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Ficolin-2 Inhibits Hepatitis C Virus Infection, whereas Apolipoprotein E3 Mediates Viral Immune Escape

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Human ficolin-2 (L-ficolin/p35) is a lectin-complement pathway activator that is present in normal human plasma and is associated with infectious diseases; however, little is known regarding the roles and mechanisms of ficolin-2 during chronic hepatitis C virus (HCV) infection. In this study, we found that ficolin-2 inhibits the entry of HCV at an early stage of viral infection, regardless of the viral genotype. Ficolin-2 neutralized and inhibited the initial attachment and infection of HCV by binding to the HCV envelope surface glycoproteins E1 and E2, blocking HCV attachment to low-density lipoprotein receptor (LDLR) and scavenger receptor B1, and weakly interfering with CD81 receptor attachment. However, no interference with claudin-1 and occludin receptor attachment was observed. The C-terminal fibrinogen domain (201–313 aa) of ficolin-2 was identified as the critical binding region for the HCV-E1–E2 N-glycans, playing a critical role in the anti-HCV activity. More importantly, we found that apolipoprotein E (ApoE), which is enriched in the low-density fractions of HCV RNA–containing particles, promotes HCV infection and inhibits ficolin-2–mediated antiviral activity. ApoE3, but not ApoE2 and ApoE4, blocked the interaction between ficolin-2 and HCV-E2. Our data suggest that the HCV entry inhibitor ficolin-2 is a novel and promising antiviral innate immune molecule, whereas ApoE3 blocks the effect of ficolin-2 and mediates an immune escape mechanism during chronic HCV infection. HCV may be neutralized using compounds directed against the lipoprotein moiety of the viral particle, and ApoE3 may be a new target to combat HCV infection. The Journal of Immunology, 2014, 193: 783–796.

Hepatitis C virus (HCV) is a major cause of liver disease and infects nearly 200 million people worldwide. Approximately 50–80% of patients are estimated to be chronically infected, which leads to liver fibrosis, cirrhosis, and eventually hepatocellular carcinoma (1). Pegylated IFN-α in combination with ribavirin is the current standard treatment; however, eventually hepatocellular carcinoma (1). Pegylated IFN-

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Abbreviations used in this article: ApoE, apolipoprotein E; CLDN1, claudin-1; GlcNAC, N-acetyl-D-glucosamine; HA, hemagglutinin; HCV, hepatitis C virus; HCVcc, hepatitis C virus cell culture; HCVpp, hepatitis C virus pseudotyped particle; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; MBL, mannann-binding lectin; MOI, multiplicity of infection; OCLN, occludin; p.i., post-infection; qRT-PCR, quantitative RT-PCR; shRNA, short hairpin RNA; SR-B1, scavenger receptor B1; TMB, tetramethylbenzidine; VSVGpp, pseudotyped particles harboring glycoprotein of the vesicular stomatitis virus.

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two types of complement lectins that can recognize the surface carbohydrate molecules of microorganisms and subsequently activate the lectin-complement system, which plays a pivotal role in innate immunity (15–17). Thee members of the human ficolin family have been characterized: t-ficolin/p35 (ficolin-2), H-ficolin (ficolin-3), and M-ficolin (ficolin-1). Similar to MBL, ficolin-2 contains a collagen-like stem structure; however, unlike MBL, ficolin-2 has a fibrinogen-like domain and a common binding specificity for N-acetyl-D-glucosamine (GlcNAc), β-(1,3)-D-glu-
can, lipotectochic acid, and various acetylated compounds (15–17). HCV contains two conserved, highly N-glycosylated envelope glycoproteins: E1 and E2 (3). Recently, we found that the binding of ficolin-2 to the N-glycans of HCV-E1–E2 glycoproteins leads to activation of the lectin-complement pathway (18). However, because little is known regarding the roles of ficolin-2 in antiviral activity, we sought to determine whether ficolin-2 possesses anti-HCV activity and to elucidate the mechanism of ficolin-2 in HCV infection.

Apolipoprotein E (ApoE) is a class of apolipoprotein found in chylomicrons and intermediate-density lipoproteins that is essential for the normal catabolism of triglyceride-rich lipoprotein constituents (19). Significant quantities of ApoE are produced in the liver and brain and, to some extent, in almost every organ. ApoE exists in three major isoforms, E2, E3, and E4, which differ from one another by a single amino acid substitution. E3 is the most common isoform and is present in 40–90% of the human population (6, 20). ApoE receptors are members of the LDLR gene family, and ApoE-containing very low-density lipoproteins are a major LDLR ligand (6, 20). ApoE3 is enriched in the low-density fractions of HCV RNA–containing particles, and HCV virions can be precipitated or neutralized by anti-ApoE and −HCV-E2 Abs, suggesting that ApoE is important to the HCV life cycle (2, 6, 21–23). Although ApoE is required for HCV pro-
virus activity, we sought to determine whether ficolin-2 possesses anti-
HCV activity and to elucidate the mechanism of ficolin-2 in HCV infection.

Materials and Methods

Reagents, Abs, and cell lines

Munman, a linear polymer of the sugar mannose from Saccharomyces cerevisiae, and GlcNAc were purchased from Sigma-Aldrich. HCV-NS3 mAbs (SO-2) were purchased from Abcam and Santa Cruz Biotechnology (Dallas, TX). ApoE3 recombinant protein was obtained from R&D Systems (Minneapolis, MN). Anti-ApoE mAb (E6D7) and goat anti-ApoE polyclonal Ab were purchased from Calbiochem (San Diego, CA). Anti-human LDLR mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human CD81 (1.3.32.22) mAb was purchased from NeoMarkers (Fremont, CA). Recombinant E2 GST and GST proteins, anti-E2 aptamer “Ab,” and anti-E2 polyclonal Abs were prepared as previously described (24, 25). A rabbit anti-ficolin-2 polyclonal Ab was prepared by our laboratory (18). The anti–ficolin-2 mAb GNS5 was purchased from Hycult Biotech (Plymouth Meeting, PA). The anti-GST mAb was purchased from EarthOx (San Francisco, CA). An HCV NS5A polyclonal Ab was provided by Dr. Jin Zhong at the Institute Pasteur of Shanghai. PE-labeled streptavidin was obtained from Sigma-Aldrich. Human hepatocellular liver carcinoma (Huh7.5.1) cells, human 293T embryonic kidney cells, and HeLa cells were cultured in complete DMEM medium with 10% (v/v) FBS (HyClone Laboratories, Logan, UT). HCVcc–JFH1 from the Huh7.5 cell line was provided by Wen-Zhe Ho from the Temple University School of Medicine (26).

