tion is also crucial for the induction of innate immunity through the activation of pattern recognition receptors (PRR), including Toll-like receptors (TLR) and RIG-I-like receptors (RLR) (44). TLR play an important role in the recognition of a wide variety of pathogens and their components, while RLR, including RIG-I and MDA5, sensitize cells in response to double-stranded RNA (dsRNA) generated by viral infection or to poly(I·C). Various proinflammatory cytokines and chemokines are activated by the PRR through the translocation of transcription factors, such as IFN regulatory factor (IRF) and NF-κB, into the nucleus and binding to their cognate promoter elements together with other transcription factors.

In this study, we have examined the role of the TLR pathway on the production of the CXC chemokines in human liver cell lines replicating HCV RNA. Among the CXC chemokines, IP-10 production was specifically enhanced in cells replicating HCV upon stimulation with conventional TLR2 ligands. Moreover, we identified CD44, a receptor for the glycosaminoglycan hyaluronan (HA) (38), as a molecule involved in IP-10 production in the HCV-replicating cells. The cell surface expression of CD44 was also upregulated in the cells harboring HCV replicons of genotypes 1b and 2a, and IP-10 production was enhanced in cells replicating HCV upon stimulation with HA. Importantly, HA also works as a ligand for TLR2 (41), and HA expression in serum is increased in CHC patients in accord with the progression of liver fibrosis (9, 30, 35, 45, 48). These results suggest that the production of IP-10 is enhanced by endogenous HA in hepatocytes in CHC patients through an interaction with TLR2 and CD44.

MATERIALS AND METHODS

Cells and viruses. Huh7OK1 cells that exhibit high susceptibility to infectious cell culture-adapted HCV clone (HCVcc) propagation (34), Huh7 cells, and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS). Huh7 cells harboring an HCV subgenomic RNA replicon of genotype 1b (Con1 strain) (28) or 2a (JFH1 strain) were cultured in DMEM supplemented with 10% FCS, 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan), and nonessential amino acids. Human normal hepatocytes and human liver sinusoidal endothelial cells were purchased from TaKaRa Bio, Inc. (Shiga, Japan) and maintained in primary hepatocytes and endothelial selective medium (TaKaRa Bio), respectively. Huh7 cells harboring a Japanese encephalitis virus (JEV) subgenomic RNA replicon (Nakayama strain) were cultured in DMEM supplemented with 10% FCS and 1 μg/ml puromycin (InvivoGen, San Diego, CA). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. The infectious RNA of the JFH1 strain was introduced into Huh7OK1 cells, and the infectious titers were expressed as focus-forming units (FFU) (46).

Plasmids and reagents. The cDNA fragment encoding CD44 was kindly provided by U. Günthert (University of Basel, Switzerland) (31) and subcloned into pcDNA3.1-C-myc-His (Invitrogen, Carlsbad, CA). The N-terminal or C-terminal deletion mutant of CD44 was amplified by PCR using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and subcloned into pcDNA3.1-C-myc-His. The cDNA fragment encoding the full-length human TLR2 was amplified by reverse transcription (RT)-PCR from total RNA of THP-1 cells and subcloned into pFlagCMV-1 (Sigma). The C-terminal deletion mutant of TLR2 was amplified by PCR using *Pfu* Turbo DNA polymerase (Stratagene) and subcloned into pFlagCMV-1. All PCR products were confirmed by sequencing with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Tokyo, Japan). Lipopolysaccharide (LPS) derived from *Salmonella enterica* subsp. *enterica* serovar Minnesota (Re-595) and peptidoglycans (PGN) derived from *Staphylococcus aureus* were purchased from Sigma. FSL-1 (a synthetic lipopeptide derived from *Mycoplasma salivarium*), Pam2CSK (a synthetic diacylated lipopeptide), Pam3CSK (a tripalmitoylated lipopeptide), phosphorothioate-stabilized human CpG (hCpG) oligodeoxynucleotides (ODN 2006) (TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT), R-837, and poly(I·C) were purchased from InvivoGen. Purified hyaluronan from human umbilical cords was purchased from Calbiochem (Darmstadt, Germany). The recombinant human alpha interferon (IFN-α) and IFN-γ were purchased from PBL Biomedical Laboratories (New Brunswick, NJ). The Quantikine ELISA (enzyme-linked immunosorbent assay) human CXCL10/IP-10 immunoassay was purchased from R&D Systems, Inc. (Minneapolis, MN). The HCV NS3/-4A protease inhibitor BILN2061 was purchased from Acme Bioscience (Belmont, CA). Lack of contamination of endotoxin (<0.01 endotoxin units/ml) in the regents, including virus stocks, recombinant proteins, and all ligands, was confirmed by using a Pyrodick endotoxin measure kit (Seikagaku Co., Tokyo, Japan).

Binding of recombinant CD44s to TLR2 or ligands. To generate a C-terminal deletion mutant of TLR2 (comprised of amino acid residues from 1 to 587) or CD44 (comprised of amino acid residues from 1 to 223), each of the cDNAs encoding N-terminally FLAG-tagged TLR2 (TLR2ΔTM) or C-terminally His-tagged CD44 (CD44ΔTM) were subcloned into pFastBac (Invitrogen), and recombinant baculoviruses possessing the cDNA were produced by using a Bac-to-Bac baculovirus expression system according to the manufacturer's instructions (Invitrogen). At 3 days after infection, TLR2ΔTM and CD44ΔTM proteins were purified from the culture supernatants and cell lysates by using column chromatography with nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA) and anti-FLAG M2 affinity gel (Sigma) according to the respective manufacturer's instructions. The protein concentrations were determined by using a Micro BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL). One hundred microliters of TLR2ΔTM (20 μg/ml), HA (50 μg/ml), PGN (40 μg/ml), LPS (40 μg/ml), or bovine serum albumin (BSA) (50 μg/ml) was added to a 96-well microtiter plate (Nunc Maxisorp P/N; Nalge Nunc International, Rochester, NY) and incubated at 4°C overnight. Nonspecific binding was blocked by incubating with phosphate-buffered saline (PBS) containing 2% FCS (PBSF) at room temperature for 1 h, followed by washing with PBS containing 0.02% Tween 20 (PBST) and incubation with various concentrations of CD44ΔTM in the PBSF at room temperature for 1 h. The wells were washed with PBST and incubated for 1 h with an antihexahistidine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and then supplemented with *o*-phenylenediamine after washing with PBST. The binding of the CD44ΔTM to TLR2ΔTM, PGN, LPS, or BSA was determined by measuring the absorbance at 450 nm.

Immunoprecipitation and immunoblotting. Cells were transfected with the plasmids by the lipofection method, harvested at 48 h posttransfection, washed three times with ice-cold PBS, and suspended in 0.4 ml lysis buffer containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 1% glycerol, and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were incubated for 30 min at 4°C and centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant was immunoprecipitated with mouse monoclonal anti-FLAG M2 and protein G-Sepharose 4B fast flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ), and the precipitates were washed with Tris-buffered saline containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, and 0.05% Tween 20 (TBST). The proteins bound to the beads were boiled in 20 μl of sample buffer, subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). These membranes were blocked with TBST containing 5% skim milk and incubated with mouse monoclonal anti-FLAG M2 or anti-hexahistidine monoclonal antibody (Santa Cruz) at room temperature for 1 h and then with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 1 h. The recombinant proteins were analyzed by SDS–12.5% PAGE under reducing conditions, stained with GelCord blue

stain reagent (Pierce), and detected by immunoblot analysis using anti-hexahistidine monoclonal antibody (Santa Cruz), anti-FLAG M2 monoclonal antibody, anti-human CD44 monoclonal antibody (clone 3C-11; Cell Signaling Technology, Inc., Beverly, MA), or anti-human TLR2 polyclonal antibody (clone TLR2.1; Santa Cruz). The stable knockdown clones (1×10^5 cells/well) were stimulated with FSL-1 (1 μg/ml), and degradation of IκBα and expression of CD44 and β-actin were determined by immunoblotting using antibodies specific to IκBα (Cell Signaling), CD44 (clone 3C-11), and β-actin (Sigma). The immune complexes and cell lysates were visualized with Super Signal West femto substrate (Pierce) and detected by using an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

DNA microarray analysis. Total RNA was extracted from Huh7 and HCV subgenomic replicon-harboring cells stimulated or not with FSL-1 (1 μg/ml) for 24 h by using an RNeasy minikit (Qiagen) and purified by using a QuickPrep mRNA purification kit (GE Healthcare Life Science, Little Chalfont, United Kingdom). Differentially expressed genes were screened with the use of a DNA microarray system, the human genome U133 plus 2.0 (Kurabo, Osaka, Japan). Data were analyzed by using the GeneChip operating software version 1.4 (Affymetrix 690036) with Microarray Suite version 5.0 (MAS 5.0). Differentially expressed genes were extracted using DNA microarray viewer (Kurabo), and then hierarchical clustering was performed by using Avadis 4.3 prophetic software (Strand Life Sciences, Bangalore, India).

