Hepatitis C virus (HCV)-mediated chronic liver disease is a global health problem, and inflammation is believed to be an important player in disease pathogenesis. HCV infection often leads to severe fibrosis/cirrhosis and hepatocellular carcinoma, although the mechanisms for advancement of disease are not fully understood. The proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 have critical roles in establishment of inflammation. In this study, we examined induction of IL-1β/IL-18 secretion following HCV infection. Our results demonstrated that monocyte-derived human macrophages (THP-1) incubated with cell culture-grown HCV enhance the secretion of IL-1β/IL-18 into culture supernatants. A similar cytokine release was also observed for peripheral blood mononuclear cell (PBMC)-derived primary human macrophages and Kupffer cells (liver-resident macrophages) upon incubation with HCV. THP-1 cells incubated with HCV led to caspase-1 activation and release of proinflammatory cytokines. Subsequent studies demonstrated that HCV induces pro-IL-1β and pro-IL-18 synthesis via the NF-κB signaling pathway in macrophages. Furthermore, introduction of HCV viroporin p7 RNA into THP-1 cells was sufficient to cause IL-1β secretion. Together, our results suggested that human macrophages exposed to HCV induce IL-1β and IL-18 secretion, which may play a role in hepatic inflammation.

Hepatitis C virus (HCV) infection leads to chronic liver disease in ~70% of infected patients, who may develop cirrhosis and hepatocellular carcinoma (HCC) during the course of infection (1). The occurrences of cirrhosis and HCC are increasing among persons infected with HCV (2). Persistent liver inflammation is strongly associated with HCV-mediated liver disease progression and also increases the risk of an aggravated immune response and fibrosis (3). HCV is a single-stranded, positive-sense RNA virus that belongs to Flaviviridae family in the Hepacivirus genus. HCV infects primarily hepatocytes and is released into the circulation. However, intercellular communication in HCV-infected liver is poorly understood. Enhanced expression of inflammatory cytokines and chemokines is crucial in disease processes, either by direct signaling or by recruiting immune cells. Kupffer cells are resident macrophages in the liver and play a pivotal role in triggering inflammation during liver diseases (4). During HCV infection, virus is sensed by pattern recognition receptors (PRRs) on Kupffer cells, which modulate the inflammatory response (5).

Inflammation is a protective immune response to ensure the repair of damaged tissue and removal of detrimental stimuli by host cells (6). Although immune cells, including macrophages and dendritic cells (DCs), play important roles, nonprofessional cells such as epithelial cells, endothelial cells, and fibroblasts also contribute to inflammation induced by microbial infection or tissue damage (7, 8). Interleukin-1β (IL-1β) and IL-18 play an important role in combating the invading pathogen as part of the innate immune response. Since HCV infection often leads to chronic disease, we wanted to examine the status of IL-1β/IL-18 in HCV-infected hepatocytes or its induction through cross talk with macrophages. IL-1β-activating platforms, known as inflammasomes, can assemble in response to the detection of endogenous host- and pathogen-associated danger molecules. The inflammasomes comprise a family of cytoplasmic PRRs collectively known as NOD-like receptors (NLRs) to sense viral nucleic acid and/or viral proteins. Once activated, NLRs form a multiprotein complex with apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC) and caspase-1 for assembly of inflammasomes, which in turn activates caspase-1 (9). NLRP1, NLRP3, and NLRC4 have been shown to sense viral infections, leading to activation of inflammasomes, and trigger the release of the proinflammatory cytokines IL-1β and IL-18. The production of IL-1β and IL-18 is a tightly regulated process which requires two distinct signals for activation and release (10). The first signal leads to NF-κB activation and synthesis of pro-IL-1β and pro-IL-18 mRNA in a Toll-like receptor (TLR) signal-dependent manner. The second signal involves activation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 into mature and biologically active IL-1β and IL-18.

