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RESEARCH PAPER

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Cross-priming for antitumor CTL induced by soluble Ag + polyI:C depends on the TICAM-1 pathway in mouse CD11c$^+$/CD8α^+ dendritic cells

Masahiro Azuma, Takashi Ebihara,† Hiroyuki Oshiumi, Misako Matsumoto and Tsukasa Seya*

Department of Microbiology and Immunology; Hokkaido University Graduate School of Medicine; Sapporo, Japan

†Current affiliation: Howard Hughes Medical Institute; Washington University School of Medicine; St. Louis, MO USA

Keywords: cross-presentation, dendritic cell, TLR3, TICAM-1 (TRIF), tumoricidal CTL

Abbreviations: APC, antigen-presenting cells; CTL, cytotoxic T lymphocytes; DAMP, damage-associated molecular pattern; DC, dendritic cells; IFN, interferon; IPS-1, IFNβ promoter stimulator-1; MDA5, melanoma differentiation associated gene 5; Mf, macrophages; NK, natural killer; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptors; PV, poliovirus; RIG-I, retinoic acid inducible gene-1; SL8, an OVA tetramer; TICAM-1, Toll-IL-1 receptor homology domain-containing molecule-1; TLR, Toll-like receptor; WT, wild-type

PolyI:C is a nucleotide pattern molecule that induces cross-presentation of foreign Ag in myeloid dendritic cells (DC) and MHC Class I-dependent proliferation of cytotoxic T lymphocytes (CTL). DC (BM or spleen CD8α$^+$) have sensors for dsRNA including polyI:C to signal facilitating cross-presentation. Endosomal TLR3 and cytoplasmic RIG-I/MDA5 are reportedly responsible for polyI:C sensing and presumed to deliver signal for cross-presentation via TICAM-1 (TRIF) and IPS-1 (MAVS, Cardif, VISA) adaptors, respectively. In fact, when tumor-associated Ag (TAA) was simultaneously taken up with polyI:C in DC, the DC cross-primed CTL specific to the TAA in a syngenic mouse model. Here we tested which of the TICAM-1 or IPS-1 pathway participate in cross-presentation of tumor-associated soluble Ag and retardation of tumor growth in the setting with a syngeneic tumor implant system, EG7/C57BL6, and exogenously challenged soluble Ag (EG7 lysate) and polyI:C. When EG7 lysate and polyI:C were subcutaneously injected in tumor-bearing mice, EG7 tumor growth retardation was observed in wild-type and to a lesser extent IPS-1$^{-/-}$ mice, but not TICAM-1$^{-/-}$ mice. IRF-3/7 were essential but IPS-1 and type I IFN were minimally involved in the polyI:C-mediated CTL proliferation. Although both TICAM-1 and IPS-1 contributed to CD86/CD40 upregulation in CD8α$^+$ DC, H2K^b-SL8 tetramer and OT-1 proliferation assays indicated that OVA-recognizing CD8 T cells predominantly proliferated in vivo through TICAM-1 and CD8α$^+$ DC is crucial in ex vivo analysis. Ultimately, tumor regresses > 8 d post polyI:C administration. The results infer that soluble tumor Ag induces tumor growth retardation, i.e., therapeutic potential, if the TICAM-1 signal coincidentally occurs in CD8α$^+$ DC around the tumor.

Introduction

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are two major effectors for antitumor cellular immunity. These effectors are driven through activation of dendritic cells (DC) and/or macrophages (Mf), which is mediated by pattern-recognition receptors (PRRs) for the recognition of microbial patterns.[1,2] Antigen (Ag) presentation and upregulation of NK cell-activating ligands are major events induced in DC/Mf in response to PRRs, which link to evoking CTL- and NK-antitumor immunity, respectively. The immune-potentiating function of specific components of the classical adjuvants are largely attributable to the ligand activity of PRRs (CpG DNA/TLR9, polyI:C/TLR3, monophosphoryl lipid (MPL) A/TLR4, Pam2/TLR2, etc.).[3] That is, the DC/Mf competent to drive effectors are generated through PRR signal in inflammatory nest where affected cells and recruited immune cells encounter exogenous or endogenous PRR ligands. Since studying the functional properties of PRRs in tumor immunity is on the way using a variety of possible ligands and cell biological analyses, immune responses reflecting the total adjuvant potential around Ag-presenting cells (APC) in local inflammatory nests are not always elucidated even in mice.

RNA-sensing PRR pathways, including TLR3-TICAM-1, TLR7-MyD88 and RIG-I/MDA5-IPS-1 participate in driving Type I IFN induction and cellular immunity in DC subsets.[1,4,5] Type I IFN and the IFNAR pathway in DC and other cells reportedly evoke and amplify T cell immunity.[5,6] TLR7 resides exclusively in plasmacytoid DC[7] whereas TLR3 mainly exists in

*Correspondence to: Tsukasa Seya; Email: seya-tu@pop.med.hokudai.ac.jp
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myeloid DC/Mf and epithelial cells.[8] They are localized on the membrane of the endosome and deliver the signal via their adaptors, MyD88 and TICAM-1.[7,8] RIG-I and MDA5 are ubiquitously distributed to a variety of mouse cells and signal the presence of cytoplasmic viral products through IPS-1.[9] Thus, TLR3 and RIG-I/MDA5 are candidates associated with DC maturation to drive effector cells.[10] Indeed, viral dsRNA analog, polyI:C, is a representative ligand for TLR3 and MDA5 and induces polyI:C-mediated DC-NK reciprocal activation.[11,12] These are also true in human DC.[13]

The point of this study is by which pathway antitumor CTL are induced for tumor regression in a mouse tumor-implant model. It has been postulated that DC present exogenous tumor Ag to the MHC Class I-restricted Ag-presentation pathway and proliferate CD8 T cells specific to the extrinsic Ag. When tumor cells provide soluble and insoluble exogenous Ag, this Class I Ag presentation occurs mostly TAP/proteasome-dependent, suggesting the pathway partly sharing with that for endogenous Ag presentation. This DC's ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation.[14] DC cross-presentation leads to the cross-priming and proliferation of Ag-specific CD8 T cells in vivo and in vitro.[14-18] A variety of PAMP[15,16] and intrinsic DAMP[17] as well as other factors including Type I IFN,[5,18] $CD4^+$ T cells[19] and NKT cells[20] augment cross-priming in tumor-bearing mice. However, by what molecular mechanism polyI:C enhances CTL induction in tumor-bearing mice remains largely unsettled.

Here, we made an EG7 tumor-implant mouse system and treated the mice with s.c.-injected ovalbumin (OVA)-containing cell lysates (Ag) and polyI:C. Spleen $CD8\alpha^+$ DC turn CTL-inducible when stimulated with Ag and polyI:C. In either case of s.c., i.p., or i.v. injection of polyI:C, the TLR3/TICAM-1 pathway predominantly participates in $CD8\alpha^+$ DC cross-priming and antitumor CTL induction. Earlier studies using non-tumor models, suggested that both TLR3 and MDA5 appeared to participate in polyI:C-dependent CTL induction.[21] TLR3 is predominantly involved in primary Ag response and Th1 skewing,[22] while MDA5 participates in secondary Ag response.[23] Importance of TLR3 in induction of cross-priming was first suggested by Schulz et al., who used OVA/polyI:C-loaded or virus-infected xenogenic (Vero) cells and mouse DC.[16] Here we demonstrate that the antitumor polyI:C activity is sustained by the TICAM-1 pathway in any route of injection in tumor-implant mice: antitumor CTL responses are mostly abrogated in TICAM-$1^{-/-}$ but not IPS-$1^{-/-}$ mice.

Results

Properties of EG7 tumor with high MHC in tumor-loading mice. The properties of the EG7 line we used are consistent with those reported previously.[24,25] It expressed high MHC Class I (H2-Kb) and no Qa-1b or Rae-1 (**Fig. S1**). The expression levels of these proteins were barely changed before and after implantation of EG7 cells into mice. Cell viability was not affected by in vitro stimulation with polyI:C only (**Fig. S1B**). However, a batch-to-batch difference of cell viability may have affected the rate of tumor growth in each mouse tumor-implant experiment.

$CD8^+$ T cells are responsible for tumor retardation by polyI:C. EG7 cells (2×10^6) were inoculated into the back of C57BL/6 (WT), and the indicated reagents were subcutaneously (s.c.) injected around the EG7 tumor (**Fig. 1A**). Growth retardation of tumor was observed by treatment with polyI:C or polyI:C plus EG7 lysate (**Fig. 1A**). EG7 lysate only had no effect on tumor regression. When $CD8\beta^+$ T cells were depleted before EG7 lysate/polyI:C treatment, polyI:C-mediated tumor growth suppression was cancelled (**Fig. 1A**), suggesting the participation of CD8 T cells in tumor growth suppression. The therapeutic potential of polyI:C appeared to be more reproducible in the presence of EG7 lysate than in the absence, judged from the increases of activated $CD8^+$ T cells (**Fig. 1B**) and cytotoxic activity (**Fig. 1C**) of LN T cells isolated from the mice sacrificed after the last therapy. Yet, the EG7 Ag could be more or less supplied from the implant tumor. $NK1.1^+$ cells did not participate in this EG7 tumor regression in this setting (data not shown).

