Research Article

Journal of Innate Immunity

J Innate Immun 2014;6:293–305
DOI: 10.1159/000355126

Received: August 12, 2013
Accepted after revision: August 19, 2013
Published online: October 29, 2013

Myeloid-Derived Suppressor Cells Confer Tumor-Suppressive Functions on Natural Killer Cells via Polyinosinic:Polycytidylic Acid Treatment in Mouse Tumor Models

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Key Words
Myeloid-derived suppressor cells · Mitochondrial antiviral signaling protein · Tumor immunotherapy · Double-stranded RNA

Abstract
Polyinosinic:polycytidylic acid (poly I:C), a synthetic double-stranded RNA, acts on myeloid cells and induces potent antitumor immune responses including natural killer (NK) cell activation. Myeloid-derived suppressor cells (MDSCs) systemically exist in tumor-bearing hosts and have strong immunosuppressive activity against antitumor effector cells, thereby dampening the efficacy of cancer immunotherapy. Here we tested what happened in MDSCs in poly I:C-treated mice. NK-sensitive syngenic tumor (B16)-bearing C57BL/6 mice were employed for this study. Intraperitoneal poly I:C treatment induced MDSC activation, driving CD69 expression and interferon (IFN)-γ production in NK cells. IFN-γ directly inhibited proliferation of B16 cells. This NK cell priming led to growth retardation of B16 tumors, although no direct tumoricidal activity was induced in NK cells. Mechanistic analysis using KO mice and function-blocking monclonal antibody revealed that MDSCs produced IFN-α via the mitochondrial antiviral signaling protein (MAVS) pathway after in vivo administration of poly I:C, and activated NK cells through the IFNAR pathway. MDSC-mediated NK cell priming was reconstituted by IFN-α in a coculture system. Either the MAVS or IFNAR signaling pathway was required for activation of MDSCs that led to growth retardation of B16 tumor in vivo. The results infer that MDSC is a target of poly I:C to prime NK cells, which exert antitumor activity to NK-sensitive tumor cells.

Introduction

The innate sensing of microbial molecular patterns results in the modulation of the cellular immune system [1–3]. This innate-adaptive linkage closely associates with suppression of infection and tumorigenesis. Many reports showed that polyinosinic:polycytidylic acid (poly I:C), a synthetic pattern of double-stranded RNA, has potent stimulatory effects on immune responses to viral infection and cancer [4–8]. Poly I:C is an agonist for pattern-recognition receptors (PRRs), Toll-like receptor 3 (TLR3) and melanoma differentiation-associated protein 5 (MDA5), which transduce signals to the adaptor molecules TICAM-1 (also known as TRIF) and mitochondrial antiviral signaling protein (MAVS; IPS-1, Cardif, VISA) [9–12]. They differentially modulate the functions of myeloid dendritic cells (DCs) and macrophages, including cytokine/IFN production and expression of surface molecules that drive effector cell activation.

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TLR3/TICAM-1 and MDA5/MAVS activate the transcription factors, NF-κB and interferon (IFN) regulatory factor 3 (IRF-3), to typically induce type-I IFN. Type-I IFN evokes subsequent activation of the IFNAR pathway, which participates in the induction of IFN-stimulated genes (ISGs) including IRF-7 [13, 14]. IRF-7 further modifies the function of poly I:C by upregulating PRRs. Thus, the activity of poly I:C immediately affects IRF-3-derived genes and secondarily upregulates genes by activation of the IFNAR pathway. These pathways are crucial for driving the effector functions of NK cells and cytotoxic T cells that result in tumor regression after poly I:C treatment [6, 15].

NK cells are important for antitumor effects not only through direct cytotoxic activity, but also indirectly, through the production of cytokines including IFN-γ [16–20]. $DX5^+$ or $NK1.1^+$ cells have been used as conventional NK cells, which have features distinct from other lymphoid cells. Optimal NK cell responses require the presence of accessory cells such as DCs or macrophages [21]. NK cells are essential for poly I:C-induced growth retardation of NK-sensitive tumors such as B16 melanomas since poly I:C treatment does not induce antitumor activity in NK cell-depleted mice [4, 5]. IFN-γ production and cytotoxic activity by NK cells are potentiated by stimulating mice in vivo with poly I:C. NK cell activation appears to have many modes and myeloid NK cell contact serves a critical factor for antitumor NK cell activation.

Myeloid-derived suppressor cells (MDSCs) belongs to myeloid lineages with potent immunosuppressive activity against antitumor immune responses in mice and humans [22, 23]. MDSCs are widely distributed at tumor sites and in the peripheral organs, spleen and lymph nodes. Defined as a $CD11b^+Gr1^+$ subset in mice, they are heterogeneous populations of early myeloid progenitors that arise in bone marrow. Recently, they have also been found to originate from hematopoietic stem and progenitor cells accumulated in the spleen under tumor-bearing conditions [24]. The immunoregulatory functions of MDSCs in cancer have been studied extensively [22, 25]. MDSCs inhibit antigen-dependent T cell proliferation through the production of immunosuppressive factors including arginase-1, reactive oxygen species and reactive nitrogen species, and the release of immunosuppressive cytokines. However, the effect of MDSCs on NK cell function in tumor-bearing hosts is controversial. The anergy of NK cells is reportedly induced by MDSCs through membrane-bound TGF-β in a tumor-implant model using 3LL, B16 and EG7 cells [26]. MDSCs derived from patients with hepatocellular carcinoma inhibit autologous NK cell activity when cocultured in vitro [27]. Splenic MDSCs in TS/A tumor-bearing mice repress NK cell cytotoxicity [28]. A subset of MDSCs expresses NKG2D ligand on the cell surface and activates NK cells through NKG2D-NKG2D ligand interaction [29]. Although MDSCs express PRRs, their contribution to the MDSC function in poly I:C-induced growth retardation of tumors has not been fully understood.

Recent studies have demonstrated that TLR stimulation could modulate the function of immunosuppressive myeloid-derived cells as well as myeloid DCs in cancer. Tumor-associated macrophages and MDSCs were converted from tumor supporters to tumoricidal effectors after treatment with TLR agonists [7, 30, 31]. It was demonstrated that CpG treatment blocks MDSC-mediated T cell suppression associated with the maturation and differentiation of MDSCs [30, 31]. In this study, we revealed that poly I:C treatment allows cancer-expanded MDSCs to prime NK cells through the MAVS and the type-I IFN signaling pathway in vivo, leading to retardation of tumor growth.

Materials and Methods

Mice and Tumor Cells

Inbred C57BL/6 wild-type (WT) mice were purchased from Clea, Japan. $TICAM\text{-}1^{-/-}$ and $MAVS^{-/-}$ mice were generated in our laboratory. $IFNAR1^{-/-}$ mice were kindly provided by T. Taniguchi (University of Tokyo). Mice of 6- to 10-weeks of age were used in all experiments that were performed according to animal experimental ethics committee guidelines of Hokkaido University. B16D8 cells were developed in our laboratory [4]. B16D8 cells were cultured at 37°C under 5% CO_2 in RPMI containing 10% FBS, penicillin and streptomycin. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). The protocol was approved by the Committee on the Ethics of Animal Experiments in the Animal Safety Center, Hokkaido University, Japan. All mice were used according to the guidelines of the institutional animal care and use committee of Hokkaido University, who approved this study as ID number 08-0290, 'Analysis of Anti-Tumor Immune Response Induced by the Activation of Innate Immunity'.

Tumor Challenge and Poly I:C Treatment

Mice were shaved at the back and injected s.c with B16D8 cells (6×10^5), 3LL cells (3×10^6) or EL4 cells (1×10^6) suspended in 200 μl PBS(−). Tumor size was measured using a caliper. Tumor volume was calculated using the following formula: tumor volume (cm^3) = (long diameter) × (short diameter)^2 × 0.4. Poly I:C (GE Bioscience) (200 μg/head) with no detectable LPS was injected i.p. as indicated. In some cases, polymixin B-treated poly I:C was used. When an average tumor volume of 0.4–0.6 cm^3 was reached, the treatment was started and was repeated every 4 days.

Cell Isolation and Culture

When tumor volume reached 1–2 cm^3, i.e. 14–18 days after tumor challenge, mice were injected i.p. with 200 μg poly I:C or PBS(–). After 4 h, $CD11b^+Gr1^+$ MDSC-like cells were isolated from splenocyte suspension or single-cell suspension from the collagenase-treated tumor of poly I:C-injected or PBS-injected mice by using biotin-conjugated anti-Gr1 monoclonal antibody (RB6–8C5) and Streptavidin Microbeads (Miltenyi) as described previously [7]. NK cells were purified from splenocytes of naïve mice by using DX5 Microbeads (Miltenyi). In these purification steps, two rounds of positive selection were performed. We routinely prepared $Gr1^+$ cells at more than 95% purity and almost 100% of $Gr1^+$ cells expressed CD11b. The purity of $DX5^+$ cells was more than 90%. Isolated $CD11b^+Gr1^+$ cells and $DX5^+$ cells were cocultured for 20–24 h. In some experiments, anti-IFNAR1 monoclonal antibody (MAR1–5A3) was added to the culture for neutralization of IFNAR1. Recombinant mouse IFN-α (R&D systems) was used for stimulation of $CD11b^+Gr1^+$ cells and NK cells.

Cells isolated from mouse spleen were incubated for 24 h and the conditioned medium was collected. Concentrations of IFN-α and IFN-γ were determined by ELISA according to manufacturer's instructions (PBL Interferon Source and eBioscience). NK cytotoxicity was determined by standard ^{51}Cr release assay as described previously [32].