Titration of infectious HCV

Infection of Huh7.5.1 cells and the titration of infectious HCV were performed as previously reported (27). The infectious supernatants were serially diluted 10-fold in complete DMEM and used to infect 106 naive Huh7.5.1 cells per well in 96-well plates (Corning). The virus was incu-

bated with the cells for 2 h at 37˚C and then supplemented with fresh complete DMEM. The level of HCV infection was determined 3 d post-infection (p.i.) by immunofluorescence staining for HCV NS5A. The viral titer is expressed as focus-forming units per milliliter of supernatant, determined by the average number of NS5A+ foci detected at the highest dilutions. The multiplicity of infection (MOI) is the average number of virus particles infecting each cell and indicates focus-forming units of virus used for infection per number of cell (28).

Amplification of HCVcc viral stocks

For large-scale generation of viral stocks, infectious supernatants were diluted in complete DMEM and used to inoculate 15% confluent Huh7.5.1 cells at an MOI of 0.01 in a T75 flask (Corning). Infected cells were trypsinized and replated before confluence at days 4–5 p.i. Supernatants from infected cells were then harvested 7–8 d p.i., filtered through a 0.22-

µm filter, concentrated 100-fold using an Amicon Ultra-4 (Millipore, Billerica, MA), and aliquoted for storage at −80˚C. The titer of the viral stock was determined as described above.

Purification of intracellular and extracellular HCV (HCVcc) virions using sucrose gradient ultracentrifugation

HCV was concentrated and purified with sucrose density-gradient ultracentrifugation according to a previously reported method (27, 28). For preparation of intracellular HCV, infected Huh7.5.1 cells were harvested ∼4 d p.i. The cells were washed in PBS three times and then repeatedly frozen and thawed from −80˚C to 37˚C three times. Supernatants from cell lysates were centrifuged at 12,000 rpm for 5 min to remove cellular debris. Pellets from supernatants were resuspended and centrifuged through a 20% sucrose cushion at 28,000 rpm for 4 h using an SW28 rotor in a Beckman Coulter Optima L-100 XP ultracentrifuge. The pellets were resuspended in 1 ml TNE buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA), loaded onto a 20–
60% sucrose gradient (12.5-ml total volume), and centrifuged at 14,000 rpm for 16 h at 4˚C in a SW41 Ti rotor (Beckman Coulter). Fractions of 1.3 ml were collected from the top of the gradient. The fractions were analyzed by quantitative RTPCR (qRT-PCR) to detect HCV RNA. To determine the infectivity titer of each fraction, fractions were titrated on Huh7.5.1 cells as described above.

Production of HCVpp

Production and infection of Huh7.5.1 cells using infectious HCV JFH1 (a genotype 2a isolate) or murine leukemia virus–based HCVpp were performed as described (29, 30). Pseudotyped particles harboring glycoproteins of the vesicular stomatitis virus (VSVGpp) on murine leukemia virus were used as a control.

HCVcc and HCVpp infection and neutralization

The HCVcc infection and neutralization experiments were essentially performed according to previously published methods (27). Huh7.5.1 cells in 24-well plates were infected with HCVcc (MOI of ∼2; 1.6 × 106 copies/ml) at 37˚C for 4 h. The supernatants were discarded, and the infected cells were washed twice with PBS and incubated in DMEM containing 10% FBS for 72 h. For neutralization of HCV infectivity, HCV was incubated with different amounts of GST–ficolin-2 or GST protein at 37˚C or mixed with ApoE (0.15 µM) for 1 h and then added to Huh7.5.1 cells. At 4 h p.i., the protein–HCV mixture was aspirated and the cells were washed twice with PBS and then incubated with DMEM supplemented with 10% FBS for 72 h. HCV was identified using FITC-conjugated streptavidin IgG and biotin-labeled anti-E2 aptamer ZE2 Ab and observed under a confocal fluorescence microscope. Alternatively, the cells were lysed and HCV RNA and protein were measured by qRT-PCR and Western blotting.

To determine the role of ficolin-2 and its isoforms ApoE2 and ApoE4 during HCV infection, HCVcc neutralization was also performed. A 3-µg sample of the plasmids pcDNA3.1-ApoE2, -ApoE3, and -ApoE4 was used to transfect Huh7.5.1 cells. After 24 h, 0.5 µM GST–ficolin-2 and GST proteins mixed with 1 × 106 copies of HCVcc at 37˚C for 1 h were added to the cells and incubated for 72 h. The expression of HCV was then detected by qRT-PCR and Western blot analysis.

The neutralizing effect of ficolin-2 on six genotypes of HCVpp was measured as previously reported (24, 29, 30).

Construction of recombinant plasmids

Full-length HCV-E1 and -E2 (GenBank accession no. M67463) were amplified and subcloned in-frame into pcDNA3.1(−MYC-His(1))A-hemagglutinin (HA) to generate the plasmids pcDNA3.1(−MYC-His(1))A-E2-HA and pcDNA3.1(−MYC-His(1))A-E1-HA, respectively. The HA tag
sequence 5’-AGCATATGCGTACGTCATAAAGGATA-3’ was cloned in-frame into pcDNA3.1(-)Myc-His(A). The sequences of the constructs were verified by DNA sequencing. The pDisplay eukaryotic expression vector from Clontech Laboratories was used to display the expressed protein on the surface of cell membranes. Full-length HCV-E2 was amplified and subcloned in-frame into pDisplay to generate the plasmid pDisplay-E2 using the E2 specific primers P1 (5’-AAAGAGTTCACCTCCTGAC-3’) and P2 (5’-TGCGGTAGCTCACCTTGATGCCC-3’). The underlined nucleotides indicate the target sequence. The pair 1 oligonucleotides were annealed and inserted into pSilencer1.0-U6 digested with Apal and HindIII to produce an intermediate plasmid. The annealed product of the pair 2 oligonucleotides was subcloned into the HindIII and EcoRI sites of the intermediate plasmid to generate the plasmid shRNA-SR-B1. All of the other shRNAs were constructed in a similar manner.

** Knockdown of HCV receptors by specific shRNAs**

For transient transfection, Huh7.5.1 cells were grown in DMEM (HyClone Laboratories) supplemented with 10% FBS. Huh7.5.1 cells (5 x 10^5) in each well of a six-well plate) were transfected with plasmids encoding sh-CD81, sh-SR-B1, sh-CLDN1, sh-OCLN, or sh-LDLR using the jetPEI transfection reagent (Polyplus Transfection, Illkirch-Graffenstaden, France) according to the manufacturer’s instructions. The efficiency of transient transfection was measured using GFP as a reporter molecule, and the percentage of transfected cells was determined (based on the number of fluorescent cells counted) using a flow cytometer. The number of GFP+ cells revealed ∼70% transfected cells.

