The Hepatitis C Virus-induced NLRP3 Inflammasome Activates the Sterol Regulatory Element-binding Protein (SREBP) and Regulates Lipid Metabolism*

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Hepatitis C virus (HCV) is a major cause of liver disease and the most prevalent cause of chronic viral hepatitis in the United States. Persistent infection with HCV is associated with chronic liver disease, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Chronic HCV infection of the liver is the primary pathologic event leading to liver dysfunction and death from cirrhosis or HCC. Chronic HCV infection is characterized by inflammation and fibrosis, which are driven in part by the pro-inflammatory cytokines and chemokines produced by infected hepatocytes. The HCV-encoded NS5B RNA-dependent RNA polymerase (RdRp) plays a critical role in viral replication and morphogenesis. The accumulation of lipid droplets in infected hepatocytes is through enhanced fatty acid synthesis and de novo sterol synthesis. In this study, we elucidate the mechanisms underlying the accumulation of lipid droplets in infected hepatocytes. We show that HCV infection of human hepatoma cells leads to the activation of the NLRP3 inflammasome, which activates SREBPs and host lipid metabolism, leading to the production of proinflammatory cytokines and chemokines.

This article has been withdrawn by Steven McRae, Jawed Iqbal, Mehuli Sarkar-Dutta, Naushad Ali, and Gulam Waris. Samantha Lane and Abhiram Nagaraj could not be reached. The ASC immunoblot from Fig. 1A was reused as HCV core in Fig. 1G. Additionally, these images were reused in Burdette et al. (2010) J. Gen. Virol. 91, 681-690, Burdette et al. (2012) J. Gen. Virol. 93, 235-246, Presser et al. (2013) PLOS One 8, e56367, and Iqbal et al. (2014) PLOS One 9, e87464. The NLRP3 immunoblot in Fig. 1D was reused from Fig. 9 of Iqbal et al. (2013) J. Biol. Chem. 288, 36994-37009, representing different experimental conditions. The actin immunoblot from Fig. 1D was also reused in Fig. 1C of Burdette et al. (2012) J. Gen. Virol. 93, 235-246, representing different experimental conditions. The actin immunoblot from Fig. 1F was reused in Fig. 1G as actin. The Journal also determined that this immunoblot was reused in the following publications representing different experimental conditions: Waris et al. (2003) J. Biol. Chem. 278, 40778-40787, Waris et al. (2005) J. Virol. 79, 1569-1580, Waris and Siddiqui (2005) J. Virol. 79, 9725-9734, Waris et al. (2007) J. Virol. 81, 8122-8130, Nasimuzzaman et al. (2007) J. Virol. 81, 10249-10257, Burdette et al. (2010) J. Gen. Virol. 91, 681-690, Burdette et al. (2012) J. Gen. Virol. 93, 235-246, and Presser et al. (2013) PLOS One 8, e56367. Gulam Waris does not agree that the actin immunoblot was reused in other publications. The SCD immunoblot from Fig. 1F was reused in Fig. 6A as actin. The mature SREBP-2 immunoblot from Fig. 3B was reused as SCD in Fig. 1G. Insig 2 of Fig. 7A was reused in Fig. 7B as Insig 1. The actin immunoblot from Fig. 7A was reused in lanes 3 and 4 of the actin immunoblot in Fig. 7B. Columns f and g were duplicated in Fig. 2A. Column a from Fig. 4C was duplicated in a of Fig. 5D. The withdrawing authors sincerely apologize to the readers.

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15). Recent studies have demonstrated that inflammasome-independent NLRP3 augments TGF-β1 signaling in the kidney epithelium and cardiac fibroblasts (12, 13). NLRP3 is also known to interact with ubiquitin ligase-associated protein SGT1, heat shock protein 90 (HSP90), and thioredoxin-interacting protein (16, 17). Typically, caspase-1 mediates the maturation of IL-1β and IL-18 in immune and non-immune cells (18). However, studies have shown that several proteins associated with the glycolytic pathway are cleaved by caspase-1, which is suggestive of a broader role of caspase-1 in addition to maturation of IL-1β and IL-18 (19). Activation of caspase-1 leads to pyroptosis of the cells infected with intracellular bacteria (20). In contrast, the ability of caspase-1 to prevent hepatocyte death during reox stress by up-regulating beclin 1 expression signifies its protective function in non-immune cells (11). Caspase-1 has also been shown to regulate the expression of NF-κB target genes through caspase-7-mediated cleavage of PARP1 (21). In addition, recent studies have implicated caspase-1 in cell survival by facilitating membrane biogenesis and cellular repair via regulation of lipid metabolism (22).