Inflammatory cells and mediators are found frequently in the local environment of tumors, and inflammation is considered a hallmark of cancer (11). Hepatocellular injury followed by inflammation and activation of the innate immune system may lead to early-stage liver fibrosis, resulting in hepatic stellate cell (HSC) activation and extracellular matrix (ECM) deposition (12). Gene expression analyses also highlighted the role of proinflammatory cytokines (e.g., IL-1β and IL-6) and chemokines (e.g., IL-8 and CCL2) as key orchestrators of the cross talk between hepatocytes and activated HSCs (13). The invasive capacity of malignant cells increases in the presence of IL-1β and IL-6 (14). In this study, we demonstrated that HCV induces IL-1β/IL-18 expression through NF-κB activation and promotes release of the proinflammatory cytokines IL-1β and IL-18 from a monocyte-derived primary human macrophage cell line, THP-1. Interestingly, Kupffer cells in-
Total RNA was isolated by using a Qiagen RNeasy minikit (qRT-PCR). RNAiMax Lipofectamine reagent according to the manufacturer's quantitation using TaqMan gene expression PCR master mix (Applied kit (Invitrogen, CA). Real-time PCR was performed with cDNA for by using random hexamers and a Superscript III reverse transcriptase spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized for mRNA expression of IL-1β or IL-18 at 72 h postinfection. Cellular 18S rRNA was used as an internal control for normalization, and the results are shown as means from triplicate samples and three independent experiments.

FIG 1 HCV infection does not induce inflammasome mediators in Huh7 cells. (A) RNA from mock- or HCV-infected Huh7 cells was isolated at 72 h, and mRNA expression was measured for NLRP3, ASC, or caspase-1 by qRT-PCR. (B) RNA from mock- or HCV-infected Huh7 cells was separately analyzed for mRNA expression of IL-1β or IL-18 at 72 h postinfection. Cellular 18S rRNA was used as an internal control for normalization, and the results are shown as means from triplicate samples and three independent experiments.

MATERIALS AND METHODS

Cells and viruses. Human hepatoma (Huh7) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich Co.) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen), 100 U of penicillin G/ml, and 100 µg of streptomycin/ml. Human monocytic THP-1 cells were cultured in RPMI 1640 medium (Sigma-Aldrich Co.) containing 2-glutamine, 2-mercaptoethanol (50 µM), 25 mM HEPES, and 10% FBS. All cells were maintained at 37°C in a 5% CO2 atmosphere. For macrophage differentiation, THP-1 cells were seeded at a density of 1 × 106 cells/ml and treated with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) at 37°C overnight. The cells were then kept in fresh complete medium for 2 days before exposure to virus. Unless mentioned otherwise, we used PMA-differentiated THP-1 cells throughout our studies. Human peripheral blood mononuclear cells (PBMCs) from healthy individuals were cultured in RPMI supplemented with 10% FBS and 1% antibiotics. Kupffer cells with 95% purity were procured (Celsis In Vitro Technologies, MD) and maintained in DMEM supplemented with 10% human AB serum and 1% antibiotics.

HCV genotype 2a (clone JFH1) was grown in Huh7 cells, as previously described (15). Virus released into cell culture supernatants was filtered through a 0.45-µm-pore-size cellulose acetate membrane (Nalgene, Rochester, NY) and quantitated as standard IU/ml. For infection, cells were incubated with cell culture-grown HCV secreted IL-1α, IL-1β, or IL-18 at different time points. We did not observe a significant change in the expression levels of inflammasome mediators in HCV-infected cells compared to the mock-infected control (Fig. 1A and B). We also examined IL-1β and IL-18 secretion in cell culture supernatants of mock- or HCV-infected Huh7 cells by ELISA and did not observe a detectable level of these two proinflammatory cytokines (data not shown). Therefore, our data suggested that HCV infection does not induce inflammasome complex formation or activates IL-1β secretion from human hepatocytes. Our results are in agreement with a recent report where inflammasome activation in HCV-infected hepatocytes was not detected (16).

HCV induces mature IL-1β/IL-18 secretion in monocyte-derived human macrophages. Since we did not observe an inflam-

cubated with cell culture-grown HCV secreted IL-1β at a much lower level than did THP-1 cells, which may explain why this proinflammatory cytokine fails to induce a strong antiviral effect for clearance upon HCV infection.
Primary human macrophages secreted IL-1 upon exposure to HCV (Fig. 2C). However, we did not observe an increased secretion of IL-18 in HCV-exposed primary human macrophages. Next, we examined the status of IL-1β and IL-18 secretions from human Kupffer cells. These cells are resident macrophages in liver and are continuously exposed to HCV from the circulation. Our results demonstrated that Kupffer cells secrete IL-1β and IL-18 when incubated with HCV (Fig. 2D). Together, these results suggested that HCV induces the secretion of the inflammatory cytokines IL-1β and IL-18 in human macrophages.

