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Hepatitis C Virus Induces Toll-Like Receptor 4 Expression, Leading to Enhanced Production of Beta Interferon and Interleukin-6

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Hepatitis C virus (HCV) induces inflammatory signals, leading to hepatitis, hepatocellular carcinomas, and lymphomas. The mechanism of HCV involvement in the host's innate immune responses has not been well characterized. In this study, we analyzed expression and regulation of the entire panel of toll-like receptors (TLRs) in human B cells following HCV infection in vitro. Among all of the TLRs (TLRs 1 to 10) examined, only TLR4 showed an altered expression (a three- to sevenfold up-regulation) after HCV infection. Peripheral blood mononuclear cells from HCV-infected individuals also showed a higher expression level of TLR4 compared with those of healthy individuals. HCV infection significantly increased beta interferon (IFN-β) and interleukin-6 (IL-6) secretion from B cells, particularly after lipopolysaccharide stimulation. The increased IFN-β and IL-6 production was mediated by TLR4 induction, since the introduction of the small interfering RNA against TLR4 specifically inhibited the HCV-induced cytokine production. Among all of the viral proteins, only NS5A caused TLR4 induction in hepatocytes and B cells. NS5A specifically activated the promoter of the TLR4 gene in both hepatocytes and B cells. In conclusion, HCV infection directly induces TLR4 expression and thereby activates B cells, which may contribute to the host's innate immune responses.

Hepatitis C virus (HCV) infection induces hepatitis, hepatocellular carcinomas, and lymphoproliferative diseases, including cryoglobulinemia and B-cell lymphomas (36). Despite increasing evidence for a pathological role of chronic inflammation in these diseases (6), little is known about the mechanism of involvement of HCV in the activation and alterations of functions of B cells and hepatocytes during acute and chronic HCV infections. The paracrine effects of cytokines on B cells and hepatocytes are important in B-cell and liver immunobiology and in the regulation of a number of B-cell and hepatocyte functions. The innate immune response involving toll-like receptors (TLRs) has been shown to play an important role in the pathogenesis of many viruses, such as respiratory syncytial virus, reovirus, coxsackievirus, and mouse mammary tumor virus (4, 19, 32, 43). These viruses induce a strong activation of inflammatory cytokine responses mediated by the activation of TLRs. Conceivably, TLR-mediated innate immune responses may also play a key role in HCV pathogenesis.

TLRs are important components of the innate immune response and are transmembrane proteins that function as pattern recognition receptors for the detection of and response to microbial ligands (40). To date, at least 10 TLRs have been identified in humans which share a signature intracellular signaling motif with the interleukin-1 (IL-1) receptor, called the TLR (Toll-IL-1R) domain, and use many IL-1β signaling components, including toll-interacting protein (Tollip), the cytoplasmic adaptor molecule MyD88, and the protein kinase IL-1βR-associated kinase (7, 15, 34). A cascade of phosphorylation/recruitment/activation events following TLR activation leads to the transcription of inflammatory and antiinflammatory cytokine genes (1). Among the TLRs, TLR4, the lipopolysaccharide (LPS)-activated TLR, is the major signal transduction protein associated with the pathophysiology of sepsis (30). LPS is a ubiquitous contaminant molecule in cirrhotic patients as a result of failure of the liver to detoxify LPS accumulated from intestinal uptake (17, 22, 28). TLR4 uses several adaptor proteins, including MyD88, MAL/TIRAP, TRIF, TRAM, and IRF-3, to engage downstream signaling proteins, and it eventually activates IκB kinase and mitogen-activated protein kinases and beta interferon (IFN-β) (13, 45). The production and secretion of IFN-β is a pivotal event that leads to a global antiviral state through paracrine IFN production and the subsequent activation of IFN-stimulated genes within the infected cells and the surrounding tissues (41). However, the suppression of virus replication by IFN is transient in some patients, and the virus often resists further therapy (41), suggesting that HCV has evolved mechanisms to disrupt the host response to IFN. Indeed, HCV NS3 protein has been shown to interfere with the functions of IRF-3 (11). IRF-3 is also a key component in the signaling pathway of several TLRs (14). Since viral proteins often counteract each other in their biological effects, the fact that HCV encodes an inhibitor of IRF-3 suggests the possibility that HCV infection may also trigger the antiviral state through the activation of TLRs (11). Therefore, it is conceivable that the innate immune pattern recognition receptors, such as TLRs, play a role in HCV-induced pathogenesis. In this study, we examined the expression and biological significance of all TLRs in HCV-infected cells. We also characterized the antiviral molecules triggered

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by the TLR induction. We identified TLR4 as a potential factor in HCV pathogenesis.

MATERIALS AND METHODS

Cell culture and virus infection. Raji cells were obtained from ATCC and grown in RPMI 1640 (Invitrogen, Carlsbad, Calif.) containing 20% fetal bovine serum (FBS). Raji cells were further used for HCV infection using culture supernatant of an HCV-producing B-cell line (SB cells) derived from an HCV^+ non-Hodgkin's lymphoma (38). A control infection (HCV^-) using UV-irradiated SB cell culture supernatant was included in all the experiments. HepG2, Huh7, and HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS. For virus neutralization, the supernatant from SB cells was incubated with a monoclonal antibody CBH5 (12) or an isotope-matched antibody RD4 at 4°C for 2 h before being used for infection.

Plasmids. The various expression plasmids were constructed by inserting HCV core, E1, E2, NS3, NS4B, NS5A, and NS5B cDNA of genotype 1a (8) behind the cytomegalovirus virus immediate-early promoter in pCDNA3.1 (Invitrogen). The reporter plasmid for analyzing the TLR4 promoter activity, pGL3-TLR4 (33), contains the human TLR4 promoter cloned into the pGL3-basic luciferase reporter gene vector (Promega). Plasmids pGL3-336, -911, -2483, -3665, and -5774, which contain various fractions of the TLR4 promoter, were kindly provided by M. Rehli and B. Beutler of the Scripps Research Institute (33).

