Hepatitis C virus replicative double-stranded RNA is a potent interferon inducer that triggers interferon production through MDA5

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The cytoplasmic RNA sensors, retinoic acid-inducible gene I and melanoma differentiation-associated gene 5, play crucial roles in innate sensing of hepatitis C virus (HCV). However, the exact identity of the IFN inducer generated during HCV infection is poorly understood. To identify the IFN inducer, we extracted the RNAs from HCV-replicating cells and introduced these into IFN signalling-competent cells to examine IFN production. RNAs isolated from HCV-replicating cells triggered robust IFN-β and IFN-λ production in Huh7 cells in a viral replication-dependent manner, preferentially through the melanoma differentiation-associated gene 5 but not through the retinoic acid-inducible gene I-mediated pathway. The IFN-inducing capacity of HCV RNA survived after calf intestinal alkaline phosphatase and ssRNA-specific S1 nuclease treatment, but was completely eliminated by dsRNA-specific RNase III digestion, suggesting that viral replicative dsRNA is an IFN inducer. Furthermore, HCV viral RNA extracted from replicating cells was sensitive to 5′-monophosphate-dependent 5′-3′ exonuclease (TER) digestion, suggesting that the HCV genome lacks a 5′-triphosphate or -diphosphate. In semi-permeabilized cells, the HCV IFN inducer primarily resided in an enclosed membranous structure that protects the IFN inducer from RNase digestion. Taken together, we identified HCV replicative dsRNA as a viral IFN inducer enclosed within the viral replication factory.

INTRODUCTION

Hepatitis C virus (HCV) chronically infects approximately 160 million people worldwide and causes hepatocellular carcinoma (Lavanchy, 2011). Approximately 80% of infected patients develop chronic infections and 15% develop acute infections (Choo et al., 1989). The mechanism by which HCV infection leads to these different consequences is not fully understood. Innate immune responses during early viral infection shape the adaptive immune response and the outcome of viral infection (Sullivan et al., 2015). Understanding the mechanisms underlying how the host innate immune system senses HCV and the virus evades host innate recognition could help define the determinants for chronic infection and assist in the development of novel antiviral treatments that evoke innate immunity.

HCV is a member of the family Flaviviridae. Its 9.6 kb positive-sense RNA genome encodes a single polyprotein that is cleaved into at least 10 individual polypeptides. The single ORF is flanked by the conserved 5′ and 3′ UTRs. The internal ribosome entry site in the 5′ UTR starts viral cap-independent translation. The 3′ UTR, involved in RNA replication, is composed of three sequential elements: a non-conserved variable region, a polyU/UC stretch and a conserved 98 nucleotide sequence (see Moradpour et al., 2007, for a review).

The pattern recognition receptors detect pathogen-associated molecular patterns. Pathogen-associated molecular patterns are associated with viruses or are generated during viral infection. The pattern recognition receptors involved in RNA virus sensing include the Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). TLRs engage MyD88 and TRIF, whereas RLRs use MAVS (also called IPS-1, VISA or Cardif) as adaptors to trigger a signalling cascade, resulting in IFN or immune modulatory cytokine production (O’Neill & Bowie, 2007). TLRs recognize viral proteins or RNAs on the cell surface or in intracellular compartments (Jensen & Thomsen, 2012). RLRs, including RIG-I and melanoma differentiation-associated gene 5 (MDA5), sense cytoplasmic viral RNAs.
HCV infection elicits robust IFN production in human, chimpanzee and cultured primary hepatocytes (Marukian et al., 2011; Su et al., 2002; Wieland et al., 2014). RIG-I recognizes the polyU/UC region within the HCV 3' UTR (Saito et al., 2008) and binds to in vitro-transcribed (IVT) viral RNA and viral genomic RNA (Arnaud et al., 2011; Schnell et al., 2012). Recent studies demonstrated that MDA5 plays a critical role in innate sensing of HCV in HepG2 cells reconstituted with miR122 and CD81 (Israelow et al., 2014), in Huh7 cells overexpressing MDA5 (Hiet et al., 2015) and in Huh7 cells (Cao et al., 2015) stably expressing MAVS–C508R that is resistant to the HCV NS3/4A cleavage (Li et al., 2005).

At present, the mechanism underlying how RLRs sense HCV during viral replication, and the determinant of viral components for innate sensing, are unknown. In this study, we established a robust innate sensing system in which RNA derived from HCV-replicating cells was introduced into innate sensing-competent cells to elicit IFN production. Using this system, we found that HCV replication generated a potent IFN inducer; HCV replicative dsRNA was an IFN inducer and probably lacked a 5' -triphosphate or -diphosphate. Furthermore, the HCV IFN inducer resided in an enclosed membranous viral replication factory. Thus, we identified a physiological HCV IFN inducer as the replicative dsRNA shielded in the viral replication factory.

