

CLEC4M-positive and CD81-negative Huh7 cells are not susceptible to JFH-1 HCVcc infection but mediate transinfection

Mariko Ishibashi · Naoko Morita · Chisato Nomura-Kawaguchi ·
Yohko Shimizu · Takaji Wakita · Mariko Esumi

Received: 18 January 2014 / Accepted: 10 June 2014 / Published online: 26 June 2014
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Abstract C-type lectin domain family 4, member M (CLEC4M), a *trans*-membrane protein specifically expressed in liver sinusoidal endothelial cells, is considered a candidate receptor for hepatotropism of hepatitis C virus (HCV). CLEC4M was previously reported to capture artificial HCVpp (pseudoparticle) and transmit it to hepatocytes (transinfection) via CLEC4M-positive cells. It is still not known whether CLEC4M acts as a receptor for HCVcc (cell-culture-produced HCV) transinfection or whether CLEC4M is an entry receptor for HCVcc. Initially, we established stably CLEC4M-positive and HCV-replication-permissive cell lines by introducing a CLEC4M expression vector into Huh7-25 cells (Huh7-25-CLEC4M) by transfection. Huh7-25 is a mutant cell line that is resistant to JFH-1 HCVcc due to the lack of expression of CD81 but

permissive for replication of JFH1 HCV RNA. When Huh7-25-CLEC4M cells were infected with HCVcc and cultured for 6 days, none were positive for infection. Next, to examine whether CLEC4M functions as a receptor for transinfection, Huh7-25-CLEC4M cells were inoculated with HCVcc and thereafter co-cultured with Huh7-it cells, which are susceptible to HCV infection. The amount of HCV RNA was increased in Huh7-it cells co-cultured with Huh7-25-CLEC4M cells, and the transinfection was inhibited in the presence of anti-CLEC4M antibody during inoculation. Thus, CLEC4M cannot substitute for CD81 as an entry receptor for JFH-1 HCVcc. It just mediates transinfection without internalization of HCVcc. CD81 is still crucial for HCV entry into hepatocytes, and CLEC4M in liver sinusoidal endothelial cells may be responsible for hepatotropism of HCV infection by trapping circulating HCV to transmit it to adjacent hepatocytes.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-014-2150-z) contains supplementary material, which is available to authorized users.

M. Ishibashi · N. Morita · C. Nomura-Kawaguchi ·
M. Esumi (✉)
Department of Pathology, Nihon University school of Medicine,
30-1 Ohyaguchikami-cho, Itabashi-ku, Tokyo 173-8610, Japan
e-mail: esumi.mariko@nihon-u.ac.jp

Y. Shimizu
Open Research Center for Genome and Infectious Disease
Control, Nihon University School of Medicine, Itabashi-ku,
Tokyo 173-8610, Japan

Present Address:
Y. Shimizu
Division of Microbiology, Center for Infectious Diseases,
Kobe University Graduate School of Medicine, Kobe, Japan

T. Wakita
Department of Virology II, National Institute of Infection
Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

Introduction

Hepatitis C virus (HCV) causes serious liver diseases including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. HCV infection is manifested mainly in human liver hepatocytes [1] and hematopoietic cells [18]. Such strict tropism of HCV infection depends on two major factors: the ability of HCV to enter a target cell and the permissibility of HCV replication inside the cell. Target cell accessibility is mediated by HCV receptors and co-receptors, which have been identified using HCV cell culture systems with CD81, scavenger receptor class B, member 1 (SR-BI), low-density lipoprotein receptor (LDLR), claudin-1 (CLDN1), and occludin (OCLN) [7, 22]. Although CD81 and OCLN are essential determinants of tropism of HCV

