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HCV Infection Induces Autocrine Interferon Signaling by Human Liver Endothelial Cell and Release of Exosomes, Which Inhibits Viral Replication

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Abstract

Background & Aims—Liver sinusoidal endothelial cells (LSECs) make up a large proportion of the non-parenchymal cells in the liver. LSECs are involved in induction of immune tolerance, but little is known about their functions during hepatitis C virus (HCV) infection.

Methods—Primary human LSECs (HLSECs) and immortalized liver endothelial cells (TMNK-1) were exposed to various forms of HCV, including full-length transmitted/founder virus, sucrose-purified Japanese Fulminant Hepatitis-1 (JFH-1), a virus encoding a luciferase reporter, and the HCV-specific pathogen-associated molecular pattern molecules. Cells were analyzed by confocal immunofluorescence, immunohistochemical, and PCR assays.

Results—HLSECs internalized HCV, independent of cell–cell contacts; HCV RNA was translated but not replicated. Through pattern recognition receptors (TLR7 and retinoic acid inducible gene 1), HCV RNA induced consistent and broad transcription of multiple interferons (IFNs); supernatants from primary HLSECs transfected with HCV-specific pathogen-associated molecular pattern molecules increased induction of IFNs and IFN-stimulated genes in HLSECs. Recombinant type I and type III IFNs strongly up-regulated HLSEC transcription of interferon λ 3 (IFNL3) and viperin (RSAD2), which inhibit replication of HCV. Compared to CD8+ T cells, HLSECs suppressed HCV replication within Huh7.5.1 cells, also inducing IFN-stimulated genes in co-culture. Conditioned media from IFN-stimulated HLSECs induced expression of antiviral genes by uninfected primary human hepatocytes. Exosomes, derived from HLSECs following stimulation with either type I or type III IFNs, controlled HCV replication in a dose-dependent manner.

Conclusions—Cultured HLSECs produce factors that mediate immunity against HCV. HLSECs induce self-amplifying IFN-mediated responses and release of exosomes with antiviral activity.

Keywords

innate immunity; immune regulation; cytokine; Toll-like receptor

Background

Hepatitis C virus (HCV) persists in up to 80% of people after acute infection, and approximately 180 million worldwide have chronic infection [1]. HCV-related liver disease is a leading cause of hepatocellular carcinoma and indication for liver transplantation. Although hepatocytes comprise the majority of the total cell population within the liver, the remaining population of non-parenchymal liver cells, including liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), hepatic stellate cells (HSCs), is diverse and multi-functional [234]. The highly-organized liver architecture allows intimate contact between the distinct cell types.

LSECs compose ~50% of non-parenchymal cells in the liver (~10^{11} cells in humans) and have been highly conserved during evolution to clear waste molecules entering the circulation[5]. Prior work in LSECs has largely focused on Toll-like receptor (TLR)-4 and its ligand, lipopolysaccaride (LPS), which is constantly present in the portal venous blood [67]. However, the precise role of LSECs in sensing other TLR ligands, such as viral products, is

just beginning to emerge [6, 8, 9]. Moreover, in addition to the TLR system, retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) have been identified as cytosolic receptors for intracellular double-stranded RNA sensing[10]. The relative contribution of TLRs and RLRs as viral sensors varies across cell types and viruses[10].

LSECs are unusual in several respects; they do not secrete an organized basement membrane and are perforated by numerous fenestrations, which are transcytoplasmic canals clustered into sieve plates[11]. LSECs are highly efficient scavengers that pinocytose particles less than 0.2 μm, allowing them to uptake virus-sized particles[12]. The recent demonstration that LSECs and not KCs clear the bulk of blood-borne human adenovirus underscores their importance during the viremic phase of any natural viral infection[12]. Expression of the C-type lectin liver/lymph node-specific ICAM-3-grabbing nonintegrin (L-SIGN) on LSECs has been previously shown to mediate capture of HCV-particles and transcytosis of the virus across the endothelial barrier, thereby concentrating infectious particles and potentially facilitating their direct contact with hepatocytes[1314]. Moreover, a recent study points to HLSEC-derived bone morphogenetic protein 4 (BMP4), negatively regulated by hepatocellular VEGF-A, in promoting hepatocyte permeability, virus particle entry, and HCV replication[15].