Since EG7 lysate contains OVA, OVA-specific T cells in draining LN and spleen of the WT mice were counted by tetramer assay after the last therapy (**Fig. S2A and B**). The numbers of tetramer-positive cells were prominently increased in LN and spleen in mice with EG7 lysate and polyI:C. We confirmed the importance of simultaneous administration of Ag plus polyI:C for OVA-specific CTL induction as in **Figure S2C**, where pure Ag (OVA) was used instead of EG7 lysate for immunotherapy. The polyI:C adjuvant function appeared to be more efficient in the mixture of pure Ag than in polyI:C alone. Tumor regression (**Fig. S2C**) and OVA-specific CTL induction (**Fig. S2D**) were clearly observed in this additional experiment. To obtain reproducible data, we employed the EG7 lysate/polyI:C combination therapy as follows.

IFN-inducing pathways are involved in PolyI:C-derived EG7 growth retardation. We next inoculated EG7 cells (2×10^6) into the back of C57BL/6 (WT), TICAM-$1^{-/-}$, IPS-$1^{-/-}$, or TICAM-1/IPS-1 double-deficient (DKO) mice (**Fig. 2**). We s.c. administered EG7 lysate with or without polyI:C around the tumor. The EG7 lysate was the soluble fraction of EG7 which removed insoluble debris by centrifugation. The EG7 lysate contained unprecipitated micro-debris and soluble Ag. No other emulsified reagent was added for immunization. Thus, the adjuvant function of polyI:C per se is reflected in the tumor growth, although polyI:C had to be injected into mice twice a week. Retardation of tumor growth was observed $>$ 8 d after immunization with EG7 lysate + polyI:C in WT mice, though no growth retardation without polyI:C (**Fig. 2A**). The polyI:C-mediated tumor growth suppression was largely abrogated in TICAM-$1^{-/-}$ (**Fig. 2B**) and to a lesser extent in IPS-$1^{-/-}$ mice (**Fig. 2C**), and completely in TICAM-1/IPS-1 DKO mice (**Fig. 2D**). Hence, TICAM-1 plays an important role in inducing polyI:C-mediated tumor growth retardation in the s.c. setting we employed.

CD8 T cell activation induced by the TICAM-1 pathway. CD8 T cell activation in the inguinal LN was tested with polyI:C + EG7 lysate in EG7 tumor-bearing mice using CD69 as

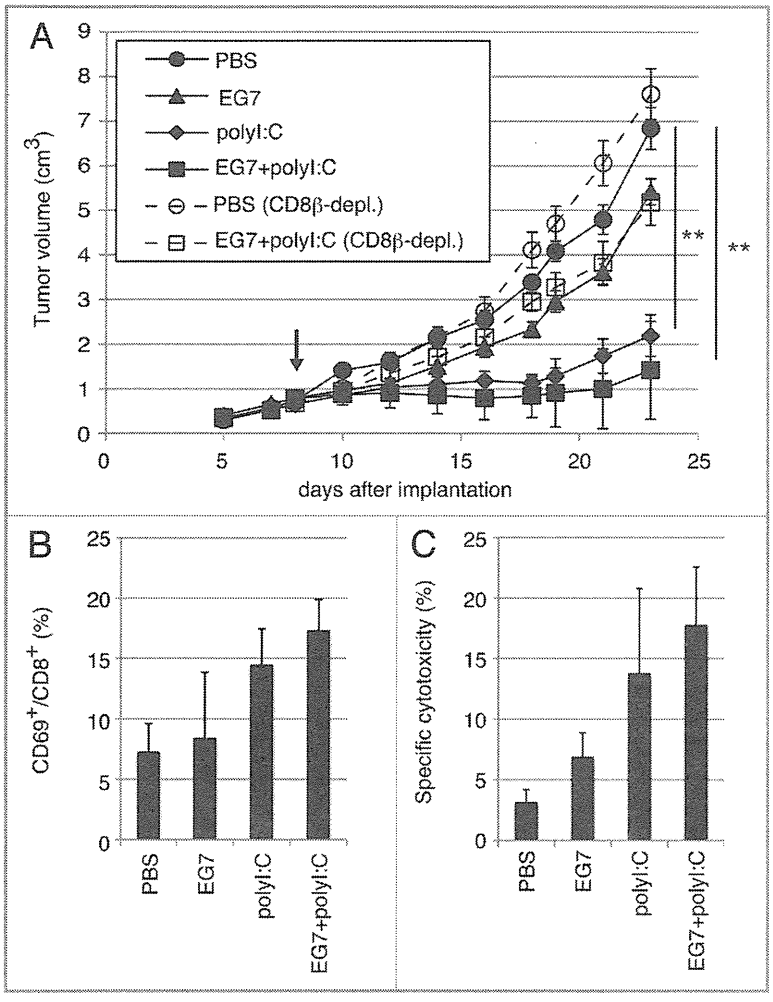

Figure 1. PolyI:C induces CTL-mediated tumor regression. (A) WT mice were challenged with EG7 cells and were treated with PBS (●), EG7 lysates (▲), polyI:C (◆) and EG7 lysates + polyI:C (■). The adjuvant therapy was started at the time indicated by the arrow and the indicated reagents injected twice per week. One of the two PBS groups (○) and one of the two EG7 lysates + polyI:C groups (□) were treated with anti-CD8β ascites in order to deplete $CD8^+$ T cells once a week. Each group had 3–5 mice. (B) Draining inguinal LNs were harvested 24 h after the last treatment and the proportion of CD69-expressing $CD8^+$ cells were counted. (C) LN cells were co-cultured with MMC-treated EG7 cells for 3 d and subjected to ^{51}Cr release assay to evaluate CTL activity. E/T = 50. All error bars used in this figure show ± SEM. Data are representative of two independent experiments. One-way analysis of variance (ANOVA) with Bonferroni's test was performed to analyze statistical significance. **, $p < 0.01$.

an activating marker. Twenty-four hours after the last polyI:C + EG7 sec.c. treatment, cells were harvested from the LN excised (**Fig. 3A**). FACS profiles of total cells from each mouse group are shown in **Fig. S3**. By combination therapy with EG7 lysate and polyI:C, T cells were activated in WT and IPS-$1^{-/-}$ mice, but the proportion of CD8 T cells was not affected by the therapy (**Fig. S4A**). Under the same conditions, T cells were barely activated in TICAM-$1^{-/-}$ mice in response to polyI:C (**Fig. 3A**). The proportion of $CD69^+$ cells are indicated in **Figure 3B**. IL-2 (**Fig. 3C**) and IFNγ (**Fig. S4B**) were highly induced in the WT and IPS-$1^{-/-}$ LN cells, while they were not induced in TICAM-$1^{-/-}$ or DKO cells. IFNγ levels were upregulated only in polyI:C-treated tumor-bearing mice, although the WT > IPS-$1^{-/-}$ profile for IFNγ production was reproducibly observed (**Fig. S4B**).

In vivo proliferation of CD8 T cells judged by tetramer assay and IFNγ induction. We next tested whether i.p. injection of polyI:C plus OVA induces CTL proliferation. PolyI:C and OVA were i.p. injected into mice and the polyI:C-dependent cross-priming of CD8 T cells were examined using the OVA tetramer assay. OVA-specific CD8 T cells were clonally proliferated in WT and IPS-$1^{-/-}$ mice, but not in TICAM-1/IPS-1 DKO and IRF-$3/7^{-/-}$ mice (**Fig. 4A**). Proliferation of OVA-specific CD8 T cells were severely suppressed in TICAM-$1^{-/-}$ mice (**Fig. 4A**), suggesting that polyI:C-mediated cross-priming of CD8 T cells largely depends on the TICAM-1 pathway followed by IRF-3/7 activation in the i.p. route. The results were reproduced in additional experiments using more mice (**Fig. 4B**) and TLR$3^{-/-}$ mice (**Fig. S5A and B**). The polyI:C cytokine response, where IFNα is IPS-1-dependent while IL-12p40 is TICAM-1-dependent, was also confirmed in serum level by polyI:C i.p. injection (**Fig. S5E**). Specific induction of IFNγ (**Fig. 4C**) was also observed in parallel with the results of **Figure 4A**.

Whether or not i.v. injection of polyI:C plus OVA induces Ag-specific CTL and cytotoxicity was next checked. OVA-specific OT-1 proliferation and cytotoxicity (**Fig. 4D and E**) were observed in in vivo analyses of WT and IPS-$1^{-/-}$ CD8 T cells but not of TICAM-$1^{-/-}$, TICAM-1/IPS-1 DKO, and IRF-$3/7^{-/-}$ mice in the i.v. setting.

