

**Figure 4. Role of CD4 and CD8 T cells in rVV-N25-treated mice.** (A) Schematic diagram depicts depletion of CD4 and CD8 T cells via treatment with monoclonal antibodies. (B) Comparison of HCV core protein expression in control, CD4-depleted, and CD8-depleted mice 28 days after immunization with LC16m8 or rVV-N25. (C, D) Histological analysis of liver samples from CD4-depleted or CD8-depleted CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice

28 days after immunization with LC16m8 or rVV-N25. The scale bars indicate 100  $\mu\text{m}$  (C) and 50  $\mu\text{m}$  (D). (E) Histological evaluation of steatosis in liver samples from CD4-depleted or CD8-depleted CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice 28 days after immunization with LC16m8 or rVV-N25. Significant relationships are indicated by a P-value. doi:10.1371/journal.pone.0051656.g004

a HCV nonstructural protein. Thus, we focused on the role of the NS2 region as the target for CD8 T cells and generated EL-4 cell lines that expressed the NS2 antigen or the CN2 antigen.

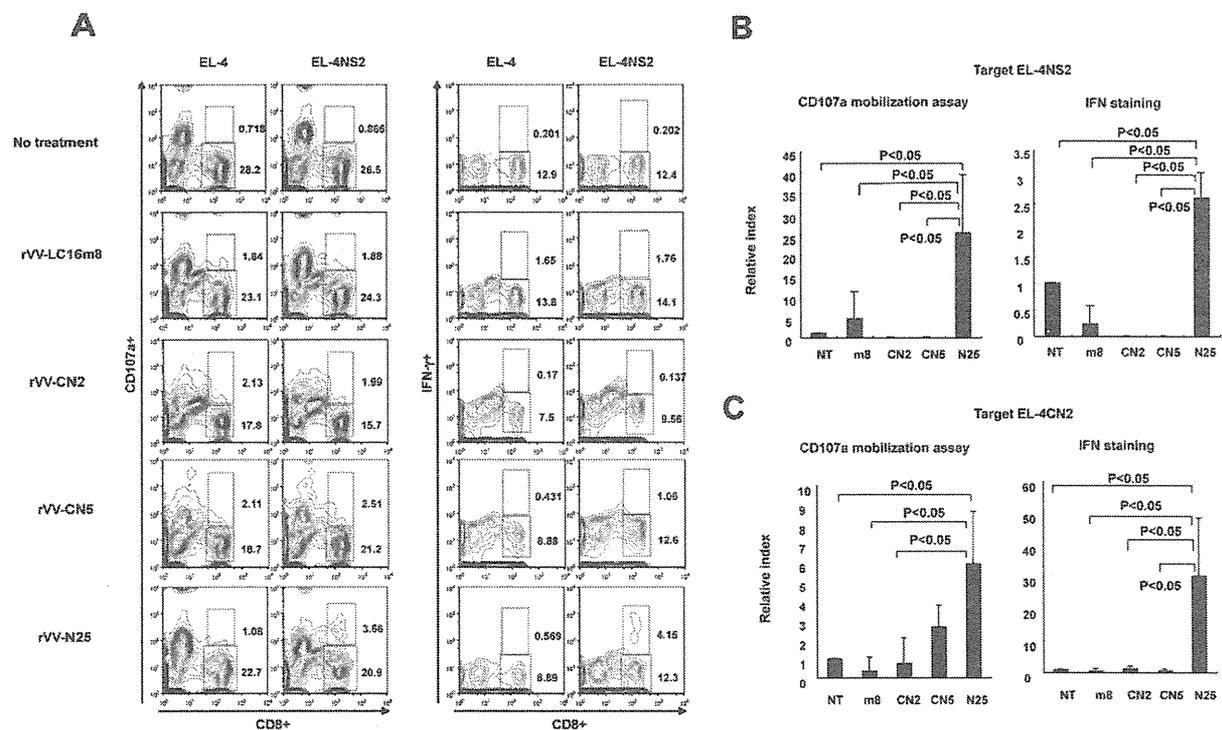
Isolated splenocytes from immunized mice were co-cultured with EL-4CN2 or EL-4NS2 cell lines for 2 weeks and analyzed.

Cytolytic cell activation can be measured using CD107a, a marker of degranulation [15]. The ratio of CD8<sup>+</sup>CD107a<sup>+</sup> cells to all CD8 T cells significantly increased in rVV-N25-treated splenocytes after co-culture with EL-4CN2 or EL-4NS2 ( $P < 0.05$ ), whereas splenocytes that had been treated with any other rVV were not detected (Figure 5A, B and C). These results indicated that rVV-N25 treatment increased the frequency of HCV NS2-specific activated CD8 T cells. Consistent with these results, the ratio of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells to all CD8 T cells for rVV-N25-treated mice was also significantly higher than that for mice treated with any other rVV ( $P < 0.05$ ). Taken together, these findings indicated that rVV-N25 induced an effective CD8 T-cell immune response and that NS2 is an important epitope for CD8 T cells.

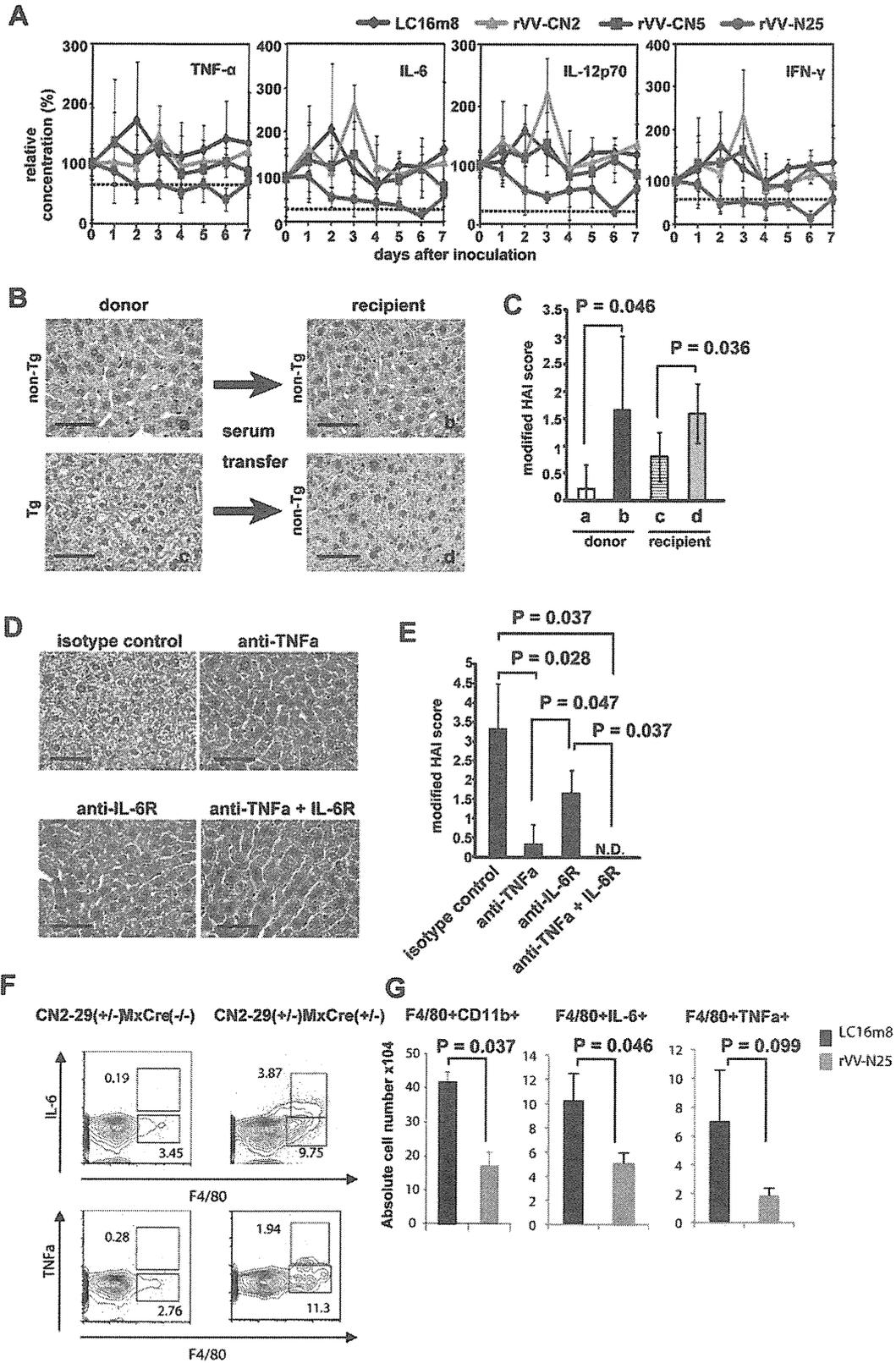
### rVV-N25 Immunization Suppressed Inflammatory Cytokines Production

To determine whether rVV-N25 treatment affected inflammatory cytokine production, we measured serum levels of inflammatory cytokines after rVV immunization. The serum levels of these inflammatory cytokines increased in the CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice (Figure 6A, Figure S5). Immunization with rVV-N25 affected serum levels of inflammatory cytokines in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice and caused a return to the cytokine levels observed in wild-type untreated mice (Figure 6A). In wild-type mice, the cytokine levels remained unchanged after immunization (Figure 6A). These results indicated that inflammatory cytokines were responsible for liver pathogenesis in the transgenic mice.

