Hepatitis C virus activates interleukin-1β via caspase-1-inflammasome complex

Dylan Burdette, Adam Haskett, Lance Presser, Steven McRae, Jawed Iqbal and Gulam Waris

Department of Microbiology and Immunology, H. M. Bligh Cancer Research Laboratories, Rosalind Franklin University of Medicine and Science, Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064, USA

Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine involved in the pathogenesis of HCV, but the sensors and underlying mechanisms that facilitate HCV-induced IL-1β proteolytic activation and secretion remains unclear. In this study, we have identified a signalling pathway leading to IL-1β activation and secretion in response to HCV infection. Previous studies have shown the induction and secretion of IL-1β through the inflammasome complex in macrophages/monocytes. Here, we report for the first time the induction and assembly of the NALP3-inflammasome complex in human hepatoma cells infected with HCV (JFH-1). We demonstrate that activation of IL-1β in HCV-infected cells involves the proteolytic processing of pro-caspase-1 into mature caspase-1 in a multiprotein inflammasome complex. Next, we demonstrate that HCV is sensed by NALP3 protein, which recruits the adaptor protein ASC for the assembly of the inflammasome complex. Using a small interfering RNA approach, we further show that components of the inflammasome complex are involved in the activation of IL-1β in HCV-infected cells. Our study also demonstrates the role of reactive oxygen species in HCV-induced IL-1β secretion. Collectively, these observations provide an insight into the mechanism of IL-1β processing and secretion, which is likely to provide novel strategies for targeting the viral or cellular determinants to arrest the progression of liver disease associated with chronic HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) often causes persistent infection in humans, which may lead to chronic hepatitis in up to 60–80% of infected adults and can progress to liver fibrosis, cirrhosis and eventually hepatocellular carcinoma (HCC) (Di Bisceglie, 1997). The HCV genome is a 9.6 kb positive-sense ssRNA molecule containing a 5’ UTR, a single ORF; and a 3’ UTR (Bartenschlager & Lohmann, 2000). The 5’ UTR contains an internal ribosome entry site (IRES), which directs cap-independent translation of a polyprotein precursor of ~3000 aa that is cleaved by viral proteases and host cell signal peptidases into mature structural proteins (core, E1 and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Bartenschlager & Lohmann, 2000; Blight et al., 2000). The study of the molecular mechanisms of HCV replication and pathogenesis have been hampered by the lack of an efficient cell culture system and small animal model. Recently, an HCV infection system has been demonstrated using HCV genotype 2a clone (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). This system allows the production of virus that can be efficiently propagated in cell culture.

One of the fundamental reactions of the innate immune response to viral infection is the processing and release of pro-inflammatory cytokines. IL-1β is a multifunctional cytokine playing important roles in inflammation, angiogenesis and the immune response (Perwez Hussain & Harris, 2007). A growing body of evidence has suggested that in response to HCV infection, multiple signalling pathways are activated that participate in the regulation of genes related to inflammation such as Cox-2, PGE2 and interferons (Gong et al., 2001; Lu et al., 2008; Waris & Siddiqui, 2005). Accumulating data suggest that pro-inflammatory cytokine elevation has a pivotal role in chronic hepatitis C (Moschen et al., 2011; Nelson et al., 1997). Recent reports have shown that IL-1β levels are higher in HCV-related liver diseases than in other forms of liver damage (Lapiński, 2001).