** Confoal fluorescence microscopic analysis**

The effect of ficolin-2 on the interactions between HCV and cellular receptors was analyzed by confocal fluorescence microscopy. HeLa cells were plated in confocal dishes (Nest) at a density of 5 x 10^5 cells/ml. After 24 h, the cells were transfected with 1 μg pEGFP-C1 (Clontech), which encodes GFP, cotransfected with shRNA-CDS1, SR-B1, CLDN1, OCLN, and LDLR into the Huh7.5.1 cells. The histogram quality and percentage of transfected cells were determined (based on the number of fluorescent cells counted) using a flow cytometer. The number of GFP+ cells revealed ∼70% transfected cells.

** Construction of short hairpin RNA expression vectors**

pSilencer1.0-U6 (Ambion) was used to construct short hairpin RNA (shRNA) expression vectors in this study. shRNAs against human CD81, SR-B1, CLDN1, OCLN, and LDLR were constructed according to previously published methods (32, 33). The 19 nt target sequences for constructing the shRNA against CD81 (GenBank accession no. NM004356.3), SR-B1 (GenBank accession no. NM183054.4), CLDN1 (GenBank accession no. NM201101.4), OCLN (GenBank accession no. BC012471), and LDLR (GenBank accession no. NM005274.7) mRNAs were 5’-UGAUUGUUGUUGCUUCCU-3’, 5’-GGACGAGGGCUUAAAGAG-3’, 5’-UAACAUAGAAGACUAAGAA-3’, 5’-GAGCCGCAUUCUUCAC-3’, and 5’-GGACGAGAUCUAAAGAA-3’, respectively. Using these shRNA-SR-B1 construct as an example, two pairs of oligonucleotides were synthesized: pair 2 oligonucleotides (5’-AGCTTGTTCTTAAAGGACGACCGTCTCGGGCC-3’ (reverse); and pair 2, 5’-AGCTTTGTTCTTAAAGGACGACCGTCTCGGGCC-3’) were designed based on the HCV sequence 5’-ATGGTTGTTCTTAAAGGACGACCGTCTCGGGCC-3’. The underlined nucleotides indicate the target sequence. The pair 2 oligonucleotides were annealed and inserted into pSilencer1.0-U6 digested with Apal and HindIII to produce an intermediate plasmid. The annealed product of the pair 2 oligonucleotides was subcloned into the HindIII and EcoRI sites of the intermediate plasmid to generate the plasmid shRNA-SR-B1. All of the other shRNAs were constructed in a similar manner.

** Construction of short hairpin RNA expression vectors**

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Ab. Color development was achieved by adding 100 μl/well tetramethylbenzidine (TMB) chromogen substrate (Sigma-Aldrich), and the reaction was stopped by adding 100 μl 0.5 M H2SO4. The absorbance at OD450 was measured with a PerkinElmer 2030 multilabel reader.

The binding ability of ficolin-2 to E2-HeLa or HeLa was measured by ELISA, as previously reported (24, 30). ELISA (96-well) plates were pretreated with polylysine (0.1 mg/ml) and then coated with 2 × 105 of each cell type at 4˚C overnight. The plates were then blocked with 2% BSA at 37˚C for 2 h. The plates were washed three times with PBST (PBS containing 0.1% Tween 20), and ficolin-2 (0.3 μM) with HCV (MOI of 0.3, 3.0 × 104 copies/100 μl) was added to each well and incubated at 37˚C for 1 h. An anti-ficolin-2 mAb (GNS, HyClone Laboratories, 1:1000) was added to each well, followed by HRP-goat anti-mouse. The absorbance at OD450 was measured as described above.

For the analysis of the blocking effect of ficolin-2 on HCV binding to the receptor-expressing HeLa cells or receptor knockdown cells, ELISA (96-well) plates were pretreated with polylysine (0.1 mg/ml) and then coated with 2 × 105 of each cell type at 4˚C overnight, followed by blocking with 2% BSA. The plates were then washed with PBST, and ficolin-2 (0.3 μM) in the presence of HCV (MOI of 0.3, 3.0 × 104 copies/100 μl) or in the presence of different concentrations of ApoE3 was added to each well and incubated at 37˚C for 1 h. A biotin-labeled anti-HCV-E2 aptamer ZE2 Ab (100 nM) (24) was added, followed by the addition of HRP-streptavidin. The absorbance at OD450 and the binding ability were measured as described above. For analysis of the interaction between ApoE3 and ficolin-2, ELISA plates were coated with ficolin-2 protein (0.15 μM, 100 μl/well) at 4˚C overnight. Then, the plate was blocked with 1% BSA at 37˚C for 1 h. After washing with PBST, different concentrations of ApoE3 protein (0.01–1.26 μM) were added and incubated at 37˚C for 1 h. Mouse anti-ApoE3 mAb (1:1000) was added and incubated at 37˚C for 1 h, followed by HRP-goat anti-mouse IgG (1:5000). Color development was achieved by adding 100 μl/well TMB chromogen substrate (Sigma-Aldrich), and the reaction was stopped by adding 100 μl 0.5 M H2SO4. The OD values at 450 nm were measured with a PerkinElmer 2030 multilabel reader.

The supernatants from plasmids pcDNA 3.1—ApoE3, –ApoE2, and –ApoE4-transfected Huh7.5.1 cells for 72 h were collected and the secreted ApoE proteins were measured using a sandwich ELISA according to previous publications (35, 36). Briefly, supernatants of each sample were loaded into a 96-well plate coated with a polyclonal anti-ApoE (goat anti-human ApoE; 1:400 dilution, Calbiochem). Following incubation at 37˚C for 1 h, the samples were removed and the plates were washed three times with PBST prior to incubation with a mouse anti-ApoE mAb (E6D7, recognizes E2, E3, and E4 isoforms of ApoE; Calbiochem) (1:2000) at 37˚C for 1 h, the samples were removed and the plates were washed three times with PBST and incubated with HRP-goat anti-mouse IgG (1:10000) at 37˚C for 1 h. The plates were then washed again three times with PBST and incubated with HRP-goat anti-mouse-IgG (1:4000) for an additional 1 h at 37˚C. The immunocomplex was reacted with TMB substrate and detected using a PerkinElmer 2030 multilabel reader. Levels of ApoE3 were normalized to a standard curve generated using ApoE3 recombinant protein obtained from R&D Systems.