Stable knockdown cell clones. The short interfering RNA (siRNA) sequences of the sense strands targeted to human CD44 (5′-GGAAAUG GUGCAUUUGGUGdTdT-3′), human TLR2 (5′-GCCUUGACCUGUC CAACAAdTdT-3′), and human MyD88 (5′-GGAGGAUUGCCAAAAG UAUdTdT-3′) were designed by using pSilencer Expression Vectors Insert Design Tool (Ambion, Austin, TX) and were introduced into the BamHI and HindIII sites of pSilencer 2.1 U6 Puro vector (Ambion). To establish stable knockdown cell clones, cells were transfected with the plasmids and drug-resistant clones were selected by treatment with puromycin (InvivoGen) at a final concentration of 1 μg/ml.

Real-time PCR. Total RNA was prepared from cells by using an RNeasy minikit (Qiagen), and the first-strand cDNA was synthesized by using a ReverTra Ace (TOYOBO, Osaka, Japan) and oligo$(dT)_{20}$ primer. Each cDNA was determined by using Platinum SYBR green quantitative PCR (qPCR) SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI PRISM 7000 (Applied Biosystems). The human IP-10, MIG, I-TAC, interleukin-8 (IL-8), HCV internal ribosome entry site (IRES), CD44, TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified using the primer pairs 5′-GGCCATCAAGAATTTACTGAAAGCA-3′ and 5′-TCT GTGTGGTCCATCCTTGGAA-3′, 5′-TTAAATTCTGGCCACAGACAA CCTC-3′ and 5′-GCAGCCAAGTCGGTTAGTGGA-3′, 5′-CTTTCATG TTCAGCATTTCTACTCC-3′ and 5′-CCTATGCAAAGACAGCGTCCT C-3′, 5′-CCCAAGGACGGAGACTTCGAT-3′ and 5′-GAAACTTGCTG TGGGTGACCAT-3′, 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CAC TCGCAAGCACCCTATCA-3′, 5′-AACCCTTGCAACATTGCCTGA-3′ and 5′-GCTTCCAGAGTTACGCCCTTGA-3′, 5′-GGAGGCAATGCTG CTGTTCA-3′ and 5′-GCCCAATATGCCTTTGTTATCCTG-3′, 5′-GAA AGCTCCCAGCAGGAACATC-3′ and 5′-GAATGAAGTCCCGCTTAT GAAGACA-3′, 5′-AGGATGATGCCAGGATGATGTC-3′ and 5′-TCAG GTCCAGGTTCTTGGTTGAG-3′, 5′-CCTGGCAAGAGCATTGTGG A-3′ and 5′-TCGTAATGGCACTCACTCTG-3′, 5′-TCTTCAACCAGAC CTCTACATTCCA-3′ and 5′-GGAACATCCAGAGTGACATCACAG-3′, 5′-GGGACCTCGAGTGTGAAGCA-3′ and 5′-CTGGAGCTCACAG GGTAGGAA-3′, and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCC ACCACCCTGTTGCTGTA-3′, respectively. The expression of the mRNA of each gene was normalized to that of GAPDH.

Reporter assay. Cells seeded onto 12-well plates at a concentration of 1.5×10^5 cells/well were transfected with 100 ng of each of the plasmids encoding the luciferase gene under the control of the IP-10, endothelial-leukocyte adhesion molecule (ELAM), and IFN-stimulated response element (ISRE) promoters and stimulated with 500 μg/ml of HA or 250 ng/ml of IFN-α at 24 h posttransfection. Luciferase activity was determined with the dual-luciferase reporter assay system (Promega, Inc., Madison, WI) and the *Renilla* luciferase reporter gene was simultaneously transfected as an internal control.

Gene silencing by siRNA. siRNAs targeted to the endogenous human CD44 (5′-GGAAAUGGUGCAUUUGGUGdTdT-3′) and negative control no. 1, which exhibits no downregulation of any human genes, were purchased from Ambion. HCV replicon-harboring cells were transfected with 100 nM siRNA by using siFector (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol.

Flow cytometry. The cell surface expression of CD44 and CD81 on Huh7 cells, cells harboring subgenomic replicons of JEV and HCV of genotypes 1b (Con1 strain) and 2a (JFH1 strain), and Huh7OK1 cells infected with HCVcc (JFH1) was analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). Cells were washed twice in PBSF and incubated with mouse monoclonal antibodies to human CD44 (clone BU52; Ancell, Inc., Bayport, MN) and human CD81 (BD Biosciences). Mouse IgG1 and IgG2b (BD Biosciences) were used as isotype controls. Cells were washed with PBSF and incubated with the phycoerythrin (PE)-conjugated anti-mouse IgG monoclonal antibody.

Statistical analysis. Results were expressed as means ± standard deviations. The significance of differences in the means was determined by Student's *t* test.

RESULTS

Expression of IP-10 is specifically increased in cells replicating HCV upon stimulation with conventional TLR2 ligands. To examine the involvement of TLR signaling pathways in IP-10 production in cells replicating HCV, we determined the level of IP-10 mRNA in the HCV replicon-harboring and Huh7 cells upon stimulation with various TLR ligands. Among the TLR ligands we examined, PGN derived from bacterial components induced a significant enhancement of IP-10 production in the replicon-harboring cells but not in Huh7 cells (Fig. 1A). A dose-dependent induction of IP-10 was observed in the replicon-harboring cells treated with PGN (Fig. 1B). The enhancement of IP-10 production was also observed in the replicon-harboring cells in response to other TLR2 ligands, including FSL-1, Pam2CSK, and Pam3CSK, which are responsive to each of the heterodimers of TLR2/-6 and TLR1/-6, in contrast to the inhibition of IP-10 production in the replicon-harboring cells upon stimulation with poly(I·C) (Fig. 1C), which was probably due to interference in the dsRNA-dependent signaling pathway by NS3/-4A protease as described previously (25). The expression of TLR2 was confirmed not only in Huh7 and HCV replicon-harboring cells but also in primary human normal hepatocytes and human liver sinusoidal endothelial cells (Fig. 1D). To further examine the expression of each of the CXC chemokines in the HCV replicon-harboring cells, the level of mRNA of the CXC chemokines was determined by real-time PCR upon stimulation with PGN or IFN-γ. Although treatment with IFN-γ suppressed the replication of HCV and induced the expression of IP-10, MIG, and I-TAC in the replicon-harboring cells, stimulation with PGN induced the expression of IP-10 but not of MIG and I-TAC in the replicon-harboring cells (Fig. 1E).

