–14]. Nevertheless, this hypothesis is unresolved in mouse cells. Once stimulated by the viral DNA/RNA, these adaptor proteins activate IFN regulatory factor (IRF)-3 and IRF-7, which induce type I IFN production [7–9]. These pattern recognition receptor-mediated early innate immune responses are crucial in controlling viral replication and spread before the initiation of more specific and powerful adaptive immune responses [8, 9, 15].

Despite numerous studies on HBV pathogenesis, the putative molecular patterns of HBV that trigger cellular responses remain unknown. A few reports have suggested that the antiviral response against HBV is mediated by the RIG-I/MAVS pathway in the cytosol and its activation is blocked by HBV polymerase in infected cells [16–18]. However, no definitive evidence in vivo is available because analysis on the gene expression and effectors required for elimination of the replicative template has been especially difficult. Since viral clearance is a multifaceted process, we hydrodynamically injected a naked HBV plasmid DNA into wild-type (WT) and gene-disrupted mice deficient in TICAM-1, MAVS, TICAM-1/MAVS, IRF-3/IRF-7, IFNAR, MyD88, or RAG2 to identify and characterize the immunological events for HBV clearance. With the availability of various gene-disrupted mice, our study allows the identification of pathways crucial for the clearance of HBV.

Materials and Methods

Animal Studies

All mice were backcrossed with C57BL/6 mice more than seven times before use. *Ticam-1*$^{-/-}$ [19] and *Mavs*$^{-/-}$ [20] mice were generated in our laboratory as described previously, while *Ticam-1*$^{-/-}$ *Mavs*$^{-/-}$ mice were generated by crossing *Ticam-1*$^{-/-}$ mice with *Mavs*$^{-/-}$ mice. *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ and *Ifnar*$^{-/-}$ mice were kindly provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). *Myd88*$^{-/-}$ mice were provided by Drs. K. Takeda and S. Akira (Osaka University, Osaka, Japan). *Rag2*$^{-/-}$ mice were kindly provided by Dr. N. Ishii (Tohoku University, Sendai, Japan). Female C57BL/6J mice were purchased from CLEA Japan (Tokyo) and used at 7–9 weeks of age. All mice were maintained under specific pathogen-free conditions in the animal facility at Hokkaido University Graduate School of Medicine (Sapporo, Japan). Animal experiments were performed according to the guidelines set by the Animal Safety Center, Japan.

Hydrodynamic Transfection of Mice with HBV1.4 Plasmid

The pTER1.4xHBV plasmid containing 1.4-genome length sequences of HBV that were previously shown to produce a similar sedimentation in sucrose density gradient centrifugation to HBV extracted from the serum of carriers [21] was used in this study. A total of 50 μg of the plasmid was injected into the tail vein of 7- to 9-week-old mice in a volume of 2.0 ml of TransIT-QR hydrodynamic delivery solution (Mirus, USA). The total volume was delivered within 3–8 s. Plasmid DNA was prepared by using an EndoFree plasmid system (Qiagen, Germany) according to the manufacturer's instructions.

Quantification of HBV DNA by Real-Time PCR

To determine the HBV DNA in the serum, 30 μl of each serum sample was pretreated with 20 units of DNase I (Roche, Germany) at 37°C overnight. The encapsidated viral DNA was extracted with the SMITEST kit (Genome Science Laboratories, Tokyo, Japan) following the manufacturer's instructions and dissolved in 20 μl of TE-buffer. The purified viral genome was quantified by real-time PCR using the SYBR green master mix (Life Technologies, USA) and the HBV-DNA-F/R primer (see suppl. table 1 for primer sequences; for all online suppl. material, see www.karger.com/doi/10.1159/000365113). Amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of