RESULTS

RNA from HCV-replicating cells elicits robust innate immune responses

To identify the IFN inducer of HCV, we used a biochemical strategy wherein RNA from virally infected cells containing a putative viral IFN inducer was introduced into innate sensing-competent cells (sensing cells) to trigger IFN production (Fig. 1a). Similar strategies have been used for influenza virus, Sendai virus and enteroviruses (Feng et al., 2012; Rehwinkel et al., 2010). This strategy bypasses viral protein-mediated innate evasion (Horner, 2014), and helps in the identification and characterization of the physiological IFN inducers produced during genuine viral replication. First, we used vesicular stomatitis virus (VSV) as a proof-of-concept model. We infected Huh7 cells with VSV, extracted total cellular RNAs from the infected cells and transfected the RNAs into naïve Huh7 cells. VSV infection in the Huh7 cells did not elicit obvious IFN production (Fig. 1b–d), probably due to innate immune evasion (Ahmed et al., 2003; von Kobbe et al., 2000). In contrast, transfection of RNA from VSV-infected cells elicited robust production of IFN-β (Fig. 1c) and IL-29, an IFN-α member (Fig. 1d) in the Huh7 cells. To rule out the possibility that the IFN induction was due to either the transfection of cellular RNA or the action of the transfection reagent, we mock-transfected or transfected total cellular RNAs from uninfected cells. The transfection reagent and these RNAs barely elicited IFN production (Fig. 1e–g). Then, we infected Huh7.5 cells with HCV Jc1G (Marukian et al., 2008), extracted RNAs from the infected cells and transfected them into Huh7 and Huh7.5 cells (Fig. 1a). HCV infection did not induce IFN production in the Huh7.5 cells (Fig. 1i, j). Transfection of RNAs from HCV-replicating cells also did not induce IFN production (Fig. 1i, j), probably due to RIG-I deficiency in the Huh7.5 cells (Sumpter et al., 2005). In contrast, transfection of the RNA into Huh7 cells induced robust production of IFN-β (Fig. 1i) and IL-29 (Fig. 1j), although the level of HCV RNA in the transfected cells was much lower than that in the infected cells (Fig. 1h).

We considered that the HCV IFN inducer would be generated during viral replication. We treated Huh7.5 cells harbouring a HCV subgenomic replicon (sgfH1) with the HCV RNA polymerase inhibitor 2CMA. After 2 days’ treatment, we transfected the RNAs from the mock- or 2CMA-treated cells into Huh7 cells. 2CMA treatment abolished viral replication (Fig. 1k). Transfection of RNAs from the mock-treated replicon cells elicited robust IFN-β (Fig. 1l) and IL-29 (Fig. 1m) production, whereas transfection of the RNA from 2CMA-treated cells did not elicit obvious IFN production (Fig. 1l, m). Therefore, RNA derived from HCV-replicating cells elicits robust IFN-β and IFN-α production in Huh7 cells.

HCV RNA is a potent IFN inducer

HCV is considered to be a poor IFN inducer (Arnaud et al., 2011). We compared the IFN stimulation capacity of HCV RNA to VSV RNA. We transfected RNAs from VSV-infected cells or HCV replicon cells in parallel into Huh7 cells at various dosages. We measured IFN production and viral RNA levels in the transfected cells. Transfection of RNAs from VSV-infected cells and HCV replicon cells triggered robust production of both IFN-β (Fig. 2b, e) and IL-29 (Fig. 2c, f). We normalized IFN RNA levels to the transfected viral RNA levels (Fig. 2a, d) to calculate the ‘relative IFN stimulation capacity’ of the individual viral RNAs. Strikingly, the HCV RNA exhibited a much stronger IFN stimulation capacity than the VSV RNA (Fig. 2g, h). Thus, HCV-replicating RNA is a potent IFN inducer.

HCV IFN inducer triggers IFN production through MDA5

Several recent studies have demonstrated that MDA5 plays an important role in sensing HCV in different reconstituted cell lines (Cao et al., 2015; Hiet et al., 2015; Israelow et al., 2014). First, we examined whether the HCV IFN inducer was sensed by MDA5 or RIG-1 in our system. We knocked...
Fig. 1. HCV-replicating RNA elicits IFN production. (a) Schematic representation of the viral RNA (vRNA) sensing system used in this study. Cells were infected with viruses (HCV and VSV) and then total cellular RNAs were extracted. After purification, a portion of the RNA was transfected into the ‘sensing cells’, and IFN production in these cells was measured. (b–d) Transfection of RNA derived from VSV-infected cells elicits IFN production in Huh7 cells. Huh7 cells were mock infected (−) or infected (+) with VSV for 10 h at an m.o.i. of 0.2. Total RNA was extracted from the infected cells and 0.8 µg fraction of the total cellular RNA was transfected into the Huh7 cells. At 10 h post-transfection, the viral RNA levels (b), IFN-β RNA levels (c) and IL-29 RNA levels (d) in the transfected cells were measured by quantitative RT-PCR. (e–g) Huh7 cells were left untreated (blank) or mock transfected with liposome (mock), total cellular RNA from Huh7.5 cells (Huh7.5), Huh7 cells (Huh7) and VSV-infected cells (VSV). At 10 h post-transfection, the viral RNA (e), IFN-β RNA (f) and IL-29 RNA (g) in the transfected cells were measured by quantitative RT-PCR. (h–j) Transfection of RNA derived from HCV-infected cells elicits IFN production in Huh7 cells. Huh7.5 cells were mock infected (−) or infected (+) with Jc1G at an m.o.i. of 1. At 3 days post-infection, total RNA was extracted from the infected cells and 0.8 µg fraction of the total cellular RNA was transfected into Huh7 and Huh7.5 cells. At 10 h post-transfection, the viral RNA levels (h), IFN-β RNA levels (i) and IL-29 RNA levels (j) were measured in the transfected cells. (k–m) Blocking of viral replication abolishes viral RNA-induced IFN production. HCV sub-genomic replicon cells (sgJFH1) were mock treated with carrier (2CMA−) or treated with 2CMA (2CMA+) at a final concentration of 2.5 µM for 2 days. Total cellular RNA was extracted and transfected into Huh7 cells. At 10 h post-transfection, viral RNA levels (k), IFN-β RNA levels (l) and IL-29 RNA levels (m) were measured in the transfected cells. Mean±SD (n=3). From (b) to (m), similar results were obtained from multiple independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