IL-1β is synthesized as an inactive cytoplasmic precursor that is processed into the biologically active mature form. The nucleotide-binding-domain leucine-rich-repeat containing molecules (NOD-like receptors, NLRs) have emerged as key players in regulating the maturation of IL-1β (Tschopp et al., 2003). Although, NALP1 and NALP3 are the best studied among the NLR family of proteins (Tschopp et al., 2003), the activation of liver-specific
NALPs has not been studied. Upon sensing signals, the NALPs (pyrin proteins), together with its adaptor protein, PYD (pyrin domain) and caspase recruitment domain (CARD)-containing protein (PYCARD) [which is also known as apoptotic speck protein containing a CARD (ASC)], regulates IL-1β maturation through the formation of a multiprotein complex called the inflammasome (Martinon et al., 2009). The inflammasome complex regulates the activation of inflammatory caspase-1 and subsequent cleavage of the IL-1β and IL-18 precursors into their functional forms. Caspase-1 is synthesized as an inactive zymogen (45 kDa) that becomes activated by cleavage to generate an enzymically active heterodimer composed of 10 and 20 kDa subunits (Martinon & Tschopp, 2004). Recently, several studies have demonstrated the role of viruses (DNA and RNA) in the activation of the inflammasome complex (Allen et al., 2009; Delaloye et al., 2009; Ichinohe et al., 2009, 2010; Kanneganti et al., 2006; Kanneganti, 2010; Muruve et al., 2008; Rajan et al., 2011; Thomas et al., 2009). NALP3 along with ASC has emerged as a potential sensing protein to activate the inflammasome complex in response to various stimuli including DNA and RNA viruses (Kanneganti, 2010).

In the present study, we demonstrate the induction, proteolytic activation and secretion of IL-1β from HCV-infected cells. Our results show that activation of IL-1β in HCV-expressing cells involves the proteolytic activation of caspase-1 in a multiprotein inflammasome complex. Next, we demonstrate that NALP3 and ASC are involved in the assembly of the inflammasome complex. Using a small interfering (si)RNA approach, we further show that induction of the inflammasome complex is involved in the activation of IL-1β in HCV-infected cells.

RESULTS

Induction and secretion of IL-1β by HCV

To demonstrate the secretion of IL-1β from HCV-infected cells, cell culture supernatants were collected at indicated time points and the release of mature IL-1β was measured using IL-1β-specific ELISA. We observed increased secretion of IL-1β (13 pg ml⁻¹) at day 2 that peaked at day 4 (44 pg ml⁻¹) in HCV-infected cells compared with mock-infected cells (Fig. 1a). To determine that the secretion of IL-1β was specific to HCV infection, Huh7.5 cells were incubated with UV-treated HCV, or incubated with cell culture supernatant collected from cells expressing JFH-1/GND (replication defective virus). We did not observe the secretion of IL-1β in these cells (Fig. 1a). However, incubation of cells with hydrogen peroxide, a known inducer of the inflammasome complex and IL-1β, induced IL-1β secretion (Fig. 1a).

Recently, intracellular accumulation of ROS, phagosomal destabilization, influenza A virus M2 ion channel protein, and adaptor protein, MyD88 in TLR7 signalling pathway have been shown to induce IL-1β secretion through the activation of the inflammasome complex (Dostert et al., 2008; Hornung et al., 2008; Ichinohe et al., 2010). To determine the signalling pathways responsible for IL-1β secretion by HCV, HCV-infected cells were incubated with bafilomycin A (BAF, an inhibitor of endosomal acidification), PDTC (pyrrolidine dithiocarbamate; antioxidant) and transfected with MyD88 siRNA. We observed about 60% reduction in IL-1β secretion in HCV-infected cells treated with PDTC alone, or with PDTC and BAF. However, HCV-infected cells treated with BAF alone, or transfected with MyD88 siRNA did not reduce IL-1β secretion. To determine if the expression of HCV p7 (ion channel protein) and HCV dsRNA induced IL-1β secretion, Huh7.5 cells were transfected with JFH-1, JFH-1/GND RNA or p7 expression plasmid. These cells were unable to induce IL-1β secretion (Fig. 1a).

