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C-type Lectins L-SIGN and DC-SIGN Capture and Transmit Infectious Hepatitis C Virus Pseudotype Particles*

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The molecular mechanisms involved in the hepatic tropism of hepatitis C virus (HCV) have not been identified. We have shown previously that liver-expressed C-type lectins L-SIGN and DC-SIGN bind the HCV E2 glycoprotein with high affinity (Lozach, P. Y., Lortat-Jacob, H., de Lacroix de Lavalette, A., Staropoli, I., Foung, S., Amara, A., Houles, C., Fieschi, F., Schwartz, O., Virelizier, J. L., Arenzana-Seisdedos, F., and Altmeyer, R. (2003) *J. Biol. Chem.* 278, 20358–20366). To analyze the functional relevance of this interaction, we generated pseudotyped lentivirus particles presenting HCV glycoproteins E1 and E2 at the virion surface (HCV-pp). High mannose *N*-glycans are present on E1 and, to a lesser extent, on E2 proteins of mature infectious HCV-pp. Such particles bind to both L-SIGN and DC-SIGN, but they cannot use these receptors for entry into cells. However, infectious virus is transmitted efficiently when permissive Huh-7 cells are cocultured with HCV-pp bound to L-SIGN or to DC-SIGN-positive cell lines. HCV-pp transmission via L-SIGN or DC-SIGN is inhibited by characteristic inhibitors such as the calcium chelator EGTA and monoclonal antibodies directed against lectin carbohydrate recognition domains of both lectins. In support of the biological relevance of this phenomenon, dendritic cells expressing endogenous DC-SIGN transmitted HCV-pp with high efficiency in a DC-SIGN-dependent manner. Our results support the hypothesis that C-type lectins such as the liver sinusoidal endothelial cell-expressed L-SIGN could act as a capture receptor for HCV in the liver and transmit infectious virions to neighboring hepatocytes.

Hepatitis C virus (HCV),[1] a member of the *flaviviridae* and genus *Hepacivirus*, is essentially transmitted during parenteral exposures to infected material such as contaminated blood or needles. How HCV reaches hepatocytes, the principal site of virus replication, is not clear. Several cellular proteins have been proposed to act as receptors for HCV on hepatocytes including CD81, scavenger receptor class B, type I, and the low density lipoprotein receptor (1–3), but these cellular proteins are not sufficient to mediate viral entry into target cells (4). Furthermore, they are expressed on several cell types *in vivo* and therefore cannot explain the hepatic tropism of HCV. HCV in blood can diffuse freely into the liver. However, it is likely that liver sinusoidal endothelial cells (LSEC) represent an obstacle to passive diffusion from the blood into the hepatic tissue. LSEC prevent access of leukocytes to hepatocytes (5) and limit the passage of molecules larger than 12 nm in diameter from the sinusoidal lumen to hepatocytes (6). Thus, passive diffusion through the fenestrated liver endothelium seems unlikely to mediate rapid and efficient hepatocyte targeting by HCV (mean diameter superior to 50 nm). LSEC seem to play a role in the capture and concentration of hepatotropic viruses such as Duck Hepatitis B virus before their transmission to neighboring hepatocytes (7). Duck Hepatitis B virus whose envelope proteins are glycosylated (8) can be detected in the LSEC of infected ducks several hours after infection. C-type lectins-like L-SIGN might be the receptor responsible for liver-specific retention of enveloped hepatotropic viruses. L-SIGN is highly expressed in LSEC (9, 10) but not in hepatocytes. Furthermore, the related molecule, DC-SIGN, is detected in the liver in Kupffer cells (11), which are immobile liver macrophages localized close to LSEC and hepatocytes.

L-SIGN and DC-SIGN act as pathogen-recognition receptors (12) and share 77% sequence homology. The principal characteristic of these C-type lectins is that they interact with mannose residues of glycoproteins in a calcium-dependent manner via their C-terminal carbohydrate recognition domain (CRD) (13, 14). Several motifs localized in their cytoplasmic domain could be involved in the internalization of the lectin by endocytosis and its recycling toward the plasma membrane (12). Several viruses including HCV bind to these lectins via their envelope glycoproteins (15–17). Recently, DC-SIGN has been shown to mediate the infection of dendritic cells (DC) and a DC-SIGN expressing cell line by the Dengue virus, another member of the *flaviviridae* and *Flavivirus* (18, 19).

HCV E1 and E2 surface glycoproteins are naturally retained in the endoplasmic reticulum via their transmembrane domains and carry 6 and 11 high mannose *N*-glycans, respectively (20–22). Previously, we and others (23–25) reported that the HCV glycoproteins and virions contained in the sera of HCV patients were captured by L-SIGN and DC-SIGN. The high mannosylated glycoform of HCV E2 binds to L-SIGN and

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[1] The abbreviations used are: HCV, hepatitis C virus; LSEC, liver sinusoidal endothelial cells; mAb, monoclonal antibody; HIV, human immunodeficiency virus; GFP, green fluorescent protein; pp, pseudoparticles; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LS, L-SIGN; DCS, DC-SIGN; DMJ, 1-deoxymannojirimycin hydrochloride; VSVG, vesicular stomatitis virus G protein; CRD, C-terminal carbohydrate recognition domain; E1, envelope glyoprotein 1; E2, envelope glyoprotein 2; CMV, cytomegalovirus; PBMC, peripheral blood mononuclear cells; FACS, fluorescence-activated cell sorter; PNGase F, peptide *N*-glycosidase F; Endo H, endoglycosidase H; RLU, relative light units; DC, dendritic cells; m.o.i., multiplicities of infection.

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DC-SIGN with high affinity (k_d = 6 and 3 nM, respectively) (23). However, due to the lack of an efficient culture system and purified HCV virions, no information is available on the glycosylation status of E1 and E2 on mature infectious HCV virions or on the functional relevance of these interactions.

In this study, we investigated the role of L-SIGN and DC-SIGN in viral entry and transmission to hepatocytes using pseudotyped lentivirus particles carrying functional E1 and E2 glycoproteins (26–28). Our results show that HCV-pp can interact with L-SIGN or DC-SIGN in a different manner compared with other viruses and that L-SIGN may act as a tissue-specific capture receptor for HCV.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Murine monoclonal antibodies (mAb) A4, A11, and H52 are directed against linear epitopes of E1 (A4) and E2 (A11 and H52) glycoproteins. Polyclonal human antibody New Lav Blot is directed against all human immunodeficiency virus (HIV) proteins including the p24 core protein. Phycoerythrin-conjugated murine mAbs anti-DC-SIGN (FAb161P), anti-L-SIGN (FAb162P), and anti-L-SIGN/anti-DC-SIGN (FAb1621P) were purchased from R&D Systems. Murine mAbs anti-L-SIGN/anti-DC-SIGN (mAb1621) and anti-L-SIGN (mAb162) were also purchased from R&D Systems. 1B10 is a DC-SIGN murine mAb directed against a conformational epitope of the DC-SIGN CRD and was described previously (16). Fluorescein isothiocyanate-conjugated mouse mAb anti-CD14 (MϕP9) and anti-CD1a (HI149) were purchased from BD Biosciences. As controls, mouse isotypic antibodies IgG2a, IgG2b, IgG1-fluorescein isothiocyanate, IgG2b-fluorescein isothiocyanate, and IgG2b-phycoerythrin were used.

Envelope Proteins, Packaging, and Transfer Vector Constructs—The p8.71 HIV packaging construct, encoding the HIV gag and pol genes, and the pTrip-GFP and pTrip-luciferase plasmids, encoding an HIV-based transfer vector containing a CMV-GFP or a CMV-luciferase internal transcriptional unit, respectively, were described previously (Fig. 1*A*) (29–31). The pCDNA3-VSVG (vesicular stomatitis virus G protein) and phCMV-cE1E2 expression vectors encode the VSVG protein and the E1 and E2 glycoproteins from a HCV 1a genotype, respectively (Fig. 1*A*) (26). The plasmid coding for the *env* gene of the JR-cerebral spinal fluid isolate was used to produce HIV pseudoparticles (HIV-pp) (32). It codes for an HIV genome in which the Nef gene has been replaced by a luciferase reporter gene.

Cell Lines and C-type Lectin Expression—Huh-7 is an adherent human hepatocytic cell line, and HEK293T is a human kidney cell line. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. BTHP1 cells are non-adherent human Raji B cells, previously misidentified as THP1 cells (33). BTHP1 cells expressing L-SIGN (BTHP1-LS) were generated by transduction with the retroviral pTrip vector expressing L-SIGN. The pCDNA3-L-SIGN (DC-SIGNR) sequence was digested by BamHI (5′) and XhoI (3′) and inserted into the pTrip vector. BTHP1, BTHP1-LS, and BTHP1 expressing DC-SIGN (BTHP1-DCS) cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. All of the products used for cell culture were from Invitrogen.