Since TICAM-1 is the adaptor for TLR3 as well as cytoplasmic helicases,[24] we confirmed the level of cross-priming being decreased in TLR$3^{-/-}$ mice and an expected result was obtained (**Fig. S5A and B**). Furthermore, in IFNAR$^{-/-}$ mice, OVA-specific CTL induction was slightly reduced compared with that in WT mice, but higher than in TICAM-$1^{-/-}$ mice (**Fig. S5C and D**). Hence, in vivo cross-presentation induced by polyI:C mostly depends on the TLR3-TICAM-1 pathway followed by transcriptional regulation by IRF-3/7 in any administration route, and is further promoted by Type I IFN presumably produced by the stromal cells through the IPS-1 pathway.[26]

IPS-1 induces DC maturation but not cross-priming in vivo. Spleen DC maturation by i.v.-injected polyI:C was tested ex vivo using $CD8\alpha^+$ DC and $CD8\alpha^-$ DC isolated from WT or KO mice with no tumor as indicated in **Figure 5A**. The maturation markers CD86 and CD40 were upregulated on both $CD8\alpha^+$ and $CD8\alpha^-$

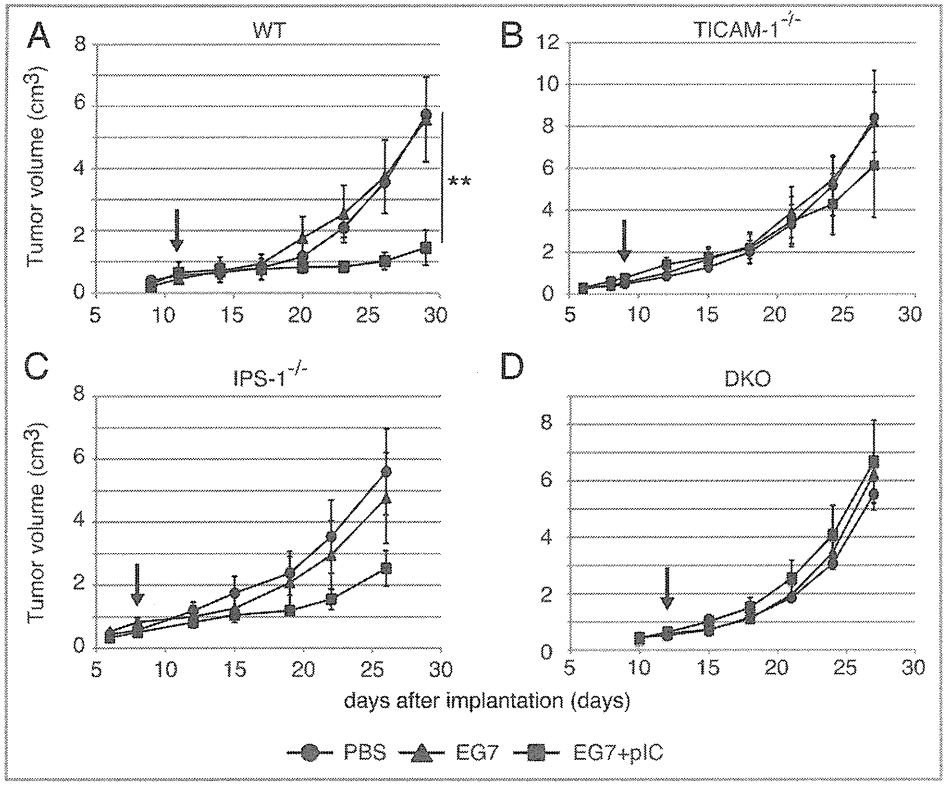

Figure 2. PolyI:C-induced tumor retardation is dependent on the TICAM-1 pathway. Antitumor effect of polyI:C on various KO mice were evaluated by using in vivo mouse tumor implant model. EG7 cells were inoculated to WT (A), TICAM-1$^{-/-}$ (B), IPS-1$^{-/-}$ (C) and DKO mice (D) on day 0. PBS (●), EG7 lysates (▲) or EG7 lysates + polyI:C (■) were s.c. administered around the tumor. The adjuvant therapies were started at the time indicated by the arrows and injected twice per week. Each group have 3–4 mice and error bar shows ± SEM. Data are representative of two independent experiments. **, $p < 0.01$

DC from WT mice when they were stimulated with OVA and polyI:C. Treatment of DC with OVA only did not induce upregulation of CD86 and CD40. Although the expression levels of CD86 and CD40 were a little less in CD8α$^+$ and CD8α$^-$ DC from TICAM-1$^{-/-}$ or IPS-1$^{-/-}$ mice than those from WT mice, both CD86 and CD40 were sufficiently upregulated even in the abrogation of either one pathway in polyI:C-injected mice. The CD86 and CD40 shifts were completely abolished in DKO mice (**Fig. 5A**). Thus, the TICAM-1 pathway participates in both potent co-stimulation and cross-priming, while the IPS-1 pathway mainly participates only in integral co-stimulation in myeloid DC.

We next assessed in vitro proliferation of OT-1 cells. CD8α$^+$ and CD8α$^-$ DC were prepared from PBS, polyI:C, OVA and OVA/polyI:C-treated mice, and mixed in vitro with CFSE-labeled OT-1 cells. WT, TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice were used for this study. OT-1 proliferation was observed with CD8α$^+$ DC but not CD8α$^-$ DC when OVA + polyI:C was injected (**Fig. 5B**). Furthermore, the OT-1 proliferation barely occurred in the mixture containing TICAM-1$^{-/-}$ CD8α$^+$ DC. Thus, OT-1 proliferation is triggered by the TICAM-1 pathway in CD8α$^+$ DC. Again, IPS-1 had almost no effect on OT-1 proliferation with CD8α$^+$ DC in this setting. In the mixture, IFNγ was produced in the supernatants of WT and IPS-1$^{-/-}$ CD8α$^+$ DC but not TICAM-1$^{-/-}$ DC by stimulation with OVA + polyI:C (**Fig. 5C**). No IFNγ was produced in the supernatants of CD8α$^-$ DC even from WT mice, which results are in parallel with those of OT-1 proliferation. In any case irrespective of tumor-bearing or not, Ag, polyI:C and the TICAM-1 pathway are mandatory for CD8α$^+$ DC to cross-prime and proliferate OVA-specific CD8 T cells.

We checked the TICAM-1- or IPS-1-specific gene expressions related to Type I IFN and MHC Class I presentation using genechip and qPCR (**Fig. S6**). PolyI:C-mediated upregulation of *Tap1*, *Tap2* and *Tapbp* messages diminished in TICAM-1$^{-/-}$ BMDC (**Fig. S6A**). The levels of these genes were hardly affected in IPS-1$^{-/-}$ BMDC (data not shown). PolyI:C-mediated upregulation was observed with MDA5 (*Ifih1*) in CD8α$^-$ and CD8α$^+$ DCs (**Fig. S6B**). Surprisingly, other factors including TLR3, TICAM-1 and MAVS messages were all downregulated in response to polyI:C in CD8α$^+$ DC (**Fig. S6B**), for the reason as yet unknown.

Effect of TLR3-mediated IFN-inducing pathway on anti-tumor CTL induction. PolyI:C is a dsRNA analog capable of incorporating into the endosome and cytoplasm by exogenous administration in vitro.[27,28] However, no evidence has been proposed that polyI:C is internalized into the endosome of