**HCV-incubated THP-1 cells induce proinflammatory cytokine secretion through activation of caspase-1.** Next, we examined the mRNA expression level of procaspase-1 in mock- or HCV-exposed THP-1 cells and observed a significant upregulation of procaspase-1 at 20 h compared to the mock-treated control (Fig. 3A). We also determined the levels of procaspase-1 (p45) and the cleaved form of caspase-1 (p20) by Western blotting in cellular lysates of mock- and HCV-exposed THP-1 cells. A reduced expression level of procaspase-1 (45 kDa) was observed in HCV-incubated THP-1 cells compared to the control (Fig. 3B). We also observed cleaved caspase-1 (20 kDa) from HCV-exposed THP-1 cells. To determine the role of caspase-1 in mature IL-1β and IL-18 secretion, mock- or HCV-exposed cells were treated with the pancaspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK), and the culture supernatants were collected. We observed a reduced level of secretion of IL-1β/IL-18 in THP-1 cells incubated with HCV (Fig. 3C). However, we did not observe a significant difference in the IL-1β or IL-18 mRNA level upon treatment with the pancaspase inhibitor. There is emerging evidence that caspase-8 is also involved in cleavage of IL-1β/IL-18 (20). However, we did not observe activation of caspase-8 under our experimental conditions. These data suggested that HCV induces caspase-1-mediated secretion of IL-1β and IL-18 from THP-1 cells.

**HCV exposure to human macrophages transcriptionally enhances IL-1β/IL-18 expression through NF-κB signaling.** To determine the transcriptional regulation of pro-IL-1β/IL-18, PMA-treated THP-1 cells were incubated with HCV at different time points, and mRNA expression was analyzed by qRT-PCR. The results demonstrated that the mRNA expression level of pro-IL-1β/IL-18 was significantly higher in THP-1 cells upon exposure to HCV than in the mock-treated control (Fig. 4A and B). As expected, transcriptional upregulation of pro-IL-1β/pro-IL-18 was observed in LPS/ATP-stimulated THP-1 cells compared to the mock-treated control (data not shown). Similar results were obtained from HCV-exposed primary human Kupffer cells (Fig. 4C and D). The transcription factor NF-κB plays an essential role in regulation of proinflammatory cytokines, interferons, and chemokines in virus-infected cells (21, 22). In response to upstream stimulatory signals, IkB-α becomes phosphorylated by cellular kinases and is degraded by the proteasome following ubiquitination. The loss of IkB-α repression then triggers the translocation of NF-κB into the nucleus to initiate target gene transcription. To determine whether transcription of IL-1β/IL-18 is upregulated through NF-κB signaling in HCV-exposed THP-1 cells, we examined the expression levels of phospho-NF-κB p65, NF-κB p65, and IkB-α by Western blotting analyses. A significant upregulation of phospho-NF-κB p65 and a concomitant reduction in IkB-α protein expression were observed in cells exposed to HCV, compared to the mock-treated control (Fig. 4E).

![Figure 2](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
suggest that the NF-κB signaling pathway is activated for IL-1β/IL-18 transcription in HCV-exposed macrophages.

To further examine whether viral RNA can stimulate IL-1β production in PMA-treated THP-1 cells, we transfected in vitro-transcribed HCV genome-length RNA into THP-1 cells and observed IL-1β production in culture supernatants (Fig. 5A). Recently, proton-selective ion channel protein M2 of influenza virus and viroporin 2B of encephalomyocarditis virus have been implicated in IL-1β secretion (23, 24). HCV p7 is an ion channel protein, is permeable to sodium and potassium (25, 26), and may be involved in HCV-mediated IL-1β production. To examine this possibility, we transfected PMA-treated THP-1 cells with in vitro-transcribed HCV core, E1-E2, E1-E2-p7, and p7 RNAs. Our result demonstrated that HCV p7 or E1-E2-p7, but not core or E1-E2, transfection induces IL-1β production (Fig. 5A). Next, we examined whether HCV p7-mediated IL-1β production occurs through signal 1 and/or signal 2. For this, THP-1 cells were cultured in isotonic medium containing 100 mM KCl or NaCl or were treated with the ion channel blocker amantadine and transfected with in vitro-transcribed HCV p7 RNA. IL-1β secretion was reduced in the presence of extracellular KCl or amantadine (Fig. 5B). On the other hand, cells cultured with extracellular NaCl did not alter HCV p7-mediated IL-1β secretion. HCV-incubated THP-1 cells treated with amantadine also showed reduced IL-1β secretion. Interestingly, a ~3-fold increase in the IL-1β mRNA level in HCV p7 RNA-transfected THP-1 cells containing KCl or amantadine was observed compared to the level in cells transfected with HCV p7 alone. Thus, HCV p7 appeared to activate both inflammasome signal 1 and 2 pathways, and further analyses should clarify the mechanisms.