infection in humans [19], these cellular proteins are ubiquitously expressed in human tissues and are not responsible for the hepatotropism of HCV infection. One liver-specific molecule that may influence the accessibility of cells to pathogens is C-type lectin domain family 4, member M (CLEC4M; also known as L-SIGN or DC-SIGNR). CLEC4M is highly expressed on liver sinusoidal endothelial cells but not on hepatocytes and dendritic cells. [5]. Soilleux and colleagues identified the cDNA sequence of CLEC4M as a gene related to that of dendritic-cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) [4]. The C-terminal end of CLEC4M possesses a carbohydrate-recognition domain and binds to mannose oligosaccharides that are present on the envelopes of pathogens [13]. CLEC4M acts as a capture receptor for viruses and pathogens, including human immunodeficiency virus type 1 (HIV-1) [5], Ebola virus [3], severe acute respiratory syndrome coronavirus (SARS-CoV) [8] and M tuberculosis [14]. CLEC4M was previously reported to mediate transinfection of HCVpp (pseudoparticle) using CLEC4M-positive cells [9, 10, 15, 16]; CLEC4M captured artificial HCVpp and transmitted it to hepatocytes. It is, however, unknown whether CLEC4M acts as a receptor for HCVcc (cell-culture-produced HCV) transinfection and whether CLEC4M is a binding receptor or an entry receptor for HCVcc. Once cells that are competent for HCV RNA replication have an entry receptor for HCVcc, they are susceptible to HCVcc infection, but cells that have only a binding receptor are unsusceptible. In the present study, we used CD81-negative and HCV-replication-permissive cells (Huh7-25) to investigate whether CLEC4M plays a role as a liver-specific HCV receptor for cisinfection and/or a receptor for transinfection.

Materials and methods

Cells

Huh7-25, Huh7-25-CD81 [2] and Huh7-it cells (a gift from Dr. Oshima, National Institute of Infection Diseases, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum. Huh7-25-CD81 cells were maintained in the complete medium containing 400 µg G418 (Invitrogen, Carlsbad, CA) per ml. Huh7-25-CD81 and Huh7-it cells were highly susceptible to HCVcc infection. Huh7-it cells were generated by Dr. Oshima from HCV replicon cells and cured by interferon treatment.

Transfection with CLEC4M expressing plasmids

Human CLEC4M-cDNA (isoform 1, accession no. NM_014257) was amplified from human liver cDNA using

the primers shown in Supplementary Table 1. The amplified cDNA was inserted into the pEF1/V5-His vector containing a Geneticin-resistance gene (Invitrogen). The nucleotide sequence of the CLEC4M construct was confirmed to be correct. Huh7-25 cells were transfected with a complex of plasmid DNA and Lipofectamine LTX (Invitrogen), and stable clones of transfected cells were established by exposure to 400 µg of G418 (Invitrogen) per ml. Stable clones transfected with pEF1/V5-His vector were used as controls.

Quantification of mRNA

Total RNA was extracted from cultured cells using TRIzol Reagent (Invitrogen) and subjected to DNase I treatment followed by cDNA synthesis as described previously [12]. The quantity of mRNA was determined by real-time PCR using SYBR Green [12]. Plasmid DNA containing a target gene was used as a standard for quantification of HCV RNA and CLEC4M mRNA. CLEC4M gene expression was normalized against the quantity of GAPDH, and the relative value was expressed in arbitrary units. Primer sequences were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are shown in Supplementary Table 1.

Immunofluorescence staining

Cells seeded on cover slips were incubated overnight at 37 °C and fixed with cold acetone for 3 min. After fixation, the cells were blocked with 1 % bovine serum albumin in phosphate-buffered saline (PBS) and incubated with the first antibody: 5 µg/ml of mouse anti-CLEC4M antibody (mAb162) (R &D systems, Inc., Minneapolis, MN) and/or 200-fold diluted human serum from an HCV-positive donor. The human anti-HCV antiserum used was characterized and found to be reactive for core, NS4B and NS5A proteins of JFH-1 HCVcc by Western blotting of the lysate from infected cells (Supplementary Fig. 1). The cells were then stained with 4 µg/ml of the secondary antibody conjugated with AlexaFluor 488 or AlexaFluor 647 (Invitrogen). The stained cells were mounted on glass slides with ProLong Gold antifade reagent containing DAPI (Invitrogen) and visualized using a BIOREVO BZ-9000 microscope (KEYENCE, Osaka, Japan), which was also used to measure the fluorescence intensity of CLEC4M proteins.