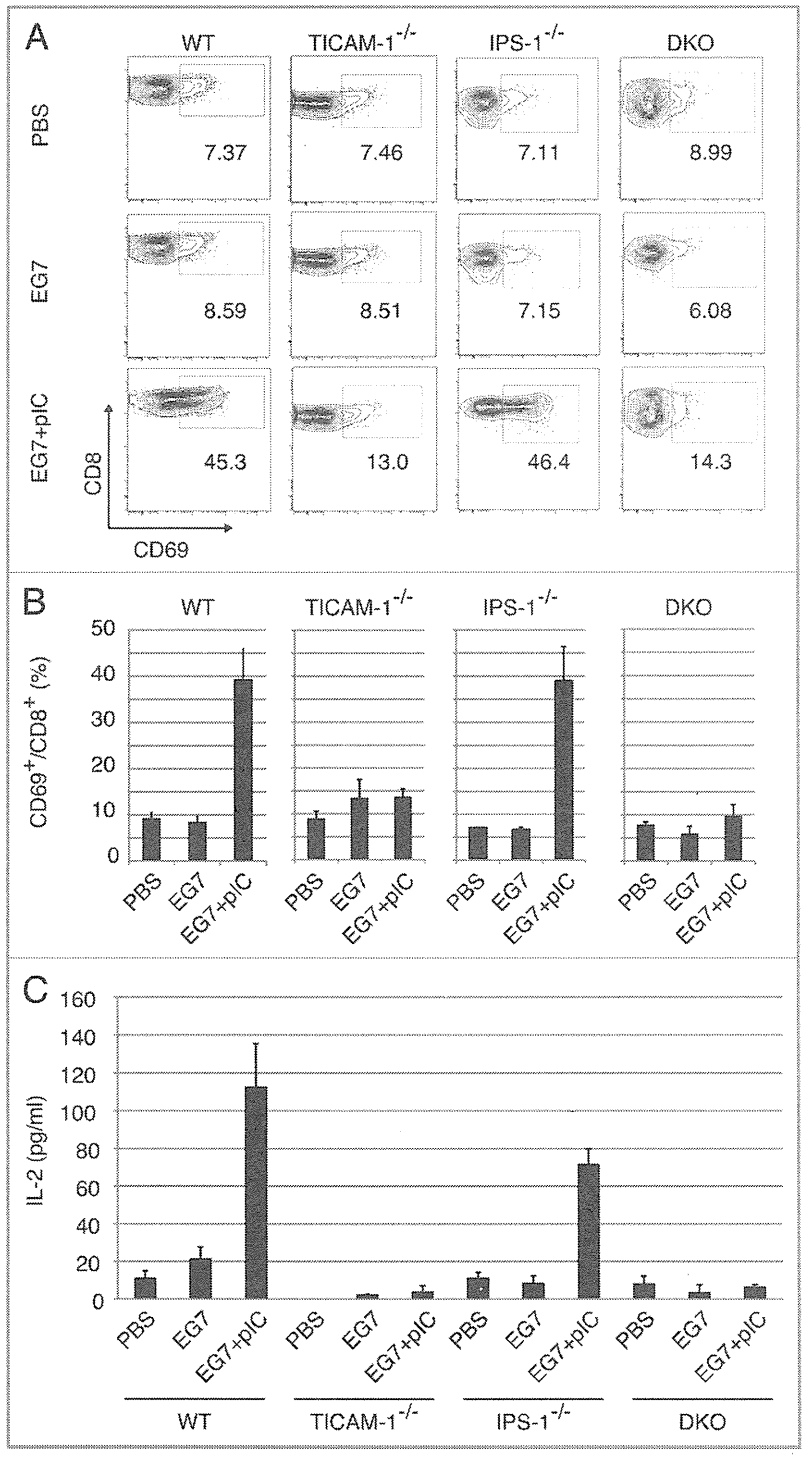

Figure 3. CD8 T cells in the draining LNs are activated through the TICAM-1 pathway by polyI:C. Draining inguinal LNs were harvested from tumor-bearing mice 24 h after the last treatment. LN cells were stained with CD3ε, CD8α and CD69, and the cells gated on $CD3\varepsilon^{+}CD8\alpha^{+}$ are shown (A). Spleen cells in each group of mice were stained separately, the CD8 levels in gated cells being variably distributed in FACS analyses. The average frequency of activated CD8 T cells defined by CD69 expression is shown (B). Alternatively, LN cells from the indicated mice were cultured for further 3 d in vitro and IL-2 production was measured by CBA assay (C).

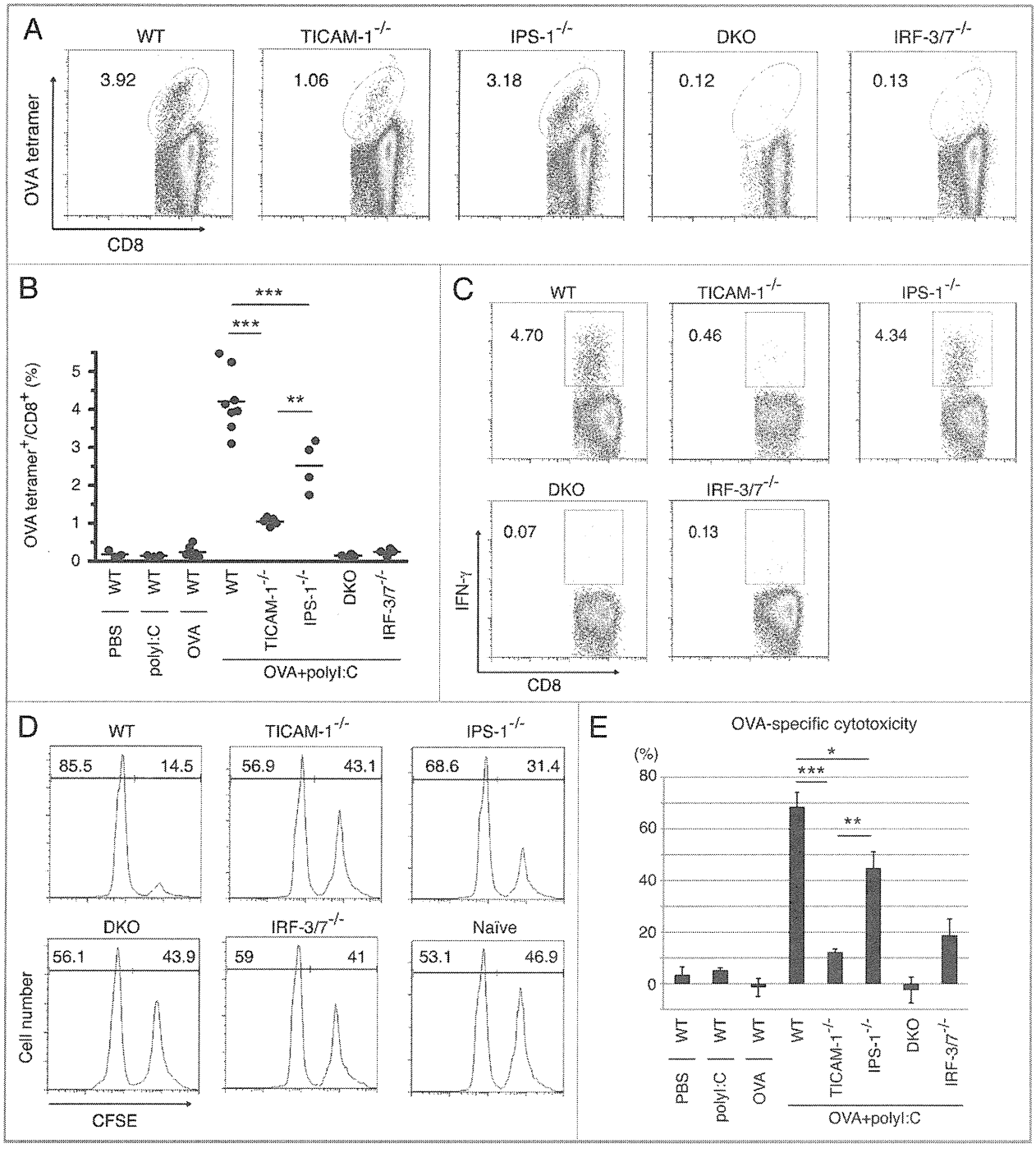

Figure 4. TICAM-1 and IRF-3/7 are essential for polyI:C-induced antigen-specific CTL expansion. WT, TICAM-1$^{-/-}$, IPS-1$^{-/-}$, TICAM-1/IPS-1 DKO and IRF-3/7$^{-/-}$ mice were i.p. administered with the combination of OVA and polyI:C. After 7days, splenocytes were harvested and stained with CD8α and OVA tetramer (A). The average percentages of OVA-specific CTL are shown (B). Alternatively, splenocytes were cultured in vitro in the presence of SL8 for 8 h and IFNγ production was measured by intracellular cytokine staining (C). To assess the killing activity, in vivo CTL assay was performed. The combinations of OVA and polyI:C were administered i.v. to each group of mice and 5 d later, cytotoxicity was measured (D). The data shown are collaborate or representative of at least three independent experiments. One-way analysis of variance (ANOVA) with Bonferroni's test was performed to analyze statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