Flow cytometry analysis
To analyze the ability of ficolin-2 protein to bind to E2-HeLa cells or HeLa cells, 0.5 μg GST–ficolin-2 or GST protein in a final volume of 100 μl PBS was incubated with 1 × 106 E2-HeLa cells or HeLa cells at 37˚C for 30 min. The cells were then washed in PBS and stained in 100 μl PBS containing 1% BSA and anti-GST mAb for 30 min. The cells were pelleted, washed, and resuspended in PBS containing 1% BSA and PE-labeled goat anti-mouse IgG Ab (1:100). After incubation, the cells were washed, and cell-bound GST–ficolin-2/GST protein was analyzed using a BD Canto II flow cyrometer (BD Biosciences).

The binding affinity of GST–ficolin–2, GST, or ApoE3 protein to target cells (E1-HeLa, E2-HeLa, HeLa) cells was measured by flow cytometry by monitoring the mean fluorescence intensity of the target cells bound to PE-labeled anti-GST or PE-labeled anti-ApoE3, as previously described (24, 29). E1-HeLa, E2-HeLa, and HeLa cells (1 × 105 cells) were preincubated with in 0.1 μM GlcNAc significantly blocked the anti-HCV activity of ficolin-2 is primarily due to its effect on viral particles rather than on an interaction with cell-surface sugars (Fig. 1B). The neutralization efficiency of ficolin-2 on HCV infection occurred in a ficolin-2 dose-dependent manner, whereas the control protein GST did not have any effect (Fig. 1C).

We further analyzed the neutralization effect of ficolin-2 on HCVpp. As shown in Fig. 1D, the infectivity of all six genotypes of HCVpp (genotypes 1a–6a) was dramatically decreased in the presence of ficolin-2 compared with the control groups. The control serum, which was the preimmunized mouse serum and did not contain anti-HCV Ab, and VSVGpp were used as negative controls. Ficolin-2 exhibited a much stronger antiviral activity against HCVpp (Fig. 1D) than against HCVcc (Fig. 1C).

Both mannan and GlcNAc competitively block the anti-HCV effect of ficolin-2
Mannan is a linear polymer of the sugar mannonse with molecular masses ranging from 34 to 62.5 kDa. GlcNAc is a monosaccharide derivative of glucose. Previous reports have shown that ficolin-2 binds to acetylated compounds and carbohydrates (e.g., GlcNAc, β-(1,3)-α-glucan) (15–17) and the heavy N-glycans of HCV E1E2 heterodimers (18). Therefore, we sought to determine whether such interactions were responsible for the anti-HCV activity of ficolin-2 by assessing whether the infectivity of HCVcc is modified in the presence of ficolin-2 (0.1 μM) preincubated with increasing amounts of mannan or GlcNAc. At 3 d p.i., the protein expression levels of HCV NS3 and the relative RNA levels of HCV were measured by Western blotting (Fig. 2A) and qRT-PCR (Fig. 2B), respectively. Both mannan and GlcNAc restored HCVcc infectivity in a concentration-dependent manner (Fig. 2A, 2B). Both 4 μg/ml mannan and 15 μg/ml GlcNAc significantly blocked the binding of ficolin-2 to cell surface–expressed E2 glycoprotein
by flow cytometry analysis (Fig. 2C). Interestingly, we found that the anti-HCV activity of ficolin-2 was more sensitive to mannan than to GlcNAc, as mannan could restore HCVcc infectivity at a much lower concentration compared with GlcNAc (Fig. 2A–C). In an indirect ELISA, virus particles from HCVcc were added and captured by GNA lectin–coated ELISA plates and incubated with ficolin-2 (0.3 μM) or control cell culture medium and different concentrations of mannan or GlcNAc, followed by extensive washing to remove unbound samples. The ficolin-bound viral particles on the ELISA plates were revealed using an anti–ficolin-2 mAb. From our data shown in Fig. 2D, 10 μg/ml (0.16–0.29 μM) mannan significantly blocked ficolin-2 binding to HCVcc, whereas 10 μg/ml (45 μM) GlcNAc (which molar concentration is almost 155- to 250-fold higher than that of 10 μg/ml mannan) did not significantly block ficolin-2 binding to HCVcc. These results suggest that the inhibitory effect of mannan on the binding of ficolin-2 to HCVcc is almost 155 to 250-fold stronger that of GlcNAc. Therefore, we proposed that the binding of ficolin-2 to the HCVcc particles was remarkably blocked by mannan and only slightly by GlcNAc (Fig. 2D, p < 0.05, versus GST–ficolin-2).

FIGURE 1. Ficolin-2 blocks HCVcc initial attachment and entry. (A) Different concentrations of ficolin-2 or GST protein were preincubated with HCVcc (left, early) or were added after HCVcc infection at 48 h p.i. (right, late). The infected cells were harvested for RNA extraction and qRT-PCR analysis. G3PDH was used as an endogenous control, and the amount of HCV mRNA was normalized based on the amount of G3PDH mRNA. *p < 0.05. **p < 0.01. (B) Huh7.5.1 cells were incubated for 1 h with HCVcc in the presence of ficolin-2 or GST protein (3 μM). At 3 d p.i., the expression of HCV-NS3 was detected by Western blot analysis; β-actin was used as an endogenous control. (C) An immunofluorescence microscopy analysis of the effects of GST–ficolin-2 or GST protein in Huh7.5.1 cells is shown as the means ± SEM from three separate experiments. *p < 0.05. (D) Recombinant ficolin-2 protein (0.15 μM) significantly inhibited HCVpp (genotypes 1a–6a) infection. A comparison of the inhibitory effects of ficolin-2 and negative controls (VSVGpp and negative serum) are shown. *p < 0.01, ficolin-2–treated group versus control serum–treated group. All of the data shown are the means ± SEM from three independent experiments. ffu, focus-forming units.

FIGURE 2. Both mannan and GlcNAc competitively block the effects of ficolin-2. Ficolin-2 (0.1 μM) was preincubated with mannan or GlcNAc, respectively, for 1 h and then further incubated with 1 × 10⁵ copies of HCVcc for 1 h. These mixtures were used to infect Huh7.5.1 cells for 4 h. The supernatants were removed and the cells were further incubated for 72 h; the relative HCV-NS3 protein and RNA expression was then detected by Western blotting (A) and real-time RT-PCR (B). (C) Flow cytometry analysis of the blocking effect of mannan or GlcNAc on ficolin-2 binding to E2-HeLa cells. (D) ELISA analysis of the blocking effect of mannan or GlcNAc on ficolin-2 binding to HCVcc E1 and E2 glycoproteins. Mannan or GlcNAc at 40 and 10 μg/ml were added, and the binding ability of GST–ficolin-2 to the E1 and E2 glycoproteins of HCV particles captured by GNA lectin was determined by ELISA using an anti–ficolin-2 mAb (GN5, HyClone Laboratories, 1:1000). *p < 0.05 compared with the GST–ficolin-2 group. All of the data are presented as the means ± SEM from three independent experiments.
Note that mannan and GlcNAc alone had no effect on HCVcc infectivity at the highest concentrations tested (15 μg/ml) (data not shown), which indicates that mannan and GlcNAc competitively block antiviral activity of ficolin-2.