Flow Cytometric Analysis

Mononuclear cells prepared from mouse spleen or tumor were treated with anti-CD16/32 (no. 93) and stained with FITC- or APC-anti-CD45.2 (no. 104), FITC- or PE-anti-CD11b (M1/70), APC- or PE-anti-GR1 (RB6–8C5), PE- or APC-anti-NK1.1 (PK136), PE-anti-CD49b (DX5), FITC-, PE- or APC-anti-CD3ε (145–2C11), FITC- or PE-anti-CD69 (H1.2F3), PE-anti-CD80 (16–10A1), PE-anti-CD86 (GL-1), PE-anti-CD40 (1C10), PE-anti-CD155 (TX56), PE-anti-CD70 (FR70), PE-anti-IL-15Ra (DNT15Ra), FITC-anti-CD150 [A12 (7D4)], and anti-RAE-1 (eBioscience and Biolegend). Samples were analyzed with a FACSCalibur instrument or FACS Aria instrument (BD Bioscience) and data analysis was performed by FlowJo software (Tree Star).

T Cell Proliferation Assay

T cell proliferation was measured by changes in fluorescence intensity using carboxyfluorescein diacetate succinimidyl ester (CFSE). Splenocytes from OT-I transgenic mice were labeled with 1 μM CFSE, placed into a round bottom 96-well plate containing $CD11b^+Gr1^+$ cells as indicated. Splenocytes were cultured in the presence of 100 nM OVA-derived peptide SIINFEKL. After 3 days, cells were harvested, stained with APC-anti-CD8α (53–6.7) and PE-anti-TCR vβ 5.1, 5.2 (MR9–4) or PE-anti-CD3ε (145–2C11), and the CFSE signal of gated lymphocytes was analyzed by flow cytometry. The extent of cell proliferation was quantified by FlowJo software (Tree Star).

Quantitative PCR Analysis

RNA was prepared with RNeasy kit (QIAGEN) or TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) with StepOne™ Real-time PCR system (Applied Biosystems). Expression of the cytokine gene was normalized to the expression of glyceraldehyde phosphate dehydrogenase (GAPDH). The following primers were used for PCR: IFNα4 forward, 5′-CTGCTGGCTGTGAGGACATACT-3′, IFNα4 reverse, 5′-AGGCACAGAGGCTGTGTTTCTT-3′, IL-15 forward, 5′-TTAACTGAGGCTGGCATTCATG-3′, IL-15 reverse, 5′-ACCT ACACTGACACAGCCCAAA-3′, IL-18 forward, 5′-GACAAA GAAAGCCGCCTCAA-3′, IL-18 reverse, 5′-ATGGCAGCCAT TGTTCCTG-3′, INAM forward, 5′-CAACTGCAATGCCACG CTA-3′, INAM reverse, 5′-TCCAACCGAACACCTGAGACT-3′, GAPDH forward, 5′-GCCTGGAGAAACCTGCCA-3′, GAPDH reverse, 5′-CCCTCAGATGCCTGCTTCA-3′. Data was analyzed by the ΔΔCt method.

Statistics

If not otherwise stated, data were expressed as arithmetic means ± SD, and statistical analyses were made by 2-tailed Student's t test. $p < 0.05$ was considered statistically significant.

Results

$CD11b^+Gr1^+$ Cells Expanded in B16 Tumor-Bearing Mice Are Immunosuppressive

$CD11b^+Gr1^+$ cells representing MDSCs accumulate in large numbers in the lymphoid tissues of tumor-bearing mice [22, 23]. We therefore investigated the spleens of mice bearing syngenic tumor cells. B16 melanoma cells, 3LL lung cancer cells or EL4 thymoma cells were s.c. injected into WT mice and, 16 days later, splenic populations of immune cells were examined in the tumor-bearing mice. The proportion of $CD11b^+Gr1^+$ cells in the spleens of B16-implanted mice was higher than that in tumor-free naïve mice, consistent with previous reports (fig. 1a). Similar profiles were obtained with the 3LL and EL4 cell lines (data not shown).

To examine whether $CD11b^+Gr1^+$ cells had immunosuppressive activity, we harvested $CD11b^+Gr1^+$ cells from the spleens of B16 tumor-implanted mice, and cocultured $CD11b^+Gr1^+$ cells with OT-I splenocytes in the presence of OVA peptide. $CD11b^+Gr1^+$ cells from tumor-bearing mice efficiently inhibited antigen-specific proliferation of $CD8^+$ OT-I T cells (fig. 1b). Therefore, $CD11b^+Gr1^+$ cells accumulated in the spleen of B16 tumor-bearing mice and had immunosuppressive functions.

We also assessed the immunosuppressive activity of $CD11b^+Gr1^+$ cells against NK cells activated by PMA/ionomycin and tested activation as level of IFN-γ production. No inhibitory effect of $CD11b^+Gr1^+$ cells on the production of IFN-γ by NK cells was observed (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000355126). Therefore, $CD11b^+Gr1^+$ cells expanded in B16 tumor-bearing mice exhibited immunosuppressive activity toward $CD8^+$ T cells but not NK cells.

In vivo Poly I:C Induces Cytokine Production and Maturation of CD11b⁺Gr1⁺ Cells

Type-I IFNs are systemically produced in tumor-bearing mice by i.p. injection of poly I:C. Poly I:C usually acts on TLR3 in myeloid/epithelial cells and MDA5 in systemic cells, leading to type-I IFN production [33]. Since CD11b⁺Gr1⁺ cells expressed both TLR3 and MDA5, we examined whether type-I IFNs were produced by CD11b⁺Gr1⁺ cells in B16 tumor-bearing mice in response to poly I:C injection. Interestingly, we found that IFN-α is produced in splenic CD11b⁺Gr1⁺ cells harvested from poly I:C-treated B16 tumor-bearing mice, but not in CD11b⁺Gr1⁺ cells unexposed to poly I:C (fig. 2a, left panel). The results were also confirmed in vitro: type-I IFNs was minimally produced in poly I:C-untreated CD11b⁺Gr1⁺ cells but robustly in poly I:C-treated cells from the spleen or tumor in direct response to poly I:C (fig. 2a, right panel). The results were reproducible with different tumor cell lines, specifically 3LL and EL4, and different sources of CD11b⁺Gr1⁺ cells

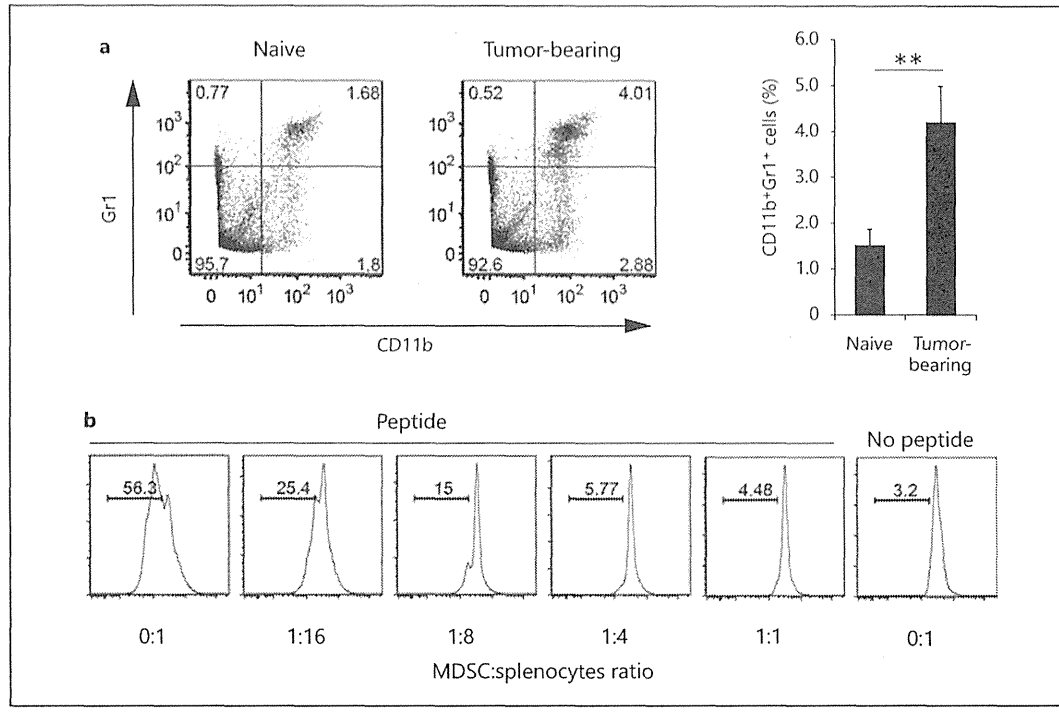
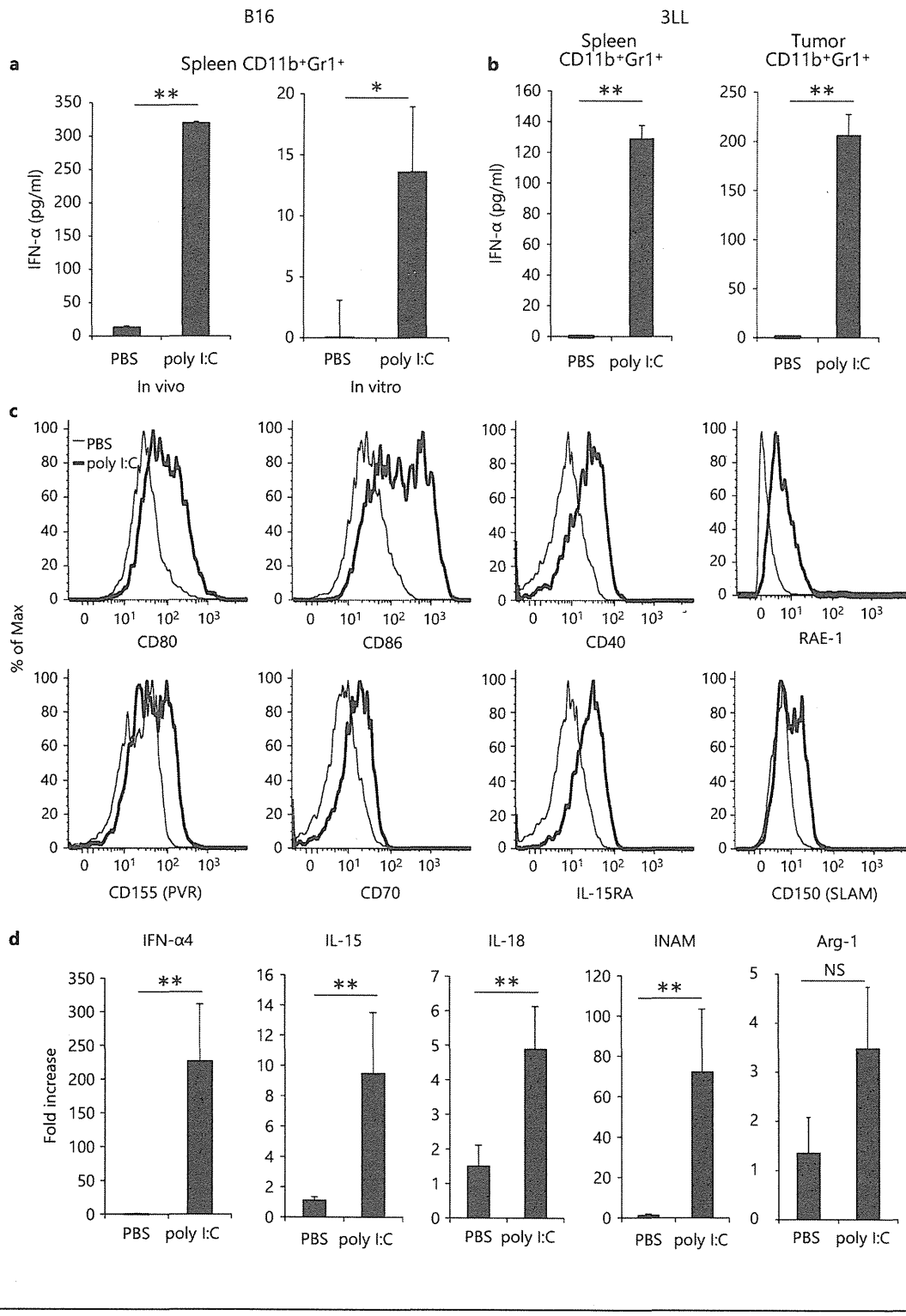