down MDA5 and RIG-I by small interfering RNA (siRNA) in Huh7 cells, and then transfected the cells with RNAs from VSV-infected cells (VSV), HCV replicon cells (HCV) or poliovirus-infected cells (PV). The knockdown (kd) efficiency was confirmed by reduction in RIG-I and MDA5 protein levels (Fig. 3a, d). The RIG-I kd markedly reduced the production of IFN triggered by a RIG-I agonist VSV RNA (Kato et al., 2006), but did not affect HCV RNA-induced IFN production (Fig. 3b, c). Conversely, the MDA5 kd severely reduced the IFN production triggered by an MDA5 agonist PV RNA (Abe et al., 2012) but did not affect VSV RNA-induced IFN production (Fig. 3e, f).
HCV replicative RNA is a potent IFN inducer

Fig. 2. RNA derived from HCV-replicating cells is a potent IFN inducer. Huh7 cells were transfected with various amounts of total RNA from VSV-infected cells (a–c) or sgJFH1 replicon cells (d–f). Total RNA from Huh7 cells was used to supplement RNA to obtain a total amount of 0.8 µg. At 10 h post-transfection, the viral RNA levels (a and d), IFN-β RNA levels (b and e) and IL-29 RNA levels (c and f) were measured in the transfected cells. (g, h) Relative IFN stimulation capacity of RNA from VSV- and HCV-infected cells was determined by normalizing IFN-β and IL-29 RNA levels to viral RNA levels, respectively. Mean±SD (n=3). From (a) to (h) similar results were obtained in two additional independent experiments.

Strikingly, HCV RNA-induced IFN production was diminished in MDA5-kd cells (Fig. 3c, f).

RIG-I plays a role in HCV sensing in reconstituted HepG2 cells (Israelow et al., 2014). We considered whether RIG-I senses the HCV IFN inducer in HepG2 cells. We knocked down MDA5 and RIG-I by siRNA in HepG2 cells (Fig. 3g, h) and then transfected the cells with viral RNAs. Similar to Huh7 cells, RIG-I kd only diminished VSV RNA-induced IFN production whereas MDA5 kd diminished PV RNA- and HCV RNA-induced IFN production (Fig. 3i, j), suggesting that the HCV IFN inducer also activated IFN production through MDA5 in HepG2 cells.

To complement the siRNA-mediated gene kd, we generated RIG-I- and MDA5-deficient Huh7 cells (Fig. 3m) and HepG2 cells (Fig. 3o) by clustered regularly interspaced short palindromic repeat (CRISPR) and then examined viral RNA-induced IFN production in these cells. Similar to siRNA-mediated kd cells, VSV RNA-induced IFN production was impaired in CRISPR-mediated RIG-I-kd cells, but not in MDA5-kd cells (Fig. 3i, n). In contrast, PV RNA- and HCV RNA-induced IFN production was impaired only in MDA5-kd cells (Fig. 3i, n).

The antiviral 2′,5′-oligoadenylate synthetase/RNase L pathway was shown to detect HCV mRNA (Han & Barton, 2002) and was proposed to mediate cleavage of HCV RNA to activate RIG-I (Malathi et al., 2010). RNase L kd did not affect HCV RNA-induced IFN production (data not shown). These data suggest that the HCV IFN inducer identified in our system is sensed by MDA5.

HCV IFN inducer is generated at late time points during viral replication

HCV induced an IFN response through RIG-I at early time points and through MDA5 at late time points (Hiet et al., 2015). As demonstrated above, we found that the IFN inducer triggered IFN production solely through MDA5 in our system. We reasoned that we had missed the RIG-I agonist produced at early time points during viral replication. We infected Huh7.5 cells with Jc1G and harvested cellular RNAs at various time points after infection and then transfected these RNAs into Huh7 and HepG2 cells (Fig. 4c, d) and HepG2 cells (Fig. 4f, g). Additionally, IFN induction was consistent with the levels of transfected HCV RNAs (Fig. 4b, e). We also examined whether the virion-associated viral genome secreted had IFN stimulation capacity. RNAs extracted from polyethylene glycol (PEG)-concentrated secreted virions (Fig. 4h) elicited no obvious IFN production (Fig. 4j, k), although the levels of the transfected RNAs were comparable to those of RNAs from sgJFH1 cells (Fig. 4i). These data suggest that the HCV IFN...
Fig. 3. HCV IFN inducer triggers IFN production through MDA5. (a–f) Huh7 cells were transfected twice with siRNA against RIG-I (a–c) and MDA5 (d–f) at 60 nM. Three days later, the cells were transfected with 0.8 µg of total RNA from the sgJFH1 cells (HCV), VSV-infected cells (VSV) or PV-infected cells (PV). At 10 h post-transfection, the viral RNA levels (b and e) and IL-29 RNA levels (c and f) were measured in the transfected cells. Mean±SD (n=3). Similar results were obtained in another independent experiment. The RIG-I (a) and MDA5 (d) protein levels were determined by Western blotting. (g–j) HepG2 cells were transfected twice with a siRNA against RIG-I or MDA5 at 60 nM. Three days later, the cells were transfected with 0.8 µg
of total RNA from the sgJFH1 cells, VSV- or PV-infected cells. At 10 h post-transfection, the viral RNA levels (l) and IL-29 RNA (j) levels were measured in the transfected cells. Mean±SD (n=3). Similar results were obtained in another independent experiment. (k–m) CRISPR-cas9-generated Huh7-RIG-I kd cells and Huh7-MDA5-kd cells were transfected with 0.8 µg of total RNA from sgJFH1 cells, VSV-infected cells or PV-infected cells. At 10 h post-transfection, the viral RNA levels (k) and IL-29 RNA levels (l) were measured in the transfected cells. Mean±SD (n=3). Similar results were obtained in another independent experiment. To help protein level detection, MDA5-kd cells and RIG-I-kd cells were treated with IFN-α (500 IU ml$^{-1}$) for 16 h and analysed by Western blotting with the indicated antibodies (m). (n, o) CRISPR-cas9-generated HepG2-RIG-I-kd cells and HepG2-MDA5-kd cells were transfected with the IFN-β promoter plasmid. After 24 h, 0.2 µg of total RNA from Huh7 cells (Huh7), VSV-infected cells (VSV), PV-infected cells (PV), sgJFH1 cells (sgJFH1) or HCVcc-infected cells (HCVcc) were supplemented with 0.6 µg of yeast tRNA and then transfected into the cells. At 12 h post-transfection, luciferase activity was measured. Mean±SD (n=3). Mock, liposome transfection. Similar results were obtained in another independent experiment. (o) The kd protein levels were analysed by Western blotting using the indicated antibodies.