We next examined the effect of HCV infection on the expression of CXC chemokines in cells upon stimulation with PGN. Huh7OK1 cells were established by the elimination of HCV RNA from the replicon-harboring cells by treatment with IFN (34). Huh7OK1 cells are highly permissive to HCVcc (JFH1 strain) in-

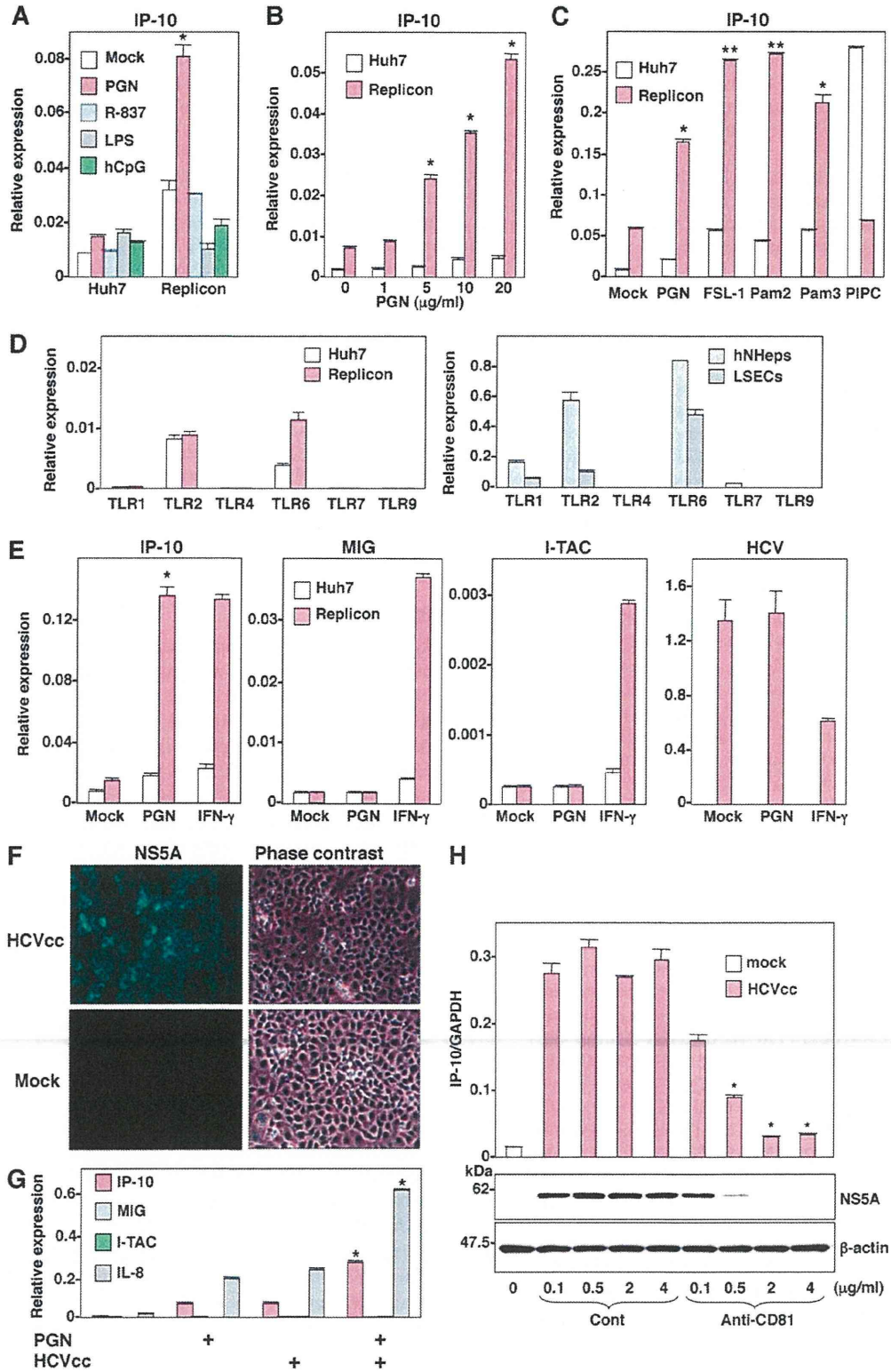

FIG 1 Enhancement of IP-10 production in the HCV-replicating cells upon stimulation with TLR2 ligands. (A) Huh7 and HCV replicon-harboring cells were stimulated with 20 μg/ml of PGN, 10 μg/ml of R-837, 20 μg/ml of LPS, or 10 μg/ml of hCpG, and the levels of IP-10 mRNA were determined by real-time PCR at 24 h after stimulation. (B) Huh7 and HCV replicon-harboring cells were stimulated with various concentrations of PGN, and the levels of IP-10 mRNA were determined at 24 h after stimulation. (C) Huh7 and HCV replicon-harboring cells were stimulated with 20 μg/ml of PGN, 1 μg/ml of FSL-1, 1 μg/ml of Pam2CSK (Pam2), 1 μg/ml of Pam3CSK (Pam3), or 50 μg/ml of poly(I·C) (PIPC), and the level of IP-10 mRNA was determined at 24 h after stimulation. (D) Total RNA was extracted from Huh7 cells, HCV replicon-harboring cells, primary human normal hepatocytes (hNHeps), and human liver sinusoidal endothelial cells (LSECs) cells, and expression levels of

fection, and most of the cells were infected within 7 days postinfection (Fig. 1F). Expression of IP-10 was induced in Huh7OK1 cells upon either stimulation with PGN or infection with HCVcc and was synergistically increased by costimulation (Fig. 1G). IL-8 is an inflammatory chemokine involved in HCV pathogenesis and a marker of the prognosis of CHC patients treated with IFN (37). In addition to IP-10, the production of IL-8 was also enhanced in Huh7OK1 cells infected with HCVcc upon stimulation with PGN (Fig. 1G). IP-10 production in Huh7OK1 cells upon infection with HCVcc was inhibited by pretreatment of cells with an anti-CD81 antibody in a dose-dependent manner in accord with the suppression of NS5A expression (Fig. 1H). These results indicate that the expression of IP-10 is specifically increased in cells replicating HCV upon stimulation with conventional TLR2 ligands.

Expression of CD44 is enhanced in cells replicating HCV and is associated with an increase of IP-10 production in response to TLR2 ligands. To determine the mechanism of enhancement of IP-10 production in cells replicating HCV upon stimulation with TLR2 ligands, the gene expression profiles in Huh7 and the replicon-harboring cells at 24 h after stimulation with a TLR2 ligand (FSL-1) were assessed by DNA microarray analysis. A variety of TLR2 ligand-inducible genes were regulated in the replicon-harboring cells upon FSL-1 stimulation (Fig. 2A). Among them, we focused on CD44 as a candidate molecule for participation in the enhancement of IP-10 production due to the significant enhancement of this molecule in the HCV replicon-harboring cells. CD44 is an adhesion molecule, a broadly distributed type I transmembrane glycoprotein, and a receptor for the glycosaminoglycan hyaluronan (HA). It has been shown that CD44 plays an important role in a variety of immunologic functions, including the adhesion, differentiation, homing, and activation of leukocytes and T cell extravasation to sites of inflammation (38). To confirm the data from the DNA microarray analysis, we examined the expression of CD44 in HCV replicon-harboring cells stimulated with TLR2 ligands. The expression of CD44 mRNA was upregulated in the replicon-harboring cells but not in Huh7 cells and was further enhanced by stimulation with FSL-1 or Pam3CSK in accord with the increase of IP-10 expression (Fig. 2B). Immunoblot analysis confirmed the enhancement of endogenous CD44 expression in the replicon-harboring cells (Fig. 2C). Furthermore, the cell surface expression of CD44 was also upregulated in the HCV replicon-harboring cells of genotypes 1b (Con1 strain) and 2a (JFH1 strain) but not in Huh7 and JEV subgenomic replicon-harboring cells (Fig. 2D), consistent with the enhancement of CD44 expression upon stimulation with FSL-1 (Fig. 2E). Furthermore, the expression of CD44 in the replicon-harboring cells was reduced by treatment with an HCV protease inhibitor (BILN2061), suggesting that enhancement of CD44 is dependent on the replication of HCV (Fig. 2F). These results suggest that the expression of CD44 is enhanced in HCV replicon-harboring cells autonomously replicating the HCV genome and is associated with an increase of IP-10 production in response to TLR2 ligands.