Fig. 1. Immunosuppressive activity of CD11b⁺Gr1⁺ cells in the spleen of B16 tumor-bearing mice. **a** WT mice were injected s.c. with B16D8 melanoma cells. The percentage of CD11b⁺Gr1⁺ cells in the spleen was determined on day 16 by flow cytometry (n = 6). Cells were gated on CD45⁺ cells. **b** CD11b⁺Gr1⁺ cells were isolated from spleens of B16 tumor-bearing mice, and cultured with CFSE-labeled OT-I splenocytes (1×10^6) at the indicated ratios. After 3 days, proliferation of CD8α⁺TCRvβ⁺ cells was measured. Data shown are representative of at least 2 independent experiments. ** $p < 0.01$.

Fig. 2. Effect of poly I:C treatment on CD11b⁺Gr1⁺ cells. **a** B16 tumor-bearing mice were injected i.p. with 200 μg poly I:C or PBS as a negative control. After 4 h, CD11b⁺Gr1⁺ cells were purified from spleens and incubated for 24 h (left panel). CD11b⁺Gr1⁺ cells isolated from spleens of B16 tumor-bearing mice were treated with 50 μg/ml poly I:C or PBS for 24 h (right panel). The concentration of IFN-α in conditioned medium was determined. **b** 3LL cells (3×10^6) were implanted into B6 WT mice and CD11b⁺Gr1⁺ cells were isolated from spleen (left panel) or tumor (right panel) after poly I:C injection as described in **a**. **c** Spleen cells were prepared from B16 tumor-bearing mice treated with poly I:C or PBS for 8 h as described in **a** and surface expression of CD80, CD86, CD40, RAE-1, CD155, CD70, IL-15RA and CD150 on CD11b⁺Gr1⁺ cells was determined. **d** CD11b⁺Gr1⁺ cells were isolated from B16 tumor-bearing mice treated with poly I:C or PBS for 4 h as described in **a** and mRNA for IFN-α4, IL-15, IL-18, INAM and arginase-1 was measured (n = 3). Data shown are representative of at least 2 independent experiments. ** $p < 0.01$, * $p < 0.05$. NS = Not significant.

(For figure 2 see next page.)



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(fig. 2b; online suppl. fig. 2a). In addition, the costimulatory molecules CD80 and CD86 on these cells were upregulated in response to poly I:C (fig. 2c). To further investigate the effect of poly I:C treatment on the function of $CD11b^+Gr1^+$ cells, we analyzed the gene expression of $CD11b^+Gr1^+$ cells isolated from B16 tumor-bearing mice, 4 h after injection with poly I:C or PBS. We found an increase in mRNA for IFN-α4, IL-15, IL-18 and INAM (fig. 2d) [21, 32]. Furthermore, in vivo poly I:C treatment for 8 h resulted in upregulation of RAE-1, PVR (CD155), CD70, IL-15RA, SLAM (CD150) and CD40 on $CD11b^+Gr1^+$ cell surface (fig. 2c). These molecules are involved in DC-mediated NK cell activation [18, 34, 35]. However, mRNA for arginase-1, which is involved in MDSC-mediated inhibition of T cell proliferation, was not increased in $CD11b^+Gr1^+$ cells (fig. 2d). These results suggest that in vivo pretreatment of mice with poly I:C effectively induces the maturation of $CD11b^+Gr1^+$ cells, resulting in enhanced expression of NK cell-activating molecules.

$CD11b^+Gr1^+$ Cells from Poly I:C-Treated Tumor-Bearing Mice Activate NK Cells

To investigate whether $CD11b^+Gr1^+$ cells from poly I:C-injected tumor-bearing mice are capable of activating NK cells, we isolated $CD11b^+Gr1^+$ cells from the spleens of tumor-bearing mice after poly I:C administration and cocultured the cells with NK cells from naïve mice. NK cells upregulated CD69 on their surface in response to the $CD11b^+Gr1^+$ cells from poly I:C-injected B16 tumor-bearing mice. However, the level of CD69 on NK cells was not changed when the cells were mixed with $CD11b^+Gr1^+$ cells from PBS-injected tumor-bearing mice (fig. 3a, left panel). $CD11b^+Gr1^+$ cells from poly I:C-injected tumor-bearing mice also induced NK cell IFN-γ production (fig. 3a, right panel). Similar results were obtained with NK cells cocultured with $CD11b^+Gr1^+$ cells from mice bearing 3LL- or EL4 cell tumors after poly I:C treatment (fig. 3b, c; online suppl. fig. 2b, c). Furthermore, $CD11b^+Gr1^+$ cells from tumors of 3LL-implant mice had a similar ability to induce IFN-γ production and CD69 expression in NK cells after poly I:C treatment (fig. 3c). IFN-γ inhibits proliferation of B16 cells in vitro without affecting the cell viability (online suppl. fig. 3; data not shown). In contrast, $CD11b^+Gr1^+$ cells did not drive a cytotoxic phenotype from NK cells (fig. 3d). In vitro stimulation of $CD11b^+Gr1^+$ cells with poly I:C did not induce NK cytotoxicity in coculture (data not shown). These results demonstrated that when poly I:C was injected into tumor-bearing mice, $CD11b^+Gr1^+$ cells acquired the ability to prime NK cells as measured by CD69 expression and IFN-γ production, but did not induce cytotoxic activity.

Type-I IFN Signaling Is Essential for NK Cell Priming by $CD11b^+Gr1^+$ Cells

Next, we investigated the mechanisms by which $CD11b^+Gr1^+$ cells primed NK cells through the in vivo administration of poly I:C. Soluble factors and membrane-associated molecules induced by poly I:C are reportedly involved in in vivo NK cell activation [15, 18]. As shown in figure 2a, $CD11b^+Gr1^+$ cells from poly I:C-treated tumor-bearing mice produced IFN-α. To examine whether type-I IFN signaling through IFNAR was involved in NK cell activation, we added anti-IFNAR1 antibodies to cultures to inhibit type-I IFN signaling by both $CD11b^+Gr1^+$ and NK cells that express IFNAR1. CD69 upregulation on NK cells and IFN-γ production induced by activated $CD11b^+Gr1^+$ cells were completely abrogated by anti-IFNAR1 antibodies (fig. 4a). These results suggest that type-I IFN signaling is essential for NK cell priming by $CD11b^+Gr1^+$ cells.

To investigate type-I IFN signaling in NK cell priming, we prepared NK cells from $IFNAR1^{-/-}$ mice. $IFNAR1^{-/-}$ NK cells were cocultured with $CD11b^+Gr1^+$ cells. $CD11b^+Gr1^+$ cells from poly I:C-stimulated WT mice stimulated CD69 expression and IFN-γ production by WT NK cells but not $IFNAR1^{-/-}$ NK cells (fig. 4b). Therefore, type-I IFN signaling in NK cells is essential for NK priming by $CD11b^+Gr1^+$ cells.

To examine whether IFNAR signaling is the only route for the induction of $CD11b^+Gr1^+$ cell-mediated NK priming, we added recombinant mouse IFN-α to cultures of NK cells or to cocultures of untreated $CD11b^+Gr1^+$ cells and WT NK cells. Recombinant mouse IFN-α in NK cell cultures resulted in induction of CD69 expression on the NK cells (fig. 4c, left panels). However, CD69 expression was minimally augmented in the NK cells cocultured with $CD11b^+Gr1^+$ cells. In contrast, NK cell IFN-γ production was clearly induced at high concentrations of IFN-α (2,000 IU/ml) and augmented by $CD11b^+Gr1^+$ cells (fig. 4c, right panel).