**HCV IFN inducer triggers IFN production independent of the 5’-triphosphate**

RIG-I recognizes the HCV genome in a sequence-dependent manner (Schnell et al., 2012) and requires the presence of a 5’-diphosphate on the viral genome (Goubau et al., 2014). To determine whether the IFN stimulation capacity of the HCV IFN inducer requires the 5’-triphosphate, we treated RNAs from HCV replicon cells with calf intestinal alkaline phosphatase (CIP) to remove 5’-triphosphate or -diphosphate. We also treated VSV RNA bearing a 5’-triphosphate (Ogino & Banerjee, 2007; Rehwinkel et al., 2010; Whelan & Wertz, 2002) and IVT-HCV RNA with an artificial 5’-triphosphate in parallel. The CIP-treated IVT RNA and VSV RNA-induced production of IFN-β (Fig. 5b) and IL-29 (Fig. 5c) was dramatically reduced. In contrast, the IFN stimulation capacity of the HCV RNA from the replicon cells was resistant to CIP treatment (Fig. 5b, c). Notably, the IFN stimulation capacity of the IVT-HCV RNA was much lower than that of HCV RNA, as evidenced by lower IFN production (Fig. 5b, c), albeit with a much higher viral RNA level (Fig. 5a).

We next attempted to explore the nature of the 5′ terminus of the HCV genome in the context of HCV replication. We used the terminator 5′-monophosphate-dependent exonuclease (TER) that digests RNA dependent on the presence of a 5′-monophosphate, to digest viral RNA from HCV replicon cells and HCV-infected cells. We also treated VSV RNA, which is supposed to include capped viral mRNAs, replication-generated 5’-triphosphate viral RNA (Ogino & Banerjee, 2007; Rehwinkel et al., 2010; Whelan & Wertz, 2002) and IVT-HCV RNA bearing a 5’-triphosphate (Fig. 5d). As expected, VSV RNA and IVT-HCV RNA were largely resistant to TER due to the presence of a cap or a 5’-triphosphate (Fig. 5e). HCV RNAs from subgenomic replicon cells and virally infected cells were readily degraded by TER (Fig. 5e). The efficiency of TER digestion was verified by efficient digestion of rRNAs bearing a 5’-monophosphate (Fig. 5f).

To demonstrate TER specificity, we treated HCV IVT RNAs with a 5’ polyphosphatase (PPase) to remove the γ- and β-phosphates and then digested the RNA with TER (Fig. 5g). PPase treatment rendered the IVT-HCV RNA sensitive to TER digestion but left capped-cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA intact (Fig. 5h, i). The HCV RNAs from replicon cells were sensitive to TER treatment in the presence or absence of PPase treatment (Fig. 5h). Taken together, these data indicate that the RIG-I agonists IVT RNA and VSV RNA are not sensitive to TER, whereas HCV RNA is sensitive to TER, suggesting that the HCV genome probably lacks a 5′-triphosphate or -diphosphate.

**HCV replicative dsRNA constitutes the physiological MDA5 agonist**

Because the HCV IFN inducer elicits IFN production through MDA5 (Fig. 3) and MDA5 detects viral dsRNA (Feng et al., 2012), we investigated whether the HCV IFN inducer is dsRNA and is sensitive to a dsRNA-specific nuclease. RNase III cleaves long dsRNA into short 12–30 bp dsRNA, whereas the S1 nuclease is a single-strand-specific endonuclease. As expected, RNase III digested artificial dsRNA poly(I:C) into short RNA fragments (Fig. 6a), whereas the S1 nuclease digested HCV IVT RNA but not poly(I:C) (Fig. 6b). We treated HCV RNAs from sgJFH1 cells with RNase III and the S1 nuclease and then transfected the RNAs into HepG2 cells and measured the induced IFN production. RNase III treatment partially digested the HCV RNA, whereas the S1 nuclease digested most of the HCV RNA (Fig. 6c). Intriguingly, RNase III nearly eliminated HCV RNA-induced IFN production whereas the S1 nuclease did not affect IFN production (Fig. 6d, e), suggesting that a small amount of HCV dsRNA remained after S1 nuclease digestion had contributed to IFN stimulation capacity.