CD44 does not participate in the IP-10 production upon stimulation with TLR2 ligands derived from bacterial components. To further examine the involvement of CD44 in IP-10 production through the TLR2 signaling pathway, we assessed the effect of knockdown of CD44 expression on the IP-10 production in HCV replicon-harboring cells. Although transduction of small interfering RNA (siRNA) targeted to an ectodomain of CD44 which is well conserved among the CD44 variant isoforms suppressed the expression of CD44, IP-10 production upon stimulation with FSL-1 in the replicon-harboring cells exhibited no reduction (Fig. 3A). Similarly, the stable knockdown of CD44 in the HCV replicon-harboring cells did not show a reduction of IP-10 production upon stimulation with FSL-1 (Fig. 3B), suggesting that CD44 expression is not involved in IP-10 production upon stimulation with the conventional TLR2 ligands derived from bacterial components. Furthermore, the degradation of IκBα upon stimulation with FSL-1 was not affected by the stable knockdown of CD44 in the replicon-harboring cells (Fig. 3C). These results suggest that CD44 is not involved in the immune activation by stimulation with TLR2 ligands derived from bacterial components in cells replicating HCV RNA.

HA participates in IP-10 production in cells replicating HCV. Previously, it has been shown that a low-molecular-weight HA derived from the human umbilical cord acts as a TLR2 ligand in primary murine macrophage cells (41). Serum HA is derived from lymphocytes, fibroblasts, and hepatic stellate cells in the liver, and elevation of serum HA is an indicator for hepatic fibrosis and dysfunction of sinusoidal endothelial cells, because most HA is degraded in the hepatic sinusoidal endothelial cells (22). To determine the role of HA in IP-10 production in human hepatoma cell lines, we established Huh7OK1 cell lines stably expressing siRNA targeted to CD44, TLR2, or MyD88. The IP-10 production upon stimulation with HA was severely impaired by the knockdown of either gene (Fig. 4), suggesting that IP-10 production upon stimulation with HA is totally dependent upon the CD44-TLR2-MyD88 axis.

The production of IP-10 in the replicon-harboring cells was enhanced in both mRNA and protein levels by stimulation with HA (Fig. 5A). In addition to IP-10, the HCV replicon-harboring cells induced the expression of IL-8 but not of other CXCR3 ligands, including MIG and I-TAC, upon stimulation with HA (Fig. 5B). The enhancement of IP-10 production by stimulation with HA was also observed in the HCV replicon-harboring cells derived from other genotypes but not in the parental Huh7 cells and JEV replicon-harboring cells (Fig. 5C). The stable knockdown of CD44 in the HCV replicon-harboring cells significantly suppressed IP-10 production upon stimulation with HA but not with IFN-α and IFN-γ (Fig. 5D). The promoter region of IP-10 in-

TLR mRNA were determined by real-time PCR. (E) Huh7 and HCV replicon-harboring cells were stimulated with 20 μg/ml of PGN or 250 ng/ml of IFN-γ, and the IP-10, MIG, or I-TAC mRNA level and HCV IRES RNA level were determined by real-time PCR at 24 h after stimulation. (F) Huh7OK1 cells infected with HCVcc at a multiplicity of infection (MOI) of 1 and incubated for 7 days were fixed with 4% paraformaldehyde–PBS, permeabilized with 0.25% saponin, and immunostained with an anti-NS5A monoclonal antibody. (G) Huh7OK1 cells infected with HCVcc were stimulated with 20 μg/ml of PGN at 6 days postinfection, and the IP-10, MIG, I-TAC, or IL-8 mRNA level was determined by real-time PCR at 24 h after stimulation. (H) Huh7OK1 cells were treated with various amounts of antibodies against isotype control IgG (Cont) or human CD81 for 2 h at 37°C and infected with HCVcc at an MOI of 1. Levels of IP-10 mRNA and expression of NS5A were determined at 6 days postinfection by real-time PCR and immunoblotting, respectively. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells or mock-infected cells.