To determine the effect of PDTC on HCV-induced ROS production, HCV-infected cells were incubated with PDTC. The results showed reduced production of ROS in the presence of PDTC (Supplementary Fig. S1, available in JGV Online). To determine the specificity of BAF, HCV-infected cells were incubated with BAF and the secretion of HCV was analysed using quantitative (Q)RT-PCR. We observed reduced secretion of HCV in the presence of BAF (Supplementary Fig. S2, available in JGV Online). To determine the effect of MyD88 siRNA on MyD88 protein expression, HCV-infected cells were transfected with MyD88 siRNA and GFP siRNA (negative control). We observed reduced expression of MyD88 protein expression in the presence of MyD88 siRNA (Supplementary Fig. S3, available in JGV Online).

To determine the effect of HCV on IL-1β transcription, total cellular RNA was extracted from mock-infected and HCV-infected cells, and the level of IL-1β mRNA was quantified by real-time RT-PCR. The results show an 11-fold increase in the levels of IL-1β mRNA in HCV-infected cells at day 2 and 26-fold at day 4 (Fig. 1b). The HCV RNA levels were 4.5-fold at day 2 and 16-fold at day 4 (Supplementary Fig. S4, available in JGV Online). Previously, NF-κB- and TLR7-mediated MyD88 signalling pathways have been shown to stimulate IL-1β transcription (Tschopp et al., 2003; Zhang et al., 2009). To determine the signalling pathways involved in IL-1β mRNA induction, HCV-infected cells were incubated with NF-κB inhibitor or transfected with MyD88 siRNA. We observed reduced expression of IL-1β mRNA expression in cells incubated with NF-κB inhibitor (73%), or transfected with MyD88 siRNA (15%) (Fig. 1b). However, GFP siRNA did not inhibit IL-1β mRNA synthesis. In addition to this, siRNA against MyD88, GFP and NF-κB inhibitor did not affect HCV RNA replication (Supplementary Fig. S4). These results collectively suggest that HCV infection can induce the transcriptional stimulation and secretion of mature IL-1β.

To demonstrate that NF-κB is constitutively activated in HCV-infected cells, cellular lysates from mock-infected and HCV-infected cells were subjected to Western blot analysis.
The results show the phosphorylation of NF-κB p65 in HCV-infected cells (Fig. 1c, lane 2). To determine the specificity of NF-κB phosphorylation, HCV-infected cells were incubated with PDTC and an NF-κB inhibitor. The results showed inhibition in NF-κB phosphorylation in the presence of PDTC or the NF-κB inhibitor (Fig. 1c, lanes 3 and 4).

**Activation of caspase-1 by HCV**

To determine if HCV-induced secretion of IL-1β was correlated with the activation of caspase-1 in HCV-infected cells, mock-infected and HCV-infected cellular lysates were subjected to Western blot analysis. Our results show increased expression of pro-caspase-1 (45 kDa) as well as higher levels of mature caspase-1 (20 kDa) at days 2 and 4 (Fig. 2a). To demonstrate the active form of caspase-1 in HCV-infected living hepatoma cells, mock-infected and HCV-infected cells were incubated with FLICA<sup>casp1</sup> (fluorochrome inhibitor of caspases). Once inside the cells, the FLICA<sup>casp1</sup> inhibitor binds covalently to the active caspase-1 (Ekert et al., 1999). Our results displayed the staining of active caspase-1 by FLICA<sup>casp1</sup> in HCV-infected cells at day 4 (Fig. 2b), which was abrogated in the presence of caspase-1 inhibitor. However, treatment with caspase-3 inhibitor...
(negative control) did not show any effect on caspase-1 staining with FLICA. To determine if the induction of caspase-1 protein was due to an increase in caspase-1 mRNA, total cellular RNA was extracted from mock-infected and HCV-infected cells, and the level of caspase-1 mRNA was quantified by real-time RT-PCR. The results show the induction of caspase-1 mRNA in HCV-infected cells at day 2 (fourfold) to day 4 (10-fold) (Fig. 2c). These results clearly indicate that HCV infection induces transcriptional stimulation and the formation of more precursor and mature forms of caspase-1.