Purification of Human Peripheral Blood Mononuclear Cells (PBMC) and Dendritic Cells—Human PBMC were isolated from healthy donors by density gradient centrifugation using Ficoll (Amersham Biosciences). Monocytes were negatively selected with magnetic beads coated with a mixture of antibodies (Miltenyi Biotec). They were seeded at 10^6 cells·ml^{-1} and subsequently cultured in fresh RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin, 50 ng·ml^{-1} recombinant human interleukin-4 (PeproTech), and 800 units·ml^{-1} recombinant human granulocyte macrophage colony-stimulating factor (Schering-Plough) for 6 days. Differentiation of dendritic cells (DC) was assessed by FACS analysis using CD14 down-regulation and CD1a up-regulation as parameters. For enhancement assays, isolated PBMC were cultured in RPMI 1640, 10% FCS medium containing phytohemagglutinin (3 μg·ml^{-1}, Murex) and interleukin-2 (10 units·ml^{-1}, Chiron) for 3 days as previously described (32). They were then seeded at 10^6 cells·ml^{-1} and subsequently cultured in fresh RPMI 1640 medium containing interleukin-2 (10 units·ml^{-1}).

Production and Purification of Pseudotyped Lentivirus Particles—HEK293T cells were seeded 1 day before transfection at 2.5×10^6 cells in a 10-cm plate in 10 ml of DMEM, 10% FCS. Medium was replaced 3 h before transfection by fresh medium (10 ml). Cells were transfected using a standard phosphate calcium transfection method (Clontech). The transfecting DNA mixture (1 ml) was composed of 8 μg of p8.71, 8 μg of pTrip-GFP or pTrip-luciferase, and 5 μg of glycoprotein(s) encoding plasmid. HCV-pp and VSV-pp correspond to lentivirus particles pseudotyped with HCV E1 and E2 glycoproteins and VSVG glycoprotein, respectively (Fig. 1*A*). For Δenv-pseudotyped lentivirus particles (Δenv-pp), the amount of envelope-encoding plasmid was replaced by the same amount of p8.71 plasmid (13 μg total). Medium was replaced 16 h after transfection by DMEM supplemented with 10% FCS (8 ml). To produce HCV-pp carrying high mannose *N*-glycans (HCV-pp^{DMJ}), α-mannosidase I and II inhibitors 1-deoxymannojirimycin hydrochloride (DMJ, 1 mM, Calbiochem) and swainsonine (5 mM, Sigma) were added. For the production of ^{35}S-labeled HCV-pp, the medium was supplemented with 200 μCi·ml^{-1} [^{35}S]cysteine and methionine (ProMix ^{35}S, Amersham Biosciences). HCV-pp were harvested in culture supernatants 24 h after transfection. Supernatants were cleared by centrifugation and filtered through 0.45-μm pore-sized membranes. HCV-pp were purified by ultracentrifugation through a 2.5-ml 20% sucrose cushion in a Beckman SW 28 rotor (28000 rpm; 2 h, 30 min; 4 °C). Virus was concentrated 100 times in FCS-free DMEM, and the p24 antigen content was determined with a commercial enzyme-linked immunosorbent assay (PerkinElmer Life Sciences).

Immunodetection of Cell Surface Antigens—L-SIGN and DC-SIGN were detected by FACS using phycoerythrin-conjugated anti-L-SIGN (FAb162P), anti-DC-SIGN (FAb161P), and anti-L-SIGN/anti-DC-SIGN (FAb1621P) antibodies. CD14 and CD1a were detected using fluorescein isothiocyanate-conjugated anti-CD14 (MϕP9) and anti-CD1a (HI149). Cells were washed in FCS-free DMEM and resuspended in A buffer (1% bovine serum albumin, 0.2% γ-globulin, and 0,1% sodium azide (all from Sigma)) followed by incubation with antibodies at a 1:100 dilution for 30 min at 4 °C. Cells were washed and fixed with paraformaldehyde (3.2%, Electron Microscopy Sciences) prior to FACS analysis (FACSCalibur, BD Biosciences) and data-processed with CellQuest software (BD Biosciences).

Glycosylation of HCV-pp Proteins—HCV-pp (6 μg of p24 equivalent) were submitted to a 20–60% sucrose density gradient (10 ml) using a Beckman SW 41 rotor (40,000 rpm; 18 h; 4 °C). Fractions (500 μl) were collected and analyzed (18 μl) directly by Western blotting.

Purified HCV-pp or HCV-pp^{DMJ} extracts (200 ng of p24 equivalent) were denatured in 0.5% SDS and 1% β-mercaptoethanol at 100 °C for 5 min followed by an overnight incubation at 37 °C in G7 buffer (50 mM sodium phosphate, pH 7.5, New England Biolabs), Nonidet P-40 buffer (1% Nonidet P-40, New England Biolabs) containing endoglycosidase H (2 milliunits of Endo H, Roche Applied Science) or peptide *N*-glycosidase F (1000 U of PNGase F, New England Biolabs). HCV-pp subjected to the same procedure in the absence of PNGase F or Endo H were used as a control.

Protein extracts were analyzed by SDS-PAGE (NuPAGE Novex Bis-Tris gels, Invitrogen) and transferred to Immobilon P membranes (Millipore). Incubation with primary antibodies A11 (anti-E2; 2 μg·ml^{-1}), H52 (anti-E2; 2 μg ml^{-1}), A4 (anti-E1; 2 μg·ml^{-1}), and New Lav Blot (anti-HIV, 1:200) was followed by incubation with an anti-mouse horseradish peroxidase-conjugated secondary antibody, NA931V (Amersham Biosciences) (1:1000). Bound antibodies were detected by exposure to enhanced chemiluminescence reagents (ECL+, Amersham Biosciences) and analyzed by a video acquisition system (Intelligent Dark Box II, Fuji) and Image Gauge software (Fuji).

Cell Infection with HCV-pp—Adherent cells (5×10^4) were plated in 12-well plates 24 h before infection in DMEM containing 10% FCS. 10^5 cells were washed three times with FCS-free medium, and 300 μl of diluted pseudotyped retroviral particles in FCS-free DMEM were added for 1 h at 37 °C under gentle agitation every 15 min without the addition of facilitating reagents such as DEAE-dextran or Polybrene. Subsequently, 300 μl of DMEM containing 10% FCS were added. 16 h later, viral inoculum was removed and replaced with 1 ml of DMEM supplemented with 10% FCS. Cells were harvested 52 h later. When GFP was used as the reporter gene, cells were fixed with paraformaldehyde (3,2%) and positive GFP cells were analyzed by FACScan (FACSCalibur, BD Biosciences). When luciferase was used as a reporter gene, cells were harvested in 150 μl of lysis B buffer (25 mM Tris phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% v/v Triton X-100, 15% v/v glycerol) and 50 μl of lysis extract were measured in substrate B buffer containing 1 mM luciferin (Sigma) and 20 mM ATP (Sigma) in a Lumat LB 9501 (Berthold) to determinate the luciferase activity.

Binding of HCV-pp and HCV-pp^{DMJ} to DC-SIGN and L-SIGN—Binding assays were performed on 10^6 BTHP1, BTHP1-LS, BTHP1-DCS, or dendritic cells in 200 μl of C buffer (RPMI 1640 medium without FCS containing 1 mM $CaCl_2$ and 2 mM $MgCl_2$) in 96-well plates.

Cells were incubated with ^{35}S-labeled HCV-pp (30 ng of p24 equivalent) for 3 h at 37 °C under gentle shaking. Virus-cell mixtures were then transferred to a new plate, and unbound radioactivity was removed by five washes with C buffer. Cell pellets were resuspended in C buffer prior to the addition of Optiphase Supermix solution (Wallac), and bound radioactivity was counted in a 1450 Microbeta Trilux γ counter (Wallac). For binding inhibition assays, cells were preincubated with inhibitors in C buffer for 30 min at 4 °C before the addition of labeled particles containing inhibitor. Inhibitors were used at a final concentration of 20 $\mu g \cdot ml^{-1}$ for mannan (Sigma), 1B10, MAb1621, and isotype control IgG or at 5 mM for EGTA (Sigma). HCV-pp-specific binding to BTHP1, BTHP1-DCS, BTHP1-LS, or dendritic cells was calculated as shown in Equation 1.

$$\text{Bound cpm from HCV-pp} - \text{Bound cpm from } \Delta\text{env-pp} \quad \text{(Eq. 1)}$$

HCV-pp and HCV-pp^{DMJ} Transmission by DC, BTHP1-LS, and BTHP1-DCS Cells—Huh-7 cells (5×10^4) were plated 1 day before transmission in 12-well plates in DMEM supplemented with 10% FCS. Before coculture, cells were washed three times with FCS-free medium and incubated in 300 μl of FCS-free DMEM. HCV-pp (150–300 ng of p24 equivalent) were incubated with BTHP1, BTHP1-LS, BTHP1-DCS, or dendritic cells (10^6) as described. Cells were resuspended in 300 μl of DMEM supplemented with 10% FCS and added to Huh-7 cells. Coculture was carried out for 68 h at 37 °C. Plates were washed three times followed by the harvesting and FACScan analysis or luciferase activity measurement as described. Virus transmission results were calculated as follows: 1) GFP(+) cells in coculture × 100/GFP(+) cells following direct infection with the same amount of HCV-pp and 2) RLU in coculture × 100/RLU following direct infection with same amount of HCV-pp.