Western blotting

Cells and human liver tissues were lysed using urea buffer (7 M urea, 2 M thiourea, 3 % CHAPS, 3 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 30 mM Tris-HCl, pH 7.4). Protein (50 µg) was treated with N-glycanase

(PNGase F) included in the Enzymatic Protein Deglycosylation Kit (Sigma-Aldrich, Saint Louis, MO). Treated and untreated proteins (20- μ g samples) were electrophoresed in an SDS-10 % polyacrylamide gel and then transferred to a nitrocellulose membrane. CLEC4M protein was detected using 0.5 μ g of goat anti-CLEC4M antibody N17 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 7.5 ng of horseradish-peroxidase-conjugated anti-goat IgG (Zymed Laboratories, San Francisco, CA) per ml. β -actin protein, used as an internal loading standard, was detected using 1 μ g of mouse monoclonal anti- β -actin antibody (Abcam, Cambridge, UK) and 0.1 μ g of horseradish-peroxidase-conjugated anti-mouse IgG (IBL, Gunma, Japan) per ml. CLEC4M and β -actin were detected using ECL Advance and Plus Western Blotting Detection Systems (GE Healthcare, Buckinghamshire, UK), respectively. Chemiluminescence was detected with a Light-Capture AE-6972 (ATTO, Tokyo, Japan) and analyzed using CS Analyzer version 2.07 software (ATTO).

Reporter assay for HCV replication

Reporter replicon RNA was transcribed in vitro from plasmid pSGR-JFH1/Luc and introduced into Huh7 cells by transfection as described previously [2]. Luciferase activity was determined by using Luciferase Assay Systems (Promega, Madison, WI) and an Infinite F200 microplate reader (TECAN, Männedorf, Switzerland).

Production of infectious HCVcc

HCVcc derived from JFH-1 was prepared as described previously [20]. To produce a large amount of HCVcc, 3.6×10^6 Huh7-it cells were inoculated with 500 μ l of HCVcc (8×10^6 HCV RNA copies/ml) for 1 h and cultured with 15 ml of the complete medium. The culture supernatant was collected at 6 days postinfection and passed through a 0.45- μ m filter. Filtered supernatant was stored at -80°C until use. The infectious titer of JFH-1 HCVcc was 9.2×10^3 focus-forming units (ffu) per ml, and the RNA titer was 2.0×10^7 copies of HCV RNA/ml.

Coinfection with HCVcc

Huh7-25-CLEC4M cells (2×10^4) were seeded on coverslips in 24-well plates and incubated overnight at 37°C . The cells were incubated with 200 μ l of HCVcc for 4 h and then cultured in 500 μ l of complete medium. At 6 days postinfection, infected cells were detected by immunofluorescence staining of HCV proteins. The infected foci were counted using fluorescence microscopy (Olympus, Tokyo, Japan). The assay was performed in triplicate.

Transinfection with HCVcc

Huh7-25-CLEC4M cells were seeded at 1×10^4 cells per well in 24-well plates. The cells were inoculated 24 h later with 200 μ l of HCVcc for 4 h, and then washed five times in wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl_2 , 2 mM MgCl_2 , 0.5 % bovine serum albumin). After washing to remove unbound HCVcc, Huh7-it cells (4×10^4 cells/500 μ l of the complete medium) were added to the cells and co-cultured for 4 h or 4 days. Then, total RNA was extracted from the co-cultured cells and the quantity of HCV RNA was determined. The co-cultured cells on cover slips were also stained for CLEC4M and HCV proteins by immunofluorescence staining. In the transinfection inhibition assay, Huh7-25-CLEC4M cells were preincubated in 200 μ l of complete medium containing MAb162 for 30 min at 37°C , followed by infection with HCVcc. MAb162 specifically recognizes the carbohydrate-recognition domain of CLEC4M. The assay was performed in triplicate.

Statistical analysis

Student's *t*-test and the Mann-Whitney *U* test were used to analyze the data. *P*-values less than 0.05 were considered statistically significant.