**Induction of NALP3 and ASC by HCV**

To demonstrate the activation of the inflammasome complex in HCV-infected cells, we first investigated the expression of the components of the inflammasome complex. We examined the expression levels of well-characterized NALPs (NALP3) and ASC proteins as well as mRNA levels in mock-infected and HCV-infected cells. Total cellular lysates and RNA were extracted and subjected to Western blot and QRT-PCR analysis. Our results showed a fourfold increase in the expression of the NALP3 protein in HCV-infected cells at day 4 (Fig. 3a, lane 3). To determine if the induction of NALP3 protein was due to an increase in NALP3 mRNA, total cellular RNA was extracted and the level of NALP3 mRNA was quantified by real-time RT-PCR. Our results show about 3.5-fold induction of NALP3 mRNA in HCV-infected cells at day 4 (Fig. 3b). Similarly, we observed the induction of ASC mRNA (~ fourfold) and protein (~ 3.7-fold) in HCV-infected cells (Fig. 3c, d). These results indicate that HCV-infected cells induce the expression of NALP3 and ASC.

![Fig. 2. Activation of caspase-1 by HCV. (a) Mock-infected and HCV-infected Huh7.5 cells were serum starved for 4 h and cellular lysates were subjected to SDS-PAGE followed by Western blot analysis using caspase-1 antibody. Lane 1, Huh7.5 lysates; lanes 2 and 3, lysates from Huh7.5 cells infected with HCV. Bottom panels represent the expression of HCV core and actin controls. (b) FLICA assay. Mock-infected and HCV-infected cells were serum starved for 4 h and treated with 50 µM caspase-1 inhibitor (z-VAD-fmk) for 1 h (iv), and 2 h (vi) or 100 µM caspase-3 inhibitor (DEVD) for 1 h (v), and 2 h (vii). The cells were incubated with the FLICA reagent and Hoescht nuclear stain for 1 h, before being visualized via fluorescence microscopy. (c) Total cellular RNA from mock-infected and HCV-infected cells were subjected to QRT-PCR using caspase-1-specific primers. The data shown here represent the means+SD of at least three independent experiments performed in triplicate. *, P<0.05 compared with mock-infected control cells.]
Activation of the inflammasome complex by HCV

To demonstrate if HCV-infected cells activate the inflammasome complex, we examined the assembly of the inflammasome complex using confocal microscopy. Mock- and HCV-infected cells were stained with antibodies against NALP3 and ASC. We observed the cytoplasmic colocalization of NALP3 with ASC in HCV-infected cells at days 2 and 4 (Fig. 4b, c). However, UV-treated HCV did not activate the inflammasome complex. The expression of HCV NS3 protein in HCV-infected cells represents the levels of HCV infection. Similarly, we examined the interaction of ASC with caspase-1 in HCV-infected cells. The results show the colocalization of ASC with caspase-1 in HCV-infected cells at days 2 and 4 (Fig. 5b, c). These results clearly indicate that HCV-infected cells induce NALP3, ASC and caspase-1 that are involved in the assembly of the inflammasome complex.

To further demonstrate the interaction between NALP3 and ASC, mock-infected and HCV-infected cellular lysates were immunoprecipitated with anti-ASC antibody and immunoblotted with anti-NALP3 antibody. The results show that ASC was able to pull-down NALP3 in HCV-infected cells (Fig. 6a, lane 2). The interaction of NALP3 and ASC was further confirmed by reciprocal co-immunoprecipitation using anti-NALP3, we observed that NALP3 was able to pull-down ASC in HCV-infected cells compared with mock-infected cells (Fig. 6c, lane 2). However, isotype mAb was unable to pull down either NALP3 or ASC (Fig. 6c, lane 2). These results clearly indicate that NALP3 indeed interacts with ASC in HCV-infected cells.