We investigated if cell-cell contact is involved in NK cell activation in cocultures of $CD11b^+Gr1^+$ cells and naïve NK cells using the Transwell system. Sufficient NK cell priming was detected when NK cells were cocultured with in vivo poly I:C-activated $CD11b^+Gr1^+$ cells. However, expression of CD69 and production of IFN-γ by NK cells was abrogated by separation of the cells by the Transwell membrane (online suppl. fig. 4a, b). These results,

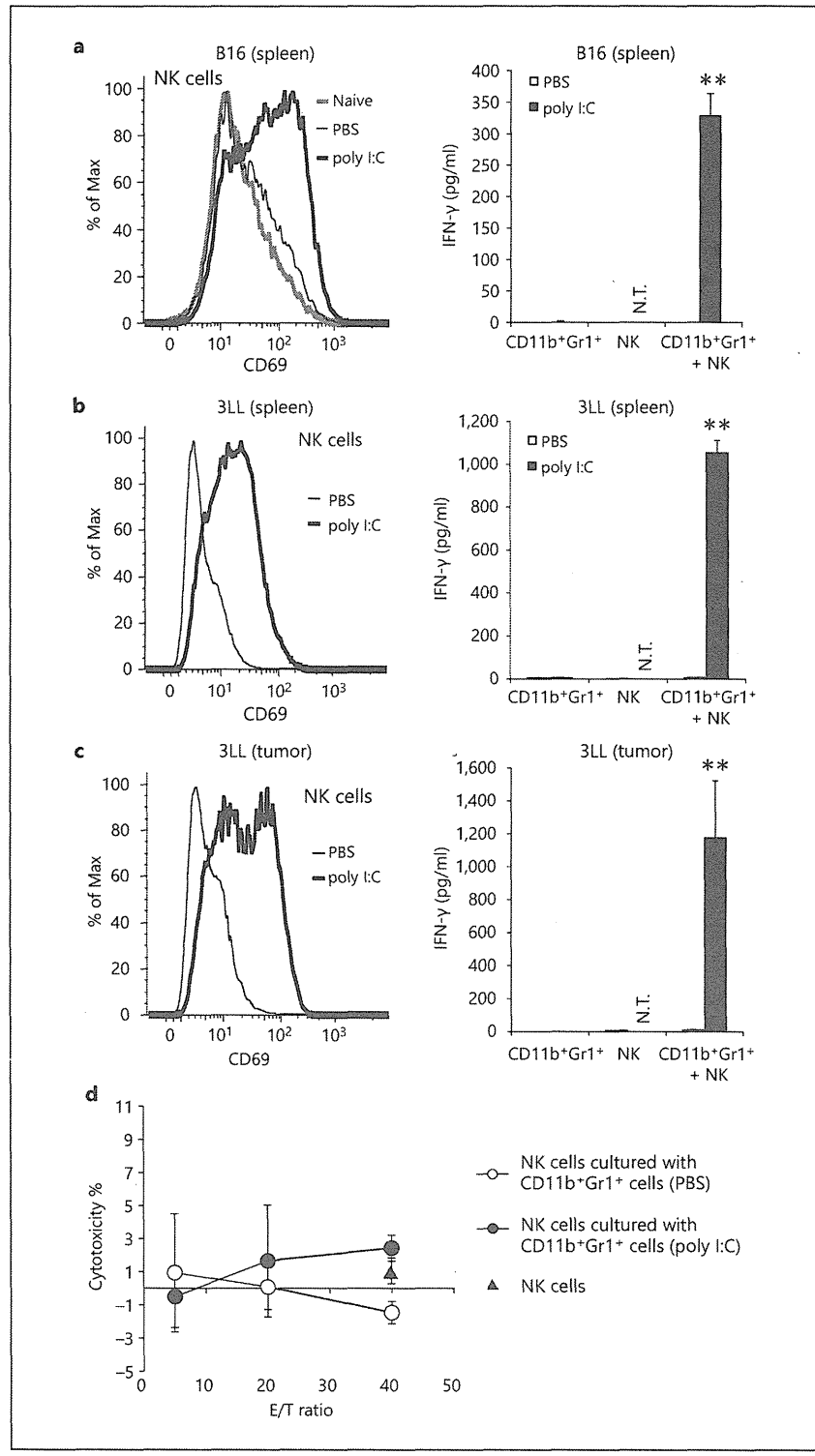

Fig. 3. NK cells are primed by $CD11b^+Gr1^+$ cells isolated from poly I:C-injected tumor-bearing mice. **a–c** $CD11b^+Gr1^+$ cells were isolated from spleens or tumors of B16 (**a**), 3LL (**b**, **c**) tumor-bearing mice pretreated with 200 μg poly I:C or PBS for 4 h and cultured with NK cells from naïve WT mice. After 24 h, CD69 expression on NK cells (**a–c**, left panels) and IFN-γ concentration in conditioned medium (**a–c**, right panels) were determined. CD69 expression of $NK1.1^+CD3\varepsilon^-$ cells is indicated (**a–c**). N.T. = Not tested. **d** Cytotoxic activity of NK cells cocultured with or without $CD11b^+Gr1^+$ cells isolated from poly I:C- or PBS-treated tumor-bearing mice was determined by standard ^{51}Cr release assay (n = 3). Triangle: NK cells not cultured with $CD11b^+Gr1^+$ cells. Data shown are representative of at least 3 independent experiments. ** $p < 0.01$.

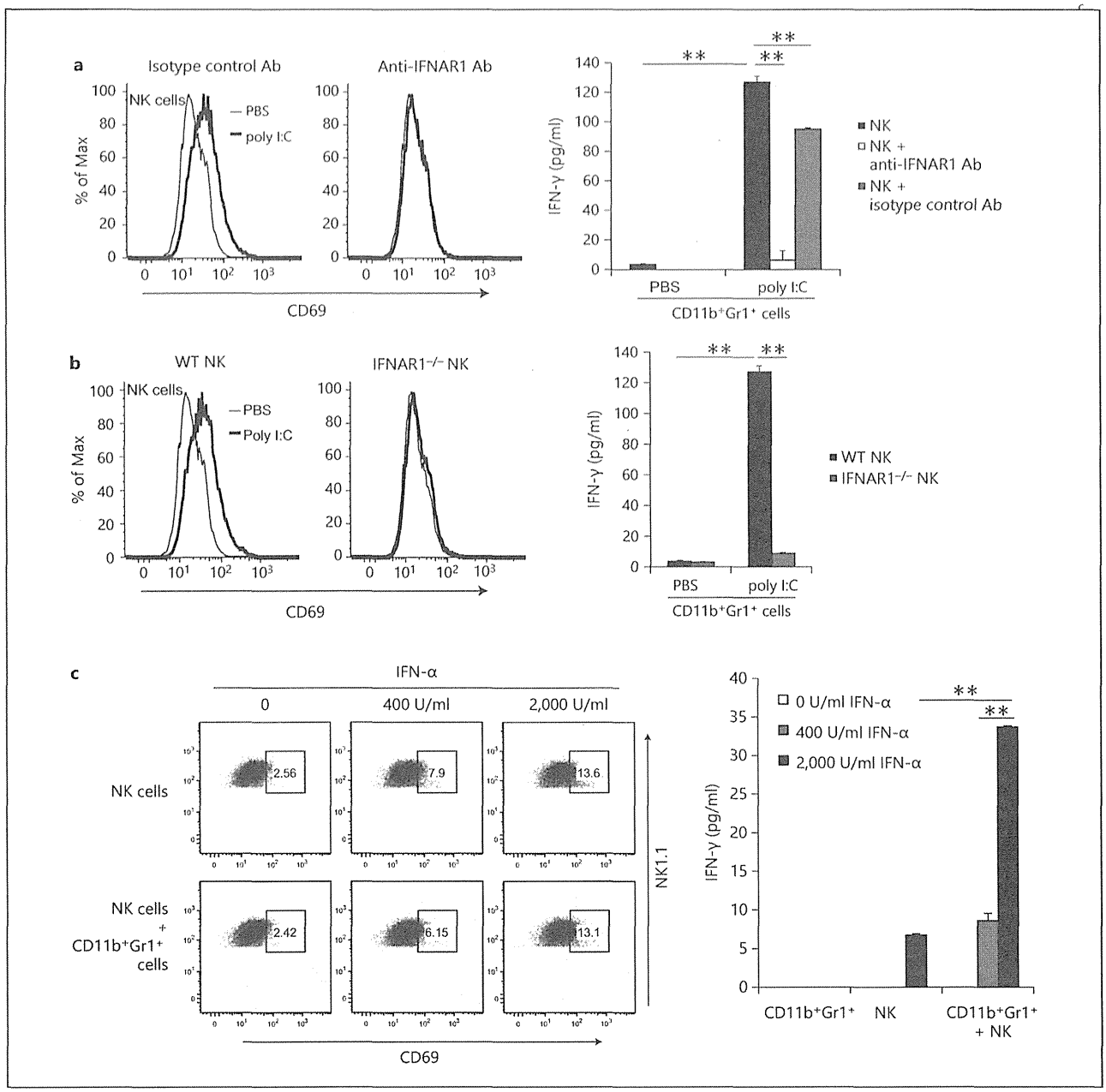

Fig. 4. Type-I IFNs from CD11b$^+$Gr1$^+$ cells and IFNAR in NK cells are indispensable for NK cell activation. **a** CD11b$^+$Gr1$^+$ cells were isolated from spleens of B16 tumor-bearing mice treated with 200 μg poly I:C or PBS for 4 h and cultured with NK cells from naïve WT mice in the presence or absence of 10 μg/ml anti-IFNAR1 antibody (Ab). After 24 h, CD69 expression on NK cells (left panels) and IFN-γ concentration in conditioned medium (right panel) was determined (n = 3). **b** CD11b$^+$Gr1$^+$ cells isolated as described in **a** were cultured for 24 h with NK cells from naïve WT mice or IFNAR1$^{-/-}$ mice, and CD69 expression (left panels) and IFN-γ production were measured (n = 3) (right panel). **c** Recombinant IFN-α was added to cultures of naïve NK cells with or without CD11b$^+$Gr1$^+$ cells from nontreated tumor-bearing mice. After incubation for 24 h, CD69 expression on NK cells (left panels) and IFN-γ concentration in conditioned medium (right panel) were determined (n = 3). CD69 expression of NK1.1$^+$CD3ε$^-$ cells is indicated (**a–c**). Data shown are representative of two independent experiments. ** $p < 0.01$.