To test the hypothesis that inflammatory cytokines were responsible for liver pathogenesis in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice, we administered transgenic mouse serum intravenously into nontransgenic mice. We observed the development of chronic hepatitis in the nontransgenic mice within 7 days after the serum transfer (Figures 6B and C). This finding was consistent with the



**Figure 5. Immunization with rVV-N25 induced CD8 T-cell degranulation, a marker for cytotoxicity, and IFN- $\gamma$  production.** (A) The numbers represent the percentage of CD107a positive cells and negative cells (left two columns) and IFN- $\gamma$ -positive cells and negative cells (right two columns). (B, C) The ratio of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells to all CD8 T cells for rVV-N25-treated mice was significantly higher than that for mice treated with any other rVV. Splenocytes ( $4 \times 10^6$  per well) were cultured with EL-4CN2 or EL-4NS2 cell lines in RPMI 1640 complete medium including 3% T-STIM<sup>TM</sup> with ConA for 2 weeks. Harvested cells were incubated for 4 h with EL-4, EL-4CN2, or EL-4NS2 in combination with PE-labeled anti-CD107a mAb and monensin in RPMI 1640 complete medium with 50 IU/mL IL-2, according to the manufacturer's instruction. After incubation, cell suspensions were washed with PBS, and the cells were further stained with APC-labeled anti-IFN- $\gamma$  mAb and Pacific blue-labeled anti-CD8 mAb. Harvested cells were stained with anti-CD107a-PE, anti-IFN- $\gamma$ -APC, or anti-CD8-Pacific blue. Results that are representative of three independent experiments are shown. Significant relationships are indicated by P-value. doi:10.1371/journal.pone.0051656.g005



**Figure 6. Immunization with rVV-N25 suppresses serum inflammatory cytokine levels.** (A) Daily cytokine levels in the serum of CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice during the week following immunization with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5. Values represent means  $\pm$  SD (n = 3) and reflect the concentrations relative to those measured on day 0. The broken lines indicate the baseline data from wild-type mice. In all cases, n = 6 mice per group. (B) Liver sections from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> and CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice. (C) Histology activity index (HAI) scores of liver samples taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup>, or CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice. (D) Liver sections from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice in which TNF- $\alpha$  was neutralized and the IL-6 receptor was blocked. The scale bars indicate 50  $\mu$ m. (E) HAI scores of liver samples taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> in which TNF- $\alpha$  was neutralized and the IL-6 receptor was blocked. Tg and non-Tg indicate CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> and CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup>, respectively. (F) Macrophages were the main producers of TNF- $\alpha$  and IL-6 in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice following poly(I:C) injection. (G) Immunization with rVV-N25 reduced the number of macrophages in liver samples from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice and suppressed TNF- $\alpha$  and IL-6 production from macrophages (Figure 6G). Significant relationships are indicated by a P-value. doi:10.1371/journal.pone.0051656.g006

hypothesis that inflammatory mediators played a key role in inducing hepatitis. Furthermore, to investigate whether TNF- $\alpha$  and IL-6 played particularly critical roles in the pathogenesis of chronic hepatitis in the transgenic mice, we neutralized TNF- $\alpha$  and blocked the IL-6 receptor in the livers of these mice. As expected, chronic hepatitis did not develop in these mice. (Figure 6D and E).

Next, to determine which cell population(s) produced TNF- $\alpha$ , IL-6, or both during continuous HCV expression in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice, we isolated intrahepatic lymphocytes (IHLs) and labeled the macrophages (the F4/80<sup>+</sup> cells) with anti-TNF- $\alpha$  and anti-IL-6 antibodies using an intracellular cytokine detection method. Macrophages in CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice produced small amounts of TNF- $\alpha$  and IL-6, while those in CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice produced much larger amounts of these cytokines (Figure 6F).

Finally, we evaluated whether rVV-N25 treatment affected the number of macrophages, cytokine production by macrophages, or both; specifically, we isolated IHLs from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice 7 days after immunization with rVV-N25 or with LC16m8. The percentage of macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>) among IHLs and IL-6 production from these macrophages were significantly lower in rVV-N25-treated mice than in control mice (Figure 6G). Though the percentage of TNF- $\alpha$ -producing macrophages was not significantly different in rVV-N25-treated and control mice (P = 0.099), rVV-N25 treatment appeared to suppress these macrophages. These results demonstrated that rVV-N25 had a suppressive effect on activated macrophages, and they indicated that this suppression ameliorated the histological indicators of chronic hepatitis.

## Discussion

Various HCV transgenic mouse models have been developed and used to examine immune response to HCV expression and the effects of pathogenic HCV protein on hepatocytes [4,16,17]. However, these transgenic mice develop tolerance to the HCV protein; therefore, examining immune response to HCV protein has been difficult.

To overcome the problem of immune tolerance in mouse models of HCV expression, we developed an HCV model in mice that relies on conditional expression of the core, E1, E2, and NS2 proteins and the Cre/loxP switching system [5,6]; we showed that the injection of an Ad-Cre vector enhanced the frequency of HCV-specific activated CD8 T cells in the liver of these mice and caused liver injury. However, the Ad-Cre adenovirus vector alone causes acute hepatitis in wild-type mice. Nevertheless, the transgenic model was useful for evaluating interactions between the host immune system and viral protein (serum ALT level over 2,000 IU/L) [5]; HCV core protein levels were reduced and expression of this protein was transient (about 2 weeks). Therefore, this Ad-Cre-dependent model cannot be used to effectively investigate immune responses to chronic HCV hepatitis.

Here, we used poly (I:C)-induced expression of Cre recombinase to generate HCV transgenic mice in order to study the effect of HCV protein and confirmed that these mice developed chronic active hepatitis—including steatosis, lipid deposition, and hepatocellular carcinoma. These pathological findings in the transgenic mice were very similar to those in humans with chronic hepatitis C; therefore, this mouse model of HCV may be useful for analyzing the immune response to chronic hepatitis. However, experimental results obtained with this mouse model may not directly translate to clinical findings from patients with HCV infection because the expression of HCV proteins was not liver specific in these mice. Furthermore, poly(I:C) injection can activate innate immune responses and, consequently, might induce temporary liver injury [18]. Additionally, poly(I:C) injection has an adjuvant effect; specifically, it stimulates TLR3 signaling [19].

To evaluate whether poly(I:C) injection caused hepatitis in CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice, we examined serum ALT levels and liver histology following poly(I:C) injection. We found that, following poly(I:C) injection, serum ALT levels in CN2-29<sup>(+/-)</sup>/MxCre<sup>(-/-)</sup> mice increased, reached a peak one day after injection, declined from day 1 to day 6, and were not elevated thereafter; this time-course indicated that poly(I:C) injection alone did not induce continuous liver injury (figure S6). Based on these findings, we believe that the effects of poly(I:C) injection in these mice did not confound our analysis of chronic hepatitis.

Immunization with rVV-N25 suppressed HCV protein levels in the liver, and this suppression was associated with ameliorated pathological chronic hepatitis findings (see Figure 3). Importantly, rVV-N25 treatment did not cause liver injury based on the serum ALT levels; therefore, this treatment was unlikely to have cytopathic effects on infected hepatocytes. These findings provided strong evidence that rVV-N25 treatment effectively halted the progression of chronic hepatitis. Immunization with plasmid DNA or with recombinant vaccinia virus can effectively induce cellular and humoral immune responses and exert a protective effect against challenge with HCV infection [20,21]. However, findings from these previous studies revealed HCV immunization of both uninfected, naïve animals and immune-tolerant animals induced a HCV-specific immune response. In the model describe here; the animals were immune competent for HCV; therefore, our findings provided further important evidence that rVV-N25 was effective in the treatment of chronic hepatitis.

In addition, we demonstrated that rVV-N25 treatment in the absence of CD4 and CD8 T cells had no effect on HCV clearance. This important observation indicated that rVV-N25-induced HCV clearance was mediated by CD4 and CD8 T cells. Many studies have shown that spontaneous viral clearance during acute HCV infection is characterized by a vigorous, broadly reactive CD4 and CD8 T-cell response. [8,22] HCV clearance and hepatocellular cytotoxicity are both mediated by CD8 antigen-specific (cytotoxic T lymphocyte) CTLs [23]. Consistent with these observations, rVV-N25 treatment effectively induced the accumulation of NS2-specific CD8 T cells, which express high levels of

CD107a and IFN- $\gamma$  in the spleen. Notably, even with rVV-N25 immunization, the frequency of activated CD8 T cells was very low, and a minimum of 2-weeks incubation was required to distinguish the difference between rVV treatments. Even if a small population of specific CD8+ T cells played a relevant role in the reduction of core protein, it is difficult to assert that the only NS2-specific CD8+ T cells were important to this reduction. However, based on the results presented in Figure 4B, we are able to conclude that at least CD8+ and/or CD4+ T cells were important to the reduction in HCV core protein. Therefore, to elucidate the mechanism of HCV protein clearance, further investigation of not only the other T cell epitopes but also other immunocompetent cells is required.