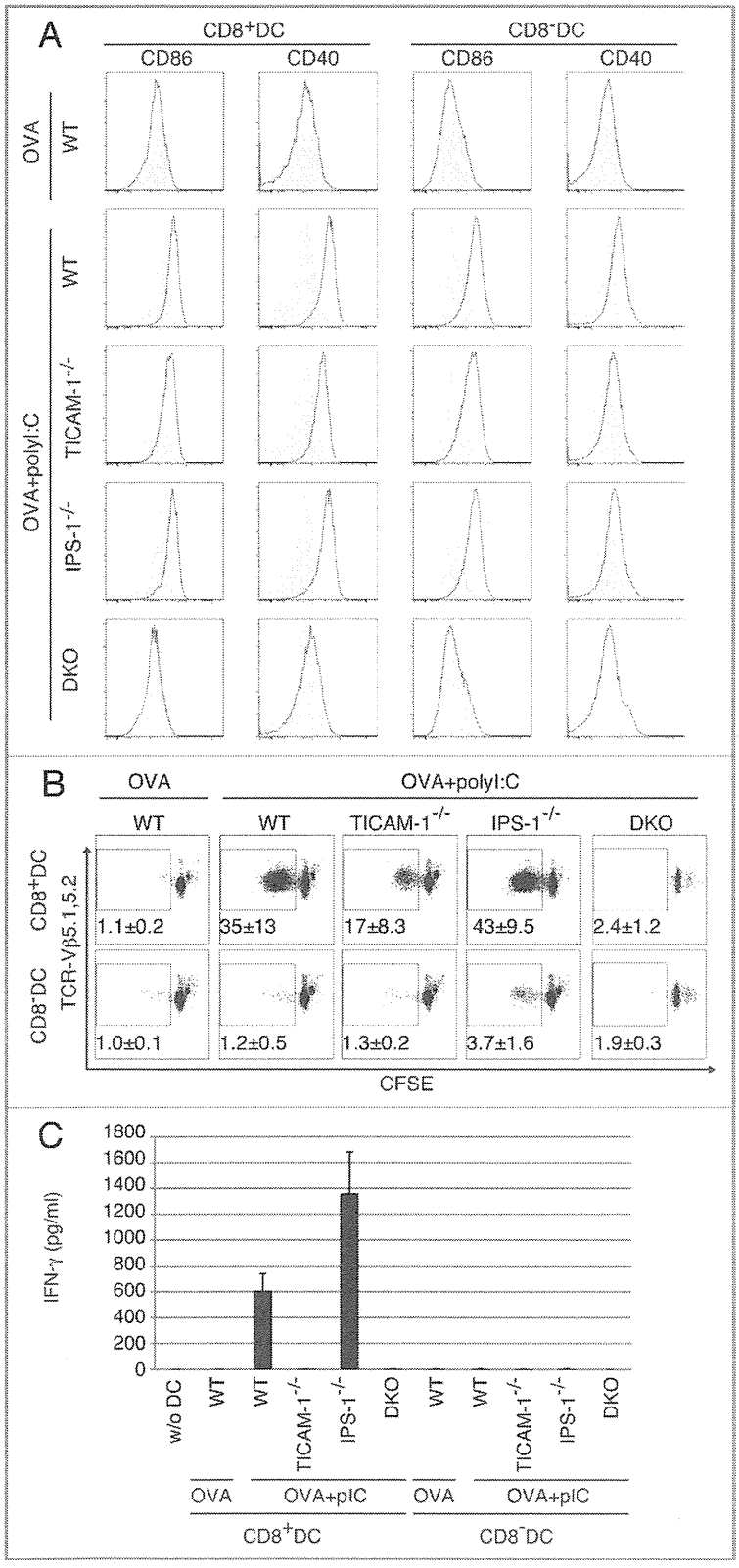

Figure 5. TICAM-1 in CD8α⁺ DC is more important than IPS-1 in polyI:C-induced cross-priming. OVA and polyI:C were administered i.v. and 4 h later, CD8α⁺ and CD8α⁻ DC were isolated from the spleen. CD86 and CD40 expressions were determined by FACS (A). Filled gray and black line show isotype control and target expression, respectively. Alternatively, CD8α⁺ and CD8α⁻ DC were co-cultured with CFSE-labeled RAG2$^{-/-}$/OT-1 T cells for 3 d. The cross-priming activity of each DC subset was determined with sequential dilution of CFSE (B) and IFNγ production (C). IFNγ was measured by CBA assay. The data shown are representative of two independent experiments. Err bar shows SD.

CD8α⁺ DC where TLR3 is expressed in vivo. Peritoneal (PEC) Mf and bone marrow-derived DC[22] usually phagocytoze polyI:C and deliver them into the endosome. In mouse CD8α⁺ DC direct internalization of polyI:C has remain unproven. Using labeled polyI:C and anti-mouse TLR3 mAb, 11F8,[22] we checked whether the exogenously-added polyI:C encountered with TLR3 in CD8α⁺ DC in vitro. TLR3 (green) was merged with TexasRed-polyI:C 30–120 min after polyI:C stimulation in the culture (**Fig. 6A**). The quantities of CD8α⁺ and CD8α⁻ DC where FITC-polyI:C was incorporated were determined by FACS analysis (**Fig. 6B**). Thus, the process by which polyI:C injected reaches the endosomal TLR3 is delineated in the CD8α⁺ DC.

Discussion

PolyI:C is an analog of virus dsRNA, and acts as a ligand for TLR3 and RIG-I/MDA5. PolyI:C has been utilized as an adjuvant for enhancement of antitumor immunity for a long time.[29] However, the mechanistic background of the therapeutic potentials of polyI:C against cancer has been poorly illustrated. It induces antitumor NK activation through DC-NK cell-to-cell interaction when CD8α⁺ DC TLR3 is stimulated in the spleen.[11] Besides myeloid cells, however, some tumor cell lines express TLR3 and dsRNA targeting tumor cells may affect the growth rate of tumors,[30] where the receptor-interacting protein (RIP) pathway is involved downstream of TICAM-1.[31] Here we showed evidence that polyI:C injection facilitates maturation of TLR3-positive CD8α⁺ DC (i.e., APC) to trigger CTL induction against exogenous soluble Ags including EG7 lysate or OVA. The TICAM-1 adaptor for TLR3 and IRF-3/7 are involved in the cross-presentation signal in CD8α⁺ DC, but the molecule/mechanism downstream of TICAM-1 that governs cross-presentation remains elusive. Since most of the tumor-associated Ags (TAA) are predicted to be liberated from tumor cells

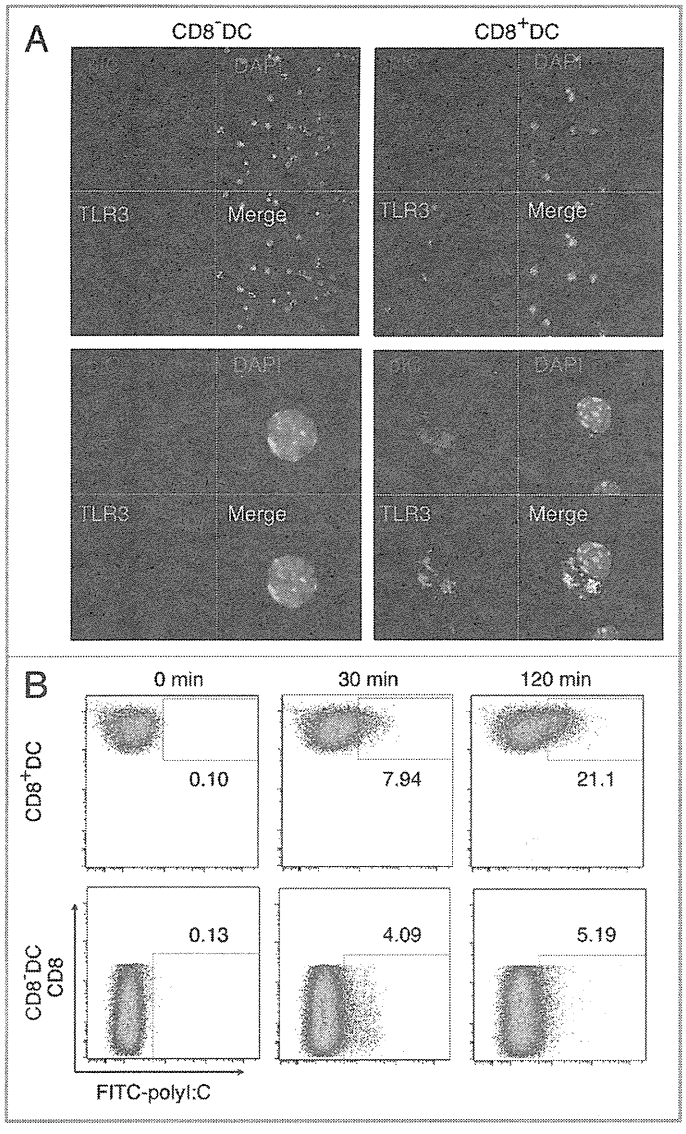

Figure 6. PolyI:C encounters TLR3 in CD8α⁺ DC. CD8α⁺ and CD8α⁻ DC were isolated by FACSAriaII and stimulated with 20 μg/ml TexasRed-polyI:C for 2 h. Then cells were stained with Alexa647-antiTLR3 and subjected to confocal microscopic analysis (A). Alternatively, splenic DC isolated by MACS were incubated with FITC-polyI:C for the time shown in figure and analyzed the degrees of polyI:C uptake by FACS (B). Data shown are the representative of three independent experiments.

as soluble Ags, the TICAM-1 pathway in CD8α⁺ DC would be crucial for driving of tumor-specific CTL around the tumor microenvironment. In any route of polyI:C injection, this is true as shown first in this study. Although TICAM-1 is an adaptor of other cytoplasmic sensors, DDX1, DDX21 and DHX36,[32] the antitumor CTL responses are merely relied on TLR3 of CD8α⁺DC in this system. Taken together with previous reports,[11,12] TICAM-1 signaling triggers not only NK activation but also CTL induction.