Enhancement of HCV-pp and HIV-pp infection by L-SIGN and DC-SIGN were tested using Huh-7 and activated PBMC target cells, respectively. Huh-7 cells (2.5×10^4) were plated in 24-well plate in DMEM and 10% FCS 24 h before transmission. BTHP1, BTHP1-LS, BTHP1-DCS, or dendritic cells (5×10^5) were incubated with low and otherwise non-infectious quantities of HCV-pp, HCV-pp^{DMJ} (50 ng of p24 equivalent) or with HIV-pp as described previously (34) for 3 h at 37 °C. The DC, BTHP1, BTHP1-LS, and BTHP1-DCS virus mixture then was directly added to Huh-7 cells or activated human T lymphocytes cells (5×10^4). Co-culture was carried out for 68 h at 37 °C. Cells were washed three times prior to lysis in buffer B and luciferase activity measurement as described previously.

RESULTS

Characterization of Functional HCV-pp—To study the interactions between HCV and L-SIGN or DC-SIGN, we produced lentivirus-pseudotyped particles carrying unmodified HCV E1 and E2 glycoproteins (Fig. 1*A*) (26–28). The E1 and E2 lentivirus pseudotype particles, HCV-pp, were biologically functional because they could infect the hepatocytic cell line Huh-7 as evidenced by the expression of either a GFP (Fig. 1*B*) or a luciferase reporter gene (Fig. 1*C*). Non-infectious lentivirus viral particles lacking surface glycoprotein (Δenv-pp) were used as negative controls. Lentivirus particles carrying the G envelope protein of VSV were used as positive controls (VSV-pp).

HCV-pp were separated on a sucrose gradient, and fractions were collected and analyzed by Western blotting. The E1 protein was distributed in fractions in which neither E2 nor HIV core protein could be significantly detected (Fig. 2*A*). The fractions in which E1 protein was detected alone might correspond to lipid droplets carrying E1 protein. Infectious HCV-pp was present only in fractions where E1, E2, and HIV p24 core protein colocalized (Fig. 2*A*). No E1 or E2 protein was detected in Δenv-pp or supernatant of cells transfected with E1 and E2 protein coding plasmids alone (data not shown).

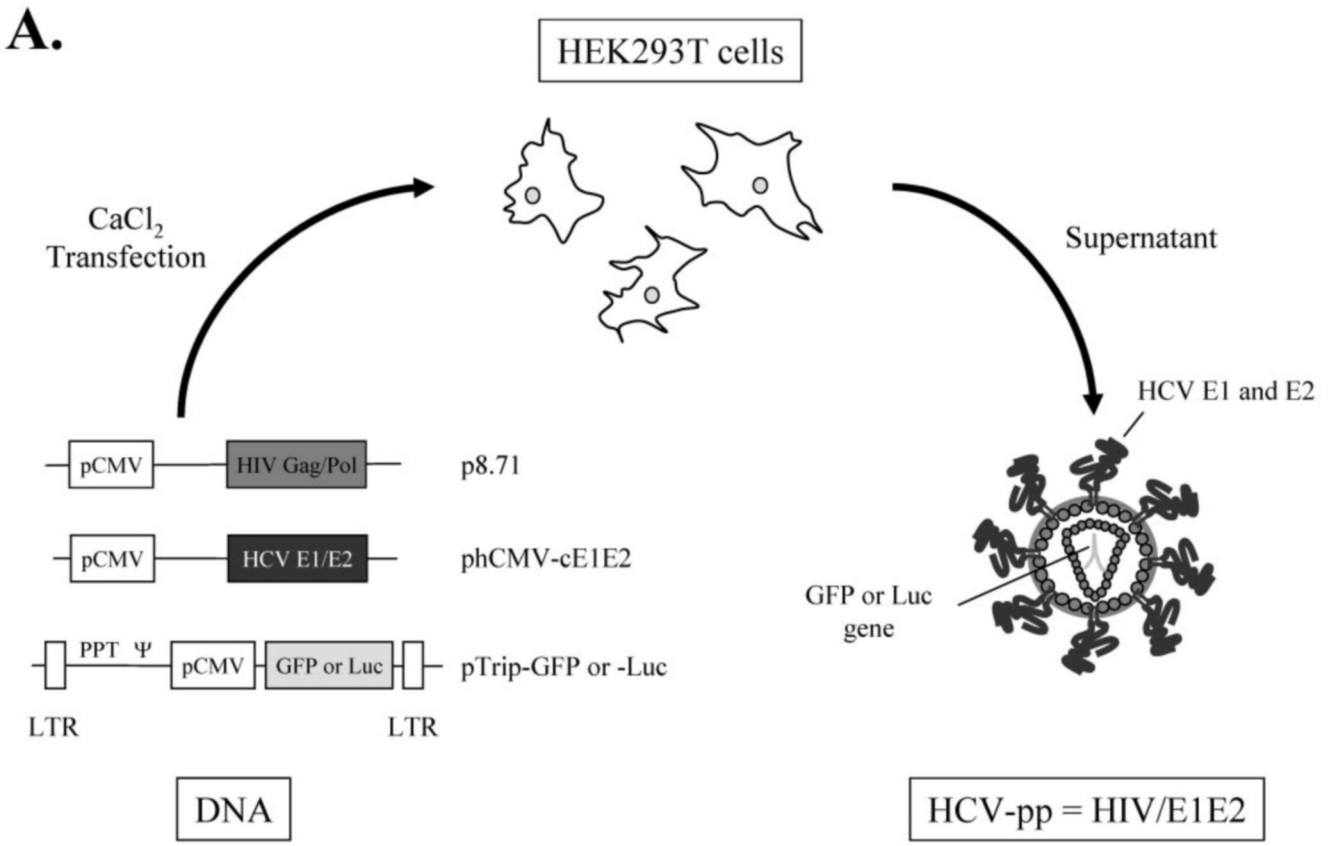
The subcellular compartment of HCV budding, *i.e.* where E1/E2 heterocomplexes interact with cores to form virus particles, and the presence of highly mannosylated or complex *N*-glycans on the HCV virion have not been determined. In agreement with a previous report (28), the HCV-pp-associated E2 is partially resistant to Endo H, indicating the *N*-glycan modification by Golgi-resident enzymes (Fig. 2*B*). To simulate a situation in which HCV *N*-glycans are not modified in the Golgi, we generated HCV-pp in the presence of swainsonine and DMJ, which inhibit Golgi α-mannosidases and therefore prevent complex glycosylation. The infectivity of these heavily mannosylated particles (HCV-pp^{DMJ}) in Huh-7 cells was similar to that of HCV-pp produced in the absence of swainsonine and DMJ (data not shown). As expected, HCV-pp^{DMJ} E2 glycoprotein remains completely sensitive to Endo H, demonstrating that it is highly mannosylated (Fig. 2*C*). Interestingly, E1 proteins on HCV-pp and HCV-pp^{DMJ} had the same glycosylation pattern (Fig. 2, *B* and *C*). We concluded that the upper band observed for E1 after Endo H treatment was a carbohydrate residue, which is not accessible to Endo H enzyme, and was not a complex carbohydrate residue. Our data suggest that during HCV-pp maturation, all of the E1 *N*-glycans and a minor fraction of E2 *N*-glycans remain inaccessible to glycosylation-modifying enzymes.

HCV-pp and HCV-pp^{DMJ} represent two glycosylation profiles of functional HCV E1 and E2 proteins that may be present on wild type virions. Therefore, HCV-pp and HCV-pp^{DMJ} are tools of choice to explore the biochemical correlations of a functional interaction of HCV with C-type lectin receptors.

HCV-pp and HCV-pp^{DMJ} Bind to L-SIGN and DC-SIGN—We previously reported that soluble HCV E2 protein binds to L-SIGN and DC-SIGN with high affinity (23). To study interactions between these lectins and functional E1 and E2 proteins on HCV-pp, L-SIGN and DC-SIGN were expressed on the surface of BTHP1 human cell line (BTHP1-LS and BTHP1-DCS, respectively) (Fig. 3*A*). Dendritic cells, which express DC-SIGN, were also differentiated from human PBMC. CD14 down-regulation and CD1a up-regulation confirmed the differentiation into DC (Fig. 8*A*). Δenv-pp and Δenv-pp^{DMJ} (produced in the presence of swainsonine and DMJ) did not bind to BTHP1 and BTHP1-LS but interacted weakly with BTHP1-DCS or dendritic cells. Δenv-pp (or Δenv-pp^{DMJ}), which does not incorporate viral glycoproteins, was used to define background values. Both ^{35}S-labeled HCV-pp and HCV-pp^{DMJ} bound to BTHP1-LS and BTHP1-DCS but not to control cells (Fig. 3*B*). Furthermore, DC-SIGN expressed on DC bound HCV-pp and HCV-pp^{DMJ} (Fig. 8*B*). However, at equimolar HIV p24, the core concentration of HCV-pp and HCV-pp^{DMJ}, a higher binding was obtained for HCV-pp^{DMJ}. This finding suggests that the presence of high mannose Endo H-sensitive glycosylation on HCV-pp^{DMJ} E2 protein enhances binding.