A unique feature of HCV is its absolute reliance on host lipids in the various stages of the viral life cycle (23). To favor its proliferation, HCV alters cellular lipid metabolism by stimulating lipogenesis, impairing mitochondrial β-oxidation and cellular lipid export, and promoting a lipid-rich intracellular environment (23, 24). This alteration of lipid homeostasis leads to the intracellular accumulation of cellular lipid inclusions, termed “lipid droplets” (LDs), that play a crucial role in the HCV life cycle, hepatic steatosis, and HCC (24–26). Sterol regulatory element-binding proteins (SREBPs) are the master regulators of lipid homeostasis, controlling the transcription of genes encoding enzymes involved in cholesterol, triglyceride, and phospholipid synthesis (27). Previously, we have demonstrated that HCV-infected human hepatic cells upregulated SREBP, the underlying mechanism by which HCV alters cellular lipid metabolism is not clearly understood. To be activated and cleaved to produce the active/mature forms, there are three SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2 (27). SREBP-1a activates all SREBP target genes, whereas SREBP-2 and SREBP-1c activate genes involved in cholesterol and fatty acid synthesis, respectively (27). SREBPs are synthesized as endoplasmic reticulum (ER)-membrane-bound precursors and exist in complex with SREBP cleavage-activating protein (SCAP) (27). SCAP is both an escort for SREBPs and a sensor of sterol. Retention of the SCAP-SREBP complex in the ER is mediated by the binding of SCAP to insulin-induced gene (Insig) proteins (29). Insig-1 and Insig-2 are membrane-bound proteins that reside in the ER and play a central role in the regulation of SREBP activation (30). When cells are depleted of cholesterol, SCAP transports SREBPs from the ER to the Golgi, where site 1 proteases (S1Ps) and site 2 proteases (S2Ps) act specifically and sequentially to release the active forms of SREBPs, which actively translocate into the nucleus and bind to the sterol response elements of the target genes.

In this study, we investigated the mechanism of increased lipid biosynthesis in cells infected with HCV. Our studies show that HCV-induced NLRP3 inflammasome activates SREBPs and stimulates lipogenic gene expression and formation of LDs. Our results demonstrate that the proteolytic activation of SREBPs in HCV-infected cells is mediated by interaction of the NLRP3 inflammasome with SCAP in the ER. We also demonstrate that caspase-1 activity is critical for SREBP activation. Collectively, these observations provide insights into the novel role of the NLRP3 inflammasome in lipid homeostasis during chronic HCV infection.

Experimental Procedures

Plasmids and Reagents—The infectious HCV J6/JFH-1 cDNA (genotype 2a) and the replication-defective HCV JFH-1/GND constructs were obtained from Dr. C. Rice (Rockefeller University, NY). Recombinant IL-1β was purchased from R&D Systems (Minneapolis, MN). The wild-type human pFLAG-NLRP3 expression vector was obtained from Dr. J. Tschopp (University of Lausanne, Switzerland). MG132, ALLM (N-Acetyl-Leu-Leu-Met-CHO), and inhibitors of caspase-1 (Z-YVAD-fmk) and caspase-3 (Ac-DEVD-CHO) were from EMD Millipore (Massachusetts, MA), and caspase-3 inhibitor#3 was from Invitrogen.

Antibodies—The antibodies used according to the protocols of the manufacturer: HCV NS3 (Virogen, MA); HCV NS5A (Biovendor, Rudolstadt, Germany); NLRP3 for immunoprecipitation (BD Biosciences, San Jose, CA); NLRP3 for IF (MBL, Woods Hole, MA); rabbit polyclonal antibody against the N-terminal 174 residues of SCAP (MBL, Woods Hole, MA); rabbit polyclonal antibody against HCV NS5A was a gift from Dr. Craig Rice (University of Pennsylvania State University). The organelle localization and subcellular immunofluorescence antibody sampler kit was from Cell Signaling Technology (Danvers, MA).

Cell Culture—The human hepatoma cell line Huh-7.5 was obtained from Dr. C. Rice (31). Huh-7.5 cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ with DMEM supplemented with 10% fetal calf serum, 100 units of penicillin/ml, and 100 μg of streptomycin sulfate/ml.

HCV Cell Culture Infection System—Fifteen micrograms of in vitro transcribed J6/JFH-1 RNA was delivered into Huh-7.5 cells by electroporation as described previously (32, 33). Cells were passaged every 3–5 days. The presence of HCV in these cells and the corresponding supernatants was determined as described previously (33). The cell-free virus was propagated in Huh7.5 cell culture as described previously (32–34). The expression of HCV protein in HCV-infected cells was analyzed by Western blotting. The HCV cell culture supernatant was collected at appropriate time points and used to infect naïve Huh7.5 cells at a multiplicity of infection of 1 for 5–6 h at 37 °C and 5% CO₂ (32, 33). The viral titer in the cell culture supernatant was expressed as focus forming units per milliliter, which was determined by the average number of HCV-NS5A-positive foci detected at the highest dilutions, as described previously (33). The cell culture supernatant collected from Huh7.5 cells expressing JFH-1/GND (replication-defective virus) was used as a negative control.

Preparation of Nuclear Extracts—Nuclear lysates were prepared from mock and HCV-infected cells. Cells were lysed in

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Preparation of Nuclear Extracts—Nuclear lysates were prepared from mock and HCV-infected cells. Cells were lysed in
Hepatitis C virus (HCV) activates the NLRP3 inflammasome, a multimeric protein complex that mediates the production of mature interleukin-1β (IL-1β) and other pro-inflammatory cytokines. This activation is driven by the assembly of NLRP3 inflammasome, an important mediator of inflammation and tissue injury.