Interestingly, rVV-N25 treatment—but not the rVV-CN2 or rVV-CN5 treatment—efficiently induced a HCV-specific activated CD8 T cells response; this difference in efficacy could have one or more possible causes. The HCV structural proteins (core, E1, and E2 proteins) in the rVV-CN construct may cause the difference; Saito et al. reported that injection with plasmid constructs encoding the core protein induced a specific CTL response in BALB/c mice [24]. Reportedly, CTL activity against core or envelope protein is completely absent from transgenic mice immunized with a plasmid encoding the HCV structural proteins, but core-specific CTL activity is present in transgenic mice that were immunized with a plasmid encoding the HCV core [21]. In contrast, when recombinant vaccinia virus expressing different regions of the HCV polyprotein were injected into BALB/c mice, only the HCV core protein markedly suppressed vaccinia-specific CTL responses [25]. Thus, the HCV core protein may have an immunomodulatory function [26]. Based on these reports and our results, we hypothesize that the causes underlying the effectiveness of rVV-N25 treatment were as follows: 1) this rVV construct included the core and envelope proteins and 2) the core protein had an immune-suppressive effect on CTL induction. Therefore, we suggest that exclusion of the core and envelope antigen as immunogen is one important factor in HCV vaccine design.

Interestingly, immunization with rVV-N25 rapidly suppressed the inflammatory response; however, immunization with either of the other rVVs did not (see Figure 6A). This result indicated that rVV-N25 may modulate inflammation via innate immunity, as well as via acquired immunity. Reportedly, Toll-like receptor (TLR)-dependent recognition pathways play a role in the recognition of poxviruses [27]. TLR2 and TLR9 have also been implicated in the recognition of the vaccinia virus [28,29]. These findings indicate that TLR on dendritic cells may modulate the immunosuppressive effect of rVV-N25 in our model of HCV infection; however, further examination of this hypothesis is required. The finding that pathological symptoms in the HCV transgenic mice were completely blocked by intravenous injection of TNF- $\alpha$  and IL-6 neutralizing antibodies indicated that the progression of chronic hepatitis depended on inflammatory cytokines in serum, rather than the HCV protein levels in hepatocytes. Lymphocytes, macrophages, hepatocytes, and adipocytes each produce TNF- $\alpha$  and IL-6 [30,31], and HCV-infected patients have elevated levels of TNF- $\alpha$  and IL-6 [32,33]. Both cytokines also contribute to the maintenance of hepatosteatosis in mice fed a high-fat diet [34], and production of TNF- $\alpha$  and IL-6 is elevated in obese mice due to the low grade inflammatory response that is caused by lipid accumulation [35]. These findings indicate that both cytokines are responsible for HCV-triggered hepatosteatosis, and anti-cytokine neutralization is a potential treatment for chronic hepatitis if antiviral therapy is not successful.

The reduction of macrophages in number might be due to the induction of apoptosis by vaccinia virus *in vitro* infection as

previously reported [36]. To understand the mechanisms responsible for the reduction of the number of macrophage, we performed another experiment to confirm whether the macrophages were infected with vaccinia virus inoculation. However, based on PCR analyses; vaccinia virus DNA was not present in liver tissue that contained macrophages (Figure S7). Furthermore, apoptosis of macrophages was not detected in liver samples (Data not shown). Based on these results, it is unlikely that the reduction in the number of macrophages was due to apoptosis induced by vaccinia virus infection. Although rVV-N25 reduced the number of macrophage, precise mechanism is still unknown. Further examination to elucidate the mechanism is required.

In conclusion, our findings demonstrated that rVV-N25 is a promising candidate for an HCV vaccine therapy. Additionally, the findings of this study indicate that rVV-N25 immunization can be used for prevention of HCV infection and as an antiviral therapy against ongoing HCV infection.

## Materials and Methods

### Ethics Statement

All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care; these guidelines conform to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006. All protocols were approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (Permit Number: 11–078). All efforts were made to minimize the suffering of the animals.

### Animals

R6CN2 HCV cDNA (nt 294–3435) [37] and full genomic HCV cDNA (nt 1–9611) [38,39] were cloned from a blood sample taken from a patient (#R6) with chronic active hepatitis (Text S1). The infectious titer of this blood sample has been previously reported [40]. R6CN2HCV and R6CN5HCV transgenic mice were bred with Mx1-Cre transgenic mice (purchased from Jackson Laboratory) to produce R6CN2HCV-MxCre and R6CN5HCV-MxCre transgenic mice, which were designated CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> and RZCN5-15<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice, respectively. Cre expression in the livers of these mice was induced by intraperitoneal injection of polyinosinic acid–polycytidylic acid [poly(I:C)] (GE Healthcare UK Ltd., Buckinghamshire, England); 300  $\mu$ L of a poly(I:C) solution (1 mg/mL in phosphate-buffered saline [PBS]) was injected three times at 48-h intervals. All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care.

### Histology and Immunohistochemical Staining

Tissue samples were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned (4- $\mu$ m thickness), and stained with hematoxylin and eosin (H&E). Staining with periodic acid–Schiff stain, Azan stain, silver, or Oil-red-O was also performed to visualize glycogen degeneration, fibrillation, reticular fiber degeneration, or lipid degeneration, respectively.

For immunohistochemical staining, unfixed frozen liver sections were fixed in 4% paraformaldehyde for 10 min and then incubated with blocking buffer (1% bovine serum albumin in PBS) for 30 min at room temperature. Subsequently, the sections were incubated with biotinylated mouse anti-HCV core mono-

clonal antibody (5E3) for 2 h at room temperature. After being washed with PBS, the sections were incubated with streptavidin–Alexa Fluor 488 (Invitrogen). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was observed using a confocal laser microscope (Laser scanning microscope 510, Carl Zeiss).

#### Generation of rVVs

The pBR322-based plasmid vector pBMSF7C contained the ATI/p7.5 hybrid promoter within the hemagglutinin gene region of the vaccinia virus, which was reconstructed from the pSFJ1-10 plasmid and pBM vector [41,42]. Separate full-length cDNAs encoding either the HCV structural protein, nonstructural protein, or all HCV proteins were cloned from HCV R6 strain (genotype 1b) RNA by RT-PCR. Each cDNA was inserted into a separate pBMSF7C vector downstream of the pBMSF7C ATI/p7.5 hybrid promoter; the final designation of each recombinant plasmid was pBMSF7C-CN2, pBMSF7C-N25, or pBMSF7C-CN5 (Figure 2). They were then transfected into primary rabbit kidney cells infected with LC16m8 (multiplicity of infection = 10). The virus–cell mixture was harvested 24 h after the initial transfection by scrapping; the mixture was then frozen at  $-80^{\circ}\text{C}$  until use. The hemagglutinin-negative recombinant viruses were cloned as previously described [42] and named rVV-CN2, rVV-N25, or rVV-CN5. Insertion of the HCV protein genes into the LC16m8 genome was confirmed by direct PCR, and expression of each protein from the recombinant viruses was confirmed by western blot analysis. The titers of rVV-CN2, rVV-N25, and rVV-CN5 were determined using a standard plaque assay and RK13 cells.

#### Statistical Analysis

Data are shown as mean  $\pm$  SD. Data were analyzed using the nonparametric Mann–Whitney or Kruskal–Wallis tests or ANOVA as appropriate; GraphPad Prism 5 for Macintosh (GraphPad) was used for all analyses. *P* values  $<0.05$  were considered statistically significant.

#### Supporting Information

**Figure S1 HAI score of liver samples taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice.**

(EPS)

**Figure S2 Lipid degeneration in samples of liver taken from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice.**

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(EPS)

**Figure S3 HCV protein expression after infection of LC16m8, rVV-CN2, rVV-N25, or rVV-CN5 into HepG2 cells.**

(EPS)

**Figure S4 Effects of treatment with rVV-N25 in RZCN5-15<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice.**

(EPS)

**Figure S5 Daily cytokine profiles of the serum from CN2-29<sup>(+/-)</sup>/MxCre<sup>(+/-)</sup> mice during the week following inoculation with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5.**

(EPS)

**Figure S6 The immune response following poly(I:C) injection in the acute phase.**

(EPS)

**Figure S7 Detection of vaccinia virus DNA in the skin, liver, and spleen after inoculation with attenuated vaccinia virus (Lister strain) or highly attenuated vaccinia virus (LC16m8 strain).**

(EPS)

**Table S1 Incidence of hepatocellular carcinoma in male and female transgenic mice at 360, 480, and 600 days after poly(I:C) injection.**

(EPS)

**Text S1 Supporting information including material and methods, and references.**

(DOCX)

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#### Author Contributions

Performed the experiments: SS KK TC Y. Tobita TO FY Y. Tokunaga. Analyzed the data: SS KK TC MK. Contributed reagents/materials/analysis tools: KT-K TW TT MM K. Mizuno YH TH K. Matsushima. Wrote the paper: SS KK MK. Study concept and design: MK.

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## Multi-Step Regulation of Interferon Induction by Hepatitis C Virus

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**Abstract** Acute hepatitis C virus (HCV) infection evokes several distinct innate immune responses in host, but the virus usually propagates by circumventing these responses. Although a replication intermediate double-stranded RNA is produced in infected cells, type I interferon (IFN) induction and immediate cell death are largely blocked in infected cells. In vitro studies suggested that type I and III IFNs are mainly produced in HCV-infected hepatocytes if the MAVS pathway is functional, and dysfunction of this pathway may lead to cellular permissiveness to HCV replication and production. Cellular immunity, including natural killer cell activation and antigen-specific CD8 T-cell proliferation, occurs following innate immune activation in response to HCV, but is often ineffective for eradication of HCV. Constitutive dsRNA stimulation differs in output from type I IFN therapy, which has been an authentic therapy for patients with HCV. Host innate immune responses to HCV RNA/proteins may be associated with progressive hepatic fibrosis and carcinogenesis once persistent HCV infection is established in opposition to the IFN system. Hence, innate RNA sensing exerts pivotal functions against HCV genome replication and host pathogenesis

MAVS has been identified as the adaptor for RIG-I and MDA5 by four independent groups, and then also known as IPS-1, Cardif or VISA (Kawai and Akira 2009). TICAM-1 has been identified as the adaptor for TLR3 and TLR4 by two independent groups, and thus also described as TRIF (Oshiumi et al. 2003). In accordance with the HUGO Gene Nomenclature Committee-approved nomenclature, here we refer to these adaptor molecules as MAVS and TICAM-1, respectively.