Effect of NALP3, ASC and caspase-1 siRNA on IL-1β secretion

To demonstrate the effect of NALP3, ASC and caspase-1 siRNA on the secretion of IL-1β, mock-infected and HCV-infected cells were transfected with siRNA against GFP, NALP3, ASC and caspase-1. To determine the effect of the specific siRNA on their target gene expression, total cellular RNA was collected and subjected to QRT-PCR. The results show a significant decrease in respective mRNA expression compared with HCV-infected cells, transfected with GFP siRNA (Fig. 7a–c).
Cell culture supernatant from siRNA-transfected cells was collected and subjected to IL-1β-specific ELISA. The results show increased secretion of IL-1β (44 pg ml⁻¹), which was reduced in HCV-infected cells transfected with NALP3, ASC or caspase-1 siRNA (Fig. 7d). However, HCV-infected cells transfected with GFP siRNA (negative control) had no effect on IL-1β secretion. These results suggest that HCV infection induces secretion of IL-1β through the assembly of the inflammasome complex. The cellular toxicity assay was performed by fluorescent measurement of the release of lactate dehydrogenase (LDH) from mock-infected and HCV-infected cells, transfected with various siRNA. We did not observe any change in the fluorescence (Fig. 7e).

**DISCUSSION**

Although induction of pro-inflammatory molecules plays an important role in the pathogenesis of HCV (Chang, 2003; Dumoulin et al., 1999; Koziel, 1999; Moschen et al., 2011), little is known about the mechanism of the HCV-mediated inflammatory processes. Inflammatory cytokines are activated by both inflammatory cells and tumour cells and their effects are important for sustaining chronic inflammation, tumour progression and inhibiting immune-mediated tumour surveillance (Gonda et al., 2009). About 15% of the global cancer burden is attributable to infectious agents and inflammation is a major component of these chronic infections (Parkin et al., 1999). Inflammation is beneficial in
appropriate amounts, but can easily become detrimental when excessive because of its tissue-damaging potential. The persistence of chronic inflammation plays a critical role in tumour growth and development (Perwez Hussain & Harris, 2007).

IL-1\(\beta\) is critical for inflammation, angiogenesis and carcinogenesis (Perwez Hussain & Harris, 2007). In mouse models of metastasis, treatment with an IL-1\(\beta\) receptor antagonist significantly decreased tumour development, moreover, mice deficient in IL-1\(\beta\) were resistant to the development of experimental metastasis, suggesting that local production of IL-1\(\beta\) aids development of metastasis (Vidal-Vanaclocha et al., 2000). In this study, we observed the transcriptional stimulation and secretion of IL-1\(\beta\) from HCV-infected cells that can aid in the development of liver disease associated with HCV infection.

Recently, the recognition of viral RNA through TLR3 and RIG-I has been shown to activate NALP3-inflammasome (Guillot et al., 2005; Poeck et al., 2010). Huh7.5 cells used in this study, do not express TLR3, and also harbour a defect in the RIG-I antiviral pathway (Li et al., 2005; Sumpter et al., 2005). Incubation of Huh7.5 cells with LPS/ATP did not induce IL-1\(\beta\) secretion (Fig. 1a). In addition, formation of dsRNA in Huh7.5 cells transfected with JFH-1 RNA did not induce IL-1\(\beta\) secretion, suggesting that secretion of IL-1\(\beta\) requires the translation/replication activities of HCV in the cytoplasm. Recently, influenza A virus M2 ion channel protein, phagosomal destabilization and ROS have been shown to activate NALP3-inflammasome (Dostert et al.,