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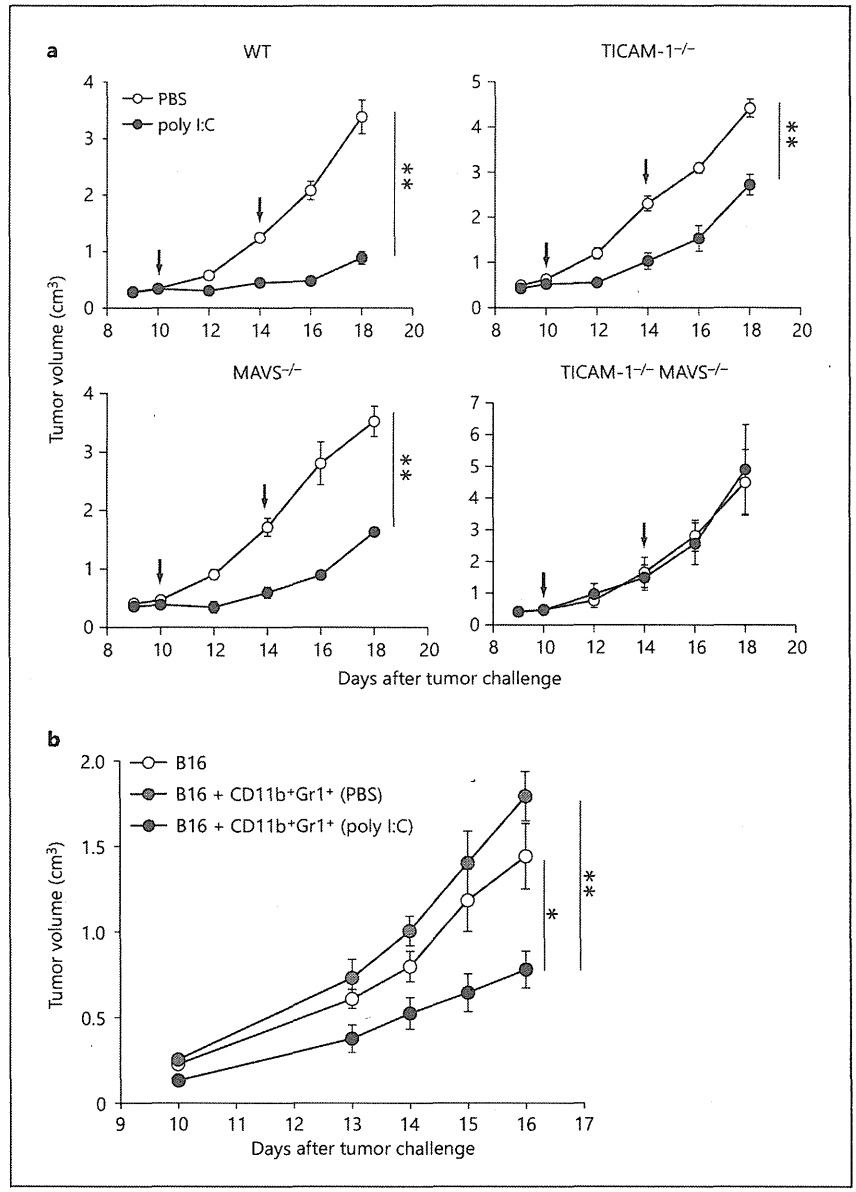

Fig. 5. Retardation of B16 tumor growth by poly I:C treatment in mouse models. **a** Both TICAM-1 and MAVS signals are involved in B16 tumor growth retardation after poly I:C therapy. B16 cells (6×10^5) were implanted s.c. into WT, TICAM-$1^{-/-}$, MAVS$^{-/-}$ and TICAM-1 and MAVS double-knockout mice. Tumor-bearing mice were treated with 200 μg poly I:C or PBS on days 10 and 14 (arrows) (n = 3–5 per group). Data are average ± SEM. **b** In vivo poly I:C-activated CD11b$^+$Gr1$^+$ cells inhibit B16 tumor growth. B16 cells (6×10^5) were mixed with or without CD11b$^+$Gr1$^+$ cells (1×10^6) from spleens of B16 tumor-bearing mice treated with 200 μg poly I:C or PBS for 4 h. Cell mixtures were implanted s.c. into WT mice on day 0 (n = 4 per group). Data are average ± SEM and are representative of 2 independent experiments. ** $p < 0.01$, * $p < 0.05$.

together with the results in figure 4, suggest that two modes of NK priming occur simultaneously in CD11b$^+$Gr1$^+$ cells: one mode is through type-I IFN production and the other is via cell-cell contact.

MAVS and IFNAR Are Required to Activate MDSCs with in vivo Poly I:C Treatment

Poly I:C induces growth retardation of B16 tumors implanted in WT mice [4–6, 36]. To determine the signaling pathway that is essential for the retardation of B16 tumor growth in vivo, we implanted B16 melanoma cells s.c. into TICAM-$1^{-/-}$ and MAVS$^{-/-}$ mice. B16 tumor growth was monitored after poly I:C injection. Marked tumor growth retardation was observed in poly I:C-treated mice (fig. 5a). The poly I:C antitumor effect was only partly abrogated in either TICAM-$1^{-/-}$ or MAVS$^{-/-}$ mice and was completely abolished in TICAM-$1^{-/-}$/MAVS$^{-/-}$ mice (fig. 5a). Therefore, both TICAM-1 and MAVS signals are involved in the antitumor activity of poly I:C, consistent with earlier reports [4, 5, 33].

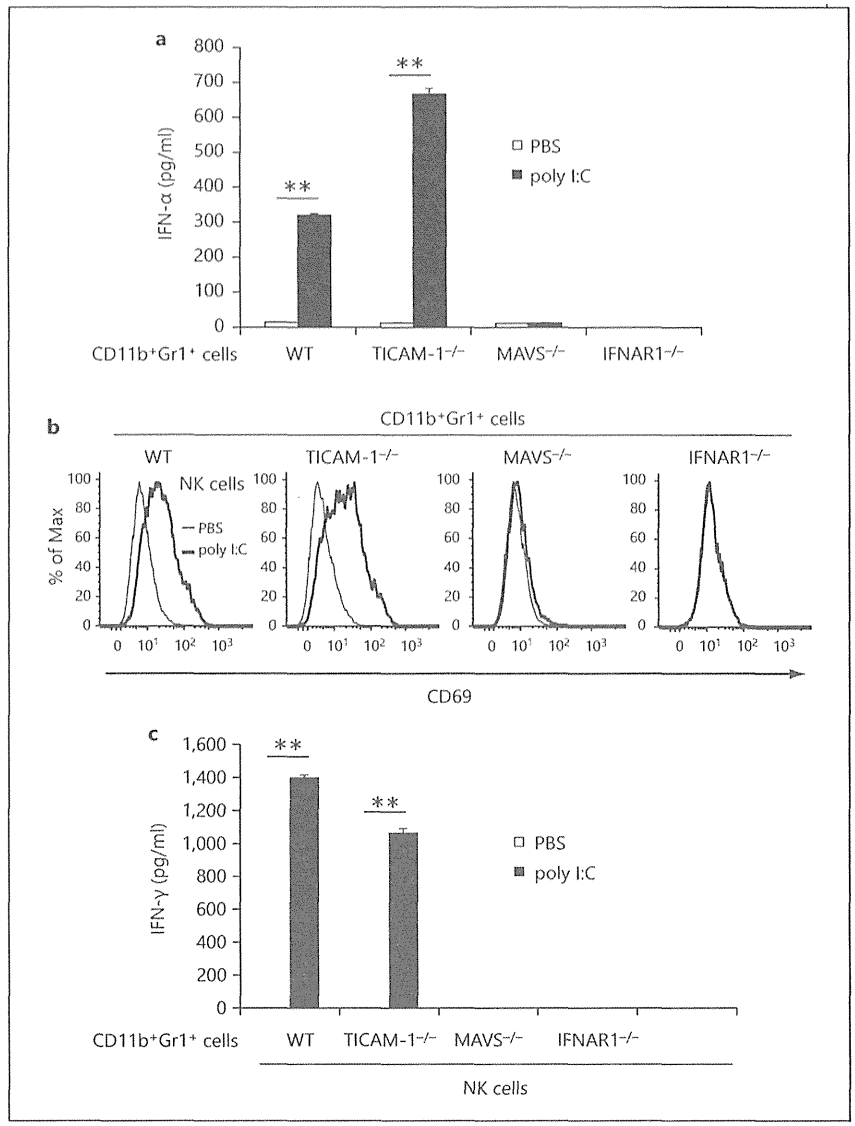

Fig. 6. MAVS and type-I IFN signaling pathways are critical for CD11b$^+$Gr1$^+$ cell activation in vivo. **a** B16 cells (6×10^5) were implanted into WT, TICAM-1$^{-/-}$, MAVS$^{-/-}$ or IFNAR1$^{-/-}$ mice. Tumor-bearing mice were treated with 200 μg poly I:C or PBS for 4 h and CD11b$^+$Gr1$^+$ cells were isolated from spleens, and allowed to stand for 24 h. IFN-α concentration in conditioned medium was determined (n = 3). **b**, **c** CD11b$^+$Gr1$^+$ cells were isolated from KO mouse lines as described in **a**, and were cultured with naïve WT NK cells for 24 h. CD69 expression on NK cells (**b**) and IFN-γ concentration in conditioned medium (**c**) were determined (n = 3). Data shown are representative of 3 independent experiments. ** $p < 0.01$.