We sought to define further the specificity of these interactions by testing known inhibitors of ligand binding to DC-SIGN or L-SIGN. The CRD-specific monoclonal antibody 1B10 inhibited the binding of HCV-pp and HCV-pp^{DMJ} to DC-SIGN, and mAb1621 directed against DC-SIGN and L-SIGN inhibited the binding of HCV-pp and HCV-pp^{DMJ} to L-SIGN (Figs. 4, *A*, and *B*, and 8*B*). In addition, these interactions were blocked by the calcium chelator EGTA (Fig. 4, *A* and *B*), confirming the calcium-dependent binding of the CRD with high mannose *N*-glycans. In contrast, we did not observe inhibition by L-SIGN-specific mAb162 (R&D) (data not shown). Interactions of HCV-pp and HCV-pp^{DMJ} with L-SIGN and DC-SIGN thus are specific to envelope proteins E1 and E2 and are increased if E2 is highly mannosylated. These results suggest a pivotal role of high mannose *N*-glycans as binding motifs on HCV E2 protein.

Expression in Cis either of L-SIGN or DC-SIGN Does Not Enhance HCV-pp Infectiveness—L-SIGN and DC-SIGN are used by several viruses, including Dengue virus, to directly infect cells. No infection of BTHP1-LS, BTHP1-DCS (Fig. 5), or dendritic cells (Fig. 8*C*) by HCV-pp was observed, in agreement with a previous report (27). Similar results were obtained with HIV-pp or HCV-pp^{DMJ}, which displays increased binding to



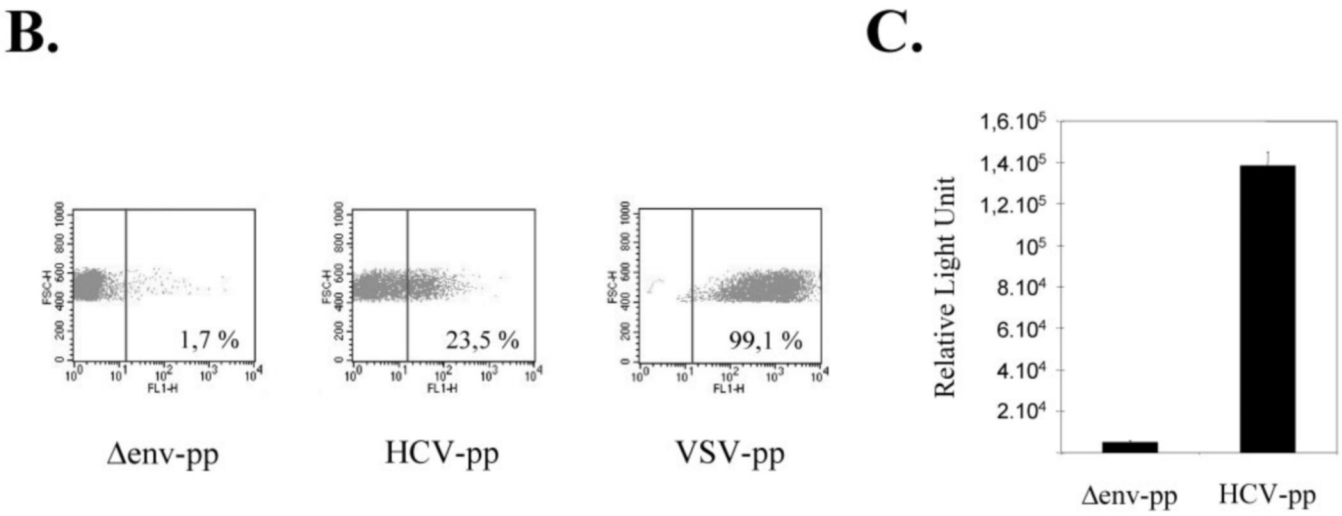


FIG. 1. **HCV E1/E2-pseudotyped lentivirus particles are biologically functional.** *A*, pseudotyped lentivirus particles were produced in HEK293T cells cotransfected with plasmids encoding HIV Gag/Pol proteins (p8.71), viral envelopes (phCMV-cE1E2 coding for HCV E1/E2 or pCDNA3-VSVG coding for VSVG protein) and a modified HIV RNA encoding the encapsidation signal (ψ) and a central GFP or luciferase transcriptional unit (pTrip-GFP or pTrip-Luc, respectively). *B*, GFP expression in HCV-pp- or VSV-pp-infected Huh-7 cells was detected by FACScan analysis. 10^5 cells were infected with 300 ng of p24 equivalent of HCV-pp. The data are representative of three independent experiments. *C*, luciferase activity in Huh-7 cells (10^5) infected by HCV-pp (300 ng of p24 equivalent). The data are expressed as RLU/mg of total protein and are representative of three independent experiments. Values are given as the mean of duplicates ± S.E.

both lectins (data not shown). Other cell lines expressing L-SIGN or DC-SIGN (HeLa and human osteosarcoma-derived cells) yielded similar results (data not shown). In contrast, these cells could be infected with Dengue virus as recently reported (data not shown) (18, 19). Retroviral particles carrying Dengue virus envelope proteins prM and E seem to act in a way similar to wild type Dengue virus (data not shown), substantiating the model of HCV-pp. Moreover, VSV-pp could infect these cells independently of the lectins. This led to the conclusion that a lack of permissiveness of BTHP1-LS or BTHP1-DCS