![Fig. 5. Similar to Fig. 4, mock-infected and HCV-infected cells were stained with ASC, caspase-1 and HCV NS3 antibodies for 1 h at RT, followed by incubation with secondary antibodies for ASC (anti-goat Alexa Fluor 546), caspase-1 (anti-rabbit Alexa Fluor 488) and NS3 (anti-mouse Alexa Fluor 633). Arrows represent the staining and colocalization of ASC with caspase-1. Bar, 10 μm.](http://vir.sgmjournals.org)
In our previous studies, we demonstrated the constitutive IL-1β mRNA expression in HCV-infected human hepatoma cells (Figs 4 and 5). These results are consistent with the recent reports of RNA viruses involved in the activation of inflammasome complex (Allen et al., 2009; Ichinohe et al., 2009; Kanneganti et al., 2006; Thomas et al., 2009). Our results provide evidence that assembly of the inflammasome complex in HCV-infected hepatoma cells requires the expression of NALP3, ASC and caspase-1. In contrast to a recent study in which ASC was localized primarily to the nucleus (Bryan et al., 2009), we have observed cytoplasmic staining of ASC and NALP3 in HCV-infected cells (Figs 4 and 5). This could be attributed to the different stimulus or cell types used in the studies. In addition, we have also determined the transcriptional stimulation of NALP3, ASC and caspase-1 (Gupta et al., 2001; Kummer et al., 2007; Masumoto et al., 2001).

The molecular mechanisms by which HCV could induce the assembly of inflammasome complex are not completely understood. Previously, several mechanisms have been reported to be involved in the activation of caspase-1 through NALP3 (Kanneganti et al., 2006; Muruve et al., 2008; Thomas et al., 2009). NALP3 along with ASC has emerged as a potential sensing protein to activate the inflammasome complex in response to various stimuli including DNA and RNA viruses (Kanneganti, 2010). HCV proteins or HCV ribonucleoprotein complex produced in the cytoplasm of the hepatocytes could be sensed directly by the sensor protein NALP3. However, there is no report that microbial products physically interact with the NLR proteins (Mackey et al., 2002). Our data supports the role of ROS in the induction of the inflammasome complex (Fig. 1). However, we did not observe the complete inhibition of IL-1β secretion by PDC treatment. The possibilities of other indirect sensing mechanisms such as RNA–protein complex produced during HCV infection with NALP3 cannot be ruled out.

In summary, our studies demonstrate a novel NALP3 inflammasome complex induction in human hepatocytes by HCV. These results provide important insight into the role of NALP3 and ASC in inflammatory processes associated with chronic HCV. We also demonstrate that the secretion of IL-1β from HCV-infected cells was reduced by inhibiting inflammasome complex assembly using siRNA against NALP3, ASC or caspase-1. Our studies yield novel insight into the design of alternative and more cost-effective strategies in the treatment of chronic HCV infection.

**METHODS**

**Plasmids, reagents and antibodies.** The infectious JFH-1 cDNA along with the replication-defective JFH-1/GND construct was obtained from Dr T. Wakita (NIID, Tokyo, Japan). HCV p7
Fig. 7. Effect of NALP3, ASC and caspase-1 siRNA on IL-1β secretion. (a–c) Mock-infected and HCV-infected cells were transfected with siRNA against GFP, NALP3, ASC and caspase-1 as described in Methods. At day 4, total cellular RNA was extracted using TRizol, cDNA was synthesized and QRT-PCR was performed using GFP-, NALP3-, ASC- and caspase-1-specific primers (Supplementary Table S1). 18S rRNA was used as an internal control. The data shown here represent the means ± SD of at least three independent experiments performed in triplicate. *, P < 0.05 compared with GFP siRNA-transfected control cells. (d) Effect of NALP3, ASC and caspase-1 siRNA on IL-1β secretion. Cell culture supernatants from siRNA-transfected mock-infected and HCV-infected cells were subjected to IL-1β ELISA. *, P < 0.05 compared with mock-infected control cells. **, P < 0.05 compared with GFP siRNA-transfected cells. (e) Cytotoxicity assay. Equal volume of cell culture medium from mock-infected and HCV-infected cells, transfected with GFP, caspase-1 and NALP3 siRNA were subjected to cytotoxicity assay using CytoTox-ONE. RFU, Relative fluorescence unit.

expression plasmid was a kind gift from Dr S. Weinmann (University of Kansas, USA).