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through modulation of the IFN system. Molecules participating in the RIG-I and Toll-like receptor 3 pathways are the main targets for HCV, disabling the anti-viral functions of these IFN-inducing molecules. We discuss the mechanisms that abolish type I and type III IFN production in HCV-infected cells, which may contribute to understanding the mechanism of virus persistence and resistance to the IFN therapy.

**Keywords** Hepatitis C virus · TLR3 · TICAM-1 (TRIF) · MAVS (IPS-1, Cardif, VISA) · Interferon-inducing pathway · Double-stranded RNA

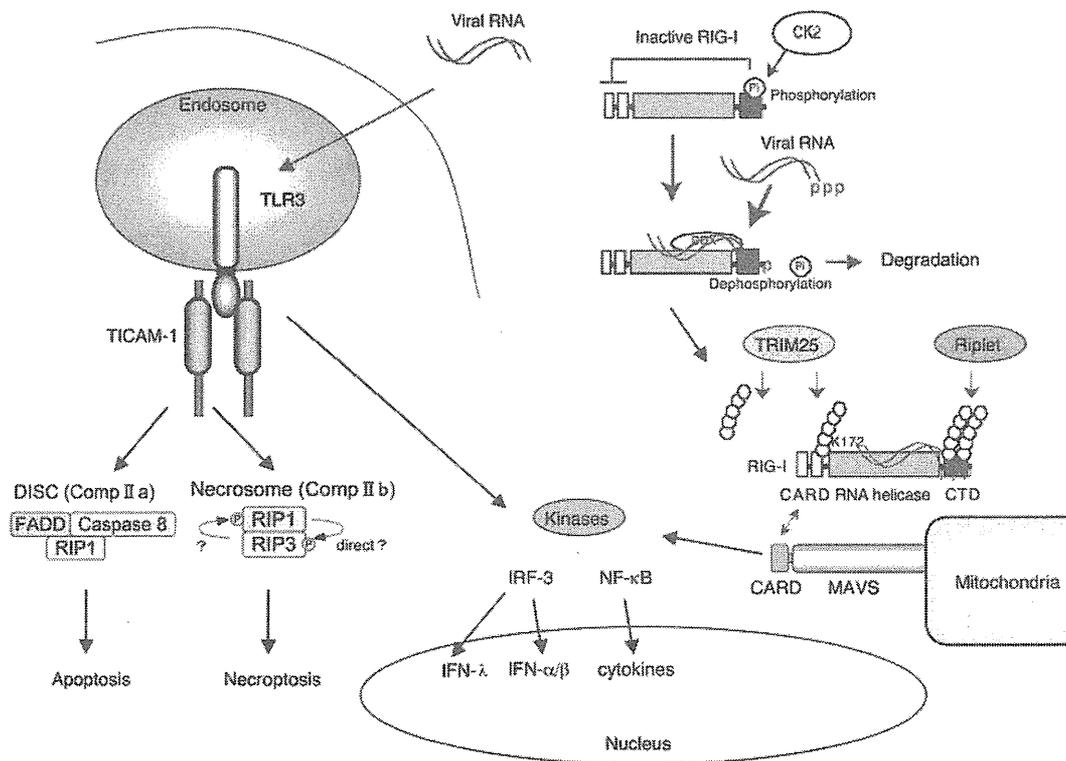
### Abbreviations

BMDC	Bone marrow-derived dendritic cells
CTL	Cytotoxic T lymphocytes
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dsRNA	Double-stranded RNA
IFN	Interferon
LD	Lipid droplet
MAM	Mitochondrial-associated endoplasmic reticulum membranes
MAVS	Mitochondrial antiviral signaling protein
Mφ	Macrophages
mRNA	Messenger RNA
NK	Natural killer
NS	Non-structural
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
STING	Stimulator of IFN genes
TICAM-1	Toll-IL-1-homology domain-containing adaptor molecule-1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	TNF- $\alpha$ receptor 1

## Introduction

Hepatitis C virus (HCV) mainly infects human hepatocytes, and triggers induction of cytokines and type I (IFN- $\alpha/\beta$ ) and type III interferons (IFN- $\lambda$ ) (Fig. 1). Although cells expressing IFN receptors respond to the released IFN and amplify type I IFN production, IFN induction is not always robust in the infected cells due to the fact that HCV proteins inhibit host IFN-inducing pathways. IFN-stimulated genes (ISGs), such as IRF-7, MAP3K14, RIG-I, IRF-2, and IRF-1 are known to inhibit HCV replication (Schoggins et al. 2011). In particular, type III IFNs are more produced than IFN- $\alpha/\beta$  in HCV-infected hepatocytes via the mitochondrial antiviral signaling protein (MAVS) pathway to induce a set of ISGs (Thomas et al. 2012). Cytokines and chemokines are released from infected hepatocytes and myeloid cells in the liver. These mediators affect the formation of inflammatory environments and modify homeostasis of the host cell community, including the recruited bystander cells. Although these scenarios generally reflect the signs of patients with HCV, what

occurs following initial virus entry into host cells remains obscure at the molecular level. HCV genome RNA is internalized via fusion and a portion of 3'-polyU/UC or 5'-triphosphate-short stem RNA acts directly as a ligand for RIG-I (Saito et al. 2008). The HCV genome functions as a messenger (m)RNA for HCV polyprotein production and, at the same time, HCV genome replicates in the cytoplasm (Lindenbach et al. 2007). Double-stranded (ds)RNA accumulating in infected cells is the main pattern molecule (PAMP) and, once liberated, provokes activation of innate immunity in myeloid cells. How host cells sense HCV RNA or dsRNA during infection and replicon transfection has been investigated, and has led to an understanding of the importance of the cytoplasmic RNA recognition pathways (Fig. 1), particularly, the MAVS pathway (Cheng et al. 2006; Li et al. 2005a, b). The current concept is that MAVS signals the kinases, TANK-binding kinase 1 (TBK1) and I $\kappa$ B kinase epsilon (IKK $\epsilon$ ), to phosphorylate IFN regulatory factors (IRF)-3 and IRF-7, resulting in the induction of type I IFN (Kawai and Akira 2009). Likewise, IRF-3 and nuclear factor (NF)- $\kappa$ B appear to participate in



**Fig. 1** Cytoplasmic and endosomal sensors for virus dsRNA in HCV infection. Live signal (*right*) and cell death signal (*left*) in response to viral dsRNA are illustrated. The live signal occurs with stimulation of TLR3 or RIG-I-like receptors and essentially induces activation of NF- $\kappa$ B to support induction of pro-inflammatory cytokines and type I/type III interferons (IFNs) (*right*). This live signal may be amplified by the function of a small amount of IFN- $\beta$  that is required for the

maintenance of homeostasis of the cellular microenvironment. In contrast, death signal occurs with TLR3: caspase 8 is a key molecule for discriminating between apoptosis and necroptosis, and its functional absence sustains the RIP1/RIP3 necrosome signal leading to necroptosis (*left*). Viral dsRNA recognition by RIG-I is induced by RIG-I ubiquitination (*right*). The two modes of K63 polyubiquitination activate RIG-I

the induction of IFN- $\lambda$  (Ding et al. 2012). Because RIG-I-like receptors are IFN-inducible genes, only trace levels are found in resting cells or those in the early stage of virus entry. Thus, how RIG-I/MDA5 captures internalized or replicating virus RNA to evoke an antiviral response in such situations remains unexplained.

There are many reports suggesting that Toll-like receptor 3 (TLR3) participates in the response to HCV dsRNA (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012) (Fig. 1). Most of the relevant studies have been performed with hepatoma cell lines due to the lack of proper systems for reproducing the HCV life cycle in culture as well as the *in vivo* animal model to examine the HCV immune responses. Cell death accompanied with a cytopathic effect is another phenotype of infected hepatocytes (Lim et al. 2012). Hepatocyte death is characterized as apoptosis, but the possible involvement of the pathway with receptor-interacting protein (RIP) kinases in infection-induced cell death has not been strictly ruled out (Fig. 1). Necrosis-like cell death (necroptosis) might cause a source of infectious virions and lead to the pathogenesis of HCV-associated liver damage. Ligands of the death receptor family, including FasL and TRAIL, are likely to associate with hepatocyte death induced by HCV infection (Bantel and Schulze-Osthoff 2003; Saeed et al. 2011; Zhu et al. 2007); however, what triggers the induction of the effector cells is still undetermined. Apart from these cell death family proteins, it is accepted that TLR3 is an activator of the RIP1 pathway (Meylan et al. 2004), which clearly participates in macrophage necroptosis (He et al. 2011). TLR3 is up-regulated in macrophages/dendritic cells (Mf/DC) in an IFN-dependent manner (Tanabe et al. 2003) and recognizes internalized virus dsRNA in the endosome of these phagocytes (Matsumoto et al. 2011). TLR3 has been characterized as an inducer of cellular immune effectors (Matsumoto et al. 2011; Seya and Matsumoto 2009). In accordance with the current dogma, natural killer (NK)-ligand up-regulation or cross-presentation of DCs that occurs with the internalization of dead cell-derived dsRNA may bridge the missing link between HCV dsRNA and TLR3-derived DC maturation (Ebihara et al. 2008).