Next, we determined the mechanisms involved in the in vivo activation of CD11b$^+$Gr1$^+$ cells by poly I:C. To investigate the signaling pathway that was important for poly I:C-induced activation of CD11b$^+$Gr1$^+$ cells in vivo, we challenged TICAM-1$^{-/-}$ and MAVS$^{-/-}$ mice with B16 melanoma cells. After tumor formation, poly I:C was injected i.p. into the mice and CD11b$^+$Gr1$^+$ cells were isolated from the spleen and were cocultured with naïve WT NK cells. CD11b$^+$Gr1$^+$ cells from tumor-bearing TICAM-1$^{-/-}$ mice produced IFN-α at levels comparable to cells from WT mice (fig. 6a). In parallel, CD69 expression on NK cells and IFN-γ production was observed in conditioned medium from mixed cultures of TICAM-1$^{-/-}$ CD11b$^+$Gr1$^+$ cells and naïve WT NK cells. The results suggest that in vivo TICAM-1 signaling is not mandatory for CD11b$^+$Gr1$^+$ cell activation to induce NK cell priming (fig. 6a–c). CD11b$^+$Gr1$^+$ cells from tumor-bearing MAVS$^{-/-}$ mice treated with poly I:C did not produce IFN-α or induce CD69 expression and IFN-γ production in NK cells (fig. 6a–c). Similar results were obtained with CD11b$^+$Gr1$^+$ cells from B16 tumor-bearing IFNAR1$^{-/-}$ mice (fig. 6a–c). These results suggest that MAVS as well as type-I IFN signaling is crucial for poly I:C-dependent NK cell priming in CD11b$^+$Gr1$^+$ cells of tumor-bearing mice.

The MAVS pathway is conserved in most cell types in mice. We examined whether NK cell priming induced by poly I:C (i.e. MAVS signal)-activated $CD11b^+Gr1^+$ cells was involved in retardation of B16 tumor growth. NK-sensitive B16 tumor cells were mixed with $CD11b^+Gr1^+$ cells isolated from poly I:C (or control PBS)-injected tumor-bearing mice, and inoculated s.c. into WT mice (fig. 5b). Significant B16 growth retardation was detected only in those tumors containing poly I:C-treated $CD11b^+Gr1^+$ cells (fig. 5b). The B16 tumors with intact $CD11b^+Gr1^+$ cells showed higher growth rates than B16 tumor cells only, which might reflect the previously-reported tumor-supporting activity of MDSCs [37]. Thus, MDSC-like $CD11b^+Gr1^+$ cells can be converted to cells with an NK-priming activity that induces growth retardation of NK-sensitive tumors in mice. NK-priming would be a condition prior to full activation of antitumor NK cells.

Discussion

We demonstrated that in vivo poly I:C treatment led to $CD11b^+Gr1^+$ MDSC maturation and cytokine production in tumor-bearing mice. Poly I:C treatment rendered tumor and spleen MDSCs competent for $DX5^+$ NK cell priming as measured by CD69 expression and IFN-γ production. Poly I:C-dependent NK priming raises through the MAVS pathway (fig. 6). Among a number of proteins that were upregulated after poly I:C treatment, type-I IFN produced by activated MDSCs was critical for NK cell priming since it activated the IFNAR pathway in NK cells. However, NK cells barely exerted direct cytotoxic activity to B16 cells in response to poly I:C-matured MDSCs. This NK activation profile resembles that of IFN-γ-producing innate lymphoid cells. Some populations of these innate lymphocytes produce IFN-γ but exhibit little cytotoxic activity [38], similar to the NK cells affected by MDSC. These findings would allow us to speculate that the production of IFN-γ without cytotoxic activity is an activation state of NK cells or innate lymphocytes where MDSCs contribute.

In tumor-bearing hosts, poly I:C treatment resulted in tumor regression. Poly I:C induces direct killing of 3LL tumor cells by M2-M1 conversion of tumor-associated macrophages [7]. The TICAM-1 signal facilitates tumor-associated macrophage conversion as well as cross-presentation by DCs leading to antigen-specific CTL induction, which is also evoked by poly I:C [8]. The action of $CD11b^+Gr1^+$ MDSCs on implant B16 tumor was tumor-supporting when MDSCs were embedded into the tumor (fig. 5b). However, once MDSCs were pretreated with poly I:C and mixed with B16 cells, tumor growth was prohibited (fig. 5b). The result suggests that MDSC has plasticity to change the function from tumor-supporting to tumor-suppressing even in vivo. Here, we highlight the first evidence of MDSCs to evoke NK cell priming, which ultimately associates with retardation of tumor growth.

Although the exact mechanism of tumor regression by MDSC-NK activation remains to be elucidated, we speculate that IFN-γ produced by the primed NK cells could evoke antitumor activity. One possibility is that IFN-γ directly inhibits the growth of a certain tumor line including B16 melanoma by inducing cell cycle arrest. IFN-γ has a synergistic effect on type-I IFNs, arresting cell cycle to cell death in some tumor cell lines independent of p53 [39]. IFN-γ also induces angiostasis, which prevents rapid tumor progression [19], and inhibits metastasis and proliferation of B16 melanoma [17, 20]. In fact, we observed that IFN-γ directly inhibits proliferation of B16 cells in vitro, suggesting that NK cell-derived IFN-γ might inhibit B16 tumor growth during poly I:C treatment (online suppl. fig. 3).

Retardation of B16 growth was partially abrogated in TICAM-$1^{-/-}$ or $MAVS^{-/-}$ mice and completely abrogated in TICAM-$1^{-/-}$, $MAVS^{-/-}$ double KO mice (fig. 5a), the two pathways contributing to in vivo poly I:C-derived tumor suppression. In addition, MDSC activation is completely abrogated in $IFNAR1^{-/-}$ mice and IFNAR in NK cells is involved in efficient IFN-γ production induced by activated MDSCs. Type-I IFN receptor signaling is crucial for growth retardation of B16 tumor in poly I:C therapy. Therefore, a variety of situations result in NK activation/priming in the therapeutic use of poly I:C in tumor-bearing mice, although IFNAR is the common factor.

Miyake et al. [5], reported that MAVS is responsible for NK cell-dependent tumor regression using $MAVS^{-/-}$ mice with poly I:C stimulation ; however, the cell types for the poly I:C response and the mechanism of induction of NK-sensitive tumor regression remain undefined. NK-activating ligands on the DC surface as well as soluble factors induced by IRF-3 and IFNAR stimulation are crucial for DC-mediated NK cell activation [15, 32]. In addition, MDSC is a cell type that specifically drives NK priming through the MAVS pathway (fig. 6). MAVS-dependent IRF-3 activation occurs through stromal cells other than DCs, and these cells including MDSCs participate in NK-sensitive tumor regression. The result of this MDSC function is in contrast to that of DCs where the TLR3/TICAM-1 pathway preferentially promotes NK cell acti-

J Innate Immun 2014;6:293–305
DOI: 10.1159/000355126

vation [4]. Specific depletion of MDSCs in preformed tumors, if possible, would enable us to confirm the functional importance of MAVS in tumor regression reported by Miyake et al. [5], who postulated that stromal cells were a source of the cell type that induces MAVS-mediated NK priming. However, the function of poly I:C via the MDSC-NK cell pathway is only a part of the total antitumor activity of poly I:C, which would be difficult to detect by tumor-size reduction. MDSCs are expanded in a late phase of implant tumor, where poly I:C may act on MDSCs and exert antitumor activity via poly I:C-stimulated MDSCs.

A recent report suggested that type-I IFNs act on accessory cells such as DCs, leading to the production of IL-15, IL-12, IL-18 and NKG2D ligands such as RAE-1. IFN-α acted on NK cells and slightly induced IFN-γ production, which was augmented in the presence of MDSCs. Cell-cell interaction between MDSCs and NK cells appears to be required for robust IFN-γ production. Induction of NK-activating ligands in association with natural NK cytotoxicity is involved in cell-cell contact-mediated NK cell activation. We found mRNA for downstream genes of IRF-3, especially IL-15, IL-18, INAM and RAE-1 elevated in MDSCs after treatment with poly I:C. However, IL-15 and RAE-1 appear not to participate in IFN-γ production by NK cells with MDSCs because neutralizing antibodies to IL-15, RAE-1 or NKG2D did not inhibit IFN-γ production by NK cells. Therefore, other molecules should be involved in NK cell activation by MDSCs. In fact, INAM or other molecules expressed on the MDSC surface sustain NK cell activation following poly I:C treatment (fig. 2; data not shown) as in bone marrow-derived cells [32].

MDSCs that have expanded in tumor-bearing hosts strongly suppress antitumor immune responses [22, 23]. Reduction of MDSC population or function is achieved by treatment with reagents that are related to improvement in tumor-specific immunity [40]. Furthermore, maturation of MDSCs can be accomplished through IFN-α production by plasmacytoid DCs or direct TLR9 stimulation, which contributes to tumor regression [30, 31]. Direct administration of IFN-α, i.e. IFN therapy, however, has not been successful as a universal therapy in cancer patients. Serious side effects are associated with high therapeutic doses of type-I IFN as well as poly I:C. Development of less toxic reagents with sufficient IRF-3/7 activation would be important for anti-MDSC cancer therapy.

Acknowledgements

We thank Drs. H. Takaki, J. Kasamatsu, K. Funami, M. Tatematsu and M. Azuma in our laboratory for their fruitful discussions. This work was supported in part by Grants-in-Aids from the Ministry of Education, Science and Culture (specified project for advanced research) and the Ministry of Health, Labor and Welfare of Japan as well as by the Akiyama Life Science Foundation (H.S.), the Takeda Science Foundation (H.S.), the Yasuda Cancer Foundation (T.S.) and the Ono Foundation (T.S.). Financial support by a MEXT Grant-in-Project 'the Carcinogenic Spiral', 'the National Cancer Center Research and Development Fund (23-A-44)', and the Japan Initiative for Global Research Network on Infectious Diseases is gratefully acknowledged.

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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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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.

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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.