Antibodies against NF-κB, phospho-NF-κB p65 and IL-1β were purchased from Cell Signalling, NALP3 was purchased from Novus Biologicals, actin was purchased from Sigma-Aldrich, ASC was purchased from MBL, N53 and N55A were purchased from Virogen. Caspase-1 antibody was purchased from Invitrogen. Caspase-1, caspase-3, NF-κB inhibitors [6-amino-4-((4-phenoxophenylethylamino)quinazoline]) and PDTC were purchased from Calbiochem-Novachem. BAF, lipopolysaccharide (LPS) and ATP were obtained from Sigma-Aldrich, protein G-Sepharose 4 fast flow beads were from GE Healthcare Bio-Sciences Corp.

Cell lines. The Huh-7.5 subline was obtained from C. Rice, Rockefeller University, USA (Blight et al., 2002). These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 100 U penicillin ml⁻¹ and 100 μg streptomycin sulfate ml⁻¹. The cells were incubated in a 37 °C incubator with 5% CO₂.

HCV cell culture infection system. The pFL-JFH1 plasmid was transcribed and delivered into Huh-7.5 cells by electroporation (Burdette et al., 2010). The cell-free virus was propagated in Huh-7.5 cell cultures as described previously (Lindenbach et al., 2005). The expression of HCV protein in HCV-infected cells was analysed using Western blot assays. The HCV cell culture supernatant was collected at appropriate time points and was used to infect naive Huh-7.5 cells at appropriate dilutions (m.o.i. of 1) for 5–6 h at 37 °C and 5% CO₂ (Burdette et al., 2010). The viral titre in cell culture supernatant was expressed as f.f.u. ml⁻¹, which was determined by the mean number of HCV-NS5A-positive foci detected at the highest dilutions as described previously (Zhong et al., 2005). The cell culture supernatants collected from Huh7.5 cells expressing JFH-1/GND (replication defective virus) were used as a negative control. Viruses were UV light (365 nm)-inactivated by irradiation for 20 min at 10 cm distance. The presence of contaminating LPS in the virus stock and cell culture supernatants was evaluated by the Limulus Amebocyte Lysate test (BioWhittaker).

IL-1β ELISA. Mock-infected and HCV-infected Huh7.5 cells were cultured for 4 days and the secreted IL-1β protein in cell culture supernatant was determined by ELISA according to the manufacturer’s protocol (R&D Systems). The cell culture supernatants were added to pre-coated wells and incubated for 2.5 h at room temperature (RT). The wells were washed, incubated with the biotinylated antibody for 1 h at RT, washed again, and then incubated with a streptavidin solution for 45 min at RT. The wells were incubated with a substrate in the dark for 30 min at RT. The reaction was terminated by adding stop solution into the wells. The absorbance was measured at 450 nm.

http://vir.sgmjournals.org
**Immunoprecipitation and Western blot analysis.** Mock-infected and HCV-infected cells were serum starved for 4 h and cellular extracts were prepared by incubating in radioimmunoprecipitation (RIPA) buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM sodium formate, 10 μl protease inhibitor cocktail (Thermo Scientific) ml⁻¹] for 45 min on ice. Cellular lysates were immunoprecipitated with anti-NALP3 or ASC antibody overnight at 4°C. The immune complexes were incubated with protein G-Sepharose for 1 h at 4°C, washed 3–4 times with RIPA buffer and boiled for 5 min in sample buffer. The samples were then subjected to SDS-PAGE. Gels were electroblotted onto a nitrocellulose membrane (Thermo Scientific) in 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were incubated overnight in blocking buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5% Tween 20, 5% non-fat dry milk], and probed with primary antibody for 1 h at RT. The membranes were then washed three times for 10 min in TBS-T (Tris-buffered saline with 1% Tween 20), followed by incubation with secondary antibody (dilution 1:20 000) for 45 min at RT. After an additional washing cycle with TBS-T, immunoblots were visualized by using the LICOR Odyssey system.