Dead cells are a source of damage-associated molecular pattern (DAMP) (Kono and Rock 2008). DAMP refers to an intracellular molecule with inflammation-inducing capacities when it is released out of the cell. DAMP does not belong to the cytokine family, but resembles PAMP in its functional properties toward activation of myeloid DCs and macrophages (Kono and Rock 2008). Its function may be associated with physiological responses related to HCV immune response in a broad sense, including regeneration and tumorigenesis. Recently, necrotic or necroptotic cell death has been closely connected with innate immune responses involving pattern sensing (Kono and Rock 2008; Nace et al. 2012).

**Table 1** Sensors for nucleic acid PAMPs and DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids (PAMP)	
Cytosolic long dsRNA	MDA5
Cytosolic 5'-PPP-RNA	RIG-I
Endosomal >140 bp dsRNA	TLR3
Nonmethylated CpG DNA	TLR9
Cytosolic dsDNA	DNA sensors <sup>a</sup>
Self-molecular patterns (DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4 <sup>b</sup>
S100 proteins	RAGE
Self-nucleic acids (DAMP)	
Self-DNA	DNA sensors <sup>a</sup>
Self-mRNA	TLR3

*HMGB1* High-mobility group box 1, *HSPs* heat shock proteins

<sup>a</sup> See Table 2

<sup>b</sup> Scavenger receptors, CD91, etc.

How HCV patterns are sensed and linked to the cellular immunity will be an intriguing issue. DAMP contains a number of cytosolic or nucleic molecules, as in Table 1, and in particular nucleic acids from infected cells. Thus, DAMP and dsRNA of viral origin are extrinsic patterns for sensors to evoke unique features of inflammation during HCV infection.

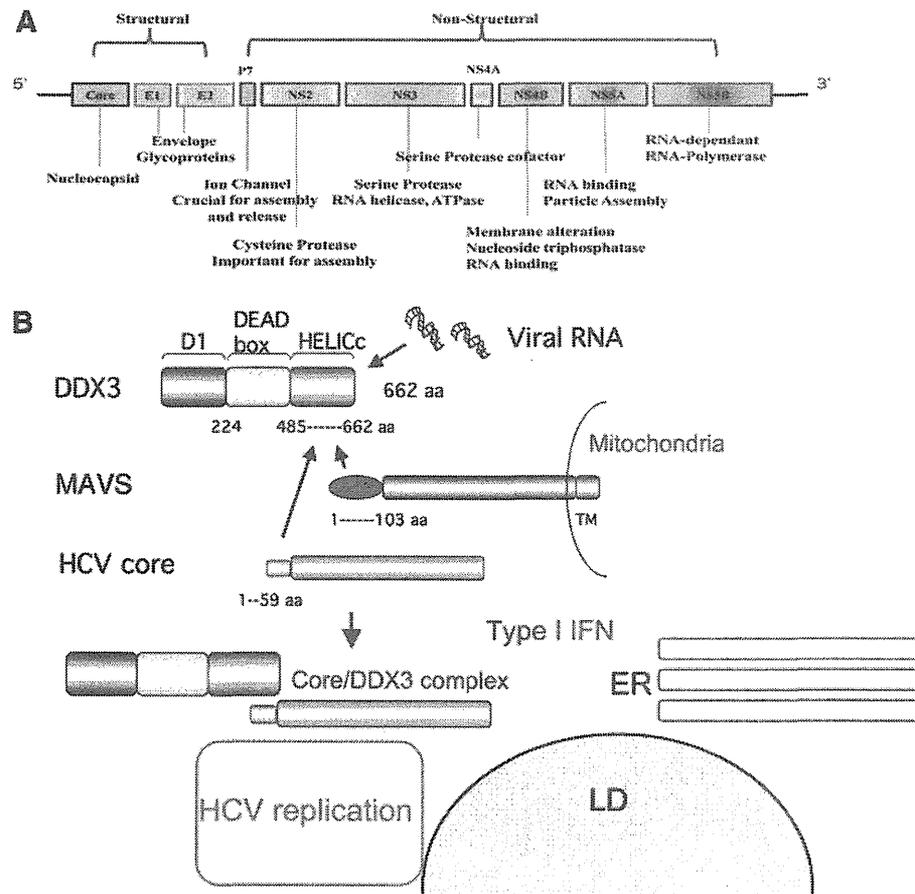
Herein, we discuss the interrelationship between these recent findings on innate immunity and HCV infection.

### Blocking IFN Induction by HCV Proteins

#### Proteolytic Control of the IFN-Inducing Pathways by NS3/4A

HCV genome RNA serves as a single mRNA that encodes ~3,000 amino acids, consisting of 10 virus proteins (Lindenbach et al. 2007). Structural proteins (core, E1, and E2) are situated at the N-terminal region of this polyprotein (Fig. 2a). The HCV polyprotein is first cleaved between 191A and 192Y by signal peptidase to separate the core protein from E1 protein and the core is retained on the endoplasmic reticulum (ER) membrane (McLauchlan et al. 2002). Then, signal peptide peptidase scissions out the core protein by cleavage at 177F and 178L from the ER membrane (Okamoto et al. 2004). E1 and E2 are also released from the remaining structural protein complex by the proteolytic function of signal peptidase (McLauchlan et al. 2002). Non-structural proteins of HCV are fragmented into functional units by NS2 and NS3/4A proteases. Hence, the release of the structural proteins precedes the mature

**Fig. 2** Two different functions of HCV core protein. HCV genome and the functions of each HCV protein. HCV core is first clipped out from the polyprotein of HCV, and later NS proteins are generated (a). HCV core protein retracts DDX3 from the MAVS–DDX3 signal complex on the mitochondria (b). DDX3 usually couples with MAVS on mitochondria and directly binds overwhelmed virus dsRNA in virus-infected cells. When the HCV core protein is produced, DDX3 binds core protein with high affinity and moves from the mitochondria to the HCV replication apparatus, where the core is recruited. The HCV replication apparatus is situated near the lipid droplet (LD) in ER. DDX3 supports HCV replication in the apparatus



processing of non-structural (NS) proteins during the HCV polyprotein processing (Lindenbach et al. 2007). Notably, two structural proteins, core and E2, exhibit regulatory functions against type I IFN induction (Florentin et al. 2012; Mulhern and Bowie 2010).

NS3/4A protease is reported to be crucial, not only for the liberation of HCV NS proteins, but also for the regulation of host anti-viral reactions by proteolytic inactivation of host cytosolic proteins, which also interfere with homeostasis of live cells. It has been reported that NS3/4A proteolytically degrades MAVS (Cheng et al. 2006; Li et al. 2005b; Loo et al. 2006). In addition, NS4B protein has been reported to target STING to repress RIG-I-mediated type I IFN induction in hepatocytes (Nitta et al. 2012). Preceding the generation of these NS proteins, HCV core (Oshiumi et al. 2010a) and E2 proteins (Florentin et al. 2012) can suppress RIG-I-mediated type I IFN production in hepatocytes and plasmacytoid DC, respectively. In particular, the generation of core protein and NS3/4A are closely associated with suppression of the HCV-mediated host IFN biological response and the promotion of HCV replication. Since core protein is produced prior to NS3/4A in HCV-infected cells, many other functions of the core are

expressed just before the proteolytic processing of HCV NS proteins within the cells.

NS3/4A cleaves MAVS (Cheng et al. 2006; Li et al. 2005b; Oshiumi et al. 2010a) and TICAM-1 (Li et al. 2005a). Hence, NS3/4A proteolytically controls at least two adaptor proteins as its substrates. In addition, Riplet is reduced in response to HCV replication (Oshiumi et al. 2010c). Whether or not Riplet is a substrate for NS3/4A is still under investigation.