**Materials and Methods**

### Hypothetical experiment setup

1. **Hypothetical buffer composition**: HEPES (pH 7.9), 10 mM KCl, 0.1 mM Na₃VO₄, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 3 mg/ml aprotinin, 1 mg/ml pepstatin, 20 mM NaF, and 1 mM DTT with 0.2% Nonidet P-40 on ice for 15 min. After centrifugation at 4 °C (13,000 rpm) for 1 min, the nuclear pellet was resuspended in high-salt buffer (hypotonic buffer with 20% glycerol and 420 mM NaCl) at 4 °C by rocking for 30 min after centrifugation. The supernatant was collected and stored at −80 °C in aliquots.

### Hypothetical Western Blotting Analysis

- **Immunoprecipitation and Western Blotting Analysis**: Cellular lysates from mock- and HCV-infected cells were prepared by incubation in radioimmune precipitation assay buffer (51 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM sodium formate, and 10 μL/ml protease inhibitor mixture (Thermo Scientific)) for 30 min on ice. Equal concentrations of cellular lysates were immunoprecipitated with the indicated antibodies overnight at 4 °C. The immune complexes were incubated with protein A-Sepharose (Invitrogen) for 1 h at 4 °C, washed three to four times with radioimmune precipitation assay buffer, and boiled for 5 min in SDS-containing sample buffer. The samples were then subjected to SDS-PAGE. Gels were electrophoresed 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, and 5% nonfat dry milk) and probed with primary antibody of interest for 1 h at room temperature. The membranes were then washed for 10 min in Tris-buffered saline with 1%Tween 20 (TBS-T), the immunoblots were visualized using the LICOR Odyssey system.

- **Laser-scanning Confocal Microscopy**: Mock- and HCV-infected cells on coverslips were fixed with 4% paraformaldehyde for 10 min, washed, and permeabilized for 5 min with 0.2% Triton X-100. Cells were then incubated with primary antibody to the specific protein for 1 h at room temperature or overnight at 4 °C, followed by incubation with Alexa Fluor-labeled secondary antibodies (Invitrogen) for 1 h. After washing with PBS, cells were mounted with anti-fade reagent containing DAPI (Invitrogen) and observed under a laser-scanning confocal microscope (Fluoview FV10i).

### Immunohistochemistry

- **Immunohistochemistry**: Liver biopsies from normal and HCV-associated cirrhosis and HCC (no history of hepatitis B virus, HIV infection, and fatty liver) were obtained from the Liver Tissue Cell Distribution System (University of Minnesota, Minneapolis, MN). Immunohistochemistry was performed according to the protocol of the manufacturer using the Leica BOND-III™ polymer refined detection system (DS 9800) at the Stephenson Cancer Center Pathology core laboratory (University of Oklahoma Health Sciences Center, Institutional Review Board (IRB) Number 3405). The tissue sections from normal and HCV-associated cirrhosis and HCC were deparaffinized and rehydrated in an automated multistainer (Leica ST5020). The tissue section slides were subjected to antigen retrieval at 100 °C for 20 min in a retrieval solution, followed by incubation in blocking solution for 1 h. The sections were stained with primary antibody for 1 h, followed by the secondary antibody (poly-HRP IgG). The detection was performed using 3,3’-diaminobenzidine tetrachloride, and counterstaining was done with hematoxylin. For double-staining, the Leica BOND-III™ polymer refined detection system (DS 9800) and Leica BOND-III™ refined red detection system (DS 9390) were used sequentially. For Western blotting analysis, frozen liver tissues were thawed in radioimmune precipitation assay buffer and crushed gently, followed by sonication and incubation on ice for 30 min. Samples were centrifuged at 4 °C, and the supernatant was collected.

### Quantitative RT-PCR

- **Quantitative RT-PCR**: Total cellular RNA was extracted from mock- and HCV-infected cells using TRIzol (Invitrogen) and treated with QIAGEN RNase-free DNase prior to RNA synthesis. The cDNA was reverse-transcribed from 1 μg of total RNA using a reverse transcription kit (Life Technologies). Quantitative RT-PCR was carried out using SYBR Green Master Mix (Life Technologies) and specific primers as described previously (3, 28, 31). 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 the ΔΔCt method as specified by the manufacturer.