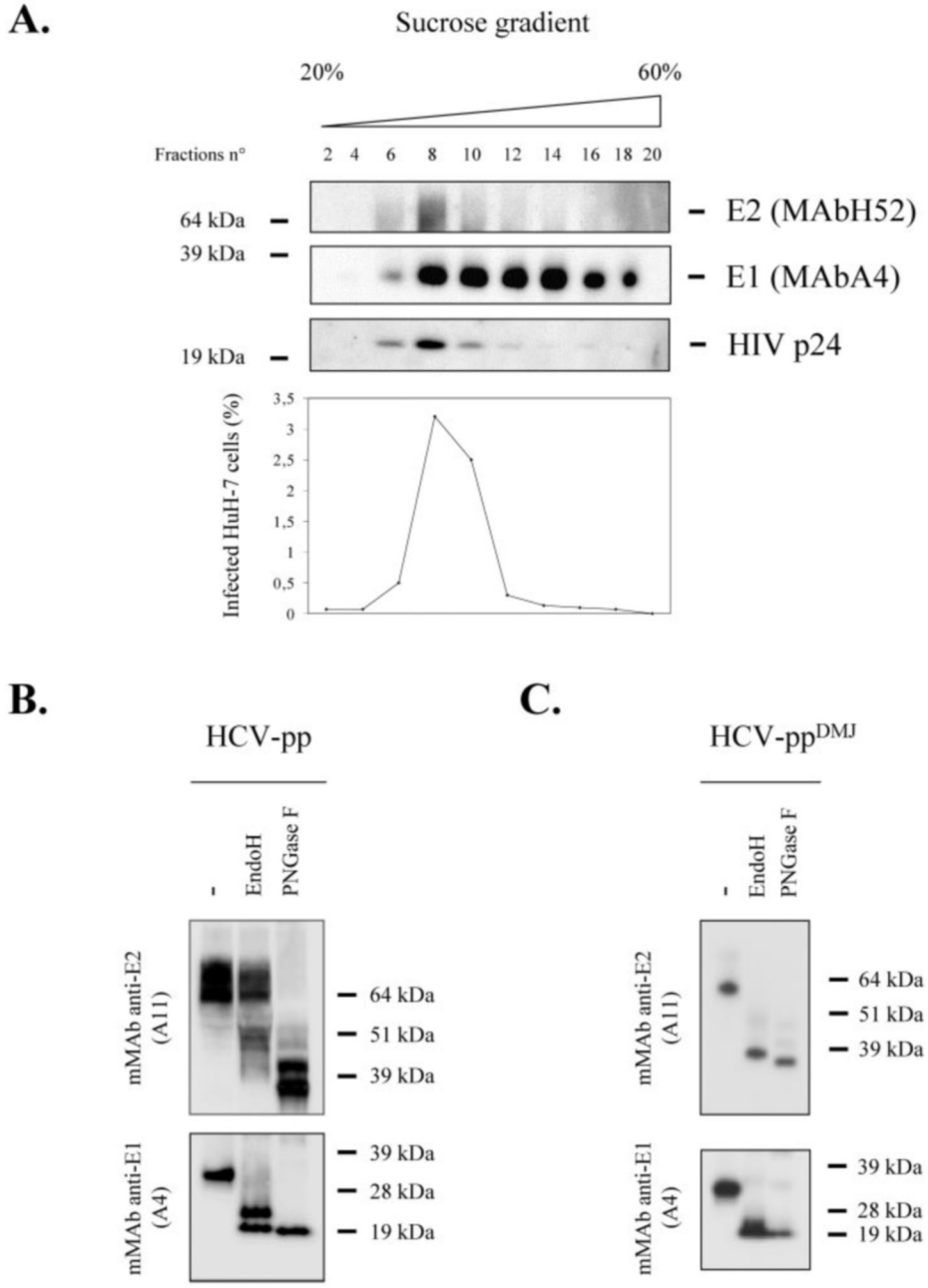


FIG. 2. **E1 and E2 glycoproteins on lentivirus particles carry high mannose *N*-glycans.** *A*, HCV-pp coding for GFP reporter gene were separated on a 20–60% sucrose gradient, and fractions were analyzed by Western blotting using E1-, E2-, and HIVp24-specific mAb and infectivity assays in Huh-7 cells. Optimal GFP expression was obtained in fractions where E1, E2, and HIV p24 core proteins colocalized. *B*, HCV-pp were subjected to digestion by Endo H or PNGase F and analysis by Western blotting. HCV-pp E2 proteins could be partially deglycosylated by Endo H and completely by PNGase F. *C*, HCV-pp produced in the presence of α-mannosidase inhibitors DMJ and swainsonine (HCV-pp^{DMJ}) were subjected to digestion by Endo H or PNGase F and analyzed by Western blotting. HCV-pp^{DMJ} E2 proteins were completely deglycosylated either by Endo H or by PNGase F. E1 protein of HCV-pp produced in the presence or absence of DMJ and swainsonine had the same glycosylation patterns.

cells to HCV-pp is because of a block at the level of virus entry. Hepatocytes and Huh-7 cells do not express L-SIGN and DC-SIGN. Expression of these lectins in these cells does not increase HCV-pp entry, whereas the infectivity of Huh-7 cells by VSV-pp remains unmodified (data not shown). Our results suggest distinct ways of DC-/L-SIGN usage by HCV and Dengue virus.

HCV-pp Captured by L-SIGN or DC-SIGN Are Transmitted to Hepatocytic Cells—We investigated whether HCV-pp captured by BTHP1-LS or BTHP1-DCS cells could be transmitted to the hepatocytic cell line Huh-7. Cells were incubated with HCV-pp (coding for the GFP or luciferase reporter gene), and unbound virus was eliminated by repeated washings prior to coculture with Huh-7 target cells. The transmission of HCV-pp captured by BTHP1-LS or BTHP1-DCS cells was confirmed with both reporter systems (Fig. 6). HCV-pp weakly interact with BTHP1 cells, because residual transmission could be observed in comparison with Δenv-pp. However, the HCV-pp transmission was on average 5-fold higher for BTHP1-LS or BTHP1-DCS compared with background transmission with BTHP1 (Fig. 6, *A* and *B*). Moreover, primary DC can efficiently transmit HCV-pp to Huh-7 cells (Fig. 8*D*). The transmission was specific, because it was inhibited by monoclonal antibodies MAb1621 and 1B10. EGTA also inhibited transmission of HCV-pp by BTHP1 cells expressing these lectins. HCV-pp transmission by DC-SIGN could also be completely inhibited by mannan, whereas transmission by L-SIGN was only weakly inhibited in the presence of mannan. The highest inhibition rate obtained for mannan was 40%. Interestingly, HCV-pp^{DMJ} carrying highly mannosylated proteins were transmitted with efficiency similar to that of HCV-pp carrying mixed glycosylation (Fig. 6*C*). The difference between HCV-pp and HCV-pp^{DMJ} resides in the modification of the E2 glycosylation pattern. Two non-exclusive hypotheses can be put forward to explain why we do not see increased transmission of HCV-pp^{DMJ}. First, a specific binding motif composed of one or more high mannose *N*-glycans could be present on E1 or E2 of HCV-pp, allowing optimal capture and transmission by DC-/L-SIGN, or second,

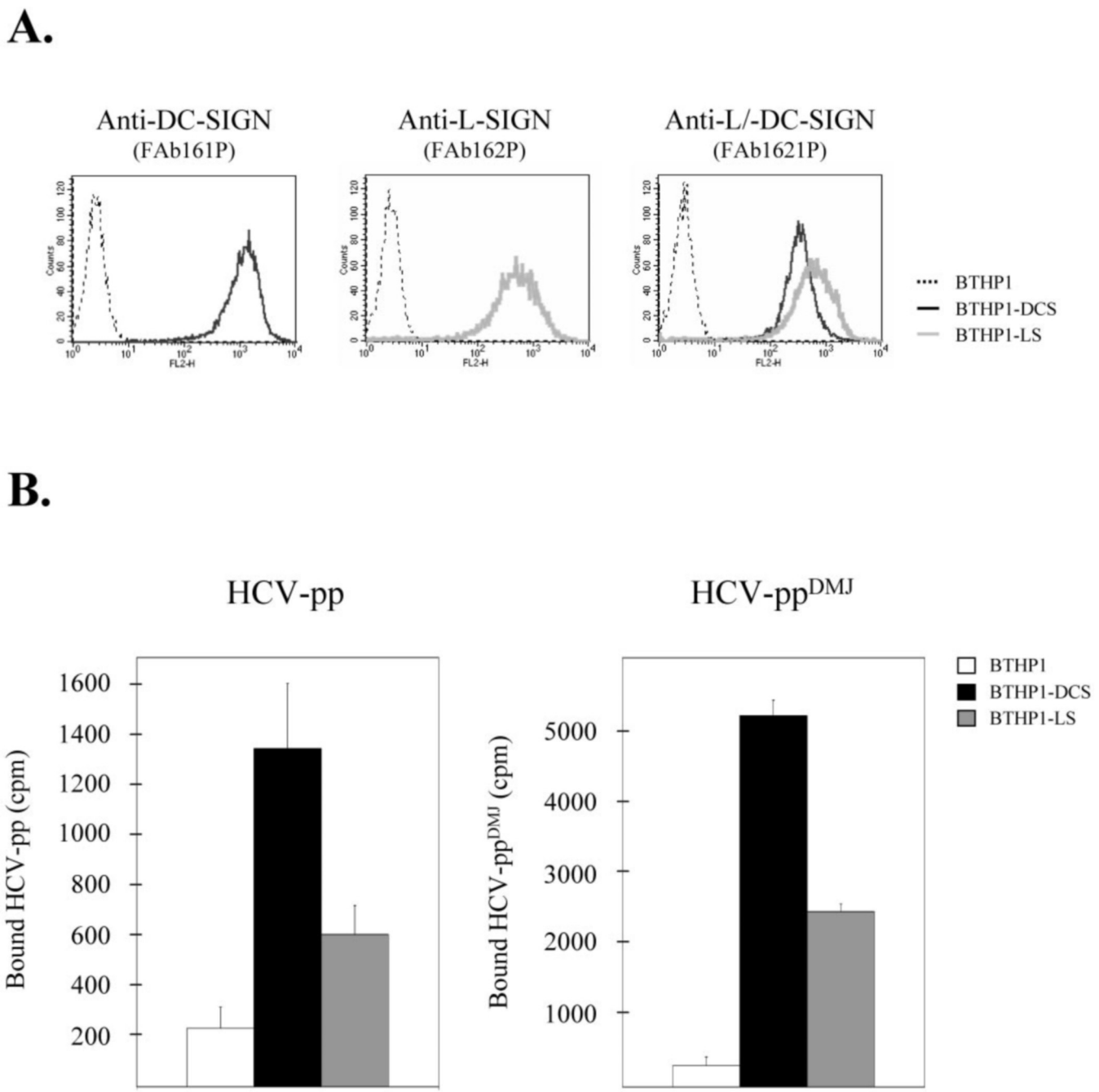


FIG. 3. **HCV-pp and HCV-pp^{DMJ} bind to C-type lectins L-SIGN and DC-SIGN.** *A*, expression of L-SIGN and DC-SIGN in BTHP1-LS and BTHP1-DCS cell lines, respectively, was detected by FACScan. *B*, ^{35}S-labeled HCV-pp and HCV-pp^{DMJ} bound to BTHP1-LS or BTHP1-DCS cells. Bound radioactivity for particles without viral envelope, Δenv-pp and Δenv-pp^{DMJ}, was subtracted from values obtained with HCV-pp and HCV-pp^{DMJ}. The data are representative of five independent experiments. Values are given as the mean of duplicates ± S.E.

the mechanism implicated in HCV-pp and HCV-pp^{DMJ} transmission is saturated in the experimental conditions used in this study.