RT-PCR analysis. Total RNA was isolated from Raji cells with the TRI reagent (MRC) following the manufacturer's instructions. PCR amplification was performed using *Taq* polymerase (Roche) for 32 cycles at 95°C for 45 seconds, 54°C for 45 seconds, and 72°C for 1 min with the previously reported primers for TLRs (26). As an internal control, reverse transcriptase PCR (RT-PCR) for β-actin was performed in all experiments. To exclude contamination of genomic DNA in the samples, controls (-RT), in which RT was replaced by water during the cDNA synthesis step, were included.

Real-time PCR (TaqMan). Quantitation of the expression levels of various genes was performed by quantitative RT-PCR based on real-time PCR. A total of 1 μg of RNA was reverse transcribed into cDNA in accordance with the manufacturer's directions (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was then performed using the ABI 7900 system (Perkin-Elmer Applied Biosystems, Foster City, CA). Sense and antisense primers and the reaction-specific probes were obtained as predeveloped TaqMan assay reagents (Perkin-Elmer). TLR4 sense primer (5′-GAACTGCAGGTGCTGGATTT-3′), antisense primer (5′-CTCTAGATTGGTCAGATTAGA-3′), and probe (5′-GTCCAGAA AAGGCTCCCAGGGCTAAAC-3′) were used in association with TaqMan PCR master mix (Perkin-Elmer), according to the manufacturer's protocols. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level in each cDNA sample was also measured as a means of normalizing cytokine mRNA expression levels. The mRNA expression data are expressed as fold induction relative to the GAPDH level.

RNA interference using siRNA. The small interfering RNAs (siRNA) used (synthesized by the USC Microchemical Core) were designed according to the guidelines of Elbashir et al. (9) with the following sequences: TLR4 sense strand, 5′-UUCGAGACUGGACAAGCCATT-3′, and TLR4 antisense strand, 5′-UG GCUUGUCCAGUCUCGAATT-3′. The sense and antisense strands of the RNA were annealed at a concentration of 80 mM in 10 mM Tris (pH 7.7)–1 mM EDTA–100 mM NaCl by heating to 90°C for 1 min and then cooling in a thermocycler at a rate of 0.1°C/s until 22°C was reached. To transfect Raji cells with siRNAs, 2×10^5 cells were washed with serum- and antibiotic-free RPMI medium, resuspended in 50 μl of serum- and antibiotic-free RPMI medium, and cultured in a 96-well tissue culture dish. A preincubated mixture of 100 pmol of siRNA and 0.8 μl of Oligofectamine (Invitrogen; 50 μl in total volume) was added to Raji cells and incubated overnight at 37°C as previously described (39). Transfection efficiency was verified with control (nonsilencing) siRNA labeled with rhodamine (QIAGEN) to be more than 80%. Cells were resuspended in 200 μl of RPMI containing 20% FBS and infected with the HCV-containing SB culture supernatant (38). To prolong the effects of siRNA, the HCV-infected, siRNA-transfected cells were retransfected with the same siRNA at days 4 and 8. The samples were harvested at 12 days after infection. A nonfunctional siRNA (Ambion Inc.) was used as a control.

Transfection and luciferase assay. Raji, HepG2, or Huh7 cells were transfected using the FuGENE6 reagent (Roche) or a Gene Pulser II (Bio-Rad) with various plasmids purified using the EndFree plasmid kit (QIAGEN). Cells were grown on six-well plates and transfected with one (0.1 μg) of the TLR4 reporter constructs, TLR4-X, P, E, H, A, K, and N, and pRL-SV40, a control *Renilla* luciferase expression plasmid (0.1 μg; Amersham Biosciences) and a viral protein expression plasmid (0.1 μg). The total DNA concentration in each transfection mixture was kept constant by adjusting with an empty vector. The TLR4 reporter gene construct pLucTLR4, containing the promoter region of TLR4, was a generous gift from M. Rehli and B. Beutler from the Scripps Research Institute (33). For stimulation by LPS, cells were incubated with LPS (10 ng/ml; SIGMA) for 6 h at 24 h after transfection. At 48 h after transfection, cells were lysed and assayed for luciferase activities using the Dual Luciferase reporter assay system according to the manufacturer's instructions (Promega). Luciferase activities were normalized to the internal control *Renilla* luciferase activity.

RESULTS

TLR gene expression in response to HCV infection of B cells in vitro. To examine the expression levels of all the known TLRs during HCV infection, we first performed semiquantitative RT-PCR of the TLR-specific mRNA in Raji cells infected with HCV (HCV^+) or UV-inactivated HCV (HCV^-), using primers specific for each of the 10 known TLRs. Most of the TLRs were expressed at an undetectable level in both infected and uninfected cells (e.g., TLRs 2, 3, 8, and 9) or at the same levels in the HCV-infected and uninfected cells (e.g., TLRs 1 and 10) (Fig. 1A). The only exception was TLR4, which was expressed approximately five times higher in the HCV-infected cells than in the cells mock infected or infected with the UV-inactivated virus (Fig. 1A). The increased expression of TLR4 mRNA was confirmed by quantitative real-time RT-PCR (data not shown) (see below).

To rule out the possibility that this increased expression of TLR4 in HCV-infected cells was an artifact of the in vitro viral infection of cell culture, we compared the TLR4 RNA in peripheral blood mononuclear cells (PBMC) derived from several hepatitis C patients and from healthy individuals. Very similar results were obtained (Fig. 1B): while almost no TLR4 RNA was detectable in healthy individuals, it was readily detected under the same conditions in the PBMC of hepatitis C patients. As a comparison, the level of TLR7 RNA was nearly the same between the infected and healthy individuals. However, one significant difference between the in vitro-infected Raji cells and PBMC from HCV^+ patients was that TLR2 was also enhanced in HCV^+ PBMC but not the in vitro-infected Raji cells. The significance between the in vitro and in vivo HCV infection is not currently understood. In the following studies, we focused on TLR4.