**DISCUSSION**

We have demonstrated that HCV induces secretion of IL-1β/IL-18 in the THP-1 cell line as well as in primary human macrophages and Kupffer cells. We have also shown that the induction of these proinflammatory cytokines occurs via the NF-κB signaling pathway, suggesting that HCV activates signal 1 in macrophages. Although hepatocytes are the primary host cell type for HCV, we did not observe secretion of IL-1β/IL-18 from infected Huh7 cells. Reactive oxygen species (ROS) production has been implicated in the induction of IL-1β (27). Impairment of autophagy in HCV-infected hepatocytes enhances ROS production (28). However, we did not observe an induction of inflammasome mediators in autophagy knockdown HCV-infected hepatocytes, suggesting that ROS may not be an effector mechanism for inflammasome induction. Human serum amyloid A (SAA), a major

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**FIG 3** THP-1 cells incubated with HCV activate caspase-1 and induce IL-1β/IL-18 secretion. (A) RNA was isolated from THP-1 cells incubated with HCV at different time points (1, 6, 20, and 48 h), and the mRNA expression level of caspase-1 was measured by qRT-PCR. Cellular 18S rRNA was used as an internal control for normalization, and the results are presented as means and standard deviations from triplicate samples and three independent experiments. (B) Western blot analysis of procaspase-1 (45 kDa) and active caspase (20 kDa) in cell extracts of mock- and HCV-exposed THP-1 cells. The membrane was reprobed with antibody to actin for comparison of protein loads. (C) PMA-treated THP-1 cells were preincubated with 10 μM the pancaspase inhibitor Z-VAD-FMK prior to incubation with HCV. Mock-treated THP-1 cells were used as a control. Cell-free supernatants were analyzed for mature IL-1β/IL-18 by ELISA. The results are shown as means and standard deviations from three independent experiments.
hepatic acute-phase protein, is highly induced during inflammatory responses primarily due to synergistic transcriptional up-regulation by proinflammatory cytokines. SAA drives IL-1β and IL-18 production and requires the NLRP3 inflammasome for IL-1β secretion by macrophages (29). We have previously observed that SAA remains unaltered in HCV protein-expressing hepatocytes (30) and could be a possibility for not activating the inflammasome in HCV-infected hepatocytes.

In the current model of the inflammasome, two distinct signals are required for activation and release of IL-1β/IL-18 (10). The first signal is triggered by PRRs and cytokine receptors, which activate NF-κB in stimulated cells to induce transcriptional activation of pro-IL-1β and pro-IL-18. A second signal then activates NLRP3, ASC, and procaspase-1, resulting in inflammasome assembly to promote cleavage of procaspase-1 and secretion of IL-1β/IL-18. Proinflammatory cytokines have been linked to a high degree of inflammation in liver diseases. Elevated levels of circulating IL-18 correlate with HCV infection (31, 32). Our results demonstrate transcriptional activation of IL-1β/IL-18 in human macrophages upon exposure to HCV. The expression level of NLRP3 is relatively low in many cell types and requires a priming signal to be induced (33). Although we observed an increase in the IL-1β/IL-18 expression level, other inflammasome mediators (NLRP3 or ASC) were not transcriptionally enhanced in our experimental system. Interestingly, we observed that IL-1β/IL-18 expression and secretion from HCV-infected THP-1 cells are more pronounced than those from Kupffer cells and PBMC-derived macrophages. Distinct regulation of IL-1β secretion from human macrophage subsets has been reported for dengue virus infection (34). We previously observed that exposure of human monocyte-derived dendritic cells (DCs) to cell culture-grown HCV significantly inhibited DC maturation and enhanced the production of IL-10 (35). Higher expression levels of IL-10 were reported to be associated with HCV persistence in chronically infected patients (36). Kupffer cells are also known to express anti-inflammatory cytokines such as IL-6 and IL-10 (37, 38),