### Cell Viability Assay

- **Cell Viability Assay**: Mock-infected cells (Huh7.5), HCV-infected cells, and HCV-infected cells transfected with various siRNA or treated with caspase-1 and caspase-3 inhibitors were placed in a 96-well plate. The cells were lysed, and ATP was quantitated according to the instructions of the manufacturer using the CellTitre Glo luminescent cell viability assay kit (Promega). The percent viability was calculated considering 100% viability for mock cells. The values represent the mean ± S.D. of three independent experiments performed in duplicate.
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**Results**

The NLRP3 Inflammasome Induces Lipogenesis in HCV-infected Cells—We have demonstrated previously that HCV stimulates lipogenesis by activating SREBPs (28). However, the underlying mechanism(s) by which HCV activates SREBPs is not clearly understood. Recently, studies have shown the role of the inflammasome complex in cell survival by facilitating membrane biogenesis and cellular repair via regulation of lipid metabolism (22). To determine whether HCV induces lipogenesis through the activation of the NLRP3 inflammasome, HCV-infected Huh7.5 cells were transfected with siRNA against each component of the inflammasome complex (i.e. siNLRP3, siASC, and sicaspase-1) or scrambled siRNA (sicontrol). Equal amounts of cellular lysates were subjected to immunoblot analysis. A marked reduction in the expression of NLRP3, ASC, and caspase-1 protein levels suggests efficient knockdown of these proteins by their corresponding siRNA. The specificity of siRNA activity was indicated by the fact that control siRNA did not inhibit the expression of these proteins (Fig. 1, A–C). HCV-infected cells transfected with siNLRP3 specifically down-regulated the expression of NLRP3 by 87% and other component (ASC) of the inflammasome complex (lane 4). This is also true for HCV-infected cells transfected with siASC compared with sicontrol (Fig. 1B, lane 4). In addition, HCV-infected Huh7.5 cells transfected with independent siNLRP3 duplex, siASC (Fig. 1B), and sicaspase-1 (Fig. 1C) specifically down-regulated the expression of NLRP3 (Fig. 1D), ASC (Fig. 1E), and caspase-1 (Fig. 1F), respectively. In all tests, p < 0.05 was considered statistically significant.

To determine the role of NLRP3 inflammasome-mediated lipogenic gene expression, lysates from mock- and HCV-infected Huh7.5 cells silenced with siNLRP3, siASC, and sicaspase-1 were subjected to immunoblot analysis. The results show increased expression of fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD) in HCV-infected cells that were reduced significantly in cells transfected with siNLRP3, siASC, and sicaspase-1 compared with sicontrol (Fig. 1G). Furthermore, we also analyzed the expression of FAS and SCD in the presence of inhibitors of caspase-1 and caspase-3 (negative control). Our results showed significantly reduced expression of FAS and SCD in HCV-infected cells treated with caspase-1 inhibitor compared with caspase-3 inhibitor (Fig. 1F, lanes 7 and 8). In addition, we also observed a significant reduction in the expression of SCD in HCV-infected cells transfected with siNLRP3#2, suggesting that siNLRP3#1 and #2 produce similar phenotypes and not likely to be the off-target effects of these siRNA (Fig. 1G). The effect of silencing of NLRP3 on SCD expression was rescued by siRNA-resistant ectopic expression of NLRP3 (pFLAG-NLRP3del) (Fig. 1H, lane 4).

To determine whether NLRP3 inflammasome-mediated induction of lipogenic genes was due to increased expression of their mRNA, total cellular RNA from the above siRNA-transfected cells were subjected to quantitative RT-PCR using 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCS)-, FAS-, and SCD-specific primers. We observed significantly higher expression of FAS mRNA (~12-fold), HMGCS mRNA (~9-fold), and SCD mRNA (~7-fold) in HCV-infected cells that was decreased in cells transfected with siNLRP3, siASC, and sicaspase-1 but not in sicontrol (Fig. 1J). In addition, treatment with caspase-1 inhibitor (Z-YVAD-fmk) but not with caspase-3 inhibitor (DEVd) also blocked HCV-induced lipogenic gene expression (Fig. 1I). Taken together, these results suggest that HCV-infected cells induce lipogenic genes through activation of the NLRP3 inflammasome and are dependent on caspase-1 activity.

The HCV-activated NLRP3 Inflammasome Induces LDs Formation—To determine the role of the HCV-induced NLRP3 inflammasome in LD formation, mock- and HCV-infected Huh7.5 cells transfected with siNLRP3, siASC, sicaspase-1, and sicontrol were stained with the neutral lipid-specific green fluorescent dye BODIPY 493/503. The results show increased staining of LDs in HCV-infected cells compared with mock-infected cells (Fig. 2A, lane E). Huh7.5 cells transfected with siNLRP3 and sicontrol were incubated with caspase-1 inhibitor (Fig. 2A, lane C) or caspase-3 inhibitor (Fig. 2A, lane D). Treatment with caspase-3 inhibitor but not with caspase-1 inhibitor results in reduced staining of LDs in HCV-infected cells (Fig. 2A, lane C). Furthermore, treatment of Huh7.5 cells transfected with siASC (Fig. 1B, lane 4) and sicaspase-1 (Fig. 1C, lane 4) did not result in accumulation of LDs (Fig. 2A, lane E). This result showing that this event is not mediated by IL-1β that is also known to stimulate cytokine production. These results suggest that HCV induces accumulation of LDs via an inflammasome-dependent mechanism(s). 