To further confirm the enhanced expression of TLR4 in HCV-infected cells, we examined the TLR4 protein level in Raji cells infected with HCV or UV-inactivated HCV at several time points after virus infection. The TLR4 protein was detected in the infected cells at days 8 and 16 postinfection, but not in the cells infected with the UV-inactivated virus (Fig. 1C). Finally, by fluorescence-activated cell sorter (FACS) assay on nonpermeabilized cells, we showed that the surface expression of TLR4 molecules increased in the HCV-infected cells. In contrast, the surface expression of CD14, which is a receptor for LPS, was not increased (Fig. 1D). These results combined indicate that HCV infection induces the expression of TLR4 in both mRNA and protein.

HCV infection induces IFN-β and IL-6. To determine if HCV infection triggered antiviral programs and inflammatory responses that are downstream responses of TLR4 stimulation, we examined various cytokines that are known to be induced by TLR4 stimulation. IFN-β and IL-6 are two such cytokines (35, 42). We performed real-time RT-PCR of the mRNAs of

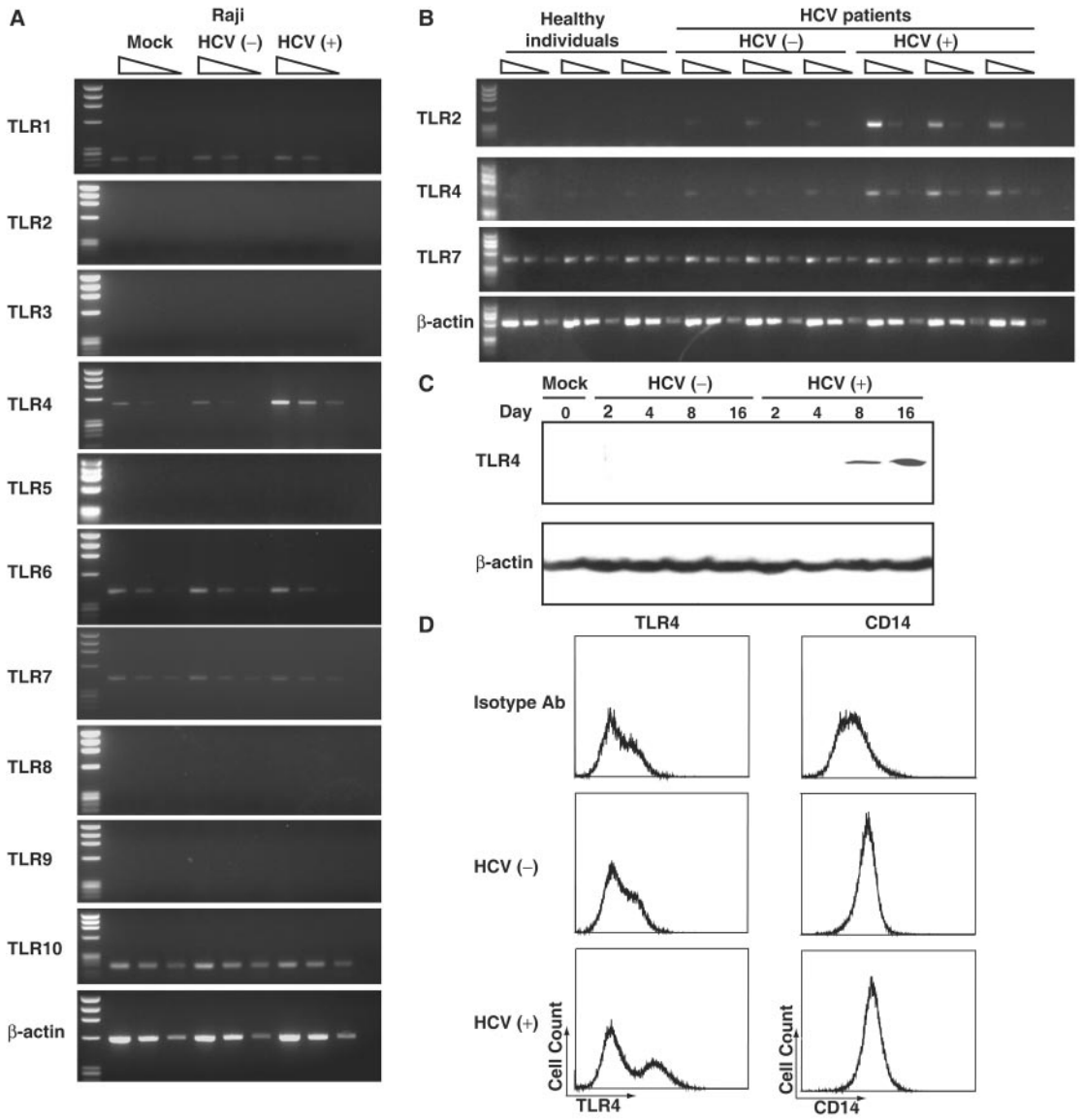

FIG. 1. Semiquantitative analysis of TLR1-10 mRNA expression in HCV-infected B cells. (A) TLR (1 to 10) mRNA expression in Raji cells mock infected or infected with HCV [HCV (+)] or UV-inactivated HCV [HCV (-)] was analyzed by semiquantitative RT-PCR using increasing dilutions (0, 5×, and 25×) of RNA samples. RNA from the infected and uninfected cells was analyzed on day 16 postinfection. Shown are the representative results from four independent experiments. (B) TLR mRNAs in PBMC of hepatitis C patients and healthy individuals. The conditions of analysis were the same as for panel A. (C) Immunoblotting of TLR4 proteins in Raji cells at various days after HCV infection. β-Actin was analyzed to verify equal loading. (D) Surface expression of TLR4 protein. Cells at 12 days after infection were stained with TLR4- or CD14-specific antibodies or an isotype antibody followed by a phycoerythrin-conjugated antibody and then subjected to FACS analysis. These results are representative of three experiments.