The aim of our study was to interrogate whether human LSECs (HLSECs) could directly uptake and sense HCV-RNA and define how the innate immune responses might control HCV replication. We found HLSECs express many of the receptors implicated in HCV attachment and entry[16] and HLSEC-to-hepatocyte contact was dispensable for uptake. HCV encoding a Luciferase reporter demonstrated early viral RNA translation. Primary HLSECs and an immortalized cell line (TMNK-1) responded to various forms of HCV-RNA, including founder/transmitted virus, sucrose-purified JFH-1, and the HCV pathogen-associated molecular pattern (PAMP; substrate for RIG-I), by marked up-regulation of Type I/III interferons (IFNs). Exogenous addition of Type I/III IFNs broadly induced IFN and interferon simulated genes (ISGs) ISG responses within HLSECs. Of the ISGs, *RSAD2* (encoding viperin) was the most robustly induced in HLSECs by IFN stimulation. Stimulation of HLSECs with either Type I/III IFNs led to secretion of exosomes that inhibit HCV replication. Collectively, these data support a previously unappreciated role for HLSECs in the innate immune response to this common disease, with potential to impact other liver-tropic infections.

Materials and Methods

Cells

Primary HLSECs were purchased from Sciencell Research Laboratories (Carlsbad, CA) [17]. TMNK-1 cells were provided by A. Soto-Gutierrez (University of Pittsburgh, PA) and the hepatoma cell line Huh 7.5.1 was obtained from Francis Chisari (Scripps Research Institute, La Jolla, CA). HLSECs were maintained in Endothelial Cell medium supplemented with 5% fetal bovine serum (FBS), 1% endothelial cell growth supplement and 1% penicillin/streptomycin (Sciencell). TMNK-1 were maintained in high glucose Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Human microvascular endothelial cells (HMEC-1) cells were

maintained as previously described[18]. Human hepatocellular liver carcinoma cell lines Huh 7.5.1 and HepG2 (ATCC, Manassas, VA) cells were maintained following manufacturer's instructions.

FACS analysis of antigen expression, western blotting, immunofluorescence, preparation and use of HCV-founder virus and HCV-PAMP, TLR and IFN stimulation, gene and protein quantitation, viral replication studies, HLSECs sorting and exosomal isolation, viral control assays, and statistical analyses are detailed in the ***Supplemental materials***.

RESULTS

Human Liver Sinusoidal Endothelial Cells (HLSECs) Express Molecules Required for Attachment, Uptake and Sensing of HCV

To investigate the expression of receptors in HLSECs, 8 different lots of primary HLSECs were stained for classical endothelial markers (**Figure 1A**). HLSECs were positive for the surface markers CD31, ICAM-1, DC-SIGN, L-SIGN, and stabilin-1, but negative for the pan-leukocyte antigen CD45. LYVE-1 (lymphatic vessel endothelial hyaluronan receptor) is constitutively expressed on LSECs and reportedly absent on other hepatic cells and conventional endothelium [11,19]. Primary HLSECs also expressed receptors implicated in HCV attachment and/or entry [LDL-R, the tretraspanin CD81, and scavenger receptor type B1 (SR-B1); **Figure 1B**]. We also verified expression of certain Pattern Recognition Receptors (PRRs) in HLSECs (**Figure 1C).** HLSECs express multiple Toll-like receptors implicated in innate antiviral immunity (TLR3/TLR7), as well as the retinoic acid inducible gene-I (RIG-I), whose expression is increased after 8 hours stimulation with pegylated-IFN-[.alpha]2 (Western blot inset). The immortalized, differentiated adult human liver endothelial cell line TMNK-1 [20] was also phenotyped (**Supplemental Figure 1**), sharing most features with primary HLSECs.