TLR3 and MDA5 are main sensors for dsRNA and differentially distributed in myeloid cells.[33,34] TLR3 is limitedly expressed in myeloid, epithelial and neuronal cells,[33] whereas MDA5 is ubiquitously expressed including non-myeloid stromal cells.[33] Several reports suggested that i.v. injection of polyI:C predominantly stimulate the stromal cells which express IFNAR,[26] thereby robust type I IFN are liberated from these cells to be a systemic response including cytokinemia and endotoxin-like shock.[35,36] Both TLR3 and MDA5 link to the IRF-3/7-activating kinases leading to the production of IFNα/β.[37,38] Once IFNα/β are released, IFNAR senses it to amplify the Type I IFN production,[39] and reportedly this amplification pathway involves cross-priming of CD8 T cells in viral infection.[18] Tumor progression or metastasis can be suppressed through the IFNAR pathway.[40] These scenarios may be right depending on the conditions employed. Our message is related to what signal pathway is fundamentally required for induction of antitumor CTL in DC. The CTL response is almost completely abrogated in TICAM-1$^{-/-}$ and IRF-3/7$^{-/-}$ mice, but largely remains in IPS-1$^{-/-}$ and IFNAR$^{-/-}$ mice when Ag and polyI:C are extrinsically administered. The results are reproducible in some other tumor-implant models (data not shown), and even in IFNAR$^{-/-}$ mice, TICAM-1-specific genes are upregulated to confer tumor cytotoxicity (**Fig. S6**, Azuma et al., unpublished data). In addition, the upregulation of these genes is independent of IPS-1 knockout in DC. Our results infer that the primary sensing of dsRNA in CD8α⁺ DC is competent to induce cross-presentation, which minimally involves the IPS-1 or IFNAR amplification pathway, at least at a low dose of polyI:C. Yet, subsequent induction of Type I IFN via the IFNAR may further amplify the cross-priming.[18,41] Further studies are needed as to which of the TICAM-1-inducible genes link to the cross-presentation in CD8α⁺ DC.

The main focus of this study was to identify the pathway for transversion of immature DC to the CTL-driving phenotype by co-administration of polyI:C with soluble Ag. The IPS-1 pathway, although barely participates in antitumor CTL driving, can upregulate CD40/CD86 co-stimulators on the membranes of splenic CD8α⁺ and CD8α⁻ DC in response to polyI:C, suggesting that MDA5 does function in the cytoplasm of splenic CD8α⁺ and CD8α⁻ DC to sense polyI:C. However, effective CTL induction happens only in CD8α⁺ DC when stimulated with polyI:C. CD8α⁺ DC express TLR3 but CD8α⁻ DC do not, and CD8α⁺ DC with no TLR3 fail to induce CTL, suggesting that integral co-stimulation by MDA5/IPS-1 is insufficient for DC to induce cross-priming of CD8 T cells: antitumor CTL are not induced until the TICAM-1 signal is provided in DC. At least, sole effect of the IPS-1 pathway and upregulation of co-stimulators on CD8α⁺ DC is limited for cross-priming and induction of antitumor CTL, which result partly reflects those in a previous report where IPS-1 and TICAM-1 harbor a similar potential for CD8 T cell proliferation when

polyI:C (Alum-containing) is employed as an adjuvant for CD8α+ DC to test proliferation of anti-OVA CTL.[21]

A question is why TICAM-1 is dominant to IPS-1 for response to exogenously-added polyI:C in CD8α+ DC. The answer is rooted in the difference of functional behavior between BMDC and CD8α+ DC. TLR3 levels are variable depending upon subsets of DC,[22] which affects DC subset-specific induction of cellular immune response. The high TLR3 expression (partly surface-expressed) is situated in CD8α+ DC before polyI:C stimulation, which is distinct from the properties of F4/80+ Mf and presumably BMDC of low TLR3 expression. The polyI:C-uptake machinery[15] appears to efficiently work in concert with the TLR3/TICAM-1 pathway in CD8α+ DC and this tendency is diminished when CD8α+ DC are pretreated with Alum + polyI:C.[21] Furthermore, there are functional discrepancies between CD8α+ splenic DC and GM-CSF-induced BMDC, which appears to reflect the difference of their TLR3 levels.[22] These results on CD8α+ DC encourage us to develop dsRNA adjuvant immunotherapy supporting TAA soluble vaccines for cancer applicable to humans, which possess the counterpart of CD8α+ DC.

There are two modes of dsRNA-mediated DC maturation, intrinsic and extrinsic modes that are governed by the IPS-1 and TICAM-1 pathways, respectively.[9,34] It is important to elucidate the in vivo qualitative difference in the two pathways in tumor-loading mice. TLR3+ DC/Mf are responsible for CTL driving via an extrinsic route in viral infection.[34] Previous data suggested that dsRNA in infectious cell debris, rather than viral dsRNA produced in the cytoplasm of Ag-presenting cells or autophagosome formation, contribute to fine tuning of DC maturation through extrinsic dsRNA recognition.[16] It is reported that dsRNA-containing debris are generated secondary to infection-mediated cell death,[41] and DC phagocytose by-stander dead cells. Likewise, soluble tumor Ags released from tumor cells usually are extrinsically taken up by APC in patients with cancer.[42] If CTL are successfully induced in therapeutic biotherapy targeted against cancer cells, this extrinsic TICAM-1 pathway must be involved in the therapeutic process.

Cross-presentation occurs in a TAP-dependent[43] and -independent fashions.[44,45] The peptides are transported by TAP into the endoplasmic reticulum (ER) and loaded onto MHC Class I for presentation at the cell surface. ER and phagosome might fuse each other for accelerating cross-presentation.[46] Another possibility is that cross-presentation occurs in early endosomes where TLR3 resides. This early endosome cross-presentation does not always depend on TAP[42-44] but requires TLR stimulation.[34] TLR4/MyD88 pathway is involved in the TAP-dependent early endosome model,[43] where recruitment of TAP to the early endosomes is an essential step for the cross-presentation of soluble Ag. These models together with our genechip analysis of polyI:C-stimulated BMDC suggested that some ER-associated proteins are upregulated in BMDC by polyI:C-TICAM-1 pathway. The results infer that the TLR3/TICAM-1 rather than the TLR4/MyD88 pathway more crucially participates in cross-presentation in response to dsRNA or viral stimuli and facilitates raising CTL antitumor immunity in APC.

Although multiple RNA sensors couple with TICAM-1 and signal to activate the Type I IFN-inducing pathway,[25] at least TLR3 in the CD8α+ DC are critical in CTL driving. CD8α+ DC are a high TLR3 expresser, while BMDC express TLR3 with only low levels.[22] CD8α- DC do not express it.[22] The Ag presentation and TLR3 levels in CD8α+ DC appear reciprocally correlated with the phagocytosing ability of DC. Although the TLR3 mRNA level is downregulated secondary to polyI:C response after maturation, this may not be related to the CD8α+ DC functions. Yet, polyI:C might interact with other cytoplasmic sensors for DC maturation.[32,47]

The route of administration and delivery methods may be important for culminate the polyI:C adjuvant function. The toxic problem has not overcome in the adjuvant therapy using polyI:C[35,36] and this is a critical matter for clinical introduction of dsRNA reagents to immunotherapy. The most problematic is the life-threatening shock induced by polyI:C. Recent advance of polyI:C study suggests that PEI-jet helps efficient uptake of polyI:C into peritoneal macrophages.[48] LC (poly-L-lysine and methylcellulose) has been used as a preservative to reduce the toxic effect of polyI:C.[49] Nanotechnological delivery of polyI:C results in efficient tumor regression.[50] There are many subsets of DC that can be defined by surface markers, and selecting an appropriate administration route can target a specific DC subset. The route for s.c. administration usually mature dermal/epidermal DC or Langerhans cells.[51,52] Some DC subsets with unique properties specialized to CTL induction would work in association with the route of polyI:C administration. Attempting to develop more harmless and efficient dsRNA derivatives will benefit for establishing human adjuvant immunotherapy for cancer.

Materials and Methods

Mice. TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice were made in our laboratory and backcrossed more than eight times to adapt C57BL/6 background.[12] IRF-3/7$^{-/-}$ and IFNAR$^{-/-}$ mice were kindly provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). TLR3$^{-/-}$ mice were kindly provided by S. Akira (Osaka University, Osaka, Japan). Rag2$^{-/-}$ and OT-1 mice were kindly provided from Drs N. Ishii (Tohoku University, Sendai, Japan). Rag2$^{-/-}$/OT-1 mice were bred in our laboratory. All mice were maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Animal experiments were performed according to the guidelines set by the animal safety center, Hokkaido University, Japan.