**Laser-scanning confocal immunofluorescence.** Mock-infected and HCV-infected cells were plated in eight-well chamber slides and serum-starved for 4 h. The cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at RT, permeabilized with 0.2% Triton X-100 and blocked for 45 min with 5% BSA in PBS. The cells were next incubated with the primary antibody for 1 h at RT, followed by incubation with secondary antibody (Molecular Probes) for 1 h. After washing with PBS, cells were mounted with an anti-fade reagent containing DAPI (Invitrogen) and observed under a laser scanning confocal microscope (Olympus Fluoview 300 fluorescence confocal microscope).

**RNA interference.** Mock-infected and HCV-infected cells were transfected with siRNA against GFP, MyD88, NALP3, ASC or caspase-1 according to manufacturer’s protocols (Santa Cruz Biotechnology and Qiagen). Each siRNA consists of pools of three to five target-specific 19–25 nt siRNA designed to knockdown the target gene expression. For GFP and caspase-1 transfections, two solutions were prepared. Solution A: 60 pmol siRNA duplex was mixed with 100 µl siRNA transfection medium. Solution B: 6 µl transfection reagent was added to 100 µl siRNA transfection medium. Solutions A and B were allowed to incubate at RT for 20 min. After 20 min, solutions A and B were combined, and incubated for 20 min at RT. The combined solutions were then added to the cells in six-well plates, and incubated for 5 h at 37°C and 5% CO₂, and the transfection solution replaced with 2 ml of complete DMEM growth medium.

NALP3 and ASC siRNAs were transfected according to the manufacturer’s protocols (Qiagen). siRNA duplex (256 ng) was diluted in 100 µl serum-free medium, along with 20 µl HiPerfect transfection reagent, the solution was incubated at RT for 10 min. The transfection solution was then added to the cells and the cells were harvested at different time points.

**FLICA assay.** Mock-infected and HCV-infected cells were plated in eight-well chamber slides and grown for 24 h. The cells were serum starved for 4 h and treated with caspase-1/caspase-3 inhibitors and then incubated with 1 × FLICA (Immunochemistry technologies) reagent for 1 h, followed by a change of medium and incubation with a Hoechst nuclear stain for 5 min. The cells were washed and analysed via fluorescence microscopy using a 488 nm filter to visualize the FLICA reagent and a 405 nm filter to visualize the Hoechst stain. Images were collected using the Metamorph software program.

**QRT-PCR.** Target genes in mock-infected and HCV-infected cells were quantified by real-time RT-PCR using the primers shown in Supplementary Table S1 (available in JGV Online). Total cellular RNA was extracted using TRIzol (Invitrogen) and DNase treated using RQ1 RNase-free DNase prior to cDNA production. The cDNA was reverse-transcribed from 1 μg total RNA using reverse transcription kit (ABI). QRT-PCR was carried out by using SYBR green master mix (ABI) and specific primer sets. 18 S rRNA was used as an internal control. Amplification reactions were performed under the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles for 10 s at 95°C and 1 min at 60°C. Relative transcript levels were calculated using ΔΔCt method as specified by the manufacturer.

**Cytotoxicity assay.** Mock-infected and HCV-infected cells were transfected with siRNA against GFP, NALP3, ASC and caspase-1. The cell culture supernatant was collected and assayed for cellular toxicity using an LDH cytotoxicity assay kit, CytoTox-ONE (Promega) according to manufacturer’s protocols.

**ACKNOWLEDGEMENTS**

We thank Dr Takaji Wakita (NIID, Tokyo, Japan) and Dr Charles Rice (Rockefeller University, NY, USA) for the generous gift of HCV genotype 2a (JFH-1) and Huh7.5 cell line. We thank Dr Robert Dickinson for help in FACS/cell sorter facility at RFUMS. This work was supported in part by the ACS Illinois Division, Inc., 09-14, NIH-R21 (AI078532), and by the Rosalind Franklin University of Medicine and Science-H.M. Bligh Cancer Research Fund to G.W.

**REFERENCES**