Results

Characterization of CLEC4M-positive cells

To investigate the potential receptor role of CLEC4M in HCV infection, we produced CLEC4M-positive cells using a CD81-negative Huh7 cell clone (Huh7-25). The Huh7-25 cell clone is highly permissive for HCV replication, but it is not susceptible to JFH-1 HCV infection because the Huh7-25 cell clone is negative for CD81 [2]. However, except for CD81, the expression level of HCV receptor genes in the Huh7-25 cell clone was similar to that of JFH-1 HCV permissive cells (Huh7-25-CD81 and Huh7-it) (Supplementary Fig. 2). Since Huh7-25 was also negative for CLEC4M mRNA, we established CLEC4M-positive cells using Huh7-25 (Huh7-25-CLEC4M). Huh7-25-CLEC4M cells produced high levels of CLEC4M mRNA and protein (Fig. 1A-C). Western blot analysis of the CLEC4M protein showed multiple bands of different sizes at >43 kDa in both Huh7-25-CLEC4M cells and human liver. These were converted to a single band of 43 kDa after treatment with N-glycanase (Fig. 1D). Thus, the glycosylation pattern of CLEC4M was different in Huh7-25-CLEC4M cells and human liver. Huh7-25-CLEC4M was similar to not only the negative control cell clone

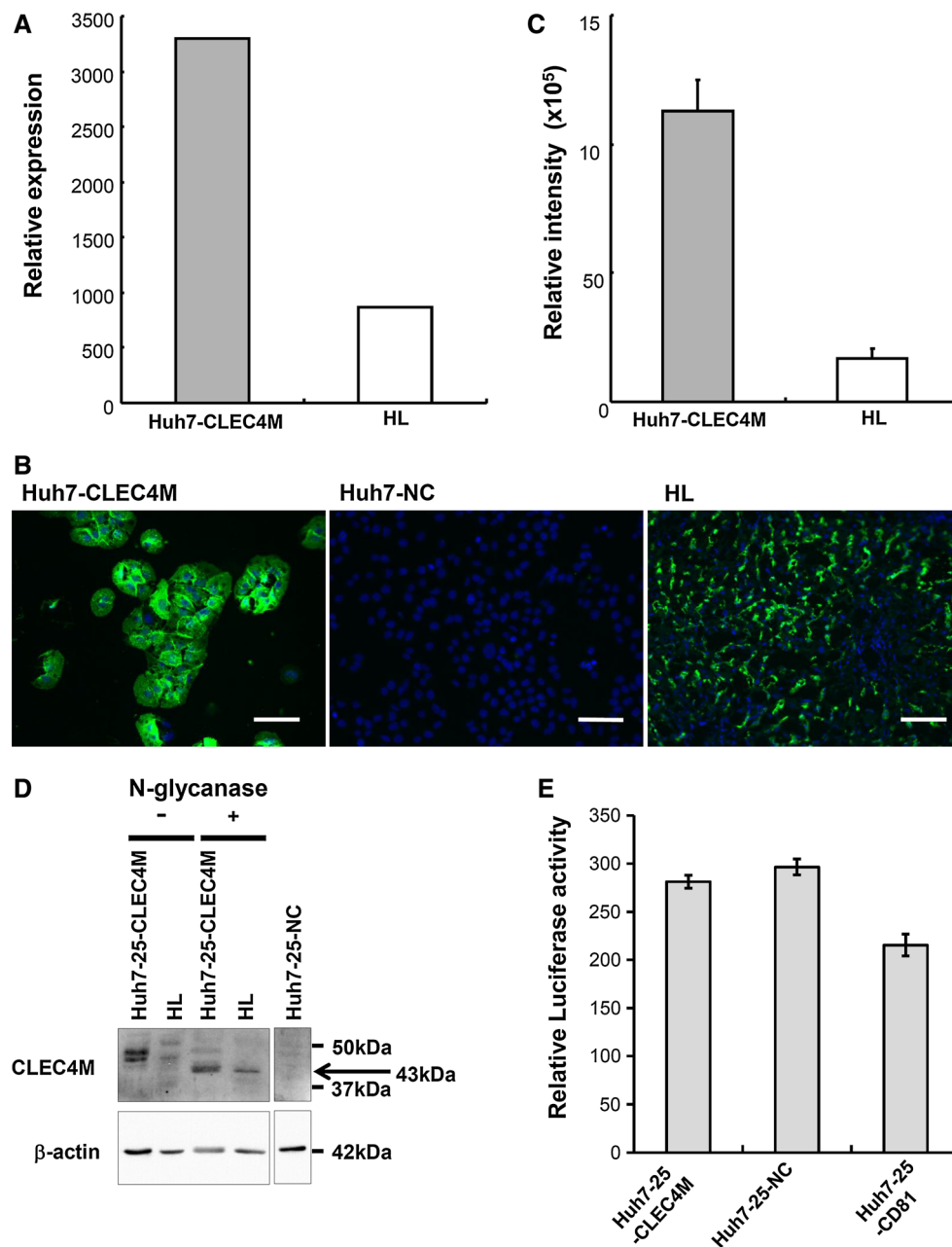


Fig. 1 Characterization of Huh7-25-CLEC4M cells. **(A)** Quantitation of CLEC4M mRNA in Huh7-25-CLEC4M cells and human liver tissue (HL). The mRNA levels were quantified by real-time PCR. Gene expression was normalized to the quantity of GAPDH used as an internal control gene, and the relative value was expressed in arbitrary units. **(B)** Immunofluorescence staining of CLEC4M. Huh7-25-CLEC4M cells were stained for CLEC4M by immunofluorescence (green) and for