IFN-β and IL-6. A small amount of IFN-β was detected in the supernatants of Raji cells; as expected, this level was enhanced by LPS stimulation (45). Both the basal level and LPS-induced IFN-β secretions were significantly higher in HCV-infected cells. (Fig. 2A, left panels). The increased secretion of IFN-β was also demonstrated by enzyme-linked immunosorbent assay (ELISA) analysis of IFN-β protein in the supernatant of HCV-infected Raji cells versus the uninfected cells (Fig. 2B). A similar increase in the HCV-infected cells was also observed for IL-6, another molecule responding to TLR4 (Fig. 2A). The most marked increase in IFN-β and IL-6 secretion occurred at day 12 postinfection; thereafter, the amounts of the secreted cytokines declined (Fig. 2C and D), indicating that enhanced cytokine expression was transient and decreased in the chronic phase of infection, such as day 32 postinfection. We also examined IFN-γ, which usually is not activated by TLR4 stimulation, and IL-8, which is known to be activated by respiratory syncytial virus-induced TLR4 stimulation (19). These two molecules were not enhanced in HCV-infected cells (Fig. 2A, right panels). To determine the kinetics of enhanced expression of IFN-β and IL-6, we quantified the mRNA expression levels of these cytokines at the different time points postinfection. The

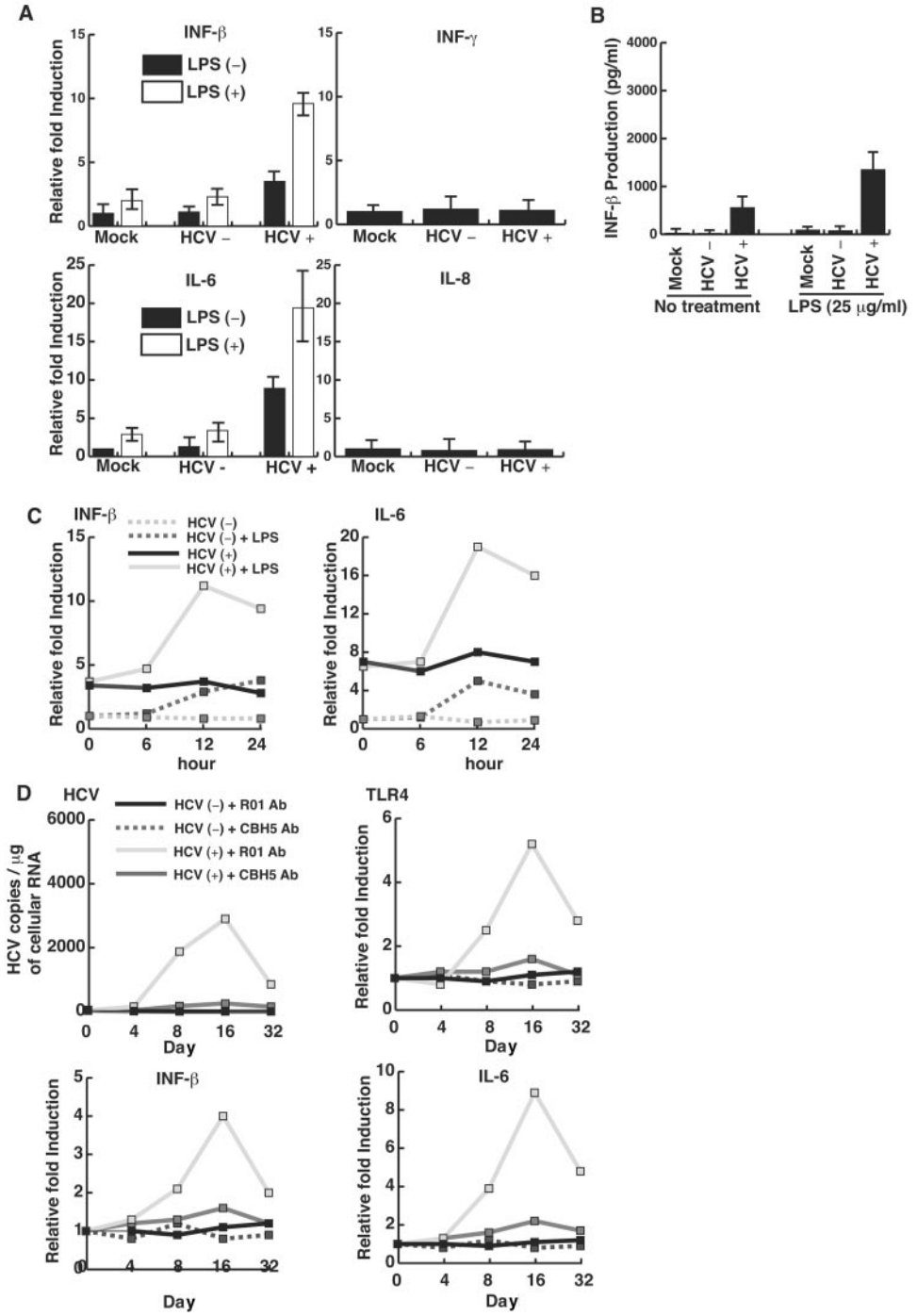

FIG. 2. HCV infection induces IFN-β and IL-6 production in B cells. (A) Quantitative analysis of cytokine mRNAs. Raji cells were infected with HCV for 12 days; half of the cells were treated with LPS for 24 h. Total cellular RNAs were analyzed by real-time RT-PCR using specific primers for IFN-β and -γ, IL-6, and IL-8. The relative level of induction in the HCV-infected cells is presented as the ratio, in the presence and absence of LPS, relative to the uninfected cells. (B) IFN-β production analyzed by ELISA. The culture supernatant from the HCV-infected cell culture was collected over a 14-day period after HCV infection. The amount of IFN-β in the culture supernatant was analyzed by ELISA. The data represent the averages of three separate experiments. (C) Kinetics of expression of IFN-β and IL-6 mRNA induction in HCV-infected Raji cells with LPS treatment. The RNA was harvested at various time points posttreatment. IFN-β and IL-6 RNA were quantified by real-time RT-PCR. (D) Time course analysis of HCV RNA titer and the effects of neutralizing antibodies against HCV on the induction of TLR4, IFN-β, and IL-6 levels at the various time points postinfection. The SB culture supernatant was treated with the various neutralizing (CBH5) or nonspecific (R04) antibodies before being used for infection of Raji cells. The infected cells were harvested at various times, up to 32 days postinfection, for RT-PCR analysis of the TLR4, IFN-β, and IL-6 RNA. HCV RNA copies were also determined by RT-PCR as previously described.

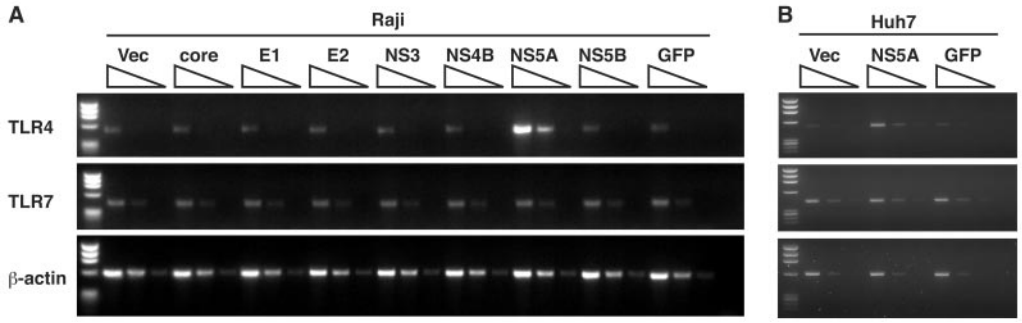

FIG. 3. NS5A induces TLR4 mRNA expression. (A) Raji cells were transfected with plasmids expressing individual HCV proteins. The TLR4, TLR7, and β-actin mRNA levels were determined by semiquantitative RT-PCR. The cDNA was serially diluted twice (0, 5-fold, and 25-fold) and used for RT-PCR. Vec, vector-transfected Raji cells. (B) Huh7 cells transfected with NS5A-expressing plasmid or control plasmids were examined for TLR4 induction by semiquantitative RT-PCR at 48 h posttransfection. TLR7 and β-actin expression served as loading controls.

enhanced expression of IFN-β and IL-6 mRNA in HCV-infected cells was at the maximum level on day 16 after infection; thereafter, it decreased at day 32 postinfection. These results indicate a two-phase antiviral response. As expected, the amounts of IFN-β and IL-6 were increased by LPS.