HCV Uptake by HLSECs does not Require Contact with Hepatocytes and is Clathrin-dependent

In order to determine whether HCV is taken up by HLSECs, we utilized the hepatoma cell line Huh 7.5.1 infected with full-length HCV JFH-1, also known as infectious cell-culture-derived HCV (HCVcc)[21]. After 5 days of infection, primary HLSECs were added to the culture for an additional 24 hours. Confocal microscopy was performed to localize NS5A, involved in the RNA replicative machinery [16], within HLSECs. Liver endothelial cells were distinguished from hepatocytes by the different morphology of the F-actin structure and by the presence of Keratin 18 filaments (**Figure 2A–2C**), the latter only expressed in Huh 7.5.1 (**Figure 2C**). With this experimental approach, we observed infected Huh 7.5.1 in direct contact with HLSEC and both cell types expressed HCV-NS5A protein within the cytoplasm (**Figure 2D**). For the first time, we were therefore able to show HCV-specific protein within HLSECs. To further address if the HCV uptake was cell-contact dependent, we cultured primary HLSECs for 24 hours with cell-free supernatant from Huh 7.5.1/JFH-1 cultures. Confocal microscopy (**Figure 2E**) and western blot analysis (**Supplemental Figure 2**) demonstrated the presence of HCV-proteins within HLECs. Thus, uptake of virus/viral proteins occurs independently of direct hepatocyte contact. Indeed, the vast majority

(>80%) of TMNK-1 demonstrated HCV-protein after 24 hours (**Figure 2F**), which was non-detectable in TMNK-1 cultured with non-HCV-infected supernatants **(Supplemental Figure 3)**. Liver endothelial cells are known to be avid and efficient scavengers able to take up virus-sized-particles[12]. To determine if clathrin-dependent endocytosis[22] was involved, primary HLSECs were pre-treated for 15 minutes with 30 μM of Pitstop2 (inhibitor of clathrin-dependent endocytosis [23]) or for 1 hour with 80 μM of Dynasore hydrate (inhibitor of the GTPase activity of dynamin[24]). After pre-treatment, cells cultured with supernatant of JFH-1-infected Huh 7.5.1 showed significant reduction of NS5A internalization compared to control cells (determined by confocal microscopy), suggesting a clathrin-dependent uptake by primary HLSECs (**data not shown**). In contrast to the Huh7 hepatocyte model system, addition of HCVcc to primary HLSECs or TMNK-1 did not result in increased HCV copy number at 24, 48 or72 hours (**data not shown**), indicating that HLSECs do not sustain replication.

HLSECs are Permissive for HCV-RNA Infection and Translation: Insights from Direct-Acting Antivirals (DAAs)

In order to examine different steps in the viral life cycle within HLSECs, we used several approaches. A cell-culture adapted JFH-1 virus expressing *Gaussia princeps* luciferase (GLuc) in frame was infected into TMNK-1 cells and the activity of secreted GLuc in culture supernatants[25] was measured at multiple intervals (2, 8, 16 and 24 hours post-infection). GLuc activity was detected within 8 hours of infection suggesting that TMNK-1 cells are permissive for HCV viral entry and genome translation. After 8 hours of infection, we could only detect background levels of GLuc activity. When sofosbuvir, an inhibitor of the viral NS5B RNA-dependent RNA polymerase, was added to the culture (30 μM)[26], we did not observe any difference in the GLuc activity. These data suggest absence of active replication in TMNK-1 cells (**Supplemental Figure 4A**). TMNK-1 cells pre-treated with the same concentration of sofosbuvir were also cultured with supernatant from Huh 7.5.1/ JFH-1 cultures. The presence of DAA in culture did not affect the detection level of HCV-NS5A within TMNK-1 cells by confocal microscopy (**Supplemental Figure 4B-C**). Taken together, these data indicate that HLSECs/TMNK-1 cells may be permissive for HCV entry and translation of the viral genome but do not sustain productive replication.