DC-SIGN expressed *in trans* was previously shown to boost HIV infection of T cells at low multiplicities of infection (m.o.i.) (17, 34). Indeed, when we used low m.o.i. of HIV-pp, DC-SIGN on BTHP1 or dendritic cells, and to a lesser extent L-SIGN on BTHP1 cells, we were able to enhance HIV infection of activated T cells (Figs. 7 and 8*E*). To test whether DC-SIGN and L-SIGN could also enhance HCV-pp infectivity, DC-SIGN and L-SIGN BTHP1 cells or dendritic cells were preincubated with a low m.o.i. of HCV-pp and then cocultured with Huh-7 cells (Figs. 7 and 8*E*). Under these experimental conditions and in contrast to HIV-pp, no enhancement of viral infectivity was observed.

DISCUSSION

The expression of L-SIGN on liver sinusoidal endothelial cells and its capacity to bind high mannose *N*-glycans on viral glycoproteins make this lectin a candidate receptor responsible for liver tropism of HCV and other enveloped hepatotropic viruses. We established previously that a soluble form of the highly mannosylated HCV E2 envelope glycoprotein binds to L-SIGN and the related C-type lectin DC-SIGN with high affinity (23). In this report, we provide insight into the functional relevance of this interaction with respect to the

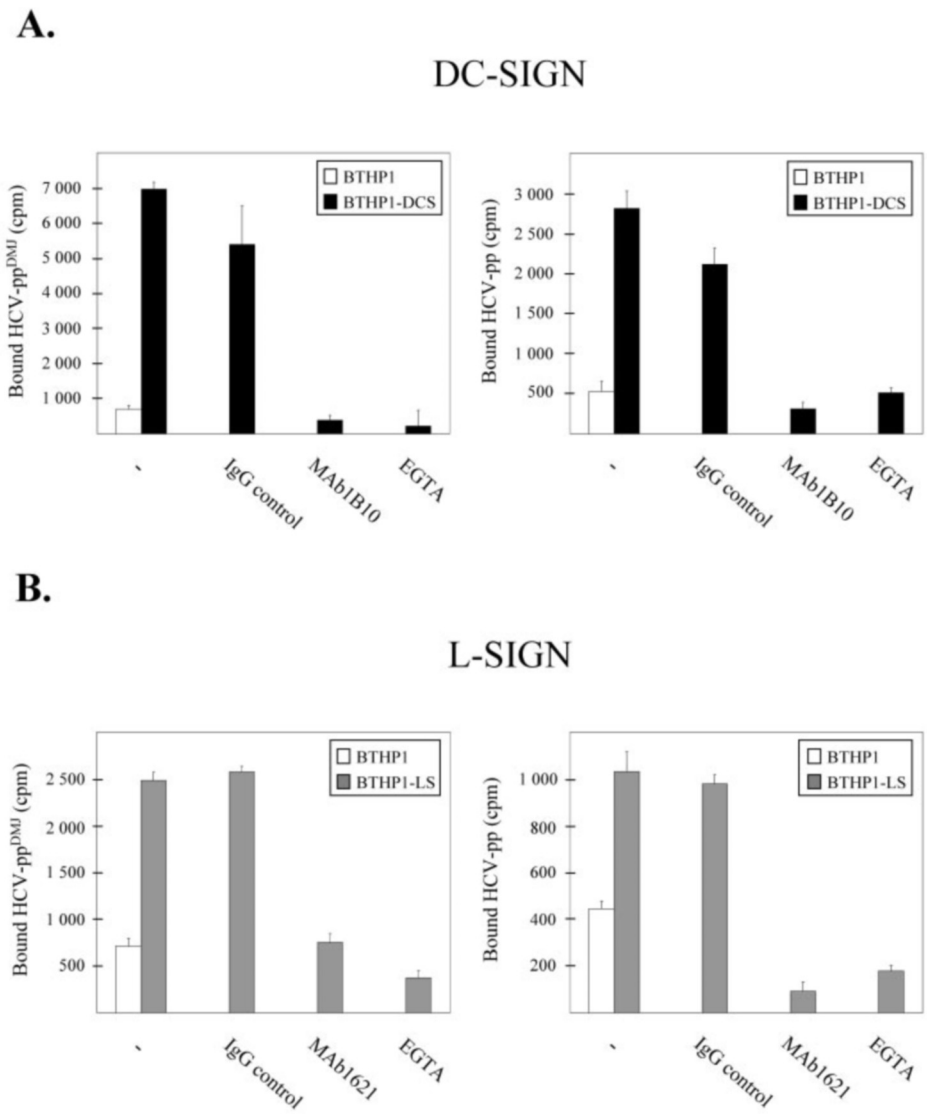


FIG. 4. **Specificity of HCV-pp and HCV-pp^{DMJ} binding to L-SIGN and DC-SIGN.** Inhibitors for C-type lectin binding were used in a binding assay with ^{35}S-labeled HCV-pp and HCV-pp^{DMJ}. *A*, specificity of binding to DC-SIGN. HCV-pp and HCV-pp^{DMJ} were incubated with BTHP1-DCS cells in the presence of EGTA (5 mM), mAb1B10 directed against the CRD of DC-SIGN (20 μg·ml^{-1}), or a control IgG2a (20 μg·ml^{-1}). *B*, specificity of HCV-pp and HCV-pp^{DMJ} binding to L-SIGN. HCV-pp and HCV-pp^{DMJ} were incubated with BTHP1-LS cells in the presence of EGTA (5 mM), mAb1621, which blocks L-SIGN (20 μg·ml^{-1}), or a control IgG2b (20 μg·ml^{-1}). Bound radioactivity for particles without viral envelope, Δenv-pp and Δenv-pp^{DMJ}, was subtracted from values obtained with HCV-pp and HCV-pp^{DMJ}. Values are given as the mean of duplicates ± S.E. The data are representative of two independent experiments.

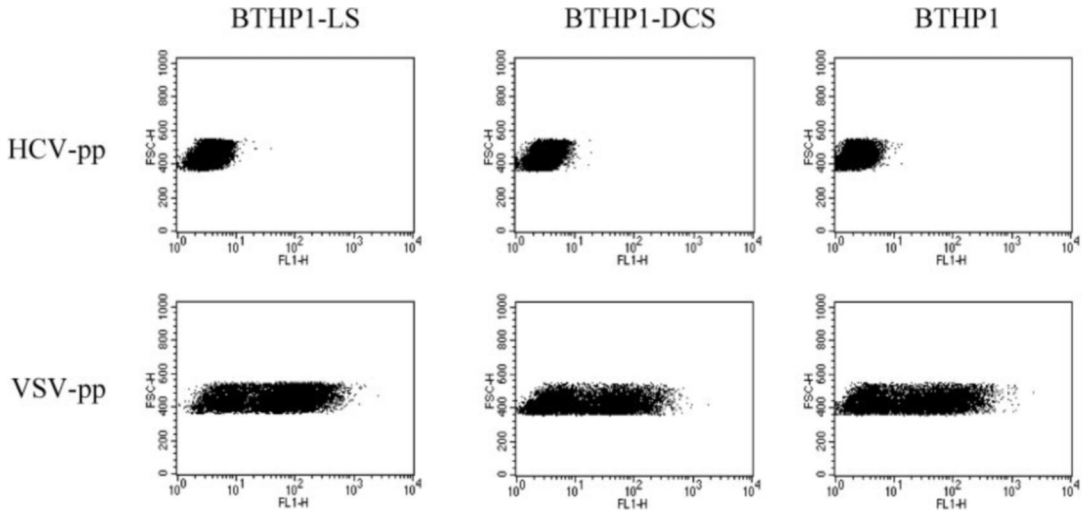


FIG. 5. **L-SIGN and DC-SIGN do not mediate entry of HCV-pp into cells.** BTHP1-LS and BTHP1-DCS cells (10^5) were infected with HCV-pp or VSV-pp (300 ng of p24 equivalent). Expression of GFP reporter gene was detected by FACS 72 h post-infection. The data are representative of more than three independent experiments.

capture of HCV by both lectins and transmission to target cells.