To further establish the specificity of HCV infection in inducing TLR4, IFN-β, and IL-6, we performed an additional experiment to rule out the possibility that UV irradiation of the SB cell culture supernatant might have inactivated cytokines present in the supernatant. We used a neutralizing monoclonal antibody, CBH5 (specific for E2 protein) (12), to inactivate HCV viral infectivity. After such a treatment, the viral infectivity was almost totally lost (Fig. 2D, upper left panel). In contrast, a control antibody, R04, did not interfere with viral infection. The viral RNA titer reached the peak level on day 16 after infection. These results indicated that the monoclonal antibody treatment effectively inhibited viral infectivity. Under such conditions, the induction of TLR4, IFN-β, and IL-6 was specifically blocked by the neutralizing antibody treatment. In addition, HCV RNA titer peaked at day 16 postinfection and decreased at day 32 postinfection, indicating that the HCV RNA level was correlated with enhanced cytokine levels. These results combined indicate strongly that HCV infection activates specifically TLR4, resulting in enhanced production and secretion of its downstream molecules, including IFN-β and IL-6.

NS5A induces expression of TLR4. To determine which viral protein is responsible for the HCV-induced overexpression of TLR4, we expressed each individual HCV protein in Raji cells and studied its effects on TLR4 mRNA expression. Among all the viral proteins examined, NS5A induced TLR4 expression by approximately 5- to 10-fold, while none of the other viral proteins did. The same results were obtained in two hepatocyte cell lines (Huh7 and HepG2) (Fig. 3B and data not shown). The degree of enhancement of the TLR4 mRNA expression was determined by real-time RT-PCR analysis to be five- to eightfold over the vector control (data not shown). Under the same conditions, TLR7 mRNA expression was not altered, indicating that HCV infection specifically up-regulates TLR4 expression. The enhanced TLR4 expression by NS5A overexpression was confirmed in the hepatocytes (Fig. 3B). These results indicate that NS5A induces TLR4 expression in both B cells and hepatocytes.

Small interfering RNA targeting *TLR4* reduced IFN-β and IL-6 induction in HCV-infected cells. To establish that the HCV-induced IFN-β and IL-6 production was mediated by TLR4 overexpression, we used the RNA interference strategy to specifically knock down TLR4 and TLR7. The TLR4- and TLR7-specific siRNAs efficiently knocked down the expression of TLR4 and TLR7 mRNAs, respectively, without affecting the other TLR mRNA species in both HCV-infected and uninfected cells (Fig. 4A to C). Correspondingly, the TLR4 protein expression was knocked down by the TLR4-specific siRNA, but not the control siRNA (Fig. 4D). These results established the validity and specificity of the TLR4 siRNA. Using this siRNA, we found that the elevated levels of IL-6 and IFN-β mRNA observed in the HCV-infected cells were significantly reduced after treatment with TLR4-specific but not TLR7-specific siRNA (Fig. 4E and F). Correspondingly, the secretion of IFN-β protein from the HCV-infected cells was significantly reduced (Fig. 4G). TLR4 siRNA did not have a significant effect on HCV replication (Fig. 4H). Therefore, the effect of the TLR siRNA on IFN-β and IL-6 was not due to the reduction of HCV replication, but represented the inhibition of the TLR4 signaling pathway. These results indicate that the HCV-induced IFN-β and IL-6 overproduction in Raji cells is mediated through TLR4 overexpression.