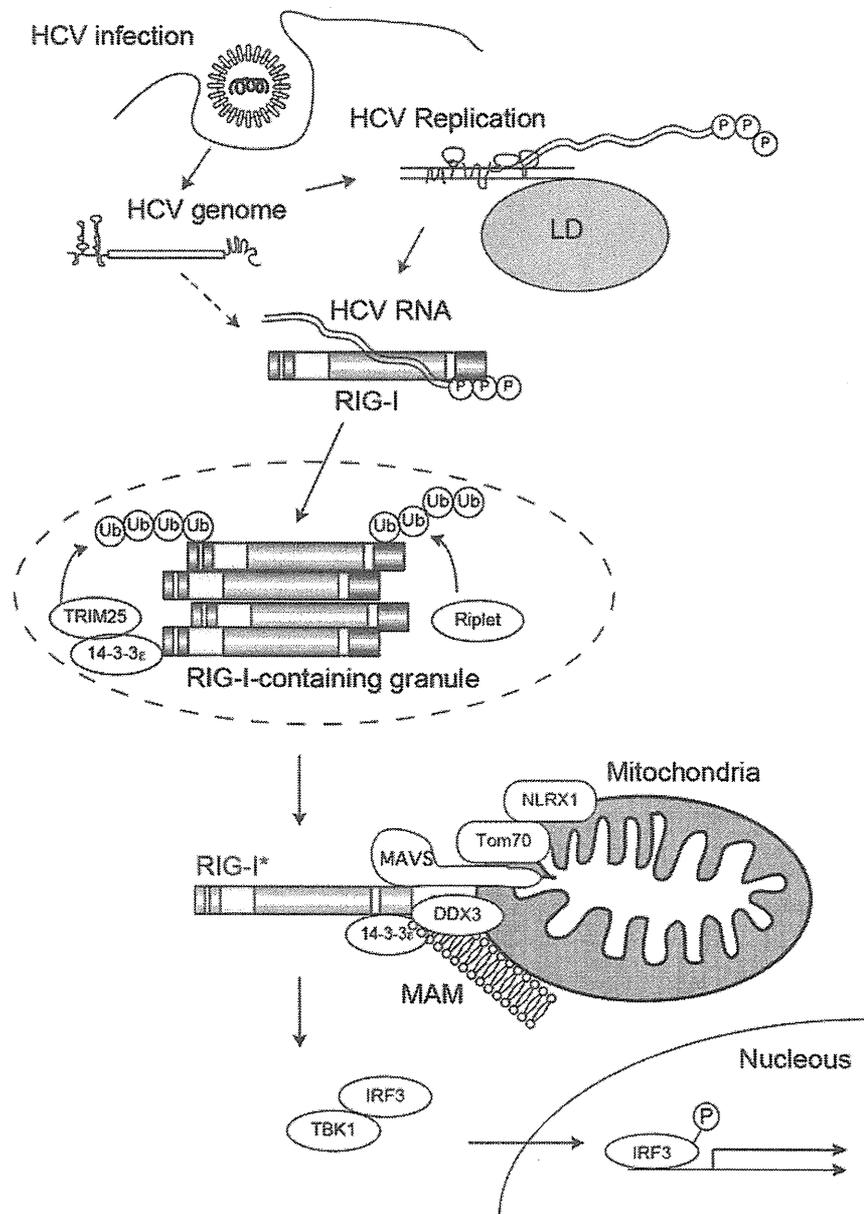
#### MAVS Inactivation by NS3/4A

The current assumption is that the RIG-I family proteins, RIG-I and MDA5, sense viral RNA to induce type I IFN and pro-inflammatory cytokines, which in turn suppress viral infection. RIG-I and MDA5 possess the N-terminal caspase activation and recruitment domain (CARD), the central DExD/H-box helicase domain, and the C-terminal RNA-binding domain (CTD) (Fig. 1). According to crystal structure analysis, the basic region of CTD binds virus dsRNA irrespective of the presence of 5'-triphosphate (Yoneyama et al. 2004), while the CARD domain participates in interaction with the adaptor.

RIG-I recognizes HCV RNA, as well as its replication intermediate, dsRNA (Saito et al. 2008). Although MDA5 recognizes long dsRNA patterns, the role of MDA5 in HCV RNA recognition is unknown. MAVS is the key adaptor for RIG-I/MDA5-mediated IFN induction in HCV infection, although it is localized in the mitochondrial outer membrane apart from intact RIG-I molecules (Seth et al. 2005). RIG-I is not quantitatively sufficient at the protein level to capture the abundant dsRNA replicating in infected cells during the early stage; other molecules have to accept the overwhelmed dsRNA in other cytoplasmic regions (Oshiumi et al. 2010b). RIG-I is initially involved in a

molecular complex (RIG-I granule), which contains many other molecules that make up a nucleocapture complex. E3 ubiquitin ligases are involved in the RIG-I granule. Ubiquitin ligases, TRIM25 (Gack et al. 2007) and Riplet (Oshiumi et al. 2009), are also situated in the RIG-I granule together with RIG-I and confer RNA-binding capacity on RIG-I through RIG-I ubiquitination (Fig. 1). TRIM25 ubiquitinates N-terminal lysines of RIG-I (Gack et al. 2007), while Riplet ubiquitinates C-terminal lysines of RIG-I (Oshiumi et al. 2009), either or both of these molecules enable RIG-I to interact with MAVS and confer mobility on mitochondria (Fig. 3). A recent report

**Fig. 3** Translocation of RIG-I from the cytoplasm to the mitochondria. RIG-I is diffusely distributed in the cytoplasm. When minute quantities of dsRNA enter the cytoplasm (dashed line), the RIG-I granule is formed with many other molecules to sense dsRNA. Once RIG-I molecules are polyubiquitinated, they form a complex with dsRNA and becomes mobile (RIG-I\*). RIG-I\* is recruited to the mitochondria to couple with MAVS. There are many other molecules associated with mitochondrial signaling. Because DDX3 captures overwhelmed dsRNA, the RIG-I-DDX3-MAVS complex allows robust IFN production in conjunction with dsRNA/DDX3. Whether DDX3 participates in IFN- $\lambda$  induction remains undetermined



speculated that after RIG-I is up-regulated and ubiquitinated in the RIG-I granule, the 14-3-3 $\epsilon$  is coupled with newly ubiquitinated RIG-I (Liu et al. 2012). Then, RIG-I moves from the granule to the mitochondrial membrane, a distinct membrane compartment linked to the ER, which is referred to as mitochondrial-associated endoplasmic reticulum membranes (MAM) (Horner et al. 2011). MAM accumulates MAVS and may coordinate MAVS signaling of innate immunity from peroxisomes (Dixit et al. 2010) and mitochondria (Seth et al. 2005), while MAVS localized to MAM serves as a molecular platform for the IFN-inducing signal. MAVS is constitutively complexed with DDX3, which serves as an acceptor of dsRNA in resting (RIG-I-insufficient) cells (Oshiumi et al. 2010b). If this is the case, the location for RIG-I ubiquitination (i.e., RIG-I granule) may differ from the site at which RIG-I interacts with the MAVS–DDX3 complex for signaling. Validating this issue will be of great interest in understanding initial viral RNA recognition.

Our previous data suggested that three forms of MAVS are detected in HCV-replicating hepatocyte lines by imaging analysis, as follows: intact MAVS, sequestered MAVS, and proteolytically liberated MAVS (Oshiumi et al. 2010a). These forms of MAVS simultaneously exist in hepatocytes expressing the HCV replicon or those infected with HCV. The MAVS proteolytically released from mitochondria appear to have decreased ability to activate IRF-3. MAVS is also diminished in some HCV-infected cells to lose its IFN-inducing function (Oshiumi et al. 2010a), suggesting that NS3/4A is a protease that determines the inactivation state of MAVS in HCV-replicating hepatocytes. A recent report suggested that Riplet is depleted during HCV replication (Oshiumi et al. 2010c),

indicating the possibility that participation of the expressed NS3/4A protease in degrading other molecules upstream of MAVS is more important for IFN regulation than clipping out of MAVS in infected cells (Fig. 1). Similarly, other factors independent of proteolytic control may be critical for dsRNA-mediated IFN inducibility, as demonstrated by Cheng et al. (2006).

#### Blocking of the DDX3-Augmented IFN Production by Core Protein

Three reports have independently showed that DEAD/H Box 3 (DDX3, also known as DBX) acts as a positive regulator for MAVS-mediated type I IFN induction (Table 2; Fig. 2b). Elevation of MAVS pathway-mediated type I IFN production by DDX3 is modally different in these three reports (Mulhern and Bowie 2010). Like RIG-I and MDA5, DDX3 is a member of the DExD/H-box family of RNA helicases and is ubiquitously expressed in a variety of cells (Kim et al. 2001). The DExD/H motif of the members in this family of proteins is predictive of a role in RNA-binding and RNA-dependent cellular processing (Schroder 2009). Schroder et al. (2008) showed that the vaccinia virus protein K7 binds DDX3 and inhibits pattern-recognition receptors-induced IFN- $\beta$  promoter activation. They suggested that DDX3 interacts with IKK $\epsilon$  to enhance IRF-3 activation, while K7 counters DDX3 activity of MAVS-mediated IFN- $\beta$  induction. This IFN-enhancing function of DDX3 in IRF-3 activation is located at the N-terminus of DDX3, which is the same region of the protein targeted by K7 for IRF-3 inhibition. Structure analysis of K7 complexed with a peptide from the N-terminus of DDX3 (Oda et al. 2009) has confirmed this finding.

**Table 2** Nucleic acid sensors related to IFN induction in innate immunity

Pattern-recognition receptors	Adaptors	Agonists (references)	Origin
MDA5	MAVS	Cytosolic long dsRNA (Yoneyama et al. 2008)	RNA viruses
RIG-I	MAVS	Cytosolic 5'-PPP-RNA (Yoneyama et al. 2008)	RNA viruses
NOD2	MAVS	Cytosolic ssRNA (Morosky et al. 2011)	RNA viruses
TLR3	TICAM-1	Endosomal >140 bp dsRNA viruses, host (Jelinek et al. 2011)	DNA/RNA viruses
TLR7/8	MyD88	Endosomal ssRNA (Uematsu and Akira 2007)	RNA viruses, bacteria
TLR9	MyD88	Nonmethylated CpG DNA (Uematsu and Akira 2007)	RNA viruses, bacteria
DDX3	MAVS	dsRNA, ssRNA (Oshiumi et al. 2013; this review)	Viruses, host
DDX1/21, DHX36	TICAM-1	dsRNA (Rathinam and Fitzgerald 2011)	Viruses?
DDX60	MAVS	dsRNA, ssRNA, dsDNA (Oshiumi et al. 2013; this review)	Viruses, host
DHX9/DHX36 MyD88	STING	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DDX41	TBK1	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DAI (ZBP1)	STING	dsDNA (Takaoka and Taniguchi 2008)	DNA viruses
IFI16	$\beta$ -Catenin	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
LRRFIP1		dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses

ssRNA single-stranded RNA

The second study indicated that DDX3 constitutively interacts with MAVS via the C-terminal region of DDX3 (Oshiumi et al. 2010b). The binding of DDX3 to MAVS is constitutive and not through the N-terminus, in contrast to the case of the virus-dependent interaction between DDX3 and IKK $\epsilon$  (Schroder et al. 2008). RIG-I-induced IFN- $\beta$  promoter reporter gene activity is inhibited by DDX3 small interfering RNA and enhanced by overexpression of DDX3 (Oshiumi et al. 2010b). Thus, DDX3 synergistically activates the IFN- $\beta$  promoter together with MAVS. The C- and N-terminal regions of the DDX3 regulate MAVS-mediated IFN induction (Hogbom et al. 2007) (Fig. 1).