Because of the lack of an *in vitro* system for growth, purification, and biochemical analysis of HCV virions, the molecular mechanisms of HCV particle formation and entry cannot be addressed using wild type HCV contained in serum from infected patients. Therefore, we generated functional lentivirus (HIV) particles in which the surface envelope glycoproteins

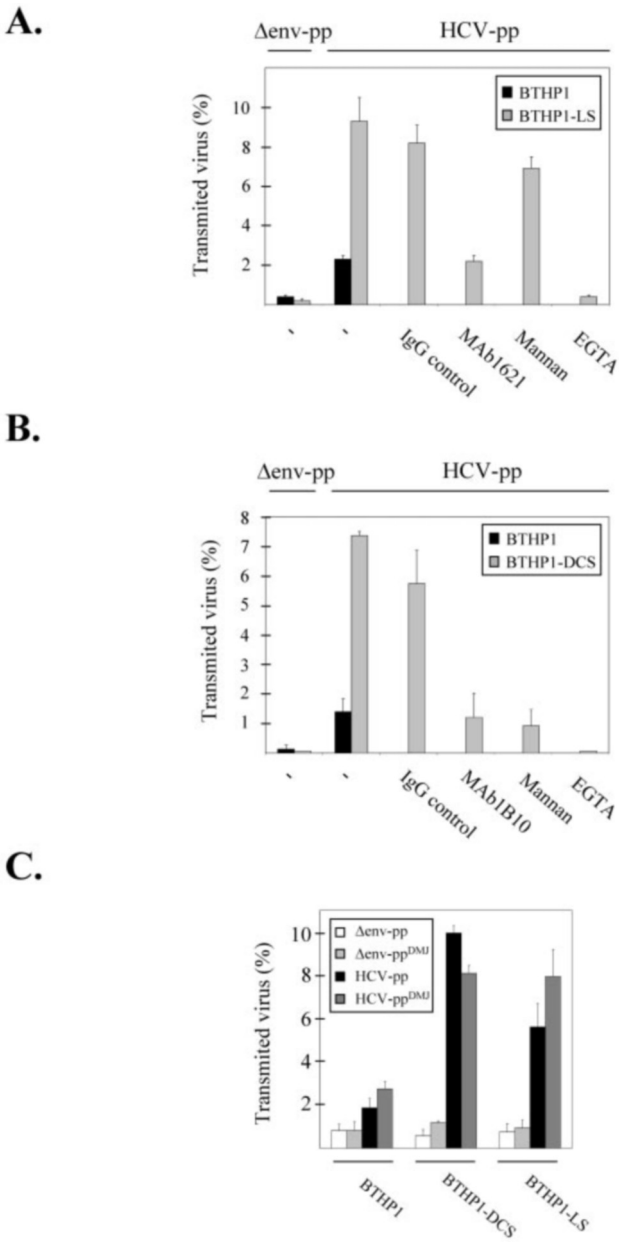


FIG. 6. **BTHP1-LS and BTHP1-DCS transmit captured HCV-pp to Huh-7 target cells.** Following binding of HCV-pp to BTHP1 cells, cells were washed five times prior to coculture with Huh-7 cells. Transmitted virus in the coculture was measured after 72 h of coculture using specific detection of a reporter gene. *A*, HCV-pp transmission by L-SIGN. Specificity was analyzed by adding the L-SIGN blocking antibody mAb1621 (20 μg·ml^{-1}), EGTA (5 mM), and mannan (20 μg·ml^{-1}) prior to HCV-pp binding. Values are given as the mean of quadruplicates ± S.E. The data are representative of three independent experiments and were obtained with HCV-pp encoding the luciferase reporter gene. The percentage of transmitted HCV-pp was determined as follows: (RLU in coculture × 100)/RLU for direct Huh-7 cells infection. The mean RLU value for directly infected Huh-7 cells was 9483 ± 2058. *B*, HCV-pp transmission by DC-SIGN. Specificity was analyzed by adding the DC-SIGN-blocking antibody 1B10 (20 μg·ml^{-1}), EGTA (5 mM) and mannan (20 μg·ml^{-1}) prior to HCV-pp binding. Values are given as the mean of duplicates ± S.E. The data are representative of three independent experiments and were obtained with HCV-pp encoding a GFP reporter gene. The percentage of transmitted HCV-pp was determined as follows: (GFP(+) cells in coculture × 100)/GFP(+) cells for direct Huh-7 cells infection. The percentage of directly infected Huh-7 cells was 21.7% ± 0.2. *C*, HCV-pp^{DMJ} transmission by L-SIGN and DC-SIGN. The capacity of transmission of HCV-pp^{DMJ}, which have an increased binding capacity (Fig. 3*B*), was compared with that of particles lacking viral envelope glycoprotein (Δenv-pp). The percentage of transmitted HCV-pp and HCV-pp^{DMJ} was determined as follows: (RLU in coculture × 100)/RLU for direct Huh-7 cells infection. The mean RLU values for directly infected Huh-7 cells were 4620 ± 90 and 3945 ± 576, respectively. Values are given as the mean of triplicates ± S.E. The data are representative of two independent experiments.

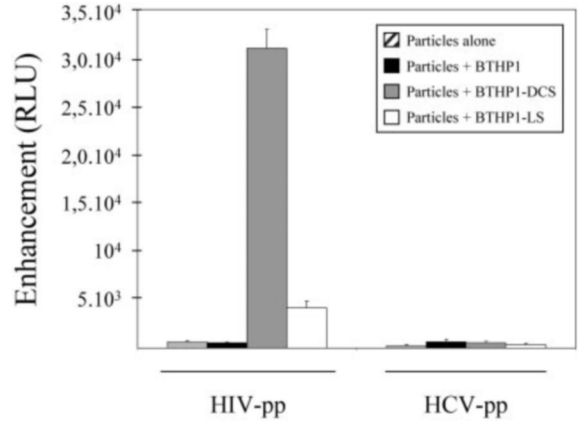


FIG. 7. **Absence of *trans*-enhancement of HCV-pp infection in the presence of BTHP1-LS or BTHP1-DCS.** Primary activated T lymphocytes and Huh-7 cells (2.5×10^4) were infected by HIV-pp and HCV-pp (50 ng of p24 equivalent), respectively, in the presence or in the absence of BTHP1, BTHP1-LS, or BTHP1-DCS cells. Infection was detected by measuring luciferase activity in Huh-7 cells or activated PBMC 72 h post-coculture. Values are given in relative light units as the mean of triplicates ± S.E. The data are representative of two independent experiments.

gp120/gp41 were replaced by HCV E1 and E2 proteins (HCV-pp) (26). We show in this study that infectivity for Huh-7 cells can only be detected in gradient fractions that contain the p24 HIV core and HCV E1 and E2 envelope proteins. These data are supported by previous studies that established that pseudotyped retroviral particles require both proteins for infection and that particles expressing either the E1 or the E2 protein do not enter target cells (26–28). Our data are in favor of a model proposing that E1 and E2 proteins are functional when associated as non-covalent heterodimers. Such heterodimers have been implicated in the native folding and maturation of the HCV envelope (35, 36). Biochemical analysis of infectious HCV-pp revealed an apparent dichotomy in glycosylation between E1 and E2. Although E1 *N*-glycans are not modified to complex Endo H-resistant *N*-glycans, E2 is almost completely Endo H-resistant, leaving only a minor fraction of *N*-glycans in a highly mannosylated state. The subcellular compartment where E1/E2 heterocomplexes are incorporated into HCV-pp is not known. This might occur in the endoplasmic reticulum or another compartment of the secretory pathway or at the plasma membrane. In the latter case, pseudotype particles incorporated E1/E2 heterodimers that have escaped endoplasmic reticulum retention and proceeded through the secretory pathway. In either setting, our results imply that some *N*-glycans in the E1/E2 heterodimer are inaccessible to sugar-modifying enzymes, resulting in the assembly of infectious HCV-pp that incorporate E1 and E2 proteins with several preserved high mannose *N*-glycans.

By analogy with other members of *flaviviridae*, it has been proposed that HCV buds into the endoplasmic reticulum. It is not known whether HCV subsequently passes through the secretory pathway and whether surface glycoproteins undergo modification of *N*-glycans from high mannosylated to complex in the Golgi. To simulate a high mannose E1/E2 glycosylation status of virus, we produced HCV-pp in the presence of α-mannosidase inhibitors (HCV-pp^{DMJ}). HCV-pp^{DMJ} carry Endo H-sensitive E2 and show similar infectivity in Huh-7 cells compared with HCV-pp, suggesting that com-