NS5A activates the TLR4 promoter. To understand the mechanism of induction of TLR4 mRNA expression by HCV infection or the NS5A protein alone, we used a reporter plasmid containing a luciferase gene under the control of the TLR4 promoter to examine whether these effects were due to regulation at the transcription level. The reporter plasmid was transfected into HCV-infected and mock-infected Raji cells, and luciferase activity was determined 48 h later. Luciferase activity was at least threefold higher in HCV-infected cells than in the uninfected cells (Fig. 5A), suggesting that HCV transactivates the TLR4 promoter in B cells. When the reporter plasmid was cotransfected with the NS5A protein into the uninfected Raji cells, a three- to fivefold induction of luciferase activity, compared to the cells transfected with the vector plasmid, was also observed. None of the other viral proteins increased the luciferase activity, indicating that the transactivation of the TLR4 promoter by HCV is most likely mediated by the NS5A protein (Fig. 5B).

To define the minimal TLR4 promoter required for the

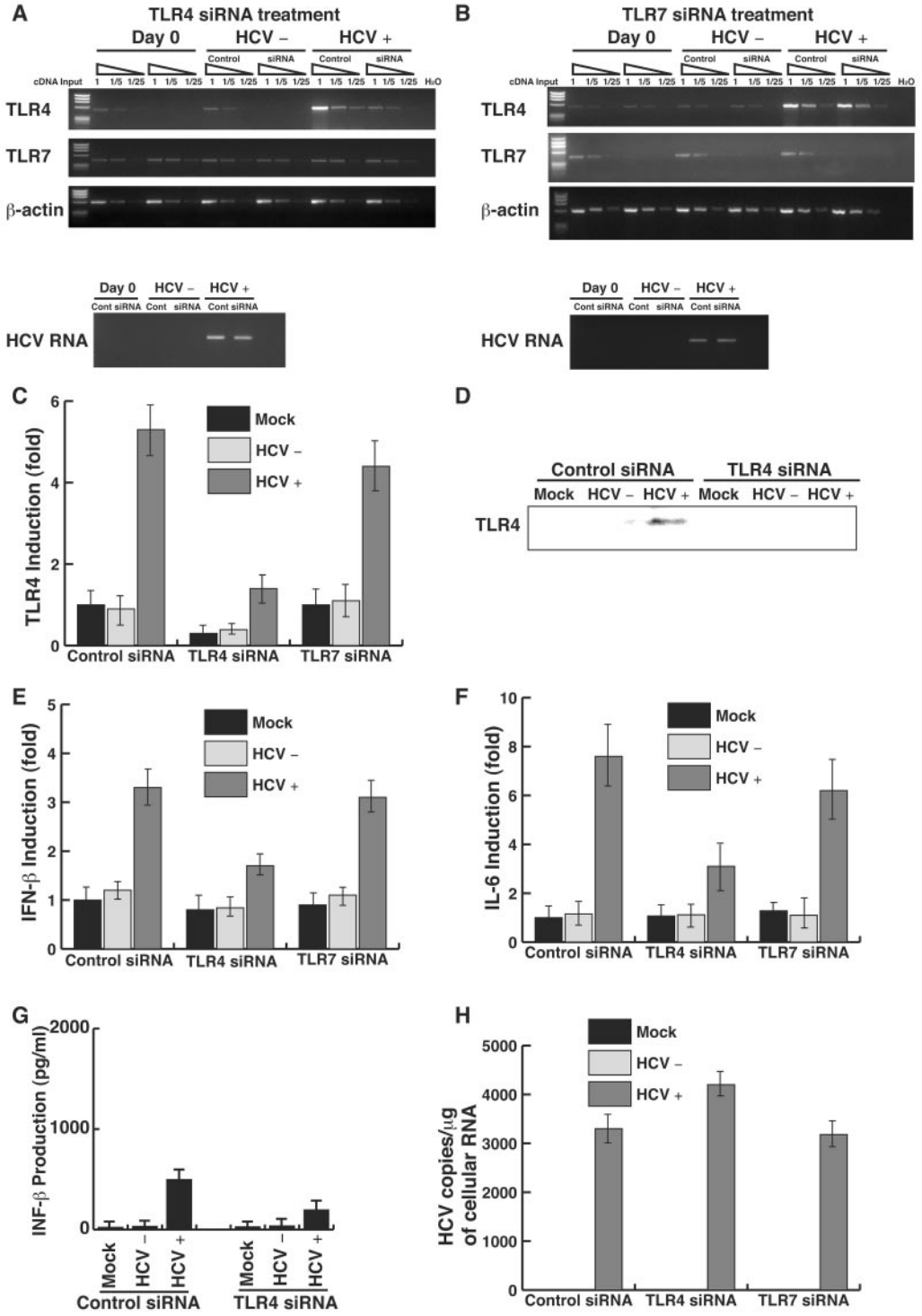

FIG. 4. Effects of siRNA against TLR4 and TLR7 on cytokine induction. (A and B) The siRNA against TLR4 and -7 specifically reduced TLR4 and TLR7 expression, respectively. Raji cells were transfected with TLR4- or TLR7-specific siRNAs and then infected with HCV at day 3 posttransfection. The RNA was harvested at 14 days after infection. Semiquantitative analysis of TLR mRNAs was performed as for Fig. 1. The presence of HCV RNA in the HCV-infected cells is shown in the lower panel. (C) TLR4 RNA expression in the siRNA-treated cells (similar to panels A and B) was determined by real-time RT-PCR. The relative ratios of TLR4 mRNA in the different cells are presented. (D) TLR protein expression in siRNA-treated Raji cells was studied by immunoblotting. (E and F) IFN-β and IL-6 RNAs in the HCV-infected, siRNA-treated cells were examined by real-time RT-PCR at 16 days postinfection. (G) IFN-β production in the supernatant of HCV-infected Raji cells treated with either the TLR4 or control siRNA. Supernatant was collected over the 14-day period postinfection and used for ELISA. (H) HCV RNA titer was quantified by real-time RT-PCR in siRNA-treated cells.