To determine the sequence of NLRP3 inflammasome activation and LD formation in HCV-infected cells, we analyzed the activation of caspase-1 and staining of LDs at various time points. Our results suggest that HCV induces activation of the NLRP3 inflammasome, which is followed by lipogenesis and LD accumulation in HCV-infected cells (data not shown).

The NLRP3 Inflammasome Activates SREBPs in HCV-infected Cells—SREBPs are known to regulate cholesterol and fatty acid biosynthesis pathways (27, 29). To determine whether the master inducers of lipid metabolism, SREBP-1 and SREBP-2, are regulated by the NLRP3 inflammasome complex in HCV-infected cells, total cellular lysates from mock- and HCV-infected cells transfected with siNLRP3, siASC, sicaspase-1, and sicontrol were subjected to Western blotting. We observed proteolytic cleavage of SREBP-1 and SREBP-2 in HCV-infected cells compared with mock-infected cells (Fig. 3, A and B, lanes 1 and 2) that were reduced in cells silenced with siNLRP3, siASC, and sicaspase-1 but not in sicontrol cells (Fig. 3, A and B, lanes 3–6). To determine the role of caspase-1 activity on SREBP-1 and SREBP-2 proteolytic activation, mock- and HCV-infected cells were incubated with inhibitors of caspase-1 and caspase-3. Our results show significantly reduced activation of SREBP-1 and SREBP-2 in the presence of caspase-1 inhibitor but not caspase-3 inhibitor (Fig. 3, C and D, lanes 3 and 4). These results suggest the role of the NLRP3 inflammasome-mediated
caspase-1 in HCV-induced proteolytic cleavage of SREBP-1 and SREBP-2 into their mature forms. To further demonstrate the activation and nuclear translocation of the mature forms of SREBPs in HCV-infected cells, cytoplasmic and nuclear lysates were subjected to Western blotting. The results show the induction of precursor SREBP-1 in HCV-infected cytoplasmic
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**FIGURE 1.** The HCV-activated NLRP3 inflammasome induces lipogenic gene expression. Mock- (Huh7.5) and HCV-infected cells (infected with HCV at a multiplicity of infection of 1 for 2 days) were transfected with sicontrol, siNLRP3, siASC, and sicaspase-1. 72 h post-transfection, cellular lysates were subjected to Western blotting using the respective antibodies. A, equal amounts of cellular lysates from mock-, HCV-, and HCV-infected cells transfected with siNLRP3 were immunoblotted with anti-NLRP3 and anti-ASC antibodies. B, cellular lysates from HCV-infected cells transfected with siASC were immunoblotted with anti-ASC and anti-NLRP3 antibodies. C, HCV-infected cells were transfected with sicontrol and sicaspase-1. D, HCV-infected cells were transfected with sicontrol and two different individual siNLRP3 duplexes (siNLRP3#1 and siNLRP3#2) that were present in the siRNA pool used above (Santa Cruz Biotechnology). Actin represents HCV infection. E, mock- (Huh7.5), HCV-, and HCV-infected cells transfected with sicontrol, siNLRP3, siASC, and sicaspase-1 at various time points or treated with caspase-1 and caspase-3 inhibitors were placed in a 96-well plate. The cells were lysed, and ATP was quantitated according to the instructions of the manufacturer using a CellTitre-Glo luminescence cell viability assay kit (Promega). The percent viability was calculated considering 100% viability for mock-infected cells compared with HCV-infected cells transfected with various siRNA or treated with caspase-1/-3 inhibitors. The values represent mean ± S.D. of three independent experiments performed in duplicate. F, equal amounts of cellular lysates from mock- and HCV-infected cells transfected with siNLRP3, siASC, and sicaspase-1 were subjected to Western blotting using anti-FAS and anti-SCD antibodies. Lane 1, mock cells; lane 2, HCV-infected cells; lanes 3–6, HCV-infected cells transfected with sicontrol, siNLRP3, siASC, and sicaspase-1, respectively; lanes 7 and 8, HCV-infected cells treated with inhibitors of caspase-1 (50 μM Z-YVAD-fmk for 2 h) and caspase-3 (100 μM DEVD for 2 h); right panel (lanes 9 and 10), basal level expression of FAS in mock cells. G, equal amounts of cellular lysates from mock- and HCV-infected cells transfected with sicontrol, siNLRP3#1, and siNLRP3#2 were subjected to Western blotting using anti-SCD antibodies. Lane 1, mock cells; lane 2, HCV-infected cells; lanes 3–5, HCV-infected cells transfected with siNLRP3#1 and siNLRP3#2, respectively. H, rescue of NLRP3 gene silencing. The wild-type NLRP3-expressing plasmid (pFLAG-NLRP3wt) and the plasmid expressing siRNA-resistant mRNA containing a deletion of the 3’ UTR of NLRP3 (pFLAG-NLRP3del) along with siNLRP3 were transfected in HCV-infected cells. The pFLAG-NLRP3del expression plasmid was generated using a site-directed mutagenesis kit according to the protocols of the manufacturer (Stratagene). Cellular lysates were subjected to Western blotting using the respective antibodies. The siNLRP3 target sequence was 5’-CAGGCTAATGATCGACTTCAA-3’ (Qiagen). I, total cellular RNA was extracted from mock- and HCV-infected cells transfected with the above siRNA and subjected to quantitative RT-PCR using FAS-, HMGCS-, and SCD-specific primers and a SYBR Green probe. The values represent mean ± S.D. of three independent experiments performed in triplicate. *, p < 0.05 compared with mock-infected Huh7.5 cells; **, p < 0.05 compared with sicontrol-transfected cells; ***, p < 0.05 compared with HCV-infected cells treated with the caspase-3 inhibitor (DEVD).