nuclei with DAPI (blue). CLEC4M on the cell surface was detected by binding of monoclonal anti-CLEC4M antibody before fixation with cold acetone. Huh7-25-NC cells were transfected with the pEF1/V5-His plasmid. Liver sinusoidal endothelial cells of HL were also positive for CLEC4M. Bar, 100 μ m. **(C)** Fluorescence

intensity of CLEC4M protein in **(B)**. Fluorescence intensity was calculated as the ratio of total CLEC4M fluorescence to the total area of the cells examined. **(D)** Western blotting of CLEC4M in Huh7-25-CLEC4M cells and HL. Several bands around 50 kDa and a single 43-kDa band were specific for CLEC4M in untreated and deglycosylated samples, respectively. **(E)** Luciferase reporter assay of HCV replication in Huh7-25-CLEC4M and -NC cells. Luciferase activity was measured 4 and 48 h after transfection with pSGR-JFH1/Luc, and the relative luciferase activity was expressed as the ratio between the two values. HCV-susceptible Huh7-25-CD81 cells were used as a positive control. The assay was performed in duplicate

(Huh7-25-NC) but also to Huh7-25-CD81 cells, which were established by transfecting Huh7-25 cells with CD81, transforming them into HCV-susceptible cells [2]

(Fig. 1E). Thus, the Huh7-25-CLEC4M cell clone was useful for determining whether CLEC4M is an entry receptor for HCVcc that can take the place of CD81.

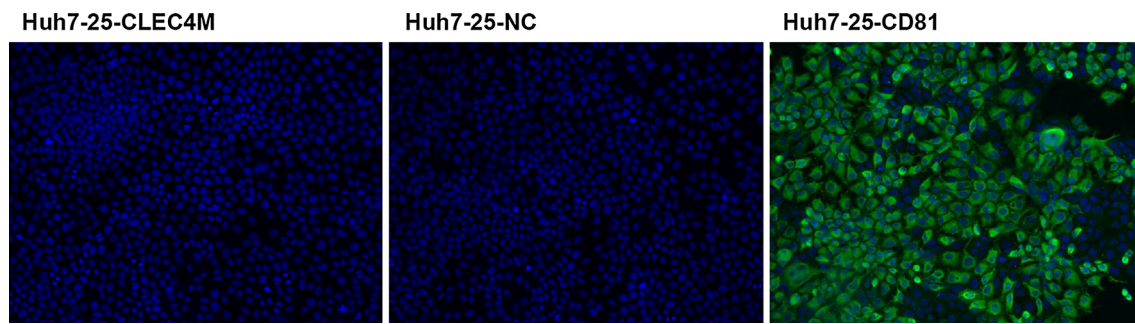


Fig. 2 Infectivity in Huh7-25-CLEC4M cells. Huh7-25-CLEC4M, -NC, and -CD81 cells were stained for HCV proteins by immunofluorescence (green) and for nuclei with DAPI (blue) 6 days

postinfection. Huh7-25-CD81 cells were used as a positive control. The assay was performed in triplicate