The C-terminal fibrinogen domain of ficolin-2 is the region critical for binding to HCV-E2

To determine which region of ficolin-2 is critical for the binding of HCV envelope glycoproteins, different deletions of both the CLR and FBG regions of ficolin-2 were constructed in a pGEX-KG background, as shown in Fig. 3A. Recombinant ficolin-2 and ficolin-2 D1, D2, and D3 proteins were prepared and quantified by SDS-PAGE and Western blot analysis (data not shown). We established stable expression of E2 in HeLa cells and confirmed that the E2 glycoprotein was expressed on the surface of E2-HeLa cells by flow cytometry (Fig. 3B). We determined that ficolin-2 did not bind to HeLa cells (Fig. 3C). We incubated the recombinant ficolin-2 protein or its mutants with E2-expressing HeLa cells or normal HeLa cells and examined the proteins that were associated with the E2 glycoprotein. The binding of ficolin-2 or its mutant proteins to the E2 glycoprotein were detected by flow cytometry. Ficolin-2 and the mutants D1 (from 113 to 313 aa) and D2 (from 201 to 313 aa) could bind to the E2 glycoprotein expressed on the surface of HeLa cells in a dose-dependent manner, whereas D3 (from 25 to 111 aa) and GST did not exhibit binding (Fig. 3D, 3E). Flow cytometry analysis confirmed that ficolin-2 and mutants D1 and D2 bound to E2-HeLa cells much more strongly than did D3 and GST (Fig. 3D, 3E). Because ficolin-2 and the mutants D1 (from 113 to 313 aa) and D2 (from 201 to 313 aa) could bind to the E2 glycoprotein, we speculated that the C-terminal FBG domain (from 201 to 313 aa) of ficolin-2 is the critical region of the HCV-E2 glycoprotein. Moreover, Western blot analysis showed that equal moles of ficolin-2 and mutants D1 and D2 could significantly inhibit HCV-NS3 protein expression and neutralize HCVcc infection, whereas D3 and GST did not exhibit neutralizing effects (Fig. 3F, 3G, *p < 0.05 compared with only HCVcc group). Real-time fluorescence qRT-PCR also showed that equal moles of ficolin-2 and mutants D1 and D2 could significantly decrease HCV RNA expression in Huh7.5.1 cells, whereas D3 and GST did not significantly block HCV infection (Fig. 3H, *p < 0.05 compared with only HCVcc group).

Ficolin-2 interferes with HCVcc binding to LDLR and SR-B1 receptors

We next sought to determine whether ficolin-2 is able to prevent the interaction of E2 with cellular receptors. HCVcc infection requires several receptors to coexist in Huh7.5.1 cells. The LDLR and SR-B1 receptors are required for the early step of HCV cell entry (7, 14), whereas the CLDN1 and OCLN receptors function in the late step of HCV entry (10–12). Huh7.5.1 cells contain all of HCV receptors and are susceptible to HCV infection; however, HeLa
cells are not an HCV target cell and are not susceptible to HCV infection. Endogenous CLDN1 was only found in HuH7.5.1 and HuH 7 cells, but not in HeLa cells (37). The endogenous ectopic HCV receptor expression levels and densities in HeLa cells are much lower than those of HuH7.5.1 cells (37–39). We established HeLa cell lines that overexpressed the receptors LDLR, SR-B1, CD81, CLDN1, or OCLN and then measured whether ficolin-2 interfered with HCVcc binding to any of these cells. shRNA expression vectors against the HCV receptors CD81, SR-B1, CLDN1, OCLN, and LDLR were constructed according to previously reported methods (32). The expression of receptors was reduced after transfection with shRNA against each receptor, as determined by SDS-PAGE and Western blot analysis using anti–SR-B1, anti-CLDN1, anti-CD81, and anti-LDLR in both HuH7.5.1 cells (Fig. 4A) and receptor overexpressing HeLa cells (Fig. 4B). Each receptor-expressing HeLa cell line was mixed with ficolin-2 plus HCVcc, and the binding ability of HCVcc to each receptor-expressing HeLa cell line was determined by ELISA. We observed that the binding ability of HCVcc to LDLR and SR-B1 receptor-expressing HeLa cells was significantly higher than to CD81, CLDN1, and OCLN receptor-expressing HeLa cells, which suggested that HCVcc binds to LDLR and SR-B1 receptors that are required for the early steps of HCV cell entry (Fig. 4C). We further found that ficolin-2 significantly reduced the interaction of HCVcc-LDLR and HCVcc–SR-B1 by 2-fold compared with cells in the absence of ficolin-2; the interaction between HCVcc and CD81 was also slightly reduced (Fig. 4C). However, the binding of HCV to CLDN1 and OCLN, which occurs in the late stages of HCV infection, was not affected (Fig. 4C). Our results are consistent with others’ reports that the binding of CLDN1 and OCLN to HCV occurs only after CD81 bound HCV particles movement, and this cell surface trafficking to the sites of CLDN1 and OCLN led to this cellular surface conformation change and exposure of CLDN1 and OCLN binding sites to HCV (17). Thus, HeLa cells that only overexpressed CLDN1 or OCLN do not lead to increased HCVcc binding to HeLa cells. Moreover, ficolin-2 did not affect the interaction between HCVcc and each receptor-expressing HeLa cell line after the cells were transfected with the respective shRNA against CD81, SR-B1, CLDN1, OCLN, and LDLR (Fig. 4C). These results suggest that ficolin-2 interferes with HCVcc binding to LDLR and SR-B1 receptors, which is required for the early steps of HCV cell entry. Ficolin-2 also interferes weakly with HCVcc binding to the CD81 receptor (Fig. 4C). Therefore, we chose LDLR, SR-B1, and CD81 as target receptors to study the blocking effect of ficolin on HCV binding to receptor-expressing cells in the following experiments.