**FIGURE 2.** The NLRP3 inflammasome induces lipid droplet formation in HCV-infected cells. A, mock- (Huh7.5), HCV-, and HCV-infected cells were transfected with sicontrol, siNLRP3, siASC, or sicaspase-1, incubated with recombinant IL-1β (20 ng/ml) for 24 h. These cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with 20 μM BODIPY 493/503 (Invitrogen) for 30 min, followed by incubation with anti-HCV NS5A antibodies (red fluorescence). Cells were visualized under a laser-scanning confocal microscope (Fluoview FV10i). DAPI was used as a nuclear stain. B, LDs were quantified manually in 15 individual cell images under various conditions using ImageJ software.

**WITHDRAWN**

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lysates and the presence of a significant amount of mature SREBP-1 in the nuclear lysates (Fig. 3C, lanes 2 and 4). In contrast, we did not detect any mature SREBP-1 in nuclear lysates of mock-infected cells (Fig. 3C, lane 3).

It is well established that the mature forms of SREBPs translocate into the nucleus and bind to the SRE of the target genes (27). To determine whether the translocation of mature forms of SREBP-1 and SREBP-2 into the nucleus is regulated by the NLRP3 inflammasome, mock- and HCV-infected cells, as described in Fig. 2, were subjected to immunofluorescence. The results show significant nuclear translocation of mature SREBP-1 and SREBP-2 in HCV-infected cells compared with mock-infected cells (Fig. 4, A and B). In contrast, we observed reduced translocation of mature SREBP-1 and SREBP-2 in HCV-infected cells transfected with siNLRP3, siASC, and sicaspase-1, or incubated with caspase-1 inhibitor but not with sicontrol or treated with caspase-3 inhibitor (Fig. 4, A–C, b, yellow dots). These results suggest the interaction of the NLRP3 inflammasome in HCV-infected cells plays a critical role in the activation and nuclear translocation of SREBPs. To demonstrate that SREBP-1 is transported to the Golgi during HCV infection, mock- and HCV-infected cells were stained with anti-SREBP-1 and anti-RCAS1 (a Golgi marker) and subjected to confocal microscopy. The results show a significant association of SREBP-1 with the Golgi in HCV-infected cells (Fig. 4C, yellow spots) compared with mock cells. In addition, we also observed a significant migration of the mature form of SREBP-1 into the nucleus of the HCV-infected cells (Fig. 4C).

The NLRP3 Inflammasome Colocalizes with SCAP in HCV-infected Cells—SCAP transports SREBPs from the ER to the Golgi. To determine whether the NLRP3 inflammasome interacts with SCAP and facilitates the transport of SREBPs from the ER to the Golgi, we performed confocal microscopy. The results show significant colocalization of NLRP3, ASC, and caspase-1 with SCAP in HCV-infected cells compared with mock-infected cells (Fig. 5, A–C, b, yellow dots).
NLRP3 inflammasome with SCAP. To determine whether the NLRP3 inflammasome associates with the ER, colocalization of NLRP3, ASC, and caspase-1 was performed with an ER marker, PDI. Mock- and HCV-infected cells were stained with anti-NLRP3, anti-ASC, caspase-1, and sicontrol or treated with inhibitors of caspase-1 (Z-YVAD-fmk) and caspase-3 (DEVD) were fixed and permeabilized as described in Fig. 2. The cells were incubated with anti-SREBP-1 and anti-SREBP-2 antibodies for 1 h at room temperature, followed by incubation with secondary antibodies for SREBP-1 (goat anti-mouse Alexa Fluor 488) and SREBP-2 (donkey anti-goat Alexa Fluor 488). DAPI was used as a nuclear stain. Arrows represent staining of SREBP-1 and SREBP-2. C, Mock and HCV-infected cells were incubated with anti-SREBP-1, anti-RCAS, and their secondary antibodies as described above. Arrows represent colocalization of SREBP-1 with the Golgi (yellow dots).
Association of the NLRP3 Inflammasome with SCAP—The association of NLRP3, ASC, and caspase-1 with SCAP was also confirmed by a protein-protein interaction approach. Cellular lysates from mock- and HCV-infected cells were immunoprecipitated with anti-SCAP, followed by Western blotting using anti-NLRP3, anti-caspase-1, and anti-SCAP antibodies. The