MDA5 detects replicative viral dsRNA from picornavirus-infected cells (Abe et al., 2012; Feng et al., 2012). We also found MDA5-sensed PV RNA as reported previously (Fig. 3f, j, l and n) (Abe et al., 2012; Feng et al., 2012). We treated the RNAs from PV-infected cells with RNase III and S1 nuclease. Similar to HCV RNA, RNase III treatment partially digested PV RNA and eliminated PV RNA-induced IFN production (Fig. 6f–h). Conversely, S1 nuclease digested most of the PV RNA without affecting its IFN
Fig. 4. RNA produced at the late time points during HCV replication elicited IFN production. (a) Schematic representation of the experimental design. Huh7.5 cells were infected with HCV Jc1G at an m.o.i. of 1 and the cells were harvested for RNA purification at various time points. Then, the RNAs were transfected into Huh7 cells (b–d) and HepG2 cells (e–g). At 10 h post-transfection, the viral RNA levels (b and e), IFN-β RNA levels (c and f) and IL-29 RNA levels (d and g) were measured in the transfected cells. Mean±SD (n=3). Representative results from three independent experiments are shown. (h) Schematic representation of virion concentration. Secreted HCV virion was collected and precipitated by addition of PEG 8000. Precipitates were lysed in TRizol. (i–k) Total RNAs from Huh7 cells (Huh7), sgJFH1 cells (sgJFH1), concentrated supernatant of Huh7.5 cells (H7.5-S) and HCVcc-infected Huh7.5 cells (H7.5-HCVcc-S) were transfected into Huh7 cells, respectively. At 10 h post-transfection, the viral RNA levels (i), IFN-β RNA levels (j) and IL-29 RNA levels (k) were measured in the transfected cells. Mean±SD (n=3). Representative results of two independent experiments are shown.
HCV replicative RNA is a potent IFN inducer

stimulation capacity (Fig. 6f–h). Thus, like picornaviruses (Abe et al., 2012; Feng et al., 2012), the HCV double-stranded replicative RNA is a MDA5 agonist.

Next, we determined the roles of the RLR sensors in the recognition of the IFN inducer after S1 nuclease digestion of HCV RNA. The S1 nuclease-treated sgJFH1 RNAs were transfected into HepG2-RIG-I-kd and HepG2-MDA5-kd cells. The RNA-induced IFN production by the S1 nuclease-treated RNA was completely abolished in the MDA5 kd cells, but not affected in the RIG-I kd cells (Fig. 6i). This result suggests that the HCV IFN inducer that is resistant to the S1 nuclease is recognized by MDA5.

### HCV IFN inducer is associated with the viral replication complex and protected by viral replication factory

HCV replication takes place in a relatively enclosed membranous web, which shields viral proteins and genomes (Miyarni et al., 2003; Quinkert et al., 2005). These membranous webs partially survive after permeabilization by digitonin.

**Fig. 5.** HCV IFN inducer triggers IFN production independently of 5'-triphosphate (5'-PPP). (a–c) Ten micrograms of total cellular RNA from Huh7 cells (Huh7), VSV-infected Huh7 cells (VSV), sgJFH1 cells (sgJFH1) and IVT-HCV Jc1G RNA (Jc1G-IVT) was treated with (+) or without (−) CIP [1 U (µg RNA)] at 37°C for 30 min, followed by extraction with the TRizol LS reagent. Then, equal amounts of purified RNA (0.8 µg) were transfected into Huh7 cells. At 10 h post-transfection, the viral RNA levels (a), IFN-β RNA levels (b) and IL-29 RNA levels (c) were measured in the transfected cells. Mean±SEM (n=4) (P<0.05, **P<0.01, unpaired t-test). Similar results were observed in another independent experiment. (d) Schematic representation of the RNA species treated with the terminator 5'-phosphate-dependent exonuclease (TER). RNA bearing a 5'-PPP, 5'-diphosphate (5'-PP) or 5'-cap remained intact after TER digestion, whereas RNAs with a monophosphate (5'-P) were readily digested by TER. (e, f) Equal amounts of total cellular RNA from VSV-infected Huh7 cells (VSV), sgJFH1 cells (sgJFH1), HCV Jc1G-infected cells (HCVcc) and IVT-HCV Jc1G RNA (Jc1G-IVT) were treated (+) or left untreated (−) with TER [1 U (µg RNA)] as described in Methods, at 42°C for 30 min. After treatment, the RNAs were subjected to quantitative RT-PCR for RNA quantification (e). Total RNA from VSV-infected cells (VSV), sgJFH1 cells (sgJFH1) and HCV Jc1G-infected cells (HCVcc) was visualized on a 1% agarose gel. The rRNA bands are indicated with arrows (f). The RNA levels in the TER-treated groups were normalized to those in the TER-untreated group. Data from duplicated samples are shown. Similar results were observed in multiple independent experiments. (g) Schematic representation of the RNA species treated with RNA 5’-polyphosphatase (PPase). RNAs bearing a 5’-PPP or 5’-PP were converted to 5’-P after PPase digestion and became sensitive to TER digestion. RNAs with a 5’-cap remained unchanged upon PPase digestion and were resistant to TER digestion. (h, i) HCV IVT RNA and total cellular RNA from sgJFH1 cells were treated with PPase before TER treatment. After treatment, viral RNA levels and GAPDH mRNA levels were measured by quantitative PCR (h), and RNA integrity was examined using a 1% agarose gel (i). Mean±SEM (n=9). Data from three independent experiments are shown.