To further confirm whether ficolin-2 is able to prevent the interaction of E2 with the cellular receptors LDLR, SR-B1, or CD81, we performed an immunofluorescence confocal microscopy analysis. LDLR-, SR-B1-, and CD81-expressing HeLa cells were incubated with HCVcc. A biotin-labeled ZE2 aptamer (ZE2, an anti–HCV-E2 glycoprotein aptamer Ab) was added to detect HCV. HCV was stained with a red PE-conjugated streptavidin, which binds to the biotin-labeled anti-E2 ZE2 aptamer Ab, and the receptors were labeled in green for HeLa cells transfected with pEGFP-LDLR, -SR-B1, and -CD81. To demonstrate the specificity of ZE2 (an anti-HCV-E2 aptamer Ab), PE-conjugated streptavidin and biotin-labeled ZE2 was added to each group. We observed that the addition of ficolin-2 decreased HCVcc binding to the cells, as displayed by less HCV-E2 (red) on the surface of the LDLR-HeLa cells (Fig. 4D, row 4 versus row 3), SR-B1-HeLa cells (Fig. 4E, row 4 versus row 3), and CD81-HeLa cells (Fig. 4F, row 4 versus row 3), indicating that ficolin-2 reduced HCVcc binding to these receptors, which is consistent the results of the ELISA (Fig. 4C). We observed that PE-labeled ZE2 did not stain the cells in all control groups and only stained HCV as shown in Fig. 4D–F, which indicated that the anti-E2 ZE2 is specific to HCV. Because E2 is both a target for ficolin-2 and anti-E2 ZE2, we further analyzed whether the anti-E2 ZE2 aptamer Ab interfered with binding or with the assay readout by steric hindrance of ficolin-2. Using flow cytometry analysis, we found that different amounts of ZE2, as indicated in Fig. 4G, did not block ficolin-2 binding to E2-HeLa cells, which indicated that ficolin-2 and the anti-E2 ZE2 aptamer Ab do not occupy overlapping binding sites of E2 glycoprotein. Ficolin-2 and ZE2 recognize different epitopes of HCV-E2 (Fig. 4G).

ApoE3 binds to both E1 and E2 glycoproteins

Our previous data convincingly showed that HCV infection causes increased expression of ficolin-2 (18, 40) and that increased ficolin-2 concentrations are associated with the efficacy of anti-viral therapy in chronic hepatitis C patients (40). Our present data confirm that ficolin-2 blocks HCV infection of hepatocytes in vitro. Because HCV-associated ApoE3 may facilitate viral entry into cells via LDLR and plays an important role in viral formation/egression and infectivity of HCV (6), we speculated that ApoE3 is involved in the effects of ficolin-2 on HCV.

First, we analyzed whether ApoE3 binds to ficolin-2 using an ELISA method. We found that ApoE3 did not directly bind to ficolin-2 (Fig. 5A). We further determined the interaction between ApoE3 and E1/E2 glycoprotein–expressing HeLa cells using flow cytometry analysis. We found that ApoE3 could bind to E2-HeLa cells and E1-HeLa cells but not to HeLa cells (Figs. 5B). Ficolin-2 protein could bind to E2-HeLa cells and E1-HeLa cells, but not to HeLa cells (Fig. 5C). The control protein GST did not bind to E1-HeLa, E2-HeLa, or HeLa cells (Fig. 5D). The following binding affinities were determined as the equilibrium $K_d$ by flow cytometry as described in Materials and Methods (41, 42): ApoE3–E2 (K$_d$, 8 ± 0.8 nM) > ficolin-2–E2 (K$_d$, 40.8 ± 4.15 nM) > ApoE3–E1 (K$_d$, 58 ± 2.5 nM) > ficolin-2–E1 (K$_d$, 172.3 ± 10.9 nM) > ApoE3–ficolin-2 (Fig. 5B, C). These results indicate that the binding affinity of ApoE3 for E2 (Fig. 5B) was stronger than that of ficolin-2 for E2 (Fig. 5C) and much stronger than that of ficolin-2 for E1 (Fig. 5C). Additionally, using flow cytometry, we found that ApoE3 bound to E2 and E1 glycoprotein–expressing HeLa cells in a dose-dependent manner (Fig. 5E) and exhibited a much higher binding affinity than ApoE3 to HeLa cells (Fig. 5E). These data further demonstrate that ApoE3 can bind to HCV-E2 and HCV-E1. Interestingly, we found that both mannain and GlcNAc blocked the binding of ApoE3 to the E1 glycoprotein (Fig. 5F) but did not block binding to the E2 glycoprotein (Fig. 5F), which suggests that ApoE3 binds to the N-glycans of E1 and the peptide portion of E2. Conversely, ApoE3 did not bind to the N-glycans of the E2 glycoprotein.

ApoE3 competitively blocks the interaction between ficolin-2 and HCV-E2

We further found that exogenous ApoE3 significantly blocked ficolin-2 binding to HCVcc (extra-HCV) in an ApoE3 dose-dependent manner using flow cytometry analysis (Fig. 6A). We also observed a certain inhibitory effect of exogenous ApoE3 on binding of ficolin-2 to intracellular HCV (intra-HCV), but with no statistical significance (Fig. 6A). The inhibitory effect of exogenous ApoE3 on ficolin-2 binding to extra-HCV was much stronger than that of ficolin-2 for E2 (Fig. 5C) and much stronger than that of ficolin-2 for E1 (Fig. 5C). Additionally, using flow cytometry, we found that ApoE3 blocked E1 and E2 glycoprotein–expressing HeLa cells in a dose-dependent manner (Fig. 5E) and exhibited a much higher binding affinity than ApoE3 to HeLa cells (Fig. 5E). These data further demonstrate that ApoE3 can bind to HCV-E2 and HCV-E1. Interestingly, we found that both mannain and GlcNAc blocked the binding of ApoE3 to the E1 glycoprotein (Fig. 5F) but did not block binding to the E2 glycoprotein (Fig. 5F), which suggests that ApoE3 binds to the N-glycans of E1 and the peptide portion of E2. Conversely, ApoE3 did not bind to the N-glycans of the E2 glycoprotein.
was then added (Fig. 6B, 6C). Furthermore, ApoE3 competitively blocked the interaction between ficolin-2 and E2 but did not block the interaction between ficolin-2 and E1 (Fig. 6D). These results also confirm that the binding affinity of ApoE3 for E2 was stronger than the binding affinity of ficolin-2 for E2.

ApoE3 promotes HCV infection and blocks the anti-HCV effect of ficolin-2

We further demonstrated that the addition of exogenous ApoE3 increased HCV-NS3 protein expression (Fig. 7A) and HCV RNA expression (Fig. 7B, versus HCVcc group, p < 0.05; Fig. 7C, versus...
ficolin-2 plus HCVcc group, \( p < 0.05 \)) in an ApoE3 dose-dependent manner and blocked the antiviral effect of ficolin-2. Preincubation of GST with HCVcc did not influence HCVcc infectivity (Fig. 7A, lane 1). A qRT-PCR analysis also revealed that ApoE3 alone (150, 300 nM) significantly enhanced HCVcc infection of Huh7.5.1 cells (Fig. 7B, ApoE3 plus HCVcc versus HCV group, \( p < 0.05 \)).