Cells. EG7 and C1498 cells were purchased from ATCC and cultured in RPMI1640/10% FCS/55 μM 2-ME/1 mM sodium pyruvate and RPMI1640/10% FCS/25 ng/ml 2-ME, respectively. Mouse splenocytes, OT-1 T cell, CD8α+ DC and CD8α- DC were harvested from the spleen and cultured in RPMI1640/10% FCS/55 μM 2-ME/10 mM HEPES.[41] B16D8 cells were cultured in RPMI/10% FCS as described previously.[12]

Reagents and antibodies. Ovalbumin (OVA) and polyI:C (polyI:C) were purchased from SIGMA and Amersham Biosciences, respectively. $OVA_{257-264}$ peptide (SIINFEKL: SL8)

and OVA ($H2K^b$-SL8) Tetramer were from MBL. Following Abs were purchased: anti-CD3ε (145-2C11), anti-CD8β (53-6.7), anti-CD11c (N418), anti-CD16/32 (93), anti-CD69 (H1.2F3) and anti-IFNγ(XMG1.2) Abs from BioLegend, anti-B220 (RA3-6B2), anti-CD4 (L3T4), anti-CD40 (1C10), anti-CD86 (GL1), and anti-MHC I-SL8 (25-D1.16) Abs from eBiosciences, anti-TCR-Vβ5.1/5.2 Ab and ViaProbe from BD Biosciences. The Rat anti-mouse TLR3 mAb (11F8) was kindly provided by David M. Segal (National Institute of Health, Bethesda, MD). To rule out LPS contamination, we treated OVA or other reagents with 200 μg/ml of Polymixin B for 30 min at 37°C before use. Texas Red- or FITC-labeled poly(I:C) was prepared using the 5' EndTag™ Nucleic Acid Labeling System (Vector Laboratories) according to the manufacturers instructions.

Tumor challenge and poly I:C therapy. Mice were shaved at the back and s.c. injected with 200 μl of 2×10^6 syngenic EG7 cells in PBS. Tumor volumes were measured at regular intervals by using a caliper. Tumor volume was calculated by using the formula: Tumor volume (cm^3) = (long diameter) × (short diameter)^2 × 0.4. A volume of 50 μl of a mixture consisting of the lysate of 2×10^5 EG7 cells with or without 50 μg of poly I:C (polyI:C) was s.c. injected around the tumor. We added no other emulsified reagent for immunization since we want to role out the conditional effect of the Ag/polyI:C. The treatments were started when the average of tumor volumes reached at 0.4–0.8 cm^3 and performed twice per week. EG7 lysate were prepared by three times freeze/thaw cycles (-140°C/37°C) in PBS, with removal of cell debris by centrifugation at 6,000 g for 10 min.[53] To deplete CD8 T cells, mice were i.p. injected with hybridoma ascites of anti-CD8β mAb. The dose of antibody and the treatment regimens were determined in preliminary studies by using the same lots of antibody used for the experiments. Depletion of the desired cell populations by this treatment was confirmed by FACS for the entire duration of the study.

Evaluation of T cell activity in tumor-bearing mice. Draining inguinal LN cells were harvested from tumor-bearing mice after 24 h from the last polyI:C treatment. The activity of T cells was evaluated by CD69 expression and IL-2/IFNγ production. These cells were stained with FITC-CD8α, PE-CD69, PerCP/Cy5.5-7AAD and APC-CD3ε. To check cytokine production, LN cells were cultured for 3 d in vitro in the presence or absence of EG7 lysates and IL-2 and IFNγ productions were determined by Cytokine Beads Array (CBA) assay (BD). To assess the cytotoxic activity of CTL, standard ^{51}Cr release assay was performed. For CTL expansion, 2.5×10^6 LN cells were co-cultured with 1.25×10^5 mitomycin C-treated EG7 cells in the presence of 10 U/ml IL-2 for 5 d. Then, LN cells were incubated with ^{51}Cr-labeled EG7 or C1498 cells for 4 h and determined cytotoxic activity. The cell-specific cytotoxicity was calculated with subtracting the cytotoxity for C1498 from for EG7 cells.

Antigen-specific T cell expansion in vivo. Mice were i.p. immunized with 1 mg of OVA and 150 μg of poly I:C. After 7 d, spleens were homogenized and stained with FITC-CD8α and PE-OVA Tetramer for detecting OVA-specific CD8 T cell populations. For intracellular cytokine detection, splenocytes were cultured with or without 100 nM OVA peptide (SIINFEKL; SL8) for 8 h and 10 μg/ml of Brefeldin A (Sigma-Aldrich) was added to the culture in the last 4 h. Then cells were stained with PE-anti-CD8α and fixed/permeabilized with Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instruction. Then, fixed/permeabilized cells were further stained with APC-anti-IFNγ. Stained cells were analyzed with FACSCalibur (BD Biosciences) and FlowJo software (Tree Star).

In vivo CTL assay. The in vivo CTL assay was performed as described.[54] In brief, WT, TICAM-$1^{-/-}$, MAVS$^{-/-}$ and IRF-$3/7^{-/-}$ mice were i.v. administered with PBS, 10 μg of OVA or OVA with 50 μg of polyI:C. After 5 d, 2×10^7 target cells (see below) were i.v. injected to other irrelevant mice and 8 h later, the OVA-specific cytotoxicity was measured by FACSCalibur. Target cells were 1:1 mixture of 2 μM SL8-pulsed, 5 μM CFSE-labeled splenocytes and SL8-unpulesed, 0.5 μM CFSE-labeled splenocytes. OVA-specific cytotoxicity was calculated with a formula: {1-(Primed [$CFSE^{high}$(%)/$CFSE^{low}$(%)]/Unprimed [$CFSE^{high}$(%)/$CFSE^{low}$(%)]} × 100.

DC preparation. DCs were prepared from spleens of mice, as described previously.[55] In brief, collagenase-digested spleen cells were treated with ACK buffer and then washed with PBS twice. Then splenocytes were positively isolated with anti-CD11c MicroBeads. $CD11c^+$ cells were acquired routinely about ≥ 80% purity. Further, to highly purify $CD8\alpha^+$ and $CD8\alpha^-$ DCs, spleen DC were stained with FITC-CD8α, PE-B220, PE/Cy7-CD11c and PerCP5.5-7AAD. $CD8\alpha^+$ or $CD8\alpha^-$ $CD11c^+B220^-$ DCs were purified on FACSAriaII (BD). The purity of the cells was ≥ 98%.

OT-1 proliferation assay. Ten micrograms of OVA with or without 50 μg of polyI:C were i.v. injected to WT, TICAM-$1^{-/-}$, IPS-$1^{-/-}$ and DKO mice. After 4 h, $CD8\alpha^+$ or $CD8\alpha^-$ DC were purified from the spleen. 2.5×10^4 $CD8\alpha^+$ or $CD8\alpha^-$ DC were co-cultured with 5×10^4 1 μM CFSE-labeled Rag$2^{-/-}$/OT-1 T cells for 3 d in 96-well round bottom plate. These cells were stained with PE-anti-TCR-Vβ5.1,5.2 and APC-anti-CD3ε and T cell proliferation was analyzed by CFSE dilution using FACSCalibur. Additionally, IFNγ in the culture supernatant was measured by CBA assay.

Statistical analysis. P-values were calculated with one-way analysis of variance (ANOVA) with Bonferroni's test. Error bars represent the SD or SEM between samples.

Disclosure of Potenial Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/oncoimmunology/article/19893/

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ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

Tsunamasa Watanabe,[1] Fuminaka Sugauchi,[2] Yasuhito Tanaka,[1] Kentaro Matsuura,[3] Hiroshi Yatsuhashi,[4] Shuko Murakami,[1] Sayuki Iijima,[1] Etsuko Iio,[3] Masaya Sugiyama,[5] Takashi Shimada,[6] Masakazu Kakuni,[6] Michinori Kohara,[7] Masashi Mizokami[5]

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[1]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[2]Department of Gastroenterology, Nagoya City Koseiin Medical Welfare Center, Nagoya, Japan
[3]Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[4]Department of Therapeutic Research, National Hospital Organization (NHO) Nagasaki Medical Center, Nagasaki, Japan
[5]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan
[6]PhoenixBio Co. Ltd., Higashi-Hiroshima, Japan
[7]Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Correspondence to
Dr Masashi Mizokami, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine 1–7–1, Kohnodai, Ichikawa 272–8516, Japan; mmizokami@hospk.ncgm.go.jp

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ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?
- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?
- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?
- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

				Dose			
Donor hepatocytes†	**No of chimeric mice**	**Inoculum**	**Test compound**	**Level (μg/kg)**	**Concentrtion (μg/ml)**	**Volume (ml/kg)**	**Frequency**
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001;