FIGURE 5. Colocalization of NLRP3, ASC, and caspase-1 with SCAP. A–C, mock- and HCV-infected cells were fixed, permeabilized, and incubated with anti-SCAP, anti-NLRP3, anti-ASC, and anti-caspase-1 antibodies for 1 h at room temperature, followed by incubation with secondary antibodies (SCAP, anti-goat Alexa Fluor 488; NLRP3, anti-mouse Alexa Fluor 546; ASC, anti-mouse Alexa Fluor 546; caspase-1, anti-mouse Alexa Fluor 546; NS5A, anti-rabbit Alexa Fluor 633). Arrows represent colocalization of SCAP with NLRP3, ASC, and caspase-1 (b). D–F, subcellular localization of the NLRP3 inflammasome complex. Mock- and HCV-infected cells were fixed, permeabilized, and incubated with ER marker protein (anti-PDI), anti-NLRP3, anti-ASC, anti-caspase-1, and anti-HCV NS5A for 1 h at room temperature. The cells were incubated with secondary antibodies (PDI, goat anti-rabbit Alexa Fluor 488; NLRP3, anti-mouse Alexa Fluor 546; ASC, anti-mouse Alexa Fluor 546; caspase-1, anti-mouse Alexa Fluor 546; NS5A, anti-rabbit Alexa Fluor 633). DAPI was used as a nuclear stain. HCV NS5A represented HCV infection. Arrows indicate colocalization of proteins. G–I, mock- and HCV-infected cells were incubated with antibodies against the marker proteins of endosomes (anti-EEA1), lysosomes (anti-LAMP1), Golgi (anti-RCAS1), anti-NLRP3, and anti-NS5A, followed by their secondary antibodies. DAPI was used as a nuclear stain. Arrows indicate colocalization of proteins.
results showed that SCAP interacted with caspase-1 in HCV-infected cells (Fig. 6A, lanes 3 and 4). Immunoprecipitation pull down NLRP3 and caspase-1 and did not show the expression of ASC, NLRP3, and caspase-1 antibodies. We observed that NLRP3, ASC, and caspase-1 were pulled down with SCAP in HCV-infected cells compared with mock-infected cells but not with an isotype control antibody (Fig. 6B, lanes 3–10). Collectively, these results suggest that the NLRP3 inflammasome interacts with SCAP in HCV-infected cells.

The NLRP3 Inflammasome Induces Degradation of Insig Proteins in HCV-infected Cells—Because Insigs are ER-resident proteins and play an important role in the activation of SREBP-1 and SREBP-2, we examined the status of Insig-1 and Insig-2 in HCV-infected cells. Mock- and HCV-infected cellular lysates were subjected to Western blotting using anti-Insig-1 and anti-Insig-2 antibodies. The results showed reduced expression of Insig-1 and Insig-2 expression in HCV-infected cells compared with mock-infected cells (Fig. 7A, lane 2). However, we did not observe any change in the expression of SCAP. Previously, it has been demonstrated that the dissociation of Insig from the ER retention complex leads to proteasome-mediated degradation of Insig (30). Our results clearly showed the degradation of Insig-1 and Insig-2 in HCV-infected cells, which was blocked by proteasome inhibitor but not by calpain inhibitor (negative control) (Fig. 7B, lanes 3 and 4), suggesting that Insig-1 and Insig-2 play critical roles in SREBP activation in HCV-infected cells.

To determine whether the interaction of the NLRP3 inflammasome/caspase-1 complex mediates the degradation of Insig proteins, we examined the status of Insig proteins in HCV-infected cells silenced with sicaspase-1. The cellular lysates were analyzed by Western blotting. The results show degradation of Insig-1 in HCV-infected cells that was blocked in cells silenced with sicaspase-1 but not in sicontrol cells (Fig. 7C, lanes 3 and 4). In addition, we observed significant silencing (68%) of caspase-1 expression in HCV-infected cells (Fig. 7C, lane 4). These results suggest a role of the caspase-1-inflammasome complex in HCV-mediated degradation of Insig proteins.

HCV Activates Caspase-1 in Hepatocytes of HCV-positive Liver Tissues—In this study, we examined caspase-1 activation as a readout of NLRP3-inflammasome activation in HCV-positive liver tissues. Caspase-1 is an effector molecule of the inflammasome complex (8, 18). We examined liver tissues from HCV-positive patients with cirrhosis (five cases) and HCC (four cases) to validate the expression and activation of caspase-1 in cell culture studies (3). Normal and HCV-positive patient liver
HCV-activated NLRP3 Inflammasome Regulates Lipid Metabolism

Figure 8. HCV activates caspase-1 in human liver tissues.
(A) HCV-positive patients with cirrhosis produced caspase-1 antibodies (brown) in liver tissues compared with normal tissues (Fig. 8A, a, HCV-positive patients with cirrhosis). The boxed areas in (b) are enlarged within the figure. B) Western blotting. Equal amounts of cellular lysates from normal (lane 1) and HCV-infected liver tissues (sample 1, cirrhosis) were subjected to Western blotting using anti-caspase-1 antibodies. Tubulin represents the protein loading control.