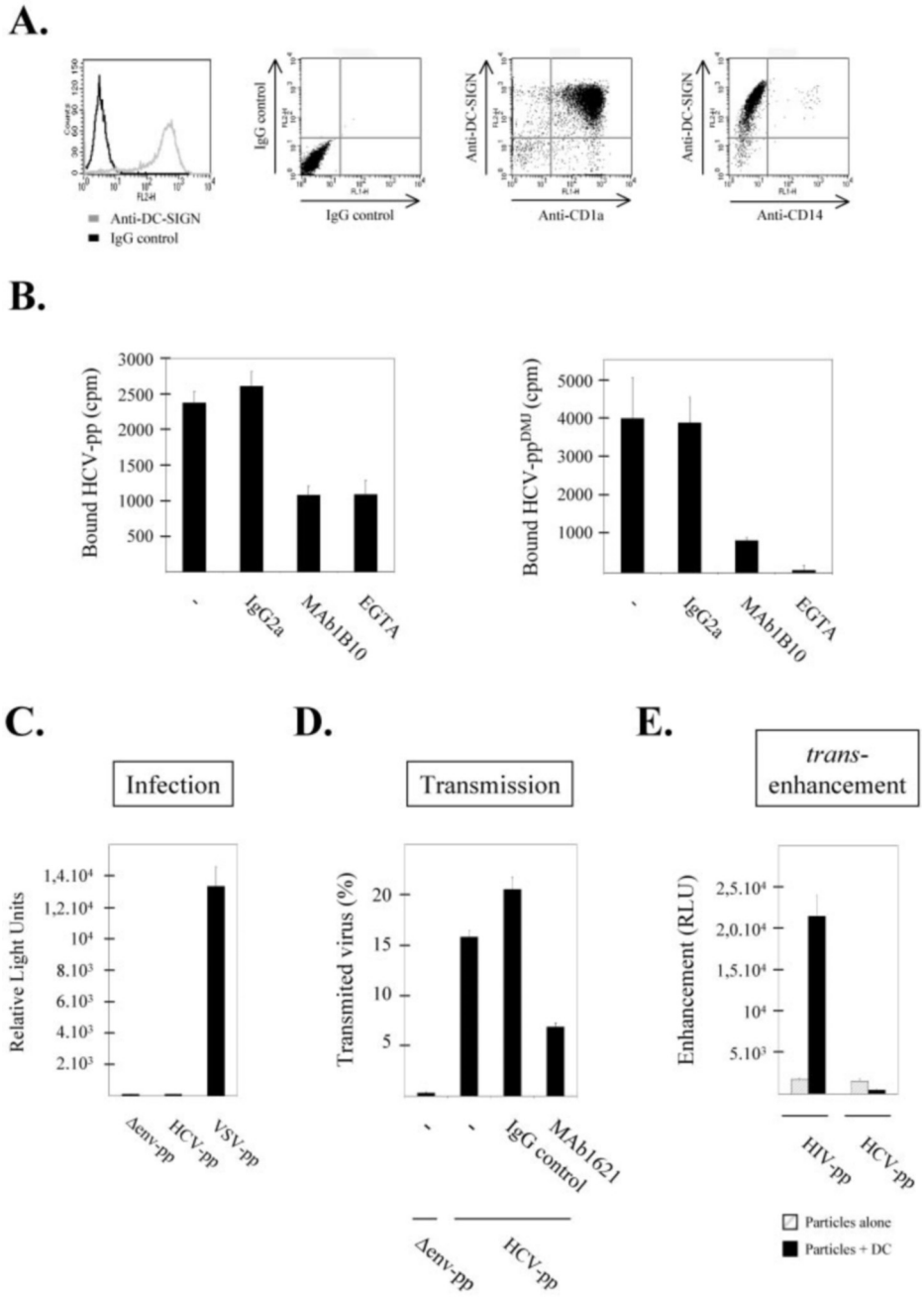


FIG. 8. **HCV-pp do not infect human primary dendritic cells but can be captured and transmitted to Huh-7 target cells.** *A*, expression of DC-SIGN in human primary DC by FACScan. CD1a up-regulation and CD14 down-regulation assess the differentiation of dendritic cells. *B*, ^{35}S-labeled HCV-pp and HCV-pp^{DMJ} bound to DC. HCV-pp and HCV-pp^{DMJ} were incubated with DC in the presence of EGTA (5 mM), DC-SIGN-blocking antibody mAb1621 (20 μg·ml^{-1}), or a control IgG2b (20 μg·ml^{-1}). Values are given as the mean of duplicates ± S.E. *C*, DC-SIGN does not mediate entry of HCV-pp into DC. Values are given as the mean of triplicates ± S.E. *D*, DC transmit captured HCV-pp to Huh-7 cells. Specificity was analyzed by adding mAb1621 (20 μg·ml^{-1}) or control IgG2b (20 μg·ml^{-1}) prior to HCV-pp binding. DC were washed and then cocultured with Huh-7 cells. Values are given as the mean of duplicates ± S.E. The percentage of transmitted HCV-pp was determined as follows: (RLU in coculture × 100)/RLU for direct Huh-7 cells infection. The mean RLU value for directly infected Huh-7 cells was 43416 ± 974. *E*, primary activated T lymphocytes and Huh-7 cells (2.5×10^4) were infected by HIV-pp and HCV-pp (50 ng of p24 equivalent), respectively, in the presence or in the absence of DC. Infection was detected by measuring luciferase activity in Huh-7 cells or activated PBMC after 72 h of coculture. Values are given in RLU as the mean of triplicates ± S.E. The data presented in *panels A–E* are representative of two independent experiments with DC from two healthy blood donors.

plex *N*-glycans are not involved in the recognition of the HCV receptor(s) on Huh-7 cells.

Our binding studies show that infectious HCV-pp attaches to L-SIGN and DC-SIGN expressed at the plasma membrane. This interaction is specific and can be blocked by several lectin binding inhibitors. However, mannan can totally inhibit the HCV-pp transmission by DC-SIGN, whereas its effect on L-SIGN is poor. Such differences have been observed for Sindbis virus (37) and suggest that carbohydrate motifs recognized by L-SIGN and DC-SIGN are different.

In contrast to other enveloped viruses, the attachment of HCV-pp or HCV-pp^{DMJ} either to DC-SIGN or L-SIGN did not enhance entry into permissive cell lines or primary human DC-SIGN(+) DC. This finding is in contrast to a previous report showing that HCV-negative strand RNA, indicating replication of the viral genome, could be detected in DC (38). However, Goutagny *et al.* (39) report that positive strand viral RNA was associated with DC in HCV-infected patients but that only a small percentage of them had negative strand viral RNA associated with DC. Moreover, LSEC (40) or primary human Kupffer cell cultures (41) that express L-SIGN or DC-SIGN, respectively (9–11), are resistant to HCV infection. This stands in sharp contrast to the permissiveness to HCV demonstrated for the neighboring hepatocytes where negative strand RNA is readily detected (42). Whether DC-SIGN or L-SIGN mediates HCV endocytosis is unknown. Should this be the case, one could speculate that the lectin routes the virus to a subcellular compartment where HCV-induced membrane fusion cannot occur and does not lead to productive infection. Based on our current findings and the reported lack of HCV infection of DC-SIGN or L-SIGN expressing cells *in vivo*, we conclude that these lectins do not mediate infection in *cis* by HCV.

DC-SIGN enhances CMV and HIV infectivity of cells that are poorly permissive originally. This phenomenon was named "*trans*-enhancement." In the HIV model, DC-SIGN expressed

by DC-enhanced T cell infection at a low m.o.i. of the virus, whereas no infection was observed in its absence (17, 34, 43). Our results extend these findings for HIV to L-SIGN but more studies are required to characterize the observed efficiency difference between the two lectins. However, no enhancement was detected when low multiplicities of HCV-pp were used. Some specific cellular proteins may be critical for this mechanism, and their absence from Huh-7 cells may abrogate enhancement.

Whereas HCV-pp differ from other viruses with respect to *cis*-infection of DC-/L-SIGN expressing cells and to DC-/L-SIGN dependent *trans*-enhancement mechanisms, infectious HCV-pp can be captured by L-SIGN and DC-SIGN and transmitted to target cells as has been described for CMV (16), HIV (17), Ebola virus (15), and severe acute respiratory syndrome coronavirus (44). Indeed, our results show that coculture of the hepatocytic cell line Huh-7 with HCV-pp-loaded L-SIGN and DC-SIGN expressing cells results in productive infection of the Huh-7 cells. A model in which LSEC play a central role in the capture of hepatotropic viruses and their transmission to hepatocytes was recently proposed (7). Breiner *et al.* (7) show that the Duck Hepatitis B virus surface protein (preS) and virions preferentially had accumulated in LSEC *in vitro* and *in vivo* in infected ducks. No productive infection by Duck Hepatitis B virus was observed in LSEC, although particles seem to be internalized by endocytosis, which could lead to the creation of a viral reservoir. In the HCV setting, upon release from LSEC, the virus might use entry receptor(s), *e.g.* CD81, scavenger receptor class B, type I, low density lipoprotein receptor (1–3), heparan sulfate (45), or the asialoglycoprotein receptor (46) for entry and productive infection of neighboring hepatocytes. Our results are in favor of a model proposing LSEC and LSIGN, respectively, as cellular and molecular effectors of hepatotropism of enveloped viruses. Moreover, HCV could use DC-SIGN on DC as a transporter to assure its dissemination, similar to a mechanism proposed for HIV. Recent studies by Yoneyama (47) indicates that myeloid DC could migrate to the liver where DC-SIGN-positive cells have previously been detected (9, 10). It remains to be clarified whether HCV is internalized into L-SIGN- or DC-SIGN-expressing cells, *e.g.* LSEC or Kupffer cells, and whether virus endocytosis would be a prerequisite for efficient transmission to hepatocytes. Both L-SIGN and DC-SIGN encompass endocytosis motifs in the cytoplasmic N terminus, but their role on HCV-pp transmission needs to be analyzed. Internalized HCV in DC-SIGN- or L-SIGN-positive cells could function as a viral reservoir for infection of neighboring hepatocytes.

We propose that in the anatomical sites where HCV replicates, C-type lectins expressed on liver sinusoidal cells may act as the capture receptor for HCV and transmit infectious virions to neighboring hepatocytes. This search should be extended to LSECtin, another C-type lectin closely related to L-SIGN and specifically expressed in LSEC (48). DC-/L-SIGN might not be the sole molecule(s) implicated in the transmission of HCV or other hepatotropic viruses.

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