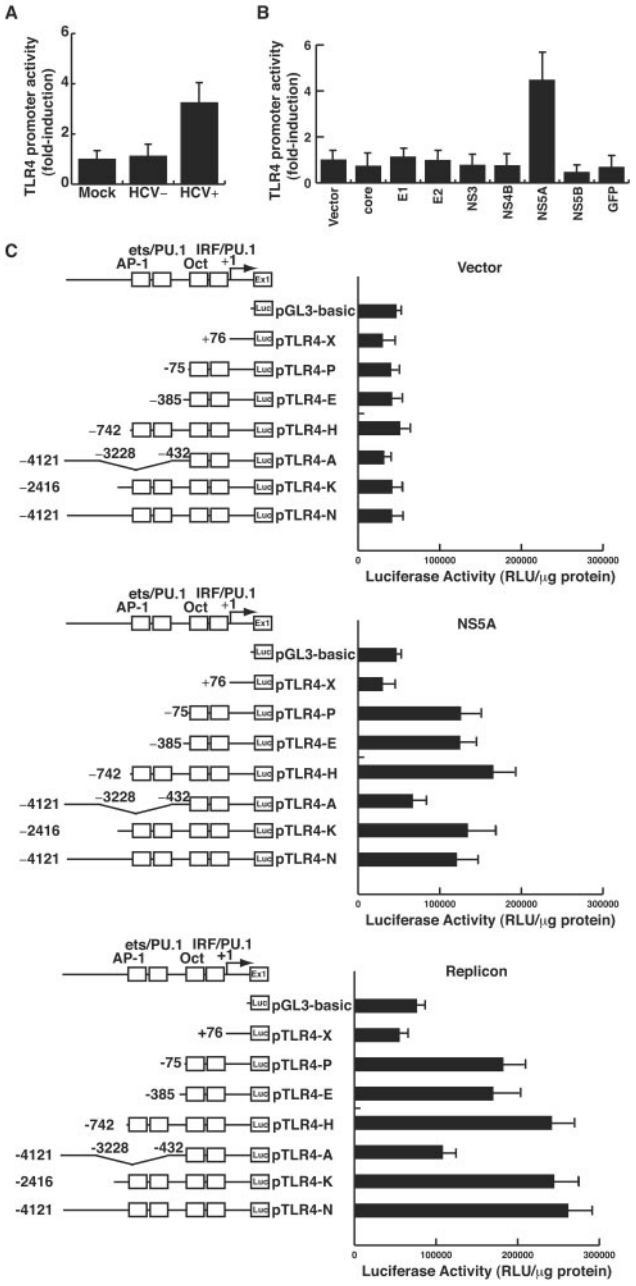

FIG. 5. Promoter analysis of TLR4 gene induction by NS5A. HCV infection activates the TLR4 promoter in Raji cells. The luciferase reporter plasmid under the control of the complete TLR4 promoter was transfected into HCV-infected Raji cells on day 14 postinfection. Luciferase activity was determined 2 days after transfection. (B) Expression of NS5A induces TLR4 in Huh7 cells. The luciferase reporter plasmid under the control of the complete TLR4 promoter was cotransfected with plasmids expressing individual HCV proteins, and the luciferase assay was performed as for panel A. (C) Minimal requirement of the TLR4 promoter. Various truncation mutants of the TLR4 promoter luciferase reporter plasmid were transfected into control Huh7 cells (vector), Huh7 cells expressing NS5A, or Huh7 cells containing the subgenomic replicon (NS3-NS5B). A luciferase assay was performed at 48 h posttransfection.

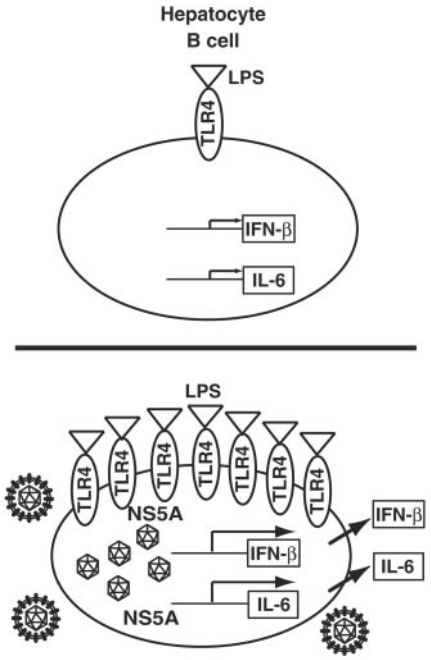

FIG. 6. Postulated mechanism of HCV-induced activation of B cells and hepatocytes mediated by TLR4 induction.

activation by HCV or by NS5A, Raji cells infected with HCV or mock infected were transfected with a series of TLR4 promoter truncation mutants that govern a reporter luciferase gene. Alternatively, Raji cells were cotransfected with the NS5A expression plasmids and one of the TLR4 promoter truncation mutants. After normalization for transfection efficiency, it was observed that the smallest truncation mutant that was still able to respond to the activation by NS5A was pTLR4-P, which contains one each of Oct-1- and IRF/PU.1-binding sites. The inclusion of the AP-1-binding site (pTLR4-H) increased the transactivation activity (Fig. 5C), suggesting that NS5A transactivation of the TLR4 promoter was mediated by PU.1, AP-1, and Oct-1. The same results were confirmed in Huh7 cells containing HCV replicon, which expresses HCV NS3 to NS5B proteins (Fig. 5C). These results together indicate that HCV causes up-regulation of the TLR4 gene at the transcriptional level by NS5A protein.