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Fig. 6. HCV replicative dsRNA constitutes the physiological MDA5 agonist. (a) Efficiency of RNase III digestion. Poly(I : C) was treated with RNase III [1 U (µg RNA)^−1] at room temperature for 1 or 5 min. The RNA was resolved on a 1 % agarose gel. (b) Efficiency of S1 nuclease digestion. Poly(I : C) and IVT RNA were treated with the S1 nuclease [20 U (µg RNA)^−1] at 30 °C for various times. The RNAs were resolved on a 1 % agarose gel. (c–e) Equal amounts of RNA from sgJFH1 cells were treated with RNase III at room temperature for 5 min or the S1 nuclease at 30 °C for 30 min. The RNAs were extracted by TRIzol LS, co-precipitated with yeast tRNA and resuspended in the same volume of DEPC-treated water. Then, equal volumes of RNA

X. Du and others
were transfected into HepG2 cells. At 10 h post-transfection, the viral RNA levels (c), IFN-b RNA levels (d) and IL-29 RNA levels (e) were measured in the transfected cells. The IFN fold induction was normalized to the yeast tRNA-transfected groups. Combined data from two independent experiments performed in triplicate are shown. (f–h) Equal amounts of RNA from PV-infected cells were treated with RNase III at room temperature for 5 min or the S1 nuclease at 30 °C for 30 min. The RNAs were extracted by Trizol LS, co-precipitated with the yeast tRNA and resuspended in the same volume of DEPC-treated water. Then, equal volumes of RNA were transfected into HepG2 cells. At 10 h post-transfection, the viral RNA levels (f), IFN-b RNA levels (g) and IL-29 RNA levels (h) were measured in the transfected cells. The IFN fold induction was normalized to the yeast tRNA-transfected groups. Mean±SD (n=3). (i) RNA from Huh7 and sgJFH1 cells was treated with the S1 nuclease as in (b) and then transfected into HepG2-MDA5-kd and HepG2-RIG-I-kd cells expressing an IFN-β promoter luciferase reporter. Luciferase activity was measured at 16 h post-transfection. Mean±SD (n=3).

DISCUSSION

RIG-I recognizes the HCV genome in a sequence-specific manner (Schnell et al., 2012). Recent studies demonstrated that MDA5 plays a crucial role in HCV innate immune recognition (Cao et al., 2015; Hiet et al., 2015; Israelow et al., 2014). The 5′-diphosphate is a prerequisite for RIG-I recognition (Goubau et al., 2014). Previous studies on RIG-I innate recognition of HCV used IVT-HCV RNA (Saito et al., 2008; Schnell et al., 2012), which may not accurately reflect the conditions in vivo. In our study we used RNA extracted from viral replicating cells, which theoretically contain viral replicative products or intermediates that act as IFN inducers. The HCV inducer was not sensitive to CIP treatment (Fig. 5a–c) and the HCV genome was readily digested by a monophosphate-dependent exonuclease (Fig. 5e), indicating that the HCV RNA extracted from the replicating cells likely lacked a 5′-triphosphate or -diphosphate. The HCV RNA polymerase NS5B synthesizes nascent viral RNA through a de novo pathway, which theoretically generates a 5′-triphosphated nascent viral RNA (Appleby et al., 2015). The absence of the 5′-triphosphate in our studies might be due to rapid processing of the 5′-triphosphate by host enzymes in vivo (Fig. 8) or the loss of the high cholesterol content in the HCV replication complex that can still be targeted by digitonin (Fiskum et al., 1980; Paul et al., 2013). After further solubilization with Triton X-100, we digested the remaining protein network (P2 fraction) with RNase A. P2 fraction-associated RNA was readily digested by RNase A (Fig. 7g), and its IFN stimulation capacity was completely abolished by RNase A digestion (Fig. 7h). These data suggest that the HCV IFN inducer is enclosed in the membranous web.

Finally, we examined whether the IFN inducer protected by the membranous web was dsRNA. We treated the digitonin-permeabilized cells with RNase III. The P1-associated viral RNA was partially resistant to RNase III digestion (Fig. 7j) and retained its IFN stimulation capacity (Fig. 7j). After Triton solubilization, the P2-associated viral RNA was partially digested by RNase III digestion (Fig. 7j) whereas the IFN stimulation capacity was completely abolished (Fig. 7j). Taken together, these data suggest that the IFN inducer is composed of dsRNA and resides in the membranous replication factory.

(Fiskum et al., 1980; Miyanari et al., 2003), whereas they are disrupted by solubilization with 1% Triton X-100 (Miyanari et al., 2003). After solubilization by 1% Triton X-100, the viral protein may remain as a protein network containing viral genomes such as yellow fever virus (Yi et al., 2012). We hypothesized that the HCV IFN inducer is the viral RNA enclosed in the membranous web and is associated with the viral nonstructural protein network. First, we permeabilized HCV replicon cells with 50 µg ml⁻¹ of digitonin. We collected the leaked cytosolic fraction (S1), and further in situ solubilized the remaining intracellular membrane fraction (P1) with 1% Triton X-100 to obtain the solubilized supernatant fraction (S2) and the remaining protein network fraction (P2) (Fig. 7a) (Yi et al., 2012). We transfected RNAs extracted from each fraction into HepH7 cells to monitor their IFN stimulation capacity (Fig. 7a). After digitonin permeabilization, the HCV nonstructural proteins NS3 and NS5A were primarily associated with the membrane fraction (P1), as were the endoplasmic reticulum marker calnexin (Fig. 7c) and the HCV RNA (Fig. 7d). Upon transfection, RNAs from the membrane fraction (P1) but not the S1 fraction elicited IFN production (Fig. 7e, f). When further solubilized by 1% Triton X-100, most of the endoplasmic reticulum marker calnexin was solubilized into the S2 fraction, suggesting efficient solubilization of the intracellular membranes. A substantial amount of the HCV NS5A and especially the NS3 proteins remained in the protein network fraction (P2) (Fig. 7c), which is similar to yellow fever virus (Yi et al., 2012). Although approximately half of the viral RNA was solubilized into the S2 fraction, the other half of the viral RNA was retained in the protein network fraction (P2 fraction) (Fig. 7d). Upon transfection these RNAs, as well as the RNAs solubilized into the S2 fraction, elicited IFN production (Fig. 7e, f). These data indicate that the HCV IFN inducer associates with viral proteins within the intracellular membranes.