In contrast, Soulat et al. (2008) demonstrated a positive role for DDX3 in IFN- $\beta$  promoter induction in another distinct manner. Specifically, DDX3 is shown to be a kinase substrate for TBK1 to synergistically enhance IFN- $\beta$  promoter activation by TBK1. Furthermore, they demonstrated that DDX3 is recruited to the IFN- $\beta$  promoter via its N-terminal region (Soulat et al. 2008). Together, these findings show that DDX3 is a positive regulator targeting the multiple sites of the RLR-induced IFN pathway. A role for DDX3 in cell cycle control and apoptosis has also been proposed in response to dsRNA (Schroder et al. 2008).

DDX3 facilitates viral replication in a variety of viruses, including HCV. The N-terminus of HCV core protein binds the C-terminus of DDX3 (Owsianka and Patel 1999). According to a recent finding, the HCV core protein participates in the suppression of DDX3-augmented MAVS signaling for IFN- $\beta$  induction (Fig. 2b), which may also be related to the function of DDX3 described in the second study mentioned before (Oshiumi et al. 2010b). Unlike the DExD/H-box helicases, such as RIG-I and MDA5, DDX3 is constitutively expressed and co-localized with MAVS around mitochondria (Table 2). However, HCV core protein interferes with DDX3 function to enhance MAVS signaling by coupling with DDX3 to dissociate it from MAVS in the mitochondria. In hepatocytes with the HCV replicon, DDX3/MAVS-enhanced IFN- $\beta$ -induction is largely abrogated, even when DDX3 is co-expressed. Whether DDX3 enhances IFN- $\lambda$  induction like RIG-I remain untested. DDX3 is spotted with minimal merging with MAVS in confocal analysis, and partly assembles in the HCV core protein located near the lipid droplet (LD) in replicon-positive hepatocytes, although in some cells MAVS is diminished or disseminated apart from mitochondria. Thus, HCV core retracts DDX3 from MAM, where RIG-I moves from the RIG-I granule to assemble together with MAVS (Figs. 2b, 3).

Our consensus is that the binding of HCV core protein to DDX3 and suppressing DDX3–MAVS complex formation are crucial for inhibition of the MAVS pathway. However, multiple functions of DDX3 may be reflected in other functional aspects of core protein; specifically, the DDX3–

core interaction is required for HCV replication (Ariumi et al. 2007). Although DDX3 promotes efficient HCV infection by accelerating HCV RNA replication, the processes appear independent of its interaction with the viral core protein (Angus et al. 2010). In addition, the association between DDX3 and core protein implicates DDX3 in HCV-related hepatocellular carcinoma progression (Chang et al. 2006). Based on its core protein association and MAVS-regulatory properties, DDX3 appears to be switched by the core protein from an HCV-suppressing (i.e., IFN-inducing) mode to an HCV replication-supporting mode (Fig. 2b). The results enable us to conclude that HCV infection is promoted by modulating the dual function of DDX3.

Evidence is accumulating that HCV assesses many steps in the IFN-inducing pathway throughout the early and late infection stages, and suppresses IFN production by multiple means. Disruption of MAVS function by NS3/4A and core protein may be crucial in HCV-infected Huh7.5 cells, even though the cells harbor dysfunctional RIG-I (Binder et al. 2007). Type I IFN suppresses tumor progression by causing expression of p53 and other tumor-suppressing agents (Takaoka et al. 2005). E2 and NS4B may affect tumor progression by controlling type III IFN induction. These unique features of the HCV proteins require further confirmation and should be in the focus of investigation regarding HCV persistence, chronic infection, and progression to cirrhosis and carcinoma.

#### Inactivation of the TICAM-1 Pathway by NS3/4A

TICAM-1 pathway has been associated with chemokine production, apoptosis, necroptosis, and IL-12p40 production in hepatoma cell lines expressing TLR3 (Li et al. 2012); however, such immune responses are predominantly absent in primary cultured cells. This might be explained by the TLR3 signaling that is likely to be shut off in most normal hepatocytes, but executed in hepatocytes of the infectious liver during chronic states of HCV infection or exposure to dsRNA stimulation. Despite the constitutive expression of the adaptor molecule TICAM-1 in human hepatocytes, only trace amount of TLR3 is being expressed in comparison to RIG-I that is commonly expressed. This gives us an insight of the role played by other cytoplasmic dsRNA sensors such as DDX1/DDX21/DHX36 (Table 2), in the activation of TICAM-1 pathway (Zhang et al. 2011). It remains unknown whether these cytoplasmic dsRNA sensors participate in IFN- $\lambda$  induction.

Although there are positive findings supporting the importance of TLR3 in the pathogenesis of HCV infection (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012), the expression level of TLR3 is still a contentious issue. TLR3 protein is undetectable by immunostaining with monoclonal antibody (TLR3.7) against huTLR3 in

uninfected human hepatocytes (Nakamura et al. 2008) or Huh-7 hepatoma cells that are commonly used for propagation of HCV (Wang et al. 2009). Only a few reports have shown evidence that TLR3 protein is weakly detected in resting primary cultured hepatocytes (Wang et al. 2009). On the other hand, several reports have suggested that the TLR3 at the messenger level was observed in cultured hepatocytes and hepatoma cell lines by real-time polymerase chain reaction (Khvalevsky et al. 2007). Since TLR3 expression is partly regulated by p53, mutated p53 in Huh-7 cells as well as other hepatocellular carcinoma cell lines may attribute to the specific lacking of the TLR3 expression. In addition, Tanabe et al. (2003) demonstrated that the lack of TLR3 expression in Huh-7 cells may be due to other transcriptional regulations.

TLR3 expression appears to be up-regulated in cultured hepatocytes in response to polyI:C (Wang et al. 2009). Similarly, TLR3 is up-regulated in hepatocytes of patients with chronic HCV infection or polyI:C-injected mice (McCartney et al. 2009; Nakamura et al. 2008). These results lead to the assumption that TLR3 can be up-regulated in hepatocytes in an infectious milieu in response to the produced dsRNA or liberated type I IFN by HCV-infected hepatocytes via the RIG-I-mediated IFN-inducing pathway. A similar mechanism of secondary induction of the TLR3 expression by type I IFN may as well occur in hepatoma cells. Thus, HCV infection or malignant transformation allows hepatocytes to turn TLR3 positive, enabling the activation of the TICAM-1 pathway by external virus dsRNA. This would explain the finding that TLR3 is highly expressed in biliary epithelial cells and certain hepatoma cell lines (Harada et al. 2007; Nakamura et al. 2008). Li et al. (2012) showed that TLR3 senses HCV infection and induces ISG expression when TLR3 is over-expressed artificially in the Huh7.5 cells that are deficient in RIG-I signaling. They demonstrated that HCV replication is partially restricted when the cells are infected at a low multiplicity. However, such protective effect is dismissive when the infection is overwhelmed at high multiplicity partly due to the limitation of the TLR3.

TLR3 has a distinct feature from RIG-I as TLR3 can potentially sense viral dsRNA released into the extracellular environment by other cells. Considering the fact that NS3/4A protease that interferes with the host anti-viral reactions is only expressed within infected cells, TLR3-mediated immune responses might be triggered in the uninfected hepatocytes or other cell types. On top of that, it has been reported that TICAM-1 is also a substrate for NS3/4A protease in hepatoma cell lines (Li et al. 2005a). Ferreón et al. (2005), has confirmed this in the corollary biochemical studies. TICAM-1 and its signal pathway are intact within the cells around infected hepatocytes

(Shimoda et al. 2011). This may contribute to the IFN responses observed in some patients.

TLR3 is highly expressed in biliary epithelial cells where biliary atresia occurs in response to the interaction between TLR3-stimulated monocytes and liver NK cells (Harada et al. 2007; Shimoda et al. 2008, 2011). However, it remains intriguing whether the up-regulated TRAIL or FasL in NK cells resulted from the IFN-signaling (Estornes et al. 2012) are responsible for the induction of cell death in hepatocytes. Nevertheless, TICAM-1-mediated cell death in some HCV-infected hepatocytes is also likely to occur via autophagy or *trans*-acting of dsRNA generated by HCV replication.