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

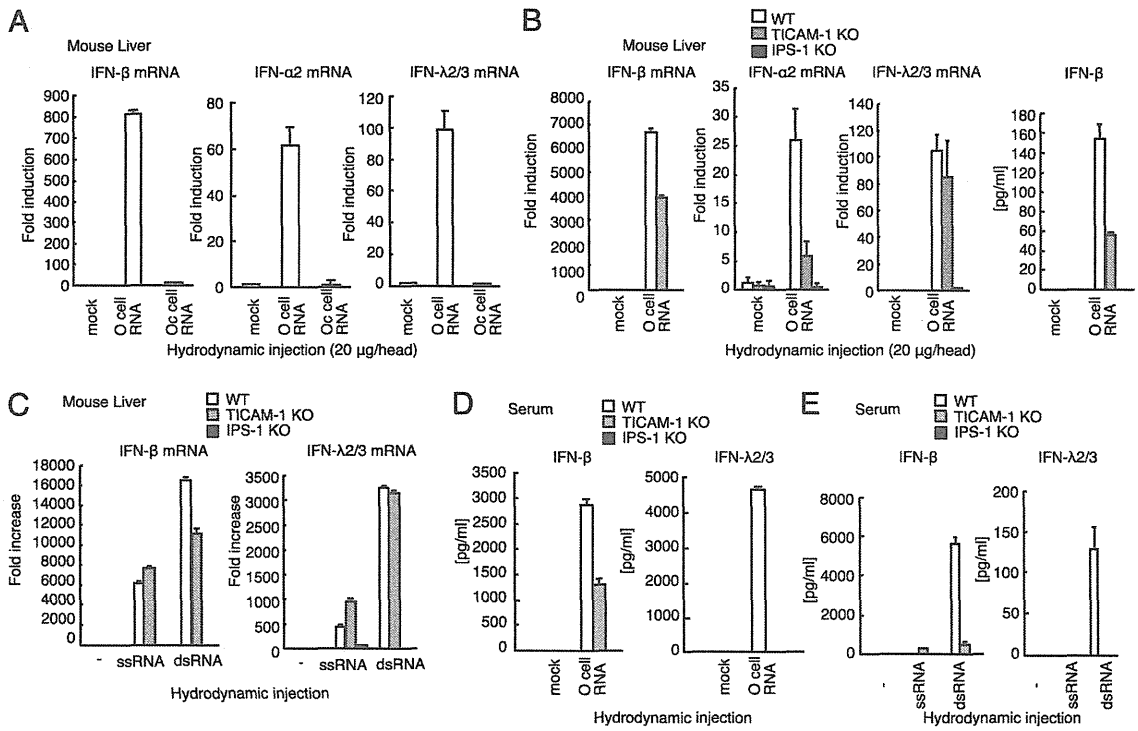

FIGURE 1. Type I and type III IFN productions in response to HCV RNA in vivo. (**A**) O cell and Oc cell RNA (20 μg) were hydrodynamically injected into wild-type mice. Six hours later, mouse livers were excised, and IFN-β, α2, and -λ2/3 mRNA levels were determined by quantitative RT-PCR. (**B**) O cell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

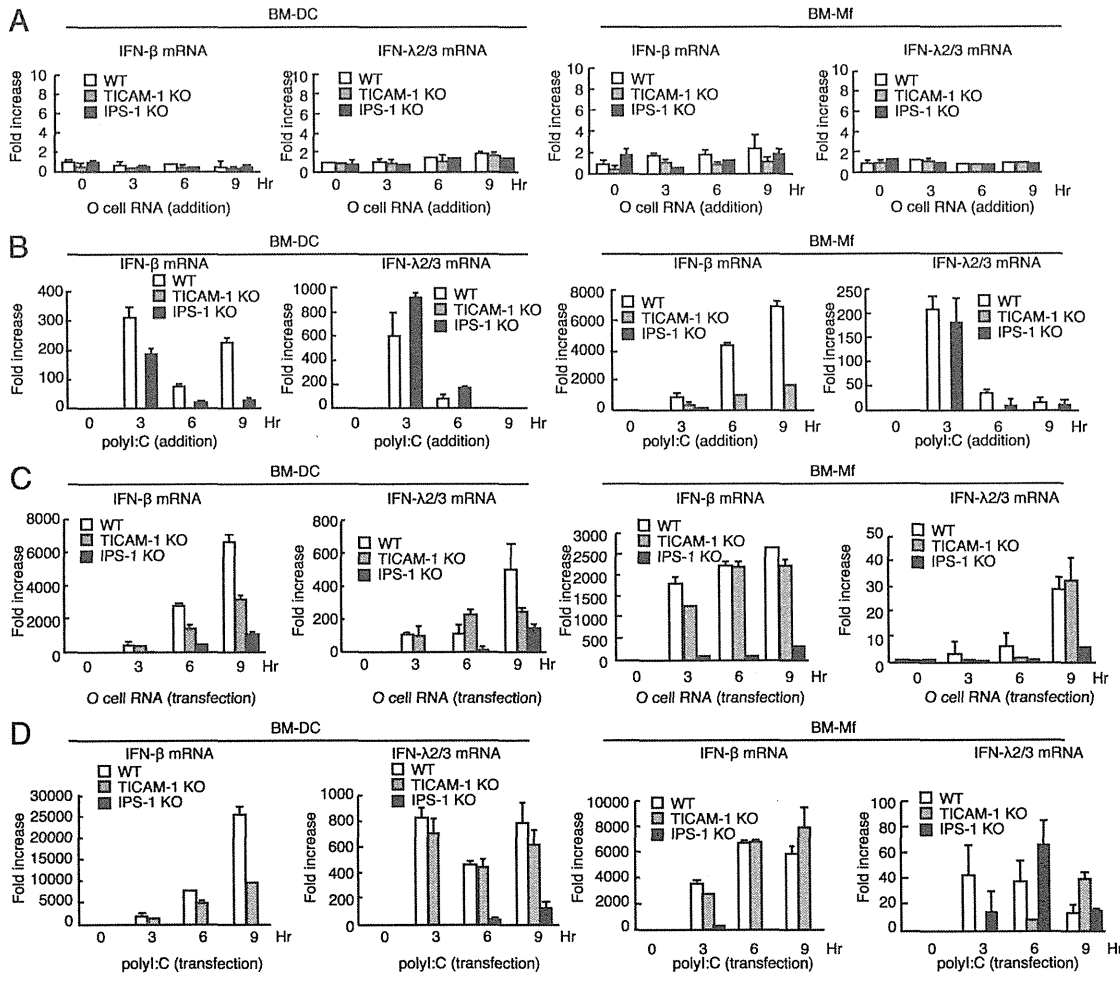

FIGURE 2. Type I and type III IFN expression in mouse DCs and Mfs in response to HCV RNA. (**A** and **B**) O cell RNA (A) or polyI:C (B) (20 μg) was added to the culture medium of BM-DCs and BM-Mfs derived from wild-type, TICAM-1 KO, and IPS-1 KO mice. IFN-β and IFN-λ2/3 mRNA levels were determined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

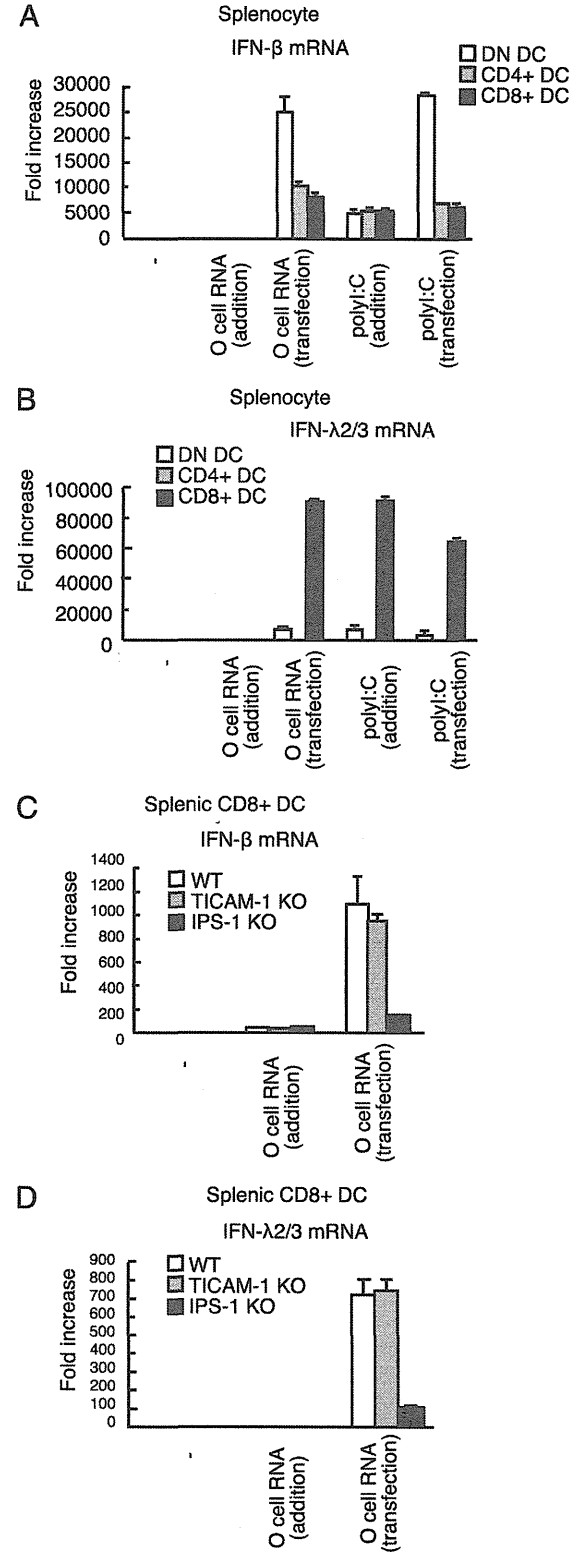

FIGURE 3. Type III IFN production by $CD8^+$ DCs. (**A** and **B**) $CD4^+$, $CD8^+$, and DN DCs were isolated from mouse spleens and stimulated with 20 μg O cell RNA without transfection or stimulated with 1 μg O cell RNA by transfection for 6 h. IFN-β (A) and -λ2/3 (B) mRNA levels were determined by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