Next, we examined whether the membranous web protected the HCV IFN inducer. We treated the membrane fraction (P1) with RNase A and extracted the RNA to measure its IFN stimulation capacity, as shown in Fig. 7a. First, the viral RNAs were partially protected by the membranous web from RNase A digestion (Miyanari et al., 2003), and these RNAs elicited IFN production (Fig. 7g, h). The incomplete protection of the viral RNA by the membranous web may reflect partial leakage of the membranous web due to the high cholesterol content in the HCV replication complex that can still be targeted by digitonin (Fiskum et al., 1980; Paul et al., 2013). After further solubilization with Triton X-100, we digested the remaining protein network (P2 fraction) with RNase A. P2 fraction-associated RNA was readily digested by RNase A (Fig. 7g), and its IFN stimulation capacity was completely abolished by RNase A digestion (Fig. 7h). These data suggest that the HCV IFN inducer is enclosed in the membranous web.
5'-triphosphate during our preparation of the RNA in vitro.
In the HCV infection scenario, RIG-I may recognize the unprocessed viral genomes as reported previously (Hiet et al., 2015; Israelow et al., 2014).

MDA5 preferentially recognizes long dsRNAs (Pichlmair et al., 2009). MDA5 recognizes picornavirus dsRNA formed by complementation of the positive- and negative-strand viral genomic RNA in a sequence-independent manner.
HCV replicative RNA is a potent IFN inducer

The HCV replication factory is a modified double-membrane vesicle (Romero-Brey et al., 2012). HCV IFN inducer was enclosed in the viral membranous replication factory (Fig. 7). This finding raises a question about how the double-membrane-vesicle-enclosed viral replicating RNA is sensed by MDA5. The HCV replication complex has a relatively long half-life of at least 12–18 h (Wölk et al., 2008). It is possible that the viral replication complexes disassemble and the replicative viral dsRNAs are exposed at late time points during viral replication and sensed by MDA5 (Cao et al., 2015; Hiet et al., 2015).

Formation of the membrane-associated replication complex is a common characteristic of almost all positive-strand RNA viruses (den Boon & Ahlquist, 2010). The membrane enclosure may shield viral replication intermediates from sensing by innate sensors. Dengue virus induces poor IFN induction in cultured cells, which is proposed to be due to enclosure of the dsRNA in an intracellular membrane, resulting in poor exposure of the dsRNA in the cytosol (Uchiha et al., 2014). RIG-I and M DA5 are excluded from HCV viral replication and assembly centres within the membranous web (Neufeldt et al., 2016). We proposed that formation of the HCV membranous viral replication complex to enclose the viral replication product might act as a means of innate immune evasion during the early life cycle stage.

**METHODS**

**Plasmids.** The HCV Jc1FLAG2 (p7-nsGluc2A) plasmid containing a secreted form of the Gaussia luciferase (Marukian et al., 2008) (renamed as Jc1G in the text) was provided by Charles Rice (The Rockefeller University, NY, USA). The sgFH1 plasmid was generated by replacing a fragment of Jc1G that was encompassed from the 5' UTR to the structural protein region with a fragment from the B7F subgenomic replicon encompassing the 5'UTR-BS-D-E-MVC internal ribosome entry site region (Yi et al., 2011). The plasmid-encoding poliovirus (PV) with a fused GFP (Teterina et al., 2010) was gifted by Charles Rice. For the CRISPR, oligos encoding subgenomic RNAs against an irrelevant target (IRR) (5’-ATA GCC ACT AAA CAC ATC AA-3'), MDA5 (5’-AAT CAG AGC CTG TTA ACT CT-3') and RIG-I (5’-CCT ACA TCC TGA GCT ACA TGG CC-3') were ligated into LentiCRISPR_v2 (Addgene, 52961). The pIFN-β Luc plasmid was kindly provided by Rongtuan Lin (McGill University, Canada) (Lin et al., 2000). The pRL-TK plasmid encoding the Renilla luciferase was obtained from Promega.

**Cell lines.** The human embryonic kidney cell line HEK-293T, Huh7 and HepG2 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China, www.cellbank.org.cn) were routinely maintained in Dulbecco’s modified medium supplemented with 10% FBS (Gibco). Huh7.5 (provided by Charles Rice) was maintained in an medium supplemented with 25 mM HEPEs (Gibco) and non-essential amino acids (Gibco). HCV sgFH1 replicon cells were generated by electroporation of IVT sgFH1 RNA into Huh7.5 cells and growth in conditioned medium supplemented with 5 μg ml⁻¹ blasticidin (Invitrogen) and maintained in conditioned medium with 0.5 μg ml⁻¹ blasticidin. For the CRISPR-mediated kd cell line, Huh7 or HepG2 cells were transduced with CRISPR lentiviruses at a dosage required to obtain approximately 60% surviving cells after growth in conditioned media supplemented with 5 μg ml⁻¹ puromycin. After 4 weeks, the surviving cells were pooled and maintained in the similar medium.