FIG 4 HCV activates the NF-κB signaling pathway in human macrophages for transcriptional upregulation of IL-1β/IL-18. (A and B) RNA from THP-1 cells incubated with HCV was isolated at different time points, and the mRNA expression level of IL-1β/IL-18 was measured by qRT-PCR. (C and D) Results from a similar analyses using RNA from Kupffer cells incubated with HCV. All qRT-PCR data shown are pools from three independent experiments and were normalized using 18S rRNA as an internal control. The results are shown as means from triplicate samples and three independent experiments. (E) Western blot analysis of phospho-NF-κB (65 kDa), total NF-κB (65 kDa), and total IkB-α (39 kDa) from lysates of control THP-1 cells with or without LPS/ATP treatment or incubated with HCV. The membrane was reprobed with antibody to β-tubulin for comparison of protein loads.
Little is known about the extracellular signals that coordinate HCV-mediated liver disease progression to the early fibrosis stage. One of the critical steps in the process of liver disease progression is activation of Kupffer cells and generation of the proinflammatory cytokines IL-1β and IL-18. We observed IL-1β/IL-18 secretion at 48 h after exposure of THP-1 cells to HCV. Sustained exposure to stimuli results in chronic inflammation and likely activates the profibrogenic effect during HCV infection (40). This observation is supported by the fact that in chronic interstitial pulmonary disease, higher levels of IL-1β secretion occur due to repetitive inhalation of silica particles, and in vivo induction of fibrosis depends upon NLRP3 and caspase-1 (41). Although chronic HCV infection leads to fibrosis, the lack of an immunocompetent small-animal model makes it difficult to examine the underlying mechanisms.

Proinflammatory cytokines play a dual role in virus infection. In acute infection, these cytokines act as an antiviral and help to clear infection. On the other hand, these cytokines may stimulate inflammatory processes in chronic infection. Therefore, it is possible that HCV-mediated activation of IL-1β/IL-18 from macrophages, along with other soluble mediators from HCV-infected hepatocytes, may activate quiescent hepatic stellate cells toward fibrosis and may be a potential target for therapeutic modalities.

FIG 5 HCV p7 protein activates IL-1β secretion from THP-1 cells. (A) THP-1 cells were transfected with in vitro-transcribed HCV core, E1-E2, E1-E2-p7, p7, and full-genome-length RNAs. Cell-free supernatants were collected after 20 h, and IL-1β secretion was determined by ELISA. The results are shown as means and standard deviations from three independent experiments. (B) THP-1 cells incubated in normal medium or medium containing 100 mM KCl, 100 mM NaCl, or 100 μM amantadine were transfected with in vitro-transcribed HCV p7 RNA. Cell-free supernatants were collected after 20 h, and IL-1β secretion was determined by ELISA. The results are shown as means and standard deviations from three independent experiments (*, P < 0.013).

which may counteract the effect of IL-1β released from HCV-exposed primary human Kupffer cells.

Virus-encoded viroporins are involved in inflammasome activation. Influenza virus M2 and encephalomyocarditis virus 2B proteins are implicated in the induction of IL-1β secretion via activation of the inflammasome (23, 24). K+ efflux is a well-known activator of the inflammasome signaling pathway (23). The HCV p7 protein is a member of the viroporin family and forms hexamers with ion channel activity (39), whose actions can drive ion flux for induction of IL-1β secretion. Our results demonstrated that HCV p7 RNA is sufficient to induce IL-1β secretion from THP-1 cells, and HCV p7-mediated IL-1β secretion is inhibited by KCl or the ion channel blocker amantadine. In fact, a recent report suggested that pretreatment with an inhibitor of potassium channels in HCV-incubated THP-1 cells reduces IL-1β maturation (16). HCV poly(U/UC) RNA transfected into THP-1 cells was shown previously to trigger IL-1β mRNA expression and secretion. Our observations, together with those of Negash et al. (16), suggest that HCV may employ multiple strategies for triggering IL-1β secretion. Further studies should reveal an in-depth mechanism for HCV-induced proinflammatory cytokine secretion.

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REFERENCES