No cisinfection of Huh7-25-CLEC4M cells with HCVcc

To examine whether CLEC4M acts as a receptor for cisinfection, Huh7-25-CLEC4M cells were inoculated with HCVcc (1.8×10^3 ffu). Six days after HCV infection, none of the Huh7-25-CLEC4M or -NC cells were positive for HCV. In contrast, 70–80 % of Huh7-25-CD81 cells were positive for HCV (Fig. 2). Thus, CLEC4M is not an entry receptor for HCV, nor does it substitute for CD81 to mediate HCV cisinfection.

Transinfection of Huh7-25-CLEC4M cells with HCVcc

Next, we investigated whether CLEC4M is involved in transinfection with HCVcc. Four hours after inoculation with HCVcc, the amount of HCV RNA in Huh7-25-CLEC4M cells was the same as in Huh7-25-NC cells (Fig. 3A). The amount of attachment of HCVcc was similar with Huh7-25-CLEC4M and -NC cells. However, the level of HCV RNA after 4 days of cultivation increased 3-fold in Huh7-it cells co-cultured with Huh7-25-CLEC4M cells, and 13-fold in comparison to the negative control (Fig. 3A). As shown by immunofluorescence staining of HCV and CLEC4M after 3 days of co-culture, HCV-positive Huh7-it cells were adjacent to the Huh7-25-CLEC4M cells (Fig. 3B). To confirm CLEC4M-dependent transinfection, Huh7-25-CLEC4M cells were treated with anti-CLEC4M antibody before inoculation with HCVcc. The amount of HCV RNA was decreased specifically by treatment with anti-CLEC4M antibody (Fig. 4), but not in the case of Huh7-it cells (CD81-positive and CLEC4M-negative cells). These results suggest that CLEC4M is a receptor for HCV transinfection.

Discussion

In this study, we showed that CLEC4M was inactive in *in vitro* cisinfection but active in transinfection with

HCVcc, and that the transinfection was mediated by a carbohydrate-recognition domain of CLEC4M. This was directly proven, for the first time, by using infectious HCVcc and particular host Huh7 cells (Huh7-25-CLEC4M) that were permissive for HCV RNA replication, negative for CD81, and positive for CLEC4M.

We directly showed that CLEC4M does not serve as an endocytotic receptor for HCV infectious particles or as a helper for HCV endocytosis in place of CD81. DC-SIGN, which is analogous to the CLEC4M protein, functions as an endocytotic receptor and has internalization motifs, such as a tri-acidic cluster, a dileucine motif and a tyrosine-based motif in the intracellular N-terminal domain [13]. The tri-acidic cluster and dileucine motif are present in the N-terminal region of CLEC4M, but the tyrosine-based motif is absent [13]. However, CLEC4M-positive Jurkat cells were susceptible to Ebola virus cisinfection [3]. CLEC4M-positive CHO cells captured SARS-CoV and incorporated, it leading to proteasome-dependent viral degradation [8]. CLEC4M-positive K562 and THP-1 cells internalized HCV virus-like particles consisting of recombinant envelope proteins E1 and E2, which differ from HCVpp [17]. Thus, the internalization by CLEC4M-positive cells is dependent on the type of cell and ligands. The early stage of infection such as attachment and entry of HCV requires particular cooperation of multiple receptors including coreceptors. Such an interaction between CLEC4M and other active receptors for HCV might be deficient in Huh7-25-CLEC4M cells.