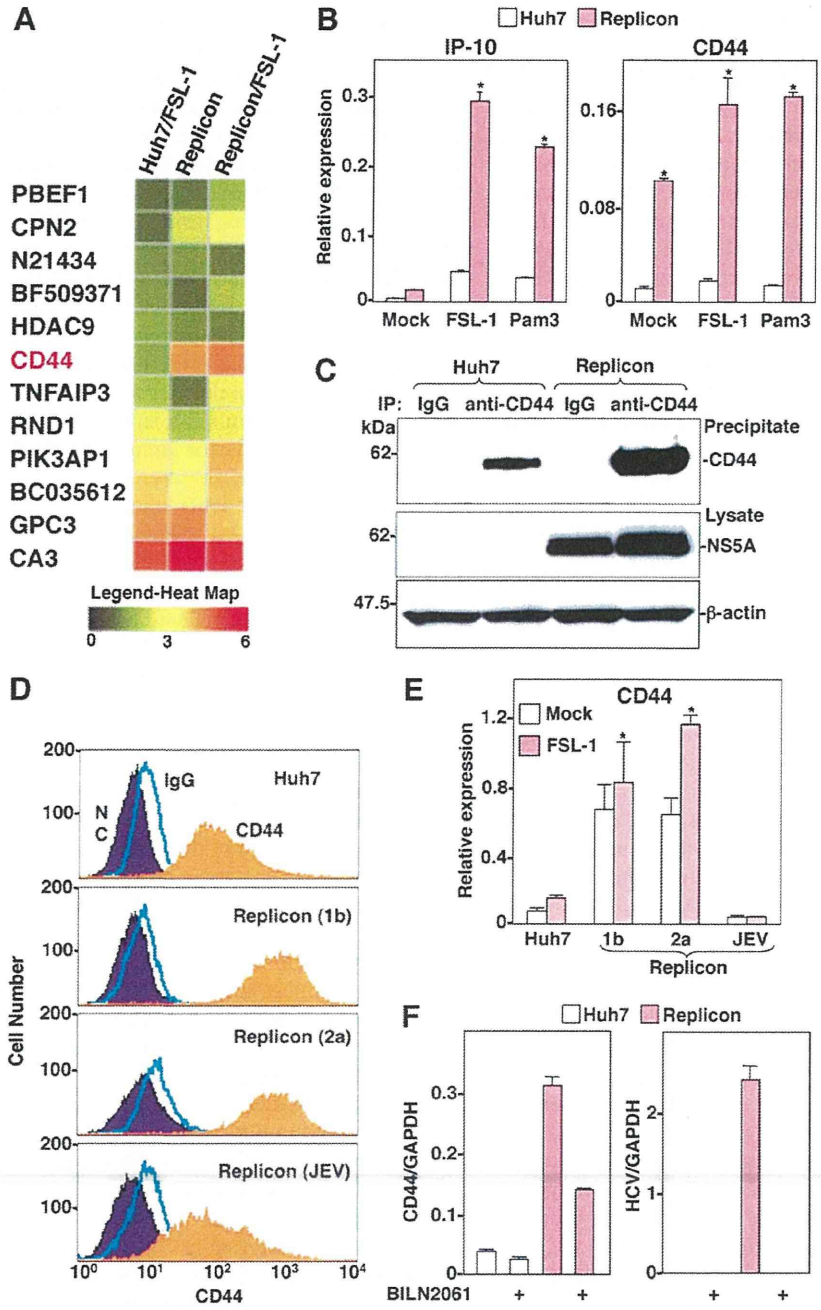

FIG 2 Expression of CD44 in HCV replicon-harboring cells is upregulated in accord with IP-10 production in response to TLR2 ligands. (A) Twelve genes in Huh7 and HCV replicon-harboring cells treated or not treated with FSL-1 were selected, and the resulting heat map is shown. PBEF1, pre-B-cell colony enhancing factor 1; CPN2, carboxypeptidase N, polypeptide 2; N21434, full-length insert cDNA YQ07B06; BF509371, unannotated protein; HDAC9, histone deacetylase 9; TNFAIP3, tumor necrosis factor, alpha-induced protein 3; RND1, Rho family GTPase 1; PIK3AP1, phosphoinositide-3-kinase adaptor protein 1; BC035612, *Homo sapiens* clone IMAGE:4183247 mRNA; GPC3, glypican 3; CA3, carbonic anhydrase III, muscle specific. (B) Huh7 and HCV replicon-harboring cells were stimulated with 1 μg/ml of FSL-1 or 1 μg/ml of Pam3CSK (Pam3), and the mRNA levels of IP-10 and CD44 were determined by real-time PCR at 24 h after stimulation. (C) Expression of CD44, NS5A, and β-actin in Huh7 and HCV replicon-harboring cells was determined by immunoblotting. IP, immunoprecipitation. (D) Cell surface expression of CD44 on Huh7 cells, HCV replicon-harboring cells derived from genotype 1b (Con1 strain) and 2a (JFH1 strain), and JEV replicon-harboring cells was determined by flow cytometry. The filled histograms of purple and orange indicate results for unstained (NC) and stained cells, respectively. Blue lines indicate results for isotype control. (E) Huh7 cells, HCV replicon-harboring cells (Con1 and JFH1 strains), and JEV replicon-harboring cells were stimulated with 1 μg/ml of FSL-1, and the level of CD44 mRNA was determined by real-time PCR at 24 h after stimulation. (F) Huh7 and HCV replicon-harboring cells were treated with 100 nM HCV protease inhibitor (BILN2061), and RNA levels of CD44 and HCV were determined at 72 h posttreatment. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$) versus the results for control cells.

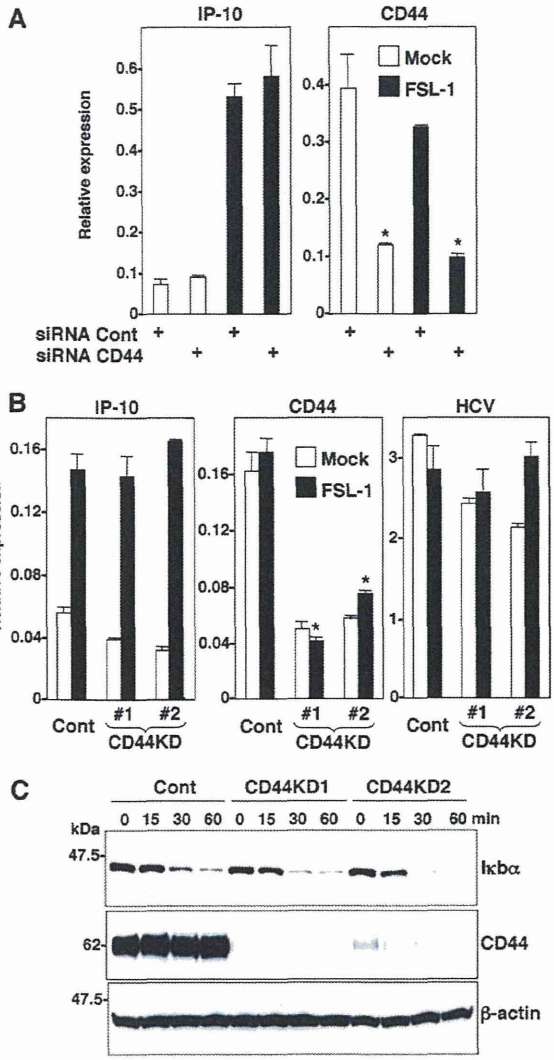

FIG 3 Effect of CD44 silencing on IP-10 production in replicon-harboring cells upon stimulation with TLR2 ligands derived from bacterial components. (A) HCV replicon-harboring cells were transfected with siRNA targeted to CD44 gene or control siRNA at a final concentration of 100 nM and stimulated with 1 μg/ml FSL-1 at 72 h posttransfection. mRNA levels of IP-10 and CD44 were determined by real-time PCR at 24 h after stimulation. (B) Stable knockdown (KD) cell clones based on the HCV replicon-harboring cells expressing siRNA targeted to CD44 gene (CD44KD #1 and #2) or control siRNA (Cont) were stimulated with 1 μg/ml of FSL-1. mRNA levels of IP-10, HCV IRES, and CD44 were determined by real-time PCR at 24 h after stimulation. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$) versus the results for cells transfected with control siRNA. (C) The stable knockdown cell clones based on the HCV replicon-harboring cells were stimulated with 1 μg/ml of FSL-1 for the times indicated, and expression of IκBα, CD44, and β-actin was determined by immunoblotting.

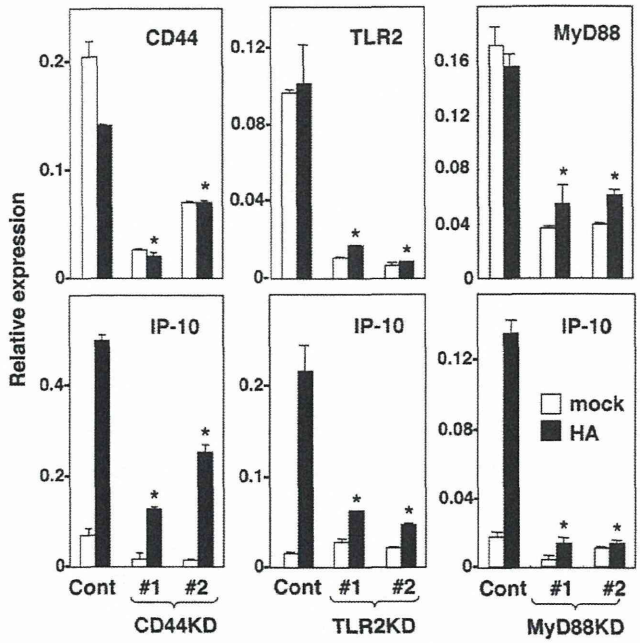

FIG 4 HA induces IP-10 production in human hepatoma cell lines through the TLR2-CD44-MyD88 axis. The stable knockdown cell clones based on the Huh7OK1 cells expressing siRNA targeted to the CD44, TLR2, or MyD88 gene (#1 and #2) or control siRNA (Cont) were stimulated with 500 μg/ml of HA. mRNA levels of IP-10, CD44, TLR2, and MyD88 genes were determined by real-time PCR at 24 h after stimulation. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$) versus the results for cells transfected with control siRNA.

cludes ISRE and two NF-κB-binding regions that are different from those of other CXCR3 ligands (13). The reporter activation assay revealed that IP-10 production in the HCV replicon-harboring cells upon stimulation with HA is dominantly regulated by an NF-κB-dependent pathway (Fig. 5E). Furthermore, activation of the IP-10 promoter upon stimulation with HA but not with IFN-α was suppressed in the stable CD44 knockdown cells (Fig. 5F).

We next examined the IP-10 expression in cells infected with HCVcc upon stimulation with HA. Although no significant increase in the cell surface expression and a slight increase of transcription of CD44 were observed in cells infected with HCVcc (Fig. 6A), IP-10 was induced in cells infected with HCVcc or treated with HA and was additively enhanced by costimulation with HCVcc and HA (Fig. 6B). Furthermore, IP-10 production upon costimulation with HCVcc and HA was decreased in the CD44 knockdown cells (Fig. 6C), whereas the expression of CD81 and viral propagation was not affected by the knockdown of CD44 (Fig. 6D and E). Collectively, these results suggest that the expression of IP-10 was also enhanced in cells infected with HCVcc upon stimulation with HA.