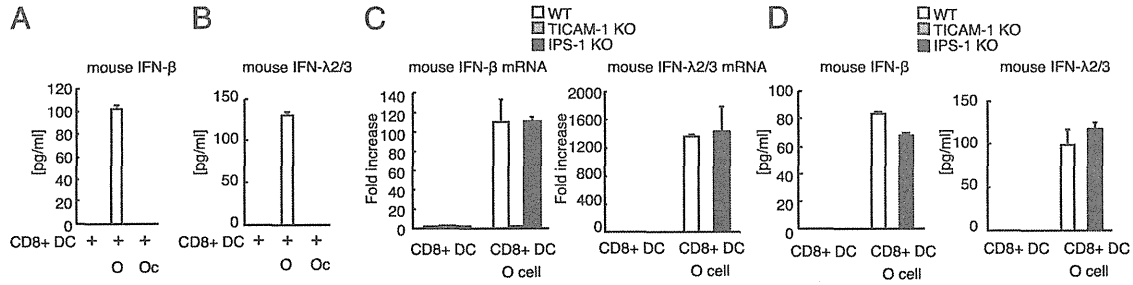

FIGURE 4. IFN-β and –λ production by $CD8^+$ DCs cocultured with hepatocytes with HCV replicons. (**A** and **B**) $CD8^+$ DCs isolated from wild-type spleens were cocultured with O cells (with HCV replicons) or Oc cells (without HCV replicons). After 24 h of coculture, IFN-β (A) and –λ2/3 (B) concentrations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

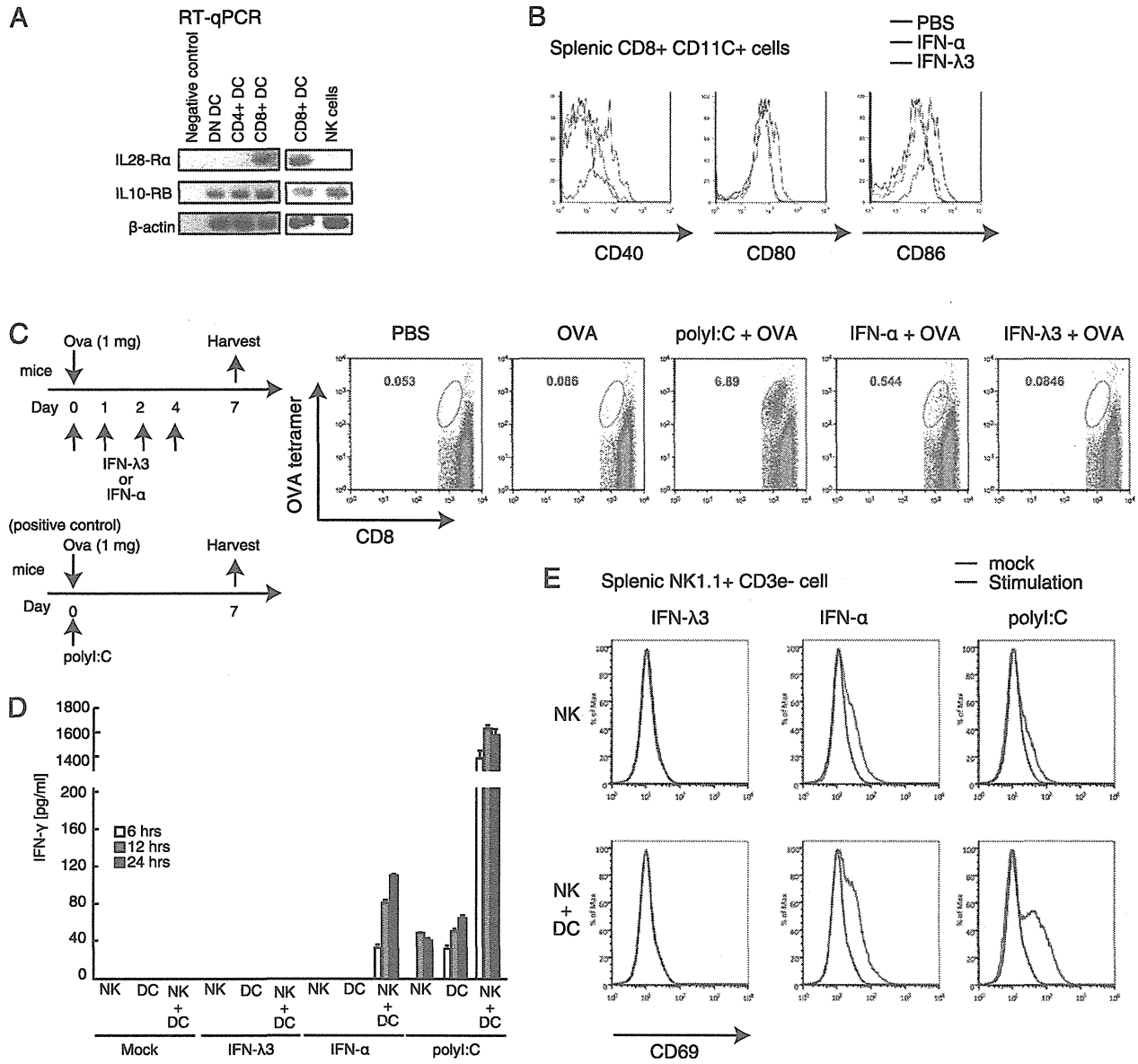

FIGURE 5. IFN-λ effects on DC functions. (**A**) DN, $CD4^+$, $CD8^+$ DCs, and NK cells were isolated from wild-type mouse spleens. IL-28Rα and IL-10RB mRNA were determined by RT-PCR. (**B**) A total of 0.5 μg IFN-λ3 or 1×10^5 IU IFN-α was i.p. injected into mice. Six hours after injection, spleen $CD8^+$ 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

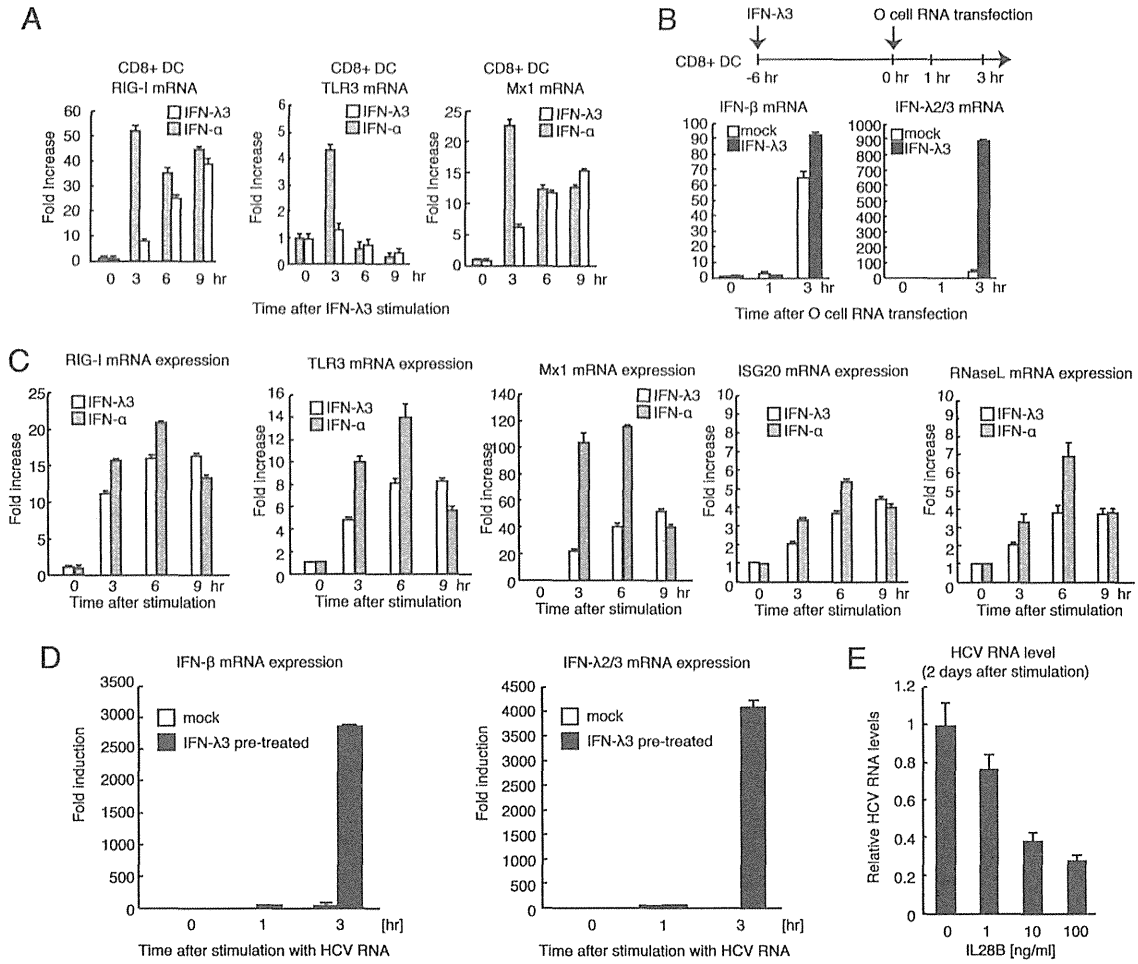

FIGURE 6. Antiviral responses induced by IFN-λ. (**A**) Mouse spleen $CD8^+$ DCs were stimulated with 100 ng/ml IFN-λ3 or 1000 IU/ml IFN-α, after which RIG-I, TLR3, and Mx1 mRNA levels were determined by quantitative RT-PCR. (**B**) Mouse spleen $CD8^+$ DCs were treated with 100 ng/ml IFN-λ3 for 6 h. 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.