### DAMP and dsRNA in HCV Pathology

Live or death signals are usually raised by viral dsRNA in virus-infected cells. Type I/type III IFNs and proinflammatory cytokines (IL-6, IL-12, TNF- $\alpha$  etc.) are liberated through IRF-3/7 and NF- $\kappa$ B activation as the output from virus-infected cells that are alive. In contrast, DAMP and cytoplasmic cytokines converted to active forms by caspase 1 eventually result from activation of inflammasome and often links to cell death events (Yeretssian 2012). TLR, Nod-like receptor and other cytosolic nucleic acid sensors are closely associated with PAMP/DAMP recognition (Table 2), therefore inflammation states are fundamentally modified by these factors (Bortolucci and Medzhitov 2010).

Replication of virus RNA allows hepatocytes to induce type III IFN, IL-7 and chemokines (Apolinario et al. 2005; Zeremski et al. 2007). HCV also regulates production of chemokines (Sillanpaa et al. 2008) and type III IFNs (Thomas et al. 2012) by infected cells. Polymorphisms around the IL-28B gene have been associated with clearance of HCV in human, indicating a role for type III IFNs rather than type I in HCV infection (Thomas et al. 2012), although little is known about the function of type III IFNs in intrinsic antiviral responses. IFNs and IL-7 released from HCV-infected hepatocytes possibly act on myeloid cells and lymphocytes expressing their receptors to induce IFN- $\gamma$  (Sawa et al. 2009). Once type I/type III IFN and IFN- $\gamma$  are systemically distributed, synergistic function of these IFNs allows the systemic cells to produce ISGs including CXCL10 (IP-10) and CCL5 (RANTES) (Larrubia et al. 2008; Zeremski et al. 2007). In addition, dsRNA-induced IL-7 forms a positive amplifying loop with T-cell-derived IFN- $\gamma$  to promote macrophage recruitment and CXCR3 ligand (CXCL10) expression by these macrophages (Andersson et al. 2009). Since CXCR3 is mainly expressed on activated T and NK cells, these cytotoxic effectors gather around the inflammatory nest as well as secondary affected organs. HCV-related extrahepatic

disorders are likely to occur in conjunction with ectopic T-cell immune response (Antonelli et al. 2008, 2009). In addition, these immunological aberrances may be modulated by viral factors. In fact, in mouse models, NS5A expression impairs clearance of other viruses from the liver due to the inhibition of IFN- $\gamma$  production (Kanda et al. 2009). By any means, induction of IFN- $\gamma$  in concert with activation of cellular immunity is a major array for live signal in HCV infection.

Necrosis-like cell death occurs contrarily in a cell type-specific manner as a result of death signal. Tumor necrosis factor (TNF)- $\alpha$  and its receptor, TNFR1, are implicated in this process. The coupling of RIP1 with RIP3, termed a necrosome, is responsible for the switching of apoptosis to necroptosis (Cho et al. 2009; He et al. 2009). Caspase 8 acts as a key protease for blocking the formation of the necrosome; the RIP1/RIP3 complex can assemble only in the absence of functional caspase 8. It has been reported that virus dsRNA often induces apoptosis in infected cells, which is known as cytopathic effect (Lim et al. 2012). TICAM-1 and RIP1 may be involved in the virus-derived apoptosis. Yet, possible involvement of RIG-I/MDA5 in cell death cannot be ruled out in some cases of viral infection (Eksioglu et al. 2011). In HCV-infected hepatocytes, how the TLR3/TICAM-1 pathway is involved in necroptotic inflammation is the next issue to be elucidated with respect to HCV pathogenesis (Fig. 1). HCV dsRNA and DAMP can be liberated from infectious hepatocytes, as well as virus particles. TNF- $\alpha$  and IL-6 are the pro-inflammatory cytokines released during HCV infection. Hence, a characteristic feature of the HCV-infected hepatocytes is that DAMP is released together with viral dsRNA from necroptotic HCV-infected cells to the surrounded environment. These factors stimulate nucleic acid sensors of myeloid DC/Mf in the draining region (Table 2). We expect that necroptosis will be of enormous interest in HCV infection following smoldering inflammation. Because each virus species harbors distinct strategies for escaping the innate dsRNA-sensing system, the physiological role of TLR3-mediated necroptosis should be analyzed in a HCV-specific fashion.

### Cellular Immunity Induced by HCV-Infected Cell Debris

Once DC/Mf responds to these unusual innate stimulators, DAMP and dsRNA, cellular immunity is provoked against HCV Ag with irregular modification by these immune enhancers during HCV infection. NK cells and cytotoxic T lymphocytes (CTL) are known to be driven in myeloid DCs by stimulation with dsRNA, and in fact are the main effectors against HCV-infected hepatocytes based on several different systems (Ebihara et al. 2008; Saeed et al.

2011; Zhu et al. 2007). DCs express NK-activating ligands by recognizing dsRNA to activate NK cells (Ebihara et al. 2007), and cell damage is reported to play a role in the regulation of NK-activating ligands (Wen et al. 2008), thereby dsRNA and DAMP are involved in the elimination of HCV-infected cells. Subsequently, DCs cross-prime CD8 CTLs through incorporation of dsRNA and HCV Ag-mounted cell debris (Jin et al. 2007). FasL and TRAIL are major effectors for the ligands of death receptors. HCV-infected cells will be eliminated if the cells express high levels of MHC class I with HCV antigen.

Our laboratory has reported that a dsRNA analog (polyI:C) has strong ability to activate NK cells in vivo (Akazawa et al. 2007; Matsumoto and Seya 2008). Two main routes for NK cell activation have been reported. First, DCs secrete several cytokines, such as IL-12, IL-18, IL-15 and IFN- $\alpha/\beta$  in response to dsRNA, and these mediators act on NK cells (Lucas et al. 2007; Matsumoto et al. 2011). Second, DCs express NK-activating ligands on their cell surface and the ligands make a balance shift to activation of NK cells through cell-cell contact (Ebihara et al. 2010). In mouse studies, IL-15 and cell-surface NK-activating ligands are crucial in polyI:C-mediated NK cell activation (Ebihara et al. 2010; Lucas et al. 2007). A main NK-activating ligand induced by polyI:C is IRF-3-inducing NK-activating molecule (INAM) (Ebihara et al. 2010). In a human system with bone marrow-derived DCs (BMDC) and HCV-infected debris (a source of dsRNA), NK cells are activated by BMDC via the TLR3-TICAM-1 pathway in BMDC (Ebihara et al. 2008). Thus, INAM may be a factor that participates in HCV-derived NK activation.

However, how dsRNA and DAMP modify the maturation of DC in an infectious milieu to induce CD4 and CD8 T cells is largely unknown because the functional properties of DAMP generated in HCV-infected hepatocytes have not been well documented (Azuma et al. 2012). Once antigens are presented on MHC class II in DCs upon internalization of infectious debris, CD4 T cells (Longhi et al. 2009), including Th1, Th2, Th9, Th17, and Tregs, are driven in a sophisticated manner. In this context, DAMP and dsRNA could act as the second signal of TLRs triggering DCs to induce cross-presentation, which leads to mounting Ag on MHC class I and subsequently induce the proliferation of CD8 T cells (CTL) (Caskey et al. 2011). Furthermore, the so-called innate lymphocytes may respond to intrinsic stimuli in an Ag-independent fashion. Thus, the function of nucleic acid sensors for DAMP and dsRNA in the presentation of exogenous antigen by DCs is an issue to be tackled (Caskey et al. 2011). Cross-presentation is enhanced by molecules, such as type I IFN and CD40, and by immune cells, including CD4 T cells, NK cells, and NKT cells (Matsumoto and Seya 2008). Yet, the role of type III IFN in T-cell cross-priming and innate lymphocyte activation are

yet unknown. TLR3/TICAM-1 is a main pathway for inducing cross-presentation in response to dsRNA in DCs (Azuma et al. 2012). PolyI:C or virus dsRNA is an example of the TLR3 ligand, and their cross-presentation-inducing activity was first described by Schulz et al. (2005). The effective adjuvancy of polyI:C has been subsequently reported by Steinman group (Caskey et al. 2011; Longhi et al. 2009); however, no report has been definitively determined which DAMPs participate in cross-presentation and possess latent cross-priming (CTL-inducing) ability.

Why HCV circumvents the host immune system of both innate and acquired arms of the immune system remains an ambiguous question. Hepatocytes stand with unique properties with lipid droplet (LD) and the bile secretion system, where hepatocytes secrete bile into canaliculi, which flows into choledochus. HCV and hepatitis B virus induce smoldering inflammation, which is believed to be a nest of carcinogenesis. These viruses have no common properties in their viral-side factors, but the host factors including hepatocytes are common bases for triggering inflammation. Thus, the host factors are undoubtedly critical in inducing infection-driven inflammation and perhaps initiation of tumor progression (Seya et al. 2012). We speculate that persistent HCV infection, followed by inflammation, is caused by the immune aberration involved in HCV infections. The main factors in the innate arm of immunity are DAMP and dsRNA, each of which is reported to associate with smoldering inflammation. However, it is unknown what occurs in the liver if this combined stimulation is constitutively exerted in HCV-infected cells and myeloid cells in the liver. Examining the function of dsRNA and DAMP on chronic HCV infection with increasing studies in innate immunity, inflammation, and cell death, will help us extending our knowledge on vaccine and adjuvants against HCV infection and tumorigenesis. Further molecular analysis will provide a hint for therapeutic strategies for patients who do not respond to IFN therapy.

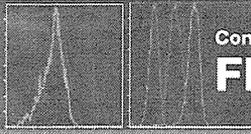
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## Cleaved/Associated TLR3 Represents the Primary Form of the Signaling Receptor

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