Because ApoE2 and ApoE4 are similar isomers of ApoE3, with only one or two amino acid differences (6), we further determined the roles of ApoE2 and ApoE4 on the effects of ficolin-2 on HCV infection. The plasmids pCDNA3.1-ApoE2, -ApoE3, and -ApoE4 were transfected into Huh7.5.1 cells. After 24 h, GST–ficolin-2 or GST proteins were preincubated with HCVcc at 37˚C for 1 h and then added into ApoE-transfected Huh7.5.1 cells. The expression of HCV-NS3 protein was detected after 72 h p.i. by Western blotting analysis. The ApoE2, ApoE3, and ApoE4 proteins were expressed in Huh7.5.1 cells at similar levels (Fig. 7D). The secreted ApoE2, ApoE3, and ApoE4 proteins in the supernatants were also detected at similar levels by Western blotting (Figs. 7D, 7E) and ELISA analysis (Fig. 7F). However, we found that ApoE2 and ApoE4 did not block the inhibitory effects of ficolin-2 on HCV RNA (Fig. 7G, versus GST–ficolin-2 plus ApoE3 group, \( p < 0.05 \)) and HCV-NS3 protein (Fig. 7H, 7I, versus GST–ficolin-2 plus ApoE3 group, \( p < 0.05 \)) levels.

The EC50 values of ficolin-2 against HCVcc-Huh7.5.1 cells in the presence and absence of 300 nM ApoE3 at 72 h were determined by immunofluorescence, as described in Materials and Methods, to be 22.57 and 6.7 \( \pm \) 0.7 \( \mu \)g/ml, respectively. Our data suggest that ficolin-2 displays a differential EC50 in the presence and absence of 300 nM ApoE3, and ApoE3 interferes with ficolin-2 independently of its ability to enhance HCV infection.

ApoE3 blocks the effect of ficolin-2 and restores HCVcc binding to LDLR and SR-B1

We further measured the effects of ficolin-2 and ApoE3 on HCV binding to its receptors. LDLR- and SR-B1–expressing HeLa cells were incubated with HCVcc plus ficolin-2 or ApoE3 and analyzed by confocal fluorescence microscopy. HCVcc was stained with a red PE-conjugated anti-E2 ZE2, and the receptors LDLR and SR-B1 were stained green in the HeLa cells transfected with pEGFP-C1-LDLR and pEGFP-C1–SR-B1. The addition of ficolin-2 decreased HCV binding to the cells, as shown by reduced HCV-E2 (red) on the LDLR-HeLa cells (Fig. 8A, row 4 versus row 3), whereas the addition of exogenous ApoE3 enhanced HCV binding to the cells, with more HCVcc found on the SR-B1–HeLa cells (Fig. 8A, row 5 versus row 4). An ELISA analysis also showed that ficolin-2 significantly blocked HCVcc binding to LDLR and SR-B1 (Fig. 8C, HCV plus...
ficolin-2 group versus HCV group, p < 0.05) and that exogenous ApoE3 blocked the binding of ficolin-2 to LDLR and SR-B1 in an ApoE3 dose-dependent manner (Fig. 8C). Although ApoE3 (600 nM) alone also significantly increased HCVcc binding to LDLR and SR-B1 (Fig. 8C, HCV plus ApoE3 group versus HCV group, p < 0.01), ficolin-2 only weakly interfered with HCVcc attachment to CD81 (Fig. 8C) and did not interfere with attachment to CLDN1 and OCLN (Fig. 8D). Ficolin-2 combined with different doses of ApoE3 had no effect on HCVcc attachment to CLDN1 and OCLN receptors (Fig. 8D). Taken together, these data suggest that exogenous ApoE3 blocked the antiviral effect of ficolin-2 and restored HCV binding to LDLR and SR-B1.

To our knowledge, this is the first report that reveals the anti-HCV effects of ficolin-2, the important role of ApoE3 in blocking the antiviral activities of ficolin-2, and the immune escape mechanism mediated by ApoE3 during chronic HCV infection. The overall model we propose based our results is shown in Fig. 9.

Discussion

In a previous study, we found that an early increase of ficolin-2 was highly correlated with hepatic inflammation and a rapid viral response in chronic HCV patients (40). In the present study, we identify ficolin-2 as a new inhibitor of HCV entry. We have demonstrated that this human lectin inhibited the entry of HCVcc (genotype 2a) and all six genotypes of HCVpp at the early stages of virus entry, regardless of the genotype.

Our present data showed that ficolin-2 blocked HCVcc entry and infection at an early stage (Fig. 1). Both LDLR and SR-B1 are required for the early steps of HCV cell entry (7, 14), whereas CLDN1 and OCLN function in the later steps of HCV entry (10–12). In the present work, we found that ficolin-2 blocked HCVcc entry at an early stage by blocking HCV binding to the early receptors LDLR and SR-B1, and weakly blocking HCV binding to CD81 (Fig. 4). Ficolin-2 did not block HCV binding to the late receptors CLDN1 and OCLN (Fig. 4).

It is likely that ficolin-2 binds to the HCV envelope glycoproteins E2 and E1 glycans and inhibits HCV entry by blocking the interaction between HCV and LDLR, SR-B1, and CD81 though steric hindrance (43, 44). The interaction between ficolin-2 and the E1 glycoprotein was weaker than the E2 glycoprotein (Fig. 5C). However, owing to the lack of a three-dimensional structure of the HCV envelope glycoproteins, the spatial relationship between HCV glycans and their receptors has not been resolved. Several recent reports have shown that lectins such as cyanovirin-N and griffithsin bind to HCV glycans and inhibit HCV entry by blocking the interaction between E2 and CD81 (44, 45). These reports also suggest that a slight increase in steric hindrance could affect the E2-receptor interaction and that the lectin ficolin-2 may be a promising drug that targets the N-glycans of HCV-E2.

Both ficolin and IFN-α are human innate immune molecules. In normal human serum, the IFN-α concentration is 2.25 ± 0.88 pg/ml (43, 46), whereas the therapeutic dose of IFN-α reaches the microgram level (180 μg/wk) in the clinical treatment of HCV (43, 46), much higher than the normal level. The mean concentration of ficolin-2 in healthy controls is ∼4.67–6.0 mg/ml (18, 40), and the EC_{50} value of ficolin-2 against HCVcc-Huh7.5.1 cells at 72 h was 6.7 ± 0.7 μg/ml. Thus, our data suggest that the ficolin-2 EC_{50} is slightly higher than or close to the mean concentration of serum ficolin-2 in normal controls, which indicates that ficolin-2 is a more effective innate immune molecule against HCV and demonstrates potential as an effective drug against HCV.