DISCUSSION

The data presented in this study indicated clearly that HCV infection induced TLR4 expression on the surface of B cells. The increased surface expression of TLR4 molecules led to increased sensitivity of the infected B cells to LPS, which, in turn, led to increased IL-6 and IFN-β production (Fig. 6). These results suggest that HCV infection can alter the innate immune response by up-regulating a toll-like receptor (TLR4) and increase B-cell responses to microbial agents in peripheral blood. The increased IL-6 production will likely lead to an increased inflammatory response, while increased IFN-β production may confer an antiviral state. The increased TLR4 expression is a result of increased transcription of the TLR4 gene and is mediated by the viral NS5A protein. Because of the

lack of a suitable in vitro HCV infection system for hepatocytes so far, we do not know whether HCV infection of hepatocytes will also lead to TLR4 induction. Nevertheless, NS5A protein did enhance TLR4 gene transcription in hepatocytes. Therefore, HCV infection may also induce TLR4 overexpression in hepatocytes. It has been known that TLR4 is expressed in many tissues, including liver, spleen, and peripheral blood leukocytes (24). Our data showed that there was an increase in the basal expression level of IFN-β and IL-6 in HCV-infected B cells even in the absence of LPS stimulation. These combined effects likely will contribute to the inflammatory response in HCV infection. This finding adds another potential effect of B-cell infection by HCV in the pathogenesis of hepatitis C.

The induction of TLR4 gene expression by NS5A is but one of the mechanisms whereby HCV affects the functions of TLR4. Several other HCV proteins have been shown to affect directly or indirectly the signal transduction pathways of a variety of TLRs. For example, the viral NS3 protein could affect the functions of IRF-3 (11), which plays a key role in the signaling of TLR4 and other TLRs. Therefore, the competing activities of the various HCV proteins on TLRs ultimately modulate the pathogenesis of HCV. Interestingly, TLR4 is the only TLR whose expression is altered by HCV infection in Raji cells in culture. However, in PBMC derived from the hepatitis C patients, there was also a significant induction of TLR2. The biological significance of this difference under the in vitro and in vivo conditions is not clear at the present time. TLR4 signaling has been reported to induce TLR2 expression via neutrophil NADPH oxidase (10). Tumor necrosis factor alpha has been reported to induce TLR2 (23). Interestingly, tumor necrosis factor alpha is also induced by HCV infection or viral E2-CD81 interaction (21, 29). Thus, it is conceivable that TLR2 is induced in vivo through the indirect effects of other viral proteins. Furthermore, even in the absence of the alteration in its expression level, it is possible that the functions of some other TLRs may be altered in HCV infection through the alteration of the signal transduction pathways of these TLRs.

Several HCV proteins inhibit antiviral responses; for example, core or NS3 protease of HCV infection has been reported to inhibit IFN-β signaling by inactivating Stat1, IRF-3, TRIF, TBK1, and Cardif (3, 11, 18, 20, 25). Paradoxically, natural HCV infection triggers a robust antiviral response, but the response is attenuated in the chronic phase. In our HCV-infected Raji cells, during the acute phase (namely, days 10 to 16 postinfection), HCV protein level is probably low and, therefore, may not completely shut down the host antiviral response. In contrast, in the chronic phase (namely, day 32 postinfection), HCV proteins accumulate in the infected cells and partially block the antiviral responses. It is necessary to separate the acute and chronic phases to understand the host antiviral responses.

It is unclear whether LPS could affect HCV pathogenesis. LPS is the most bioactive component of the cell membrane of gram-negative bacteria. It has been reported that in viral hepatitis, there is an increased concentration of endotoxin in the body (5, 17, 45), which could be the result of the defects in hepatic detoxification of endotoxin derived from the intestine (17). As a result, the inflammatory response of B cells is likely to be further enhanced by LPS in HCV infection. We found that even in the absence of LPS, there was an enhanced transcription of TLR4 and corresponding increase of IFN-β and IL-6 production and secretion following HCV infection. IL-6 acts on hepatocytes to induce synthesis of acute-phase proteins, including C-reactive protein (CRP), mannose-binding lectin (MBL), acting as an opsonin and activating complement, and fibrinogen (2). Mice expressing IL-6 from a transgene often develop fully transformed monoclonal plasmacytoma, including c-*myc* gene rearrangement, from massive polyclonal plasmacytosis with autoantibody production and glomerulonephritis, which resembles the autoimmune diseases observed in NZB/W F_1 mice or systemic lupus erythematosus patients (16, 37), suggesting that the dysregulated IL-6 gene can trigger polyclonal plasmacytosis, resulting in the generation of malignant plasmacytoma. IL-6 overexpression enhances B-cell differentiation, leading to plasma cell-induced overproduction of immunoglobulin genes (31). Interestingly, enhanced expression of IL-6 in chronic hepatitis C patients has been reported (27).

We have investigated the mechanism of the induction of TLR4 gene transcription in HCV infection. NS5A directly or indirectly acts on the promoter of the TLR4 gene. Downstream of the TLR4 is a cascade of signal transduction events from MyD88 to IRF-3, thereby activating NF-κB, which regulates the promoters of the interferon-stimulated response element. Interestingly, NS5A has been reported to activate NF-κB (44). Preliminary data from our laboratory have shown that NF-κB is indeed activated in NS5A-expressing cells in an LPS-dependent manner.

In conclusion, HCV, through the action of its NS5A protein, induces expression of TLR4, leading to enhanced IFN-β and IL-6 production and secretion, particularly in response to LPS. These results indicate that HCV infection induces an inflammatory response and antiviral state at the same time through the effects on TLR4 expression. Thus, TLRs likely play a role in HCV pathogenesis.

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