To clarify physiological relevance of CLEC4M to HCV tropism, it is necessary to examine human liver sinusoidal cells (LSECs) for cis- and transinfection with HCVcc. Although we demonstrated that CLEC4M of human liver had a different glycosylation pattern from that of Huh7-25-CLEC4M cells (Fig. 1D), Ludwig and coworkers showed that HCV virus-like particles bound to LSECs of human liver tissue sections *in situ* [17]. Thus, a different glycosylation pattern of CLEC4M on LSECs may not affect the attachment of HCV. Lai and colleagues demonstrated that

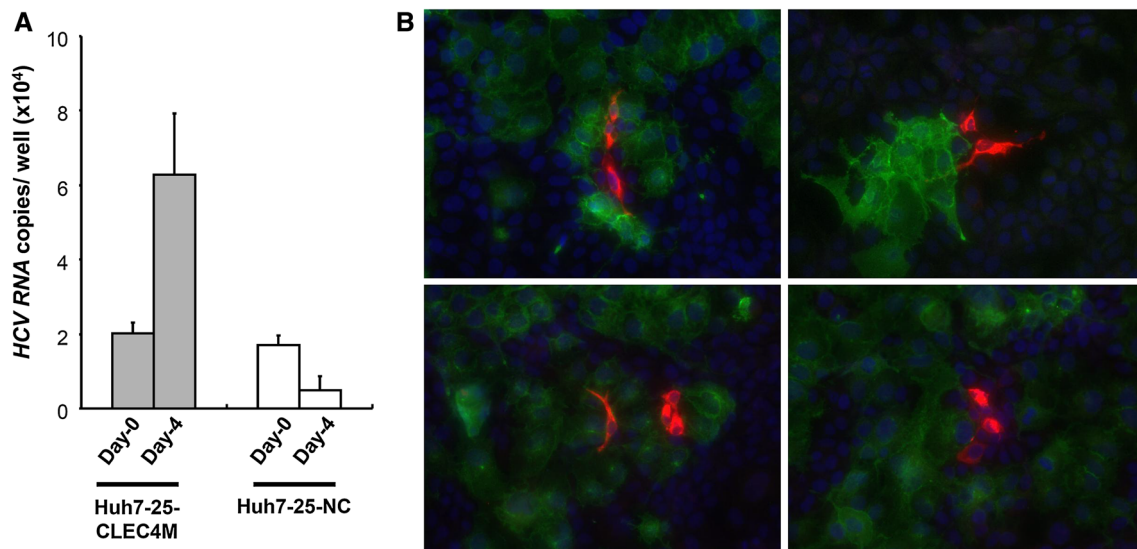


Fig. 3 CLEC4M-mediated transfection of Huh7-it cells with JFH-1 HCVcc. **(A)** Quantitation of HCV RNA 4 h (day 0) and 4 days (day 4) postinfection. Huh7-25-CLEC4M and -NC cells were incubated with JFH-1 HCVcc for 4 h at 37 °C. After being washed, cells were co-cultured with Huh7-it cells. The cells were harvested 4 days later, and HCV RNA was measured by real-time PCR. Bars indicate

standard deviation of triplicate experiments. **(B)** Immunofluorescence staining of CLEC4M and HCV protein. Co-cultured cells were stained for CLEC4M (green) and HCV protein (red) by immunofluorescence and for nuclei (blue) with DAPI. The experiment was performed in triplicate