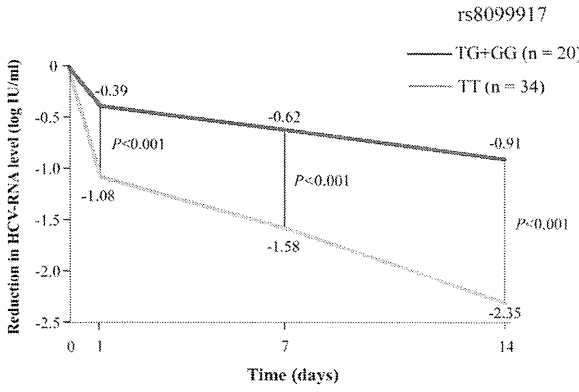

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

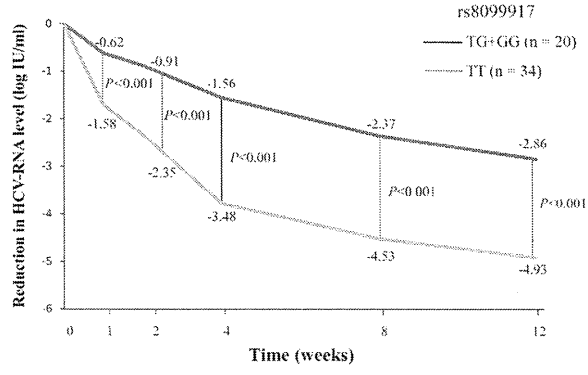

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

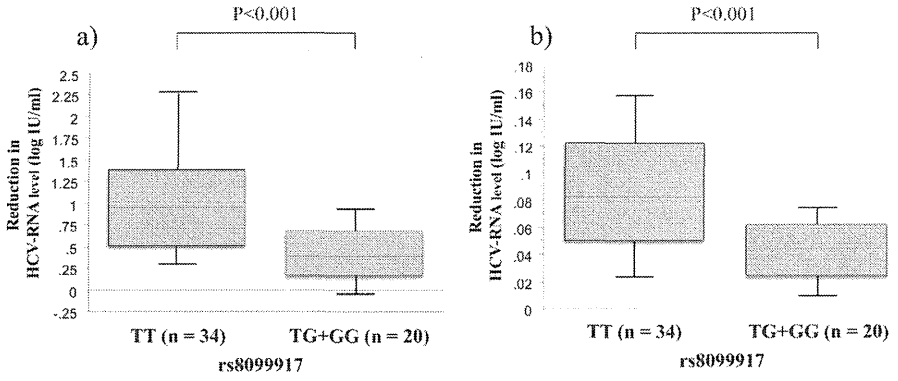

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

−3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94 ±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

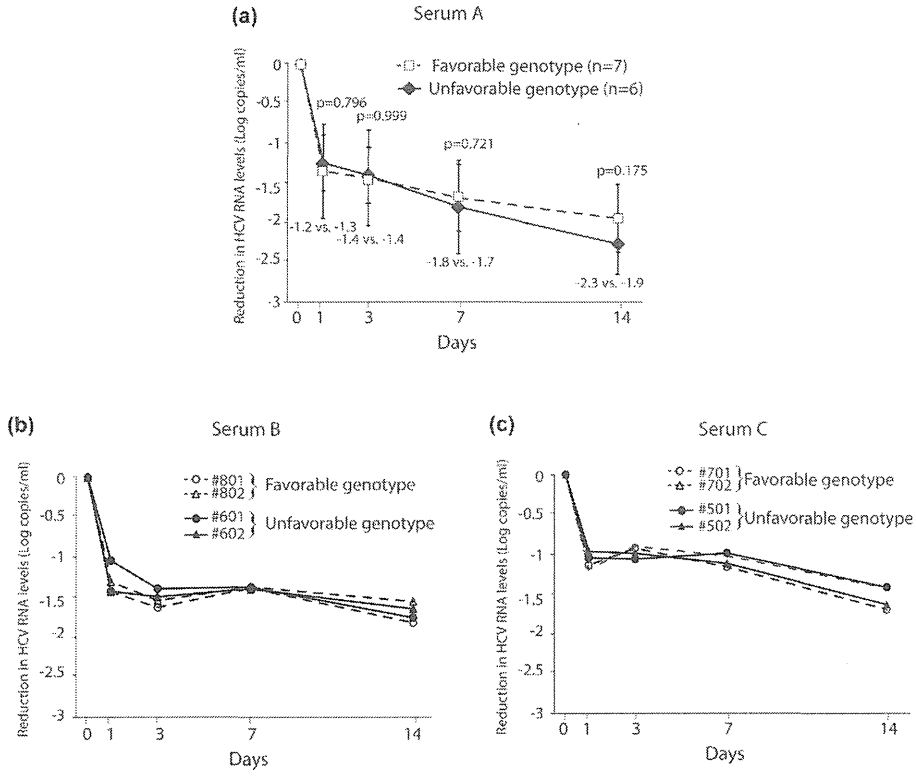

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG

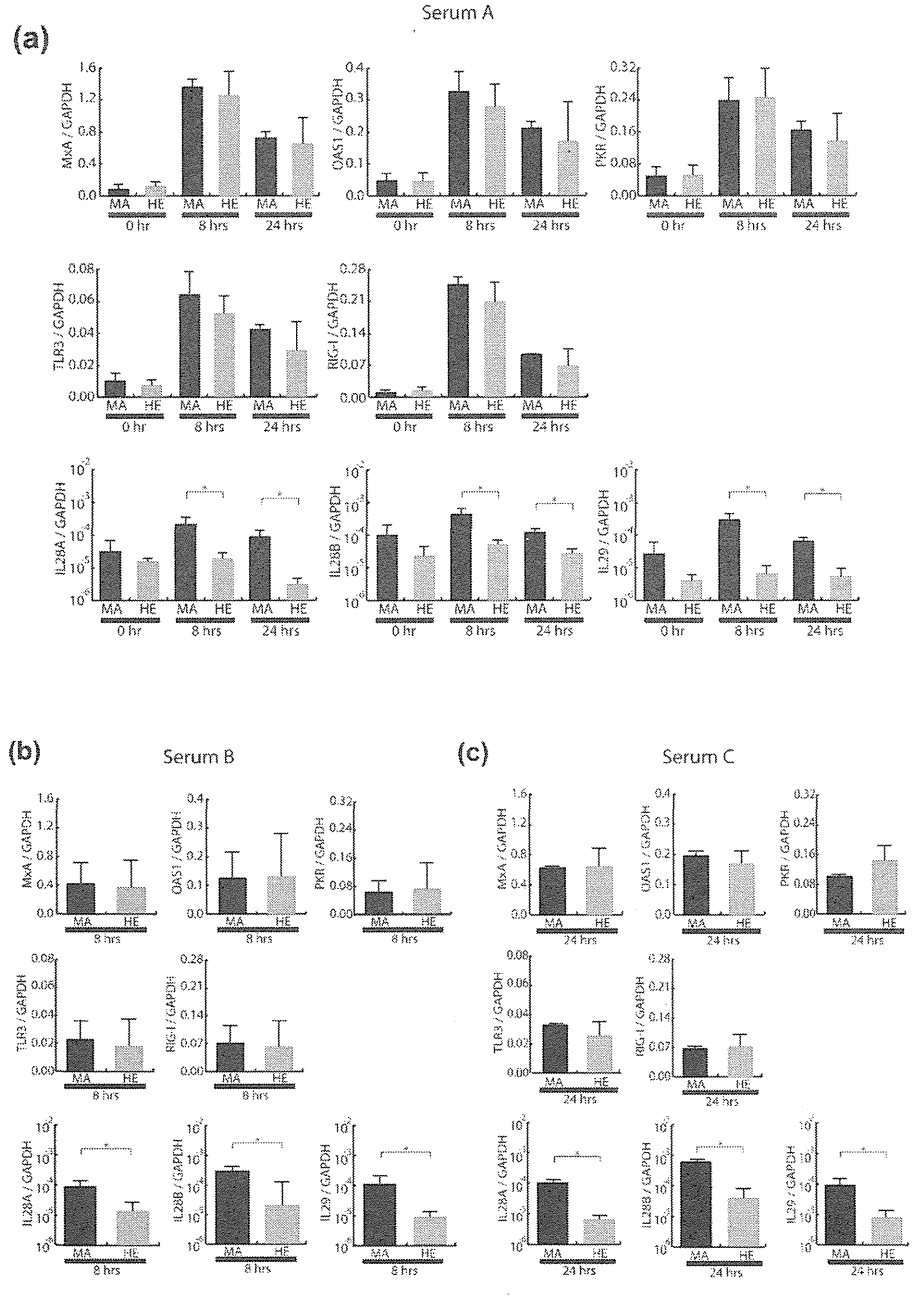

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean+SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable *IL28B* genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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