CD44 and TLR2 interact through their extracellular domains. To gain more insight into the IP-10 production by stimulation with HA through TLR2 and CD44, we determined the regions responsible for the interaction between CD44 and TLR2. The CD44 gene contains at least 20 exons, and various isoforms are generated through variable splicing of the internal 10 exons (Fig. 7A) (38). The wild-type and a mutant CD44 lacking the intracellular domain (CD44ΔTM; consists of amino acid residues from 1 to 223) but not a mutant missing the extracellular domain (CD44ΔEC; consists of amino acid residues from 223 to 361) were coprecipitated with TLR2 by immunoprecipitation analysis (Fig. 7B). A TLR2 mutant lacking the transmembrane region (TLR2ΔTM; consists of amino acid residues from 1 to 587) but not a mutant missing the extracellular domain (TLR2ΔEC; consists of amino acid residues from 588 to 784) exhibits a weak but substantial interaction with CD44 (Fig. 7C), indicating that CD44

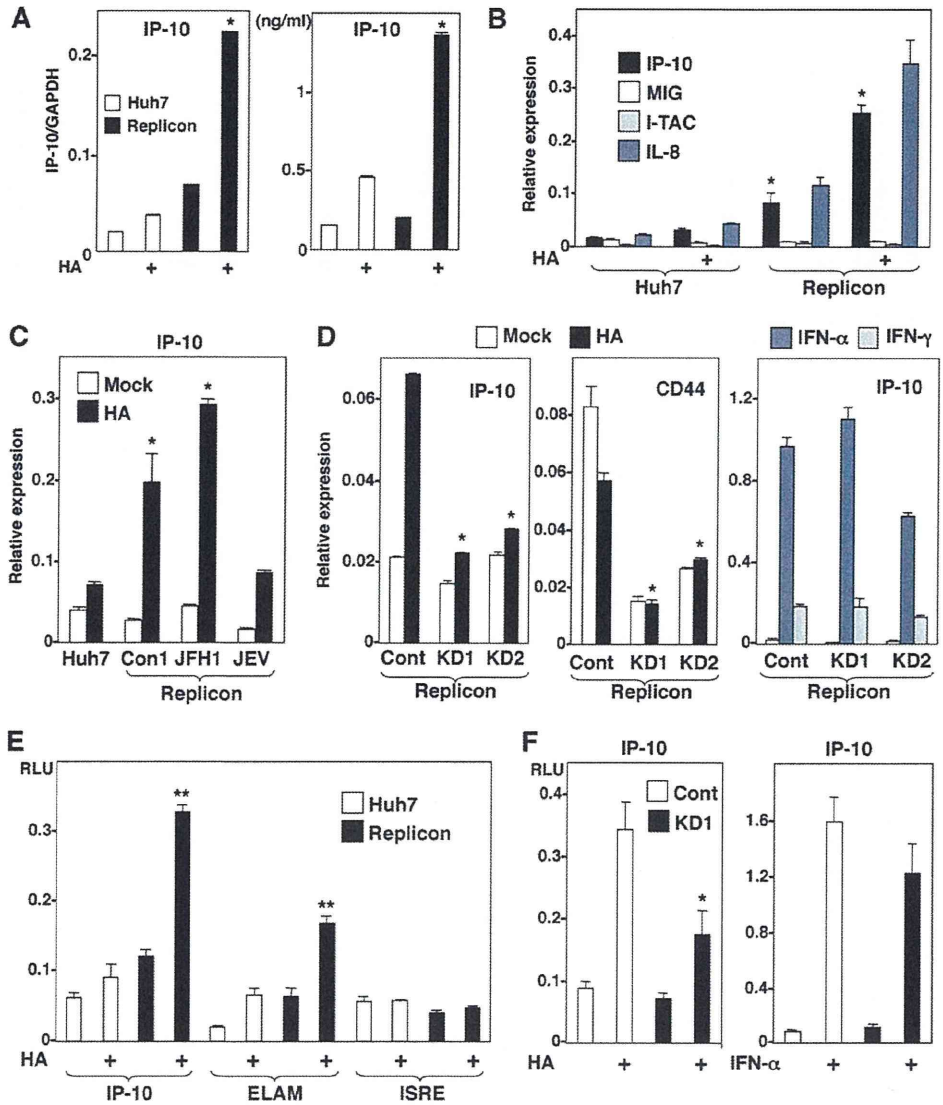

FIG 5 HA participates in IP-10 production in cells replicating HCV. (A) Huh7 and HCV replicon-harboring cells transfected with a plasmid encoding a FLAG-tagged TLR2 were stimulated with 500 μg/ml of HA, and IP-10 mRNA levels (left) and production of IP-10 in culture supernatants (right) 24 h after stimulation were determined by real-time PCR and sandwich ELISA, respectively. (B) Huh7 and HCV replicon-harboring cells were stimulated with 500 μg/ml of HA, and mRNA levels of IP-10, MIG, I-TAC, and IL-8 were determined by real-time PCR at 24 h after stimulation. (C) Huh7 cells, HCV replicon-harboring cells (Con1 and JFH1 strains), and JEV replicon-harboring cells were stimulated with 500 μg/ml of HA, and the level of IP-10 mRNA was determined by real-time PCR at 24 h after stimulation. (D) Stable knockdown cell clones based on the HCV replicon-harboring cells expressing siRNA targeted to the CD44 gene (KD1 and KD2) or control siRNA (Cont) were stimulated with 500 μg/ml of HA or 250 ng/ml of IFN-α and IFN-γ. mRNA levels of IP-10 and CD44 were determined by real-time PCR at 24 h after stimulation. (E) Huh7 and HCV replicon-harboring cells were transfected with each of the reporter plasmids encoding a firefly luciferase gene under the control of the IP-10, ELAM, or ISRE promoter together with a plasmid encoding a *Renilla* luciferase gene under the thymidine kinase (TK) promoter and stimulated with 500 μg/ml HA at 24 h posttransfection. Relative luciferase units (RLU) were determined after standardization with the expression of *Renilla* luciferase at 24 h after stimulation. (F) Stable knockdown cell clones based on the HCV replicon-harboring cells expressing siRNA targeted to the CD44 gene (KD1) or control siRNA (Cont) were transfected with a reporter plasmid encoding a firefly luciferase gene under the control of the IP-10 promoter together with a plasmid encoding a *Renilla* luciferase gene under the TK promoter and stimulated with 500 μg/ml HA or 250 ng/ml IFN-α at 24 h posttransfection. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells or cells transfected with control siRNA.

and TLR2 interact through their extracellular domains. Interestingly, the interaction between CD44 and TLR2 was enhanced by stimulation not only with HA but also PGN (Fig. 7D), although a TLR2 ligand (FSL-1) induces IP-10 production in cells replicating HCV through a CD44-independent pathway, as shown in Fig. 3. To further clarify the direct interaction between CD44 and TLR2, the extracellular domains of His-tagged CD44 (CD44ΔTM) and FLAG-tagged TLR2 (TLR2ΔTM) were expressed in insect cells. Purified samples were examined by Coomassie staining and immunoblotting (Fig. 7E). The CD44ΔTM applied in coats to the microplates exhibited binding to TLR2ΔTM but not to BSA in a dose-dependent manner (Fig. 7F, left). Furthermore, both PGN