Discussion

In recent years, activation of the inflammasomes has been implicated in various chronic diseases and in the clearance of several viruses (35–38). However, the role of the inflammasome complex in HCV pathogenesis is incompletely understood. In addition to various infections, abnormal lipid metabolism has been strongly linked to chronic inflammation in a mouse obesity model (39). Recent studies have implicated the inflammasome complex/caspase-1 in cell survival by facilitating membrane biogenesis and cellular repair via regulation of lipid metabolism (22). Consistent with this observation, our studies clearly provide a link between chronic inflammatory pathways and host lipid metabolism during HCV infection. We show that activation of the NLRP3 inflammasome in HCV-infected cells causes the activation of SREBPs and induces lipogenesis and LD formation, cellular events critical for HCV proliferation and liver disease progression in patients with chronic HCV.

In this study, our observation of caspase-1 activation in HCV-infected cells, the effector molecule of the inflammasome, is interesting in view of the current understanding of chronic HCV pathogenesis. Several studies from other groups have demonstrated the NLRP3 inflammasome activity in liver samples of patients with chronic HCV (40–42). These studies clearly established that the NLRP3 inflammasome is activated in patients infected with HCV.

It has been shown that HCV also activates the NLRP3 inflammasome in hepatic macrophages and monocytes (4–7). However, in these reports, activation of the NLRP3 inflammasome in human hepatoma cells or primary hepatocytes by HCV was not observed. The failure to observe inflammasome activation could be due to infection with a low multiplicity of infection of 0.1 and reliance on the detection of mature forms of IL-1β and IL-18 in cell culture supernatants. Recent in vivo studies have shown that non-immune cells, such as hepatocytes, express and activate the inflammasome complex but do not secrete adequate/detectable amounts of IL-1β and IL-18 compared with immune cells, suggesting that activation of the inflammasome complex in epithelial cells is likely to be involved in cytokine-independent functions (11–15). Our findings suggest that, unlike in immune cells, in human hepatocytes (epithelial cells), HCV modulates the NLRP3 inflammasome differently according to its specific niche to alter lipid metabolism, leading to LD accumulation and liver disease pathogenesis (Fig. 9). NLRP3 is known to interact with several proteins to modulate various cellular functions (12–17). Apart from cleavage/maturation of IL-1β and IL-18, caspase-1 has been shown to cleave several proteins, suggesting a broader role of the NLRP3 inflammasome/caspase-1 in addition to maturation of cytokines (19).

Our results show that the induction of lipogenic genes (HMGCS, SCD, and FAS) is mediated by activation of the NLRP3 inflammasome in HCV-infected cell lines (Fig. 1). HMGCS and FAS are critical enzymes involved in the biosynthesis of cholesterol and fatty acids, respectively (27, 29). SCD is a micro-
somal enzyme required for the biosynthesis of oleate and palmitoleate, which are the major monounsaturated fatty acids of membrane phospholipids, triglycerides, and cholesterol esters (41). The LD core contains triglycerides and cholesterol esters covered by a phospholipid monolayer (42). These results suggest a role of HMGCS, FAS, and SCD in NLRP3 inflammasome-mediated lipogenesis and LD formation. The promoters of the HMGCS, SCD, and FAS genes have been shown to contain functional binding sites for SREBPs (27, 29, 41).

Previously, we have shown activation of SREBPs in HCV-infected cells (28). However, the underlying mechanisms by which HCV is able to override the cholesterol-dependent physiological regulation of SREBP activation remain unclear. In another study, the investigators have shown activation of SREBP by HCV NS4B via the Akt pathway (43). The authors have shown phosphorylation of the mature form of SREBP after the cleavage steps by S1P and S2P in the Golgi. However, our results demonstrate how activation of the NLRP3 inflammasome by HCV infection induces the proteolytic cleavage of SREBPs prior to translocation of their mature forms into the nucleus. There are two steps in the activation of SREBP: proteolytic cleavage of SREBP in the ER/Golgi and posttranslational modification of SREBP prior to the translocation of mature forms into the nucleus (27, 29). Our results are consistent with previous studies demonstrating the role of the inflammasome complex/caspase-1 in activating SREBPs to promote lipid biogenesis and cell survival in response to bacterial pore-forming toxins (22). In contrast, another study has shown activation of the NLRP3 inflammasome by SREBP-2 in endothelial cells in the context of atherosclerotic lesions in a mouse model (44).

In normal cells, SCAP, SREBPs, and Insig proteins form a complex in the ER membrane (27, 29). When cells are depleted...
masome is clearly the novel aspect of this study. Collectively, our results highlight the implications of metabolic abnormalities in liver diseases and provide a conceptual framework to develop novel strategies for combating chronic liver diseases associated with HCV infection.

**Author Contributions**—G. W. conceived, designed, and performed the experiments, analyzed the data, and wrote the paper. G. W., S. M., J. I., M. S. D., S. L., and A. N. designed, performed, and analyzed the experiments. N. A. provided technical assistance and contributed to the preparation of Fig. 8. All authors reviewed the results and approved the final version of the manuscript.

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**References**


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