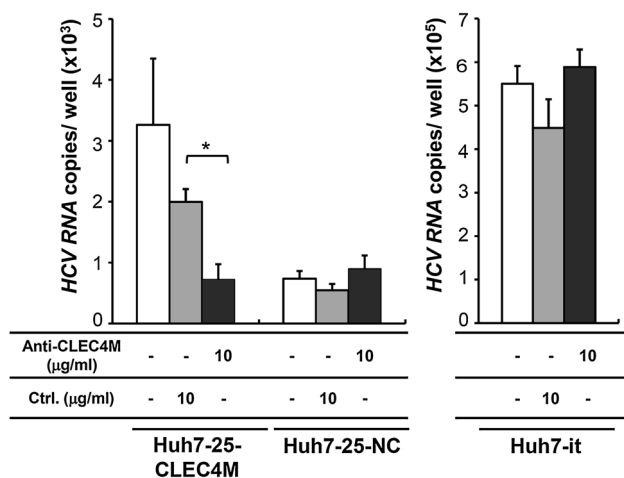


Fig. 4 Inhibition of CLEC4M-mediated transfection with JFH-1 HCVcc by anti-CLEC4M antibody. Huh7-25-CLEC4M, -NC, and Huh7-it cells were pre-incubated with anti-CLEC4M antibody for 30 min before incubation with JFH-1 HCVcc for 4 h at 37 °C. After being washed, the cells were co-cultured with Huh7-it cells for 3 days, and HCV RNA was measured. As CD81-positive Huh7-it cells were permissive for HCV entry and highly susceptible to HCV replication, the level of HCV RNA 3 days post-incubation was 100-fold higher than in Huh7-25-CLEC4M cells. Bars indicate standard errors of triplicate experiments. Ctrl., immunoglobulin isotype control; *, $P < 0.05$

CLEC4M was expressed on human LSECs and functioned in binding HCV E2 glycoproteins but that primary human LSECs did not support HCVpp entry [15]. Thus, CLEC4M on LSECs is not an entry receptor for HCV. Therefore,

LSECs should be resistant to HCVcc infection, and LSECs were indeed found to be resistant to HCVcc when co-cultured with J6/JFH-infected Huh-7.5 cells [15]. It is still unknown whether LSECs are permissive for HCV replication or not. Recently, Fletcher and associates showed that LSECs were not infected with HCVpp because expression of SR-BI and CLDN1 in the cells was very low [11]. They found that human brain endothelial cells expressed functional receptors that supported entry and replication of HCVcc. These results suggest that LSECs are unsusceptible to HCV infection due to the lower abundance of a few co-receptors on these cells. LSECs possibly function as a mediator of transfection with HCV.

Several investigators have shown that CLEC4M mediates transfection with HCVpp [9, 10, 16]. We showed in the present study that CLEC4M actually mediated transfection with bona fide HCVcc. However, the attachment of HCVcc to Huh7-25-NC cells was apparently similar to that of Huh7-25-CLEC4M (Fig. 3A). Nonspecific binding may be responsible for the attachment to Huh7-25-NC cells, which is qualitatively different from that of Huh7-25-CLEC4M cells. Such nonspecific binding is probably too weak to mediate transfection of HCVcc to adjacent Huh7-it cells. The lack of internalization in this case indicates that transfection might occur through the release of captured HCVcc to adjacent hepatocytes. In the case of human liver *in vivo*, transfection mediated by LSECs can occur with or without internalization. In duck liver *in vivo*, duck hepatitis B virus was captured by

LSECs, internalized into LSECs and then transferred to adjacent hepatocytes [6]. HIV-1 was also incorporated into dendritic cells by DC-SIGN and then transmitted to CD4 T cells via exosomes [21]. Thus, these viruses were temporarily pooled in reservoir cells to escape from host immune evasion. It is still unknown whether CLEC4M on human LSECs internalizes HCV and whether human LSECs are permissive for HCV RNA replication.

In conclusion, we demonstrate that CLEC4M is a capture receptor for JFH-1 HCVcc but cannot substitute for CD81 as an entry receptor. Therefore, CLEC4M does not mediate cisinfection but does mediate transinfection without internalization of JFH-1 HCVcc. These findings and others suggest that CLEC4M on LSECs is responsible for the hepatotropism of HCV infection by capturing and releasing HCV to adjacent hepatocytes, but not by serving as a receptor for HCV cisinfection.

Acknowledgments The authors thank Dr. M. Oshima, National Institute of Infection Diseases, Tokyo, Japan, for providing us with Huh7-it cells. This work was supported by a grant for “Open Research Center” Project for Private Universities: matching fund subsidy from MEXT (2005), Nihon University Joint Research Grant 06-023, Grant-in-Aid for Scientific Research (C) 22590350 from MEXT (2010), and “Strategic Research Base Development” Program for Private Universities subsidized by MEXT (2010). English editorial assistance was provided by NAI Inc. and funded by Nihon University School of Medicine 50th Anniversary Fund Research Grant (2012).

Conflict of interest All authors declare that they have no conflict of interest.

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