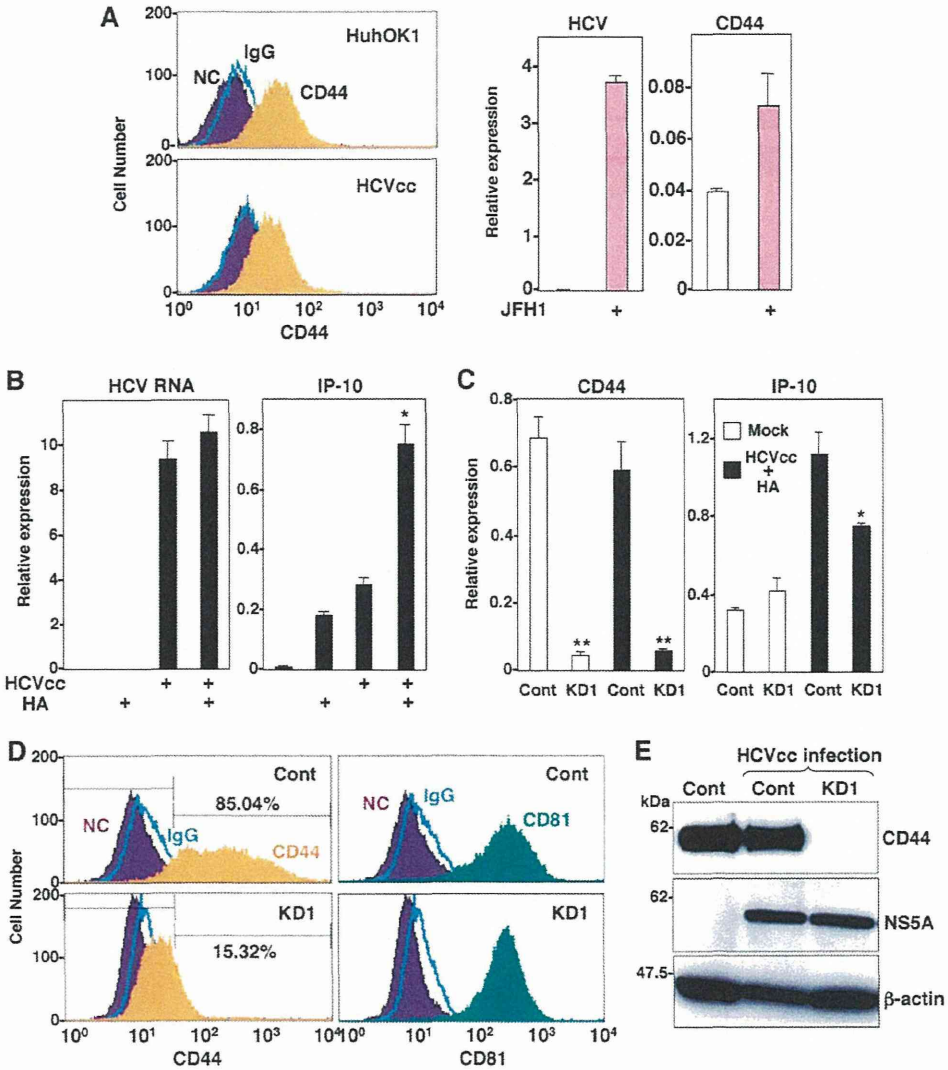

FIG 6 Enhancement of IP-10 production in HCVcc-infected cells upon stimulation with HA. (A) Left, cell surface expression of CD44 on Huh7OK1 cells infected with HCVcc at an MOI of 1 and incubated for 7 days was determined by using antibody that recognizes an ectodomain of CD44 (BU52 clones) and analyzed by flow cytometry. The filled histograms of purple and orange indicate unstained and stained cells, respectively. Blue lines indicate isotype control. Right, levels of CD44 mRNA and HCV RNA in Huh7OK1 cells infected with HCVcc. (B) Huh7OK1 cells infected with HCVcc at an MOI of 1 were stimulated with 500 μg/ml HA at 6 days posttransfection, and IP-10 mRNA and HCV RNA were determined at 24 h after stimulation. (C) Stable knockdown cell clones based on Huh7OK1 cells expressing siRNA targeted to CD44 gene (KD1) or control siRNA (Cont) and infected with HCVcc at an MOI of 1 were stimulated with 500 μg/ml HA at 6 days postinfection, and mRNA levels of CD44 and IP-10 at 24 h after stimulation were determined by real-time PCR. (D) Cell surface expression of CD44 and CD81 on the KD1 and control siRNA cells upon infection with HCVcc at an MOI of 1 was determined by flow cytometry at 7 days postinfection. The filled histograms of purple, orange, and green indicate results for unstained, CD44-positive, and CD81-positive cells, respectively. Blue lines indicate results for isotype control. (E) The KD1 and control siRNA cells were infected with HCVcc at an MOI of 1, and expression of CD44, NS5A, and β-actin at 7 days postinfection was determined by immunoblotting. Data from real-time PCR were normalized to the amount of GAPDH mRNA. Asterisks indicate significant differences ($^*P < 0.05$) versus the results for cells transfected with control siRNA or mock-infected cells.

and HA also bound to CD44ΔTM in a dose-dependent manner, in contrast to a weak interaction with LPS (Fig. 7F, right). These results suggest that IP-10 is induced in cells replicating HCV upon stimulation with HA through an engagement of the extracellular domains of CD44 and TLR2.

DISCUSSION

It has been shown that the expression of CXC chemokines is closely linked to the outcome of antiviral therapy in CHC patients. Successful antiviral therapy is associated with an increase in circulating CXCR3$^+$ CD8$^+$ T cells and the reduction of IP-10 and MIG expression in serum (24). A high level of IP-10 in the plasma of CHC patients has been shown to be an important negative prognostic biomarker of combination therapy with pegylated IFN and ribavirin (3, 5, 40). Furthermore, a recent study suggests that the truncated IP-10 processed by an endogenous DPP4 in the plasma of CHC patients works as an IP-10 receptor antagonist (4). However, the molecular mechanisms of the production of IP-10 in CHC patients have not yet been characterized.