Our data revealed that both mannan and GlcNAc, which are ligands for the ficolin-2 FBG domain, could competitively inhibit the anti-HCV activity of ficolin-2 in a dose-dependent manner (Fig. 2). Mass spectrometry has clearly shown that all 11 glycosylation sites on the HCV-E2 protein are occupied by both high-mannose glycans and complex-type oligosaccharides (47), suggesting that the sugars attached to the E2 glycopeptides are primarily mannose and secondarily GlcNAc or other sugars. These reports are in agreement with our present results, in which mannan
ApoE is a component of both HCV lipoparticles and Huh7.5.1 cells. HCV particles are packaged as lipoviroparticles. ApoE3 is enriched in the low-density fractions of HCV particles, essential for binding to LDLR, and is important for HCV infectivity (6, 20). In the present work, it was found that exogenous ApoE3 could bind to E2 and E1 glycoproteins in a dose-dependent manner (Fig. 5B, 5E). We demonstrated that ApoE3 could block ficolin-2 binding to E2, even when ficolin-2 was preincubated with E2-HeLa cells for 2 h and ApoE3 was then added (Fig. 6B, 6C). Furthermore, ApoE3 did not directly bind to ficolin-2 (Fig. 5A). Our data strongly demonstrated that the binding affinity of ApoE3 for E2 was much stronger than ficolin-2 for E2 and confirmed that ApoE3 competitively blocked the interaction between ficolin-2 and HCV-E2.

ApoE is polymorphic, with three major isoforms: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158) (49). Although these allelic forms differ from each other by only one or two amino acids at positions 112 and 158, these differences alter the ApoE structure and function. Functional ApoE gene polymorphisms may be a determinant of the outcome of HCV infection (32, 50, 51). ApoE2 and ApoE4 protect against the severe liver disease caused by

FIGURE 7. ApoE3 promotes HCV infection and blocks the anti-HCV effect of ficolin-2. (A) Western blot analysis of the effects of ApoE3 and ficolin-2 on HCV-NS3 expression in HCVcc-infected Huh7.5.1 cells using an anti-NS3 mAb (B and C) Real-time RT-PCR analysis of HCV-NS3 expression in the presence of GST–ficolin-2 or ApoE3 or GST–ficolin-2 plus ApoE3 in HCVcc-infected Huh7.5.1 cells. (D) Plasmids pcDNA 3.1- ApoE3, -ApoE2, and -ApoE4 were used to transfect Huh7.5.1 cells. After 72 h of transfection, cells were harvested and the expressions of ApoE3, ApoE2, and ApoE4 in the cell lysates and supernatants of Huh7.5.1 cells were determined using a polyclonal anti-Apol by Western blotting analysis. (E) Densities of ApoE3, ApoE2, and ApoE4 proteins expressed in the supernatants shown in (D) were normalized based on the density of the housekeeping protein β-actin. (F) The cell culture supernatants were collected at 72 h and the secreted ApoE3, ApoE2, and ApoE4 proteins were determined by specific ELISA. The effects of ApoE3, ApoE2, and ApoE4 on ficolin-2 anti-HCV infection activity were analyzed by real-time qRT-PCR (G) and Western blotting using anti–HCV-NS3 mAb (H). (I) Densities of NS3 protein shown in (H) were normalized based on the density of the housekeeping protein β-actin. All data shown are the means ± SEM of three independent experiments.

(which is primarily composed of high mannose) and, to a lesser extent, GlcNAc could competitively block the interaction between ficolin-2 and HCV-E2 N-glycans and inhibit the anti-HCV activity of ficolin-2.

We found that HCVpp was more sensitive to ficolin-2-mediated antiviral effects (Fig. 1). This result indicates that mature packaged HCVcc contains more ApoE than do unpackaged immature HCV and HCVpp, which lack apoproteins. Because HCVpp is not associated with lipoproteins, we propose that ApoE3 may competitively bind to HCVcc envelope glycoproteins and interfere with the interaction between ficolin-2 and HCVcc. The results shown in Fig. 5 demonstrated that the binding affinity of ApoE3 for E2 was stronger than ficolin-2 for E2 and much stronger than ficolin-2 for E1. Therefore, HCVpp is more sensitive to the antiviral effects of ficolin-2 than HCVcc. Recently, Urichard and colleagues (48) reported that another cholesterol-uptake receptor, NPC1 L1, is a novel HCV entry factor. Whether ficolin-2 can block HCVcc binding to the NPC1 L1 receptor and whether ApoE3 can block the antiviral effect of ficolin-2 and restore HCVcc binding to NPC1 L1 remain to be further investigated.
HCV, whereas the ApoE3 allele is associated with persistent HCV infection. Our data show that ApoE3 blocks the neutralization of HCVcc by ficolin-2, but ApoE2 and ApoE4 did not (Fig. 7D, 7E). Abs targeting several different apolipoproteins exhibited neutralizing activity against lipoprotein-associated viruses. ApoE is detected in the low-density fractions of HCV, and Abs specific to ApoE can precipitate HCV virions (32, 50, 51). ApoE3 but not ApoB is required for formation/egression and is required for HCV infectivity though interactions with LDLR (25). As this association of host elements cannot evoke an immune attack against HCV particles, lipoprotein association may favor chronic HCV infection by increasing virus-specific infectivity and by shielding the virus from humoral immune responses. Previous data, as well as our own, suggest that lipoproteins play an important role in HCV infection and that ApoE3 mediates immune escape from antiviral innate immunity, promoting HCV infection. This could explain why increased concentrations of ficolin-2 in HCV patients (40) still result in chronic infection.

**FIGURE 8.** ApoE3 blocks the effects of ficolin-2 and restores HCVcc binding to LDLR and SR-B1. (A and B) Immunofluorescent detection of E2 and SR-B1 or LDLR receptor expression in SR-B1 or LDLR plasmid-transfected HeLa cells in the presence of ficolin-2 or ficolin-2 plus ApoE3 using confocal fluorescence microscopy. Original magnification ×600. (C and D) Exogenous ApoE3 restored HCV binding to its receptor and reversed the blocking effects of ficolin-2, as shown by ELISA. **p < 0.01.

**FIGURE 9.** A potential model of the ficolin-2 antiviral effects and ApoE3-mediated immune escape.
Proof-of-concept studies suggest that the HCV entry inhibitor ficolin-2 is a novel and promising antiviral innate immune molecule that increases the preventive and therapeutic arsenal against HCV infection. Our research also suggests that HCV may be neutralized using compounds directed against the lipoprotein moiety of the viral particle and that ApoE3 may be a new target to combat HCV infection.

Disclosures
The authors have no financial conflicts of interest.

References