**Viruses.** Cell-cultured HCV (HCVcc) was generated as previously described and titrated in Huh7.5 cells by limiting dilution (Lindenbach et al., 2005). The VSV stock was obtained from the Shanghai Public Health Clinical Center. For PV production, the plasmid was linearized and purified for in vitro transcription. Then, the IVT RNA was electroporated into Huh7.5 cells. The supernatants were collected 15 h post-electroporation and passed through a 0.45 μm filter. To generate CRISPR lentiviruses, HEK293T cells were seeded onto six-well plates at a density of 7×10⁴ cells well⁻¹. The next day, the CRISPR plasmids were transfected with the VSV-G plasmid and gap-pol plasmid at a 2:1:3 ratio.

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**Fig. 8.** Proposed model for the generation of the HCV IFN inducer during viral replication. (a) HCV replication takes place within the viral replication complex, which is a virus-modified membranous web. The membranous web protects viral replicase components from accessibility of RNase. Upon solubilization by Triton, the membranous web is disrupted and the viral replicating intermediate is exposed. (b) HCV replicates its genome by a de novo pathway, which theoretically generates a 5'-triphosphate on the nascent viral RNAs. The 5'-triphosphate RNAs might be processed by an unknown mechanism, which renders the HCV RNA refractory to RIG-I recognition. Similar to other positive-strand RNA viruses, HCV probably uses dsRNA as a template in the strand displacement reaction. The dsRNA generated during replication constitutes the IFN inducer that is resistant to S1 nuclease digestion.
ratio. At 48 h post-transfection, the medium was harvested and filtered through a 0.45 µm syringe filter.

**Antibodies and inhibitors.** Anti-MDA5 antibody (D74E4; Cell Signaling) was used at 1: 100–1: 500 dilution; anti-RIG-I antibody (Alme-1; Enzo Life Sciences) was used at 1: 1000 dilution; anti-calnexin antibody (610523; BD) was used at 1: 2000 dilution; anti-β-actin antibody (A1978; Sigma) was used at 1: 5000 dilution; anti-NS5A (9E10) (gifted by Charlie Rice) was used at 1: 2000 dilution; anti-NS3 antibody (217-A; Virogen) was used at 1: 2000 dilution; and 2CMCA (sc-283467; Santa Cruz) was dissolved in DMSO to make a 20 mM stock.

**RNA preparation.** Total cellular RNAs from virally infected cells harvested at 10 h (VSV), 12 h (PV) and 72 h (HCV) or replicon cells were subjected to real-time PCR (SYBR Premix Ex Taq Tli RNaseH Eraser (Perfect Real Time) from TaKaRa. The cDNA samples were reversely transcribed using the PrimeScript RT Reagent kit with gDNA Eraser (Perfect Real Time) and with RNA 5' and reverse 3'-phosphate-dependent exonuclease (TER) [1 U (µg RNA)–1] in Cutsmart buffer (provided by NEB) for 30 min at 37 °C; and with RNA 5' PPase (RP8092; Epicentre) [4 U (µg RNA)–1] for 30 min at 37 °C. For in situ permeabilization or solubilization, the permeabilized or solubilized RNA from each fraction was resuspended in the same volume of DEPC water. Then an equal volume of RNA was collected and supplemented with Huh7 RNA to obtain a total amount of 0.8 µg if needed.

**Quantitative RT-PCR.** RNA was purified from the TRIzol extraction and reversely transfected using the PrimeScript RT Reagent kit with gDNA Eraser (Perfect Real Time) from Takara. The cDNA samples were subjected to real-time PCR (SYBR Premix Ex Taq Tli RNaseH Plus) using the gene-specific primers listed below. The fold induction was calculated by comparison to the control groups based on the 2-ΔΔC_{t} method (Livak & Schmittgen, 2001). GAPDH was used as a housekeeping gene for loading normalization. The primer pairs used in this study were as follows: GAPDH, forward 5' -GAT ATC GTG GAA GGA CTC ATG A-3' and reverse 3' -GAT ATC GTG GAA GGA CTC ATG A-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3' and reverse 5' -GAT GTG ATC TGT CTG GTC GGA-3'; IFN-β, forward 5' -GAT TCA TCT AGC ACT GGC TGG-3' and reverse 5' -CTT CAG GAT ATG CAG AAT CC-3'; IL-29, forward 5' -GAT GTG ATC TGT CTG GTC GGA-3'

**RNA interference.** Duplex Stealth siRNAs were purchased from Invitrogen. The siRNA sequences targeting human MDA5 and RIG-I were as follows: siMDA5-1, 5'-CCC UUA UCA UUA UUC AUG AUG AAC UCC-3'; siMDA5-3, 5'-GGG CAU GGA GAU UAA CUC AGA A-3'; siRIG-I-1, 5'-GAA UCU UAG UGA UGA UCC AUG UCC A-3' and reverse 5'-CCA CAG AAU CUU GUG AAC AAC CUA A-3'. The negative-control siRNA duplex was obtained from Invitrogen. The cells were transfected twice with siRNAs at a final concentration of 60 nM using the Lipofectamine RNAiMAX Transfection reagent (Invitrogen) with the forward transfection protocol provided by the manufacturer.

**Dual-luciferase reporter assay.** HepG2 cells (1 × 10^5 cells) seeded onto a 24-well plate were cultured overnight and then transfected with the IFN reporter plasmids pIFN-β-Luc and Renilla luciferase for normalization using the Fugene HP (Roche) transfection reagent according to the manufacturer’s instructions. The next day, the cells were transfected with 0.8 µg of the indicated RNAs using the TransIT-mRNA Transfection kit (Mirus) as described above. At 10 h post-transfection, the cells were lysed with passive lysis buffer and luciferase activity was assessed using a dual-luciferase assay kit (Promega).
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