In this study, we suggested that CD44 is involved in the IP-10

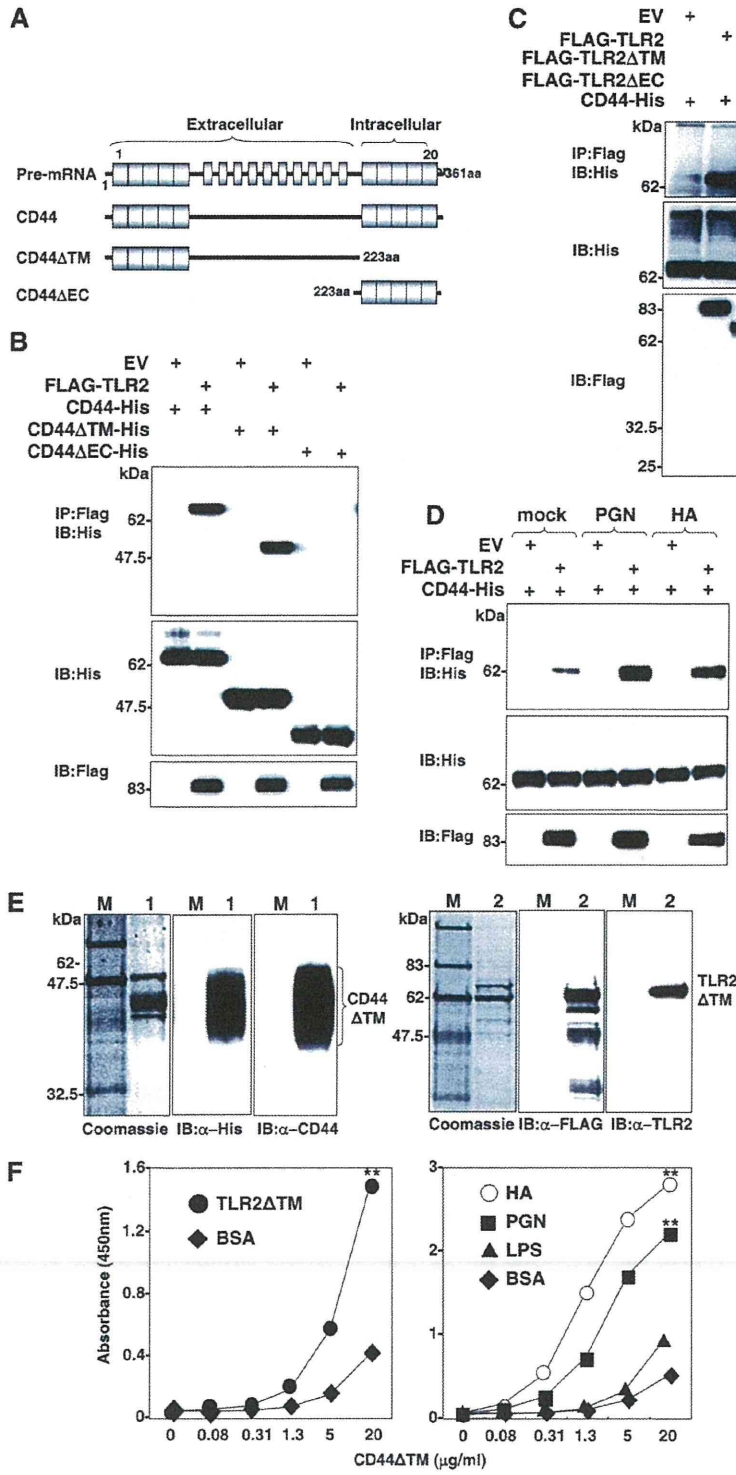

FIG 7 CD44 and TLR2 interact through their extracellular domains. (A) Structures of CD44 and its mutants used in this study. (B) FLAG-TLR2 was coexpressed with CD44-His, its mutants, or empty vector (EV) in 293T cells and immunoprecipitated with anti-FLAG antibody, and the precipitates were determined by immunoblotting (IB) with anti-His antibody. (C) CD44-His was coexpressed with FLAG-TLR2, its mutants, or empty vector in 293T cells and subjected to immunoprecipitation and immunoblotting using the appropriate antibodies. (D) Flag-TLR2 was coexpressed with CD44-His in 293T cells, stimulated with 20 μg/ml PGN or 500 μg/ml HA at 36 h posttransfection, and subjected to immunoprecipitation and immunoblotting using the appropriate antibodies at 24 h after stimulation. (E) Purified CD44ΔTM (lanes 1) and TLR2ΔTM (lanes 2) were examined by Coomassie staining and immunoblotting using antitag and specific antibodies. M denotes molecular mass markers. (F) Microtiter wells were coated with 20 μg/ml TLR2ΔTM (closed circles), 50 μg/ml HA (open circles), 40 μg/ml PGN (closed squares), 40 μg/ml LPS (closed triangles), or 50 μg/ml BSA (closed diamonds) at 4°C overnight and then incubated with the indicated concentrations of CD44ΔTM at room temperature for 1 h. The binding of CD44ΔTM was determined by measuring the absorbance at 450 nm. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for wells treated with BSA.

production upon stimulation with HA through an engagement with TLR2 and that the enhancement of CD44 expression was observed in the HCV replicon-harboring cells of genotypes 1b and 2a but not in cells infected with HCVcc (genotype 2a, JFH1 strain). We do not know the reason why CD44 expression was enhanced in the replicon-harboring cells but not in cells infected with HCVcc, despite the identical origin of the viral genome. Continuous replication of the HCV genome might be required for the enhancement of CD44 expression in the replicon-harboring cells autonomously replicating the HCV genome, in contrast to HCVcc-infected cells exhibiting distinct cytopathic effects. To clarify the role of CD44 in the IP-10 production in cells infected with HCV in more detail, we have to await the establishment of a robust and reliable *in vitro* replication system of various HCV genotypes, especially genotypes 1b and 1a, which are associated with progressive liver injury and persistent infection.

The cellular sources of CXCR3 ligands in CHC patients would be liver parenchymal cells, hepatic stellate cells, and sinusoidal endothelial cells within the liver and infiltrated immunocompetent cells, such as lymphocytes, macrophages, and dendritic cells. We have shown previously that production of IP-10 was enhanced in the macrophage cell lines stably expressing HCV NS5A proteins in response to various TLR ligands, in contrast to the impairment of most proinflammatory cytokines and chemokines (1; also unpublished data). Although replication of HCV in the immunocompetent cells is conflicting (8, 20, 29, 39, 43), it might be feasible to speculate that IP-10 is produced in the immunocompetent cells of CHC patients.

Upon tissue injury, high-molecular-weight HA, a ubiquitously distributed extracellular matrix component, is degraded into low-molecular-weight HA, which in turn activates an inflammatory response, although the precise receptor targeted for this response is still controversial (19). On the other hand, it has been reported that CD44 is dispensable for chemokine production by stimulation with HA in macrophages (18). Interestingly, in HCV-replicating cells, IP-10 production upon stimulation with HA but not with FSL-1 requires CD44. These results suggest that IP-10 production by stimulation with endogenous TLR2 ligands may be regulated by at least two different pathways in hepatocytes of CHC patients, through CD44-dependent and -independent pathways in response to HA and ligands derived from the intestinal microbiota, respectively. The increase of HA expression in accord with the progression of liver fibrosis in CHC patients may participate in the CD44-dependent IP-10 induction. On the other hand, HCV core and NS3 proteins have been shown to induce immune activation in immunocompetent cells through a TLR2-dependent signaling pathway, suggesting that HCV proteins also participate in immune activation as exogenous ligands (6, 7). We tried to neutralize the IP-10 induction in the HCV replicon-harboring cells by using monoclonal antibodies against CD44 and TLR2. However, these antibodies exhibited no significant inhibition of IP-10 production upon stimulation with HA (data not shown), probably due to lack of inhibition of the interaction between ligands and receptors. Furthermore, pretreatment with PGN exhibited no effect on the binding of HA to CD44 (data not shown), suggesting that the TLR2 agonist and HA bind to different regions of CD44. Further studies are needed to clarify the relationship between TLR2 and CD44 for IP-10 production in the HCV-replicating cells.

In contrast to our observations, it has been reported that the induction of CXC chemokines, particularly I-TAC, was significantly enhanced in HCV-replicating cells following stimulation with either IFN-γ or TNF-α and that stimulation with both had a synergistic effect (14). Although we confirmed that the expression of all of the CXC chemokines was significantly induced by stimulation with IFN-γ alone and costimulation with TNF-α in the HCV replicon-harboring cells (Fig. 1E and data not shown), only IP-10 was induced by stimulation with PGN or HA, suggesting that IP-10 is produced in HCV-replicating cells in a ligand-specific manner. The synergistic increase of I-TAC by the activation of IRF3 through a dsRNA-dependent signaling pathway has also been reported (13); however, it is difficult to reconcile the selective increase of I-TAC production by the dsRNA-mediated innate immune response because of the inhibition of the signaling pathway by the HCV NS3/-4A protease (25). Our data indicated that IP-10 production induced by HA or PGN is dependent upon the TLR2–MyD88–NF-κB axis, suggesting that the activation of NF-κB upon stimulation with HA plays a crucial role in the IP-10 production in cells replicating HCV. Although both the IP-10 and I-TAC promoters contain the ISRE, an increase in IP-10 production from stimulation with HCV RNA (5′ untranslated region), poly(I·C), IFN-γ, or TNF-α was not observed (13). Among the CXC chemokines, only IP-10 has two NF-κB-binding elements in the promoter, and the activation of IP-10 by stimulation with HA was mainly regulated by NF-κB but not ISRE in cells replicating HCV (Fig. 5E). These results strongly supported our notion that the selective increase of IP-10 production by stimulation with HA is dominantly regulated by the activation of NF-κB in the HCV-replicating cells.

CD44 variants have been implicated in many biological processes, including hematopoiesis, chronic inflammation, and metastatic spread of cancer cells (10, 38), and are useful markers in the diagnosis and prognosis of the progression of human tumors (11, 15). In chronic HCV infection, HA has been shown to be involved in HCV pathogenesis, while the participation of the specific CD44 variants has not been studied yet. The CD44v8 to -v10 variants have been shown to directly associate with TLR2 through the cytoplasmic domain and negatively regulate the inflammatory response in macrophages and mouse embryonic fibroblasts (21). Furthermore, it has been shown that the expression of CD44 contributes to the suppression of TLR4-mediated inflammation through the induction of the negative regulator in alveolar and peritoneal macrophages (27). The expression of TLR and CD44 variants varies among cell types, and the expression pattern of the molecules might determine the inflammatory response in cells infected with HCV. Further studies are needed to clarify the involvement of each of the CD44 variants in the pathogenesis of HCV.

Intervention to reduce the expression of endogenous HA and to inhibit the interaction between CD44 and TLR2 may provide a novel therapeutic measure for CHC patients exhibiting no response to the current pharmaceutical intervention.

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