Intracellular HCV-RNA Induces Type I and Type III Interferons in HLSECs

The fact input virus was translated but GLuc activity was rapidly reduced to background level suggested the possibility of a robust innate immune response limiting replication with HLSECs. To investigate the responses elicited by intracellular sensing of HCV-viral RNA without potential contaminants from HCVcc supernatants, we used four different approaches. First, viral-RNA (vRNA) of a full-length infectious molecular clone of HCV known to efficiently transmit infection, transmitted founder (T/F) virus identified by single genome sequencing and phylogenetic inference[2728], was added to cultured TMNK-1. The T/F vRNA (genotype 1a) induced significant up-regulation of Type I/III IFNs (black bars) that was reduced by chloroquine (white bars), a lysosomotropic agent that prevents endosomal acidification, thus inhibiting TLR signaling (**Figure 3A**). We found that sucrose-purified HCV-virus[29] induced significant transcription of multiple IFNs in TMNK-1 cells

(**Figure 3B**). These data indicate that HCV-RNA is taken up directly by HLSECs and induces innate immune responses.

Next, we *in vitro* transfected full-length JFH-1 into TMNK-1 cells in the absence or presence of a specific TLR7 antagonist, IRS661[30]. JFH-1 induced highly robust *IFNB*, and IFNLs transcription (**Figure 3C**), and TLR7 inhibition decreased but did not totally abrogate their expression (**Figure 3D**), suggesting other antiviral signalling pathways might be involved. Accordingly, we transfected primary HLSECs with the HCV-genome 3′ non-translated poly-U/UC tract, previously shown to function as the HCV-PAMP substrate of RIG-I, the cytosolic PRR for HCV[31, 32] HCV-PAMP induced strong up-regulation of *IFNB* and IFNL genes (**Figure 4A**) and significantly higher secretion of IFN-β, IFN-λ, and IP-10 (CXCL10, marker of HCV infection[33]) compared to control (**Figure 4B**). To further confirm the contribution of RIG-I to the transcriptional up-regulation of IFNs within HLSECs, we pre-treated TMNK-1 cells with Antarctic phosphatases to remove 5'-triphosphate groups required for RIG-I signaling[34,35]. Phosphatase pre-treatment significantly decreased PAMP-induced IFN transcription (**Supplemental Figure 5**).

To determine specificity of innate immune response profiles, the primary HLSECs were also stimulated with a synthetic TLR3 ligand (poly I:C) or with a TLR4 ligand (LPS). Poly I:C, an analogue of viral dsRNA, induced only modest *IFNA1* up-regulation (**Figure 4C**). LPS exclusively induced up-regulation of *TNFA* and *IL6* (**Figure 4D**). From these experiments, we conclude that the robust up-regulation of Type I/III IFNs observed in HLSECs with HCV transfection is distinct from TLR3 or TLR4 stimulation.

IFNs Induce Robust *IFNL3* and ISGs from HLSECs

IFNs are known to induce the expression of hundreds of antiviral genes, a process termed *cell-autonomous immunity*[36] that results in a "feed-forward" self-amplifying loop[37]. Considering the reported antiviral potency of Type III IFNs against HCV replication[33], we investigated the expression at the gene level of IFNL receptor components (*IL28RA* and *IL10RB*) in primary HLSECs. The ratio of the receptor components was higher in primary HLSECs relative to the HepG2 cell line (**Supplemental Figure 6**).

Given the robust induction of IFNs within HLSECs after HCV-RNA sensing, we tested IFNs downstream effects by treating primary HLSECs with pegylated-IFN-α2 (used in standard antiviral therapy), IL-28A (IFNL2), IL-28B (IFNL3), or IL-29 (IFNL1). As shown in **Figure 5A**, *IFNL3* was the most consistently-induced IFN mRNA in primary HLSECs. Interestingly, among the Type III IFNs, *IFNL3* has demonstrated the greatest *in vitro* antiviral potency against HCV replication[33,38]. Only one of the primary HLSECs came from a patient with the *IL-28B* CC genotype, precluding statistical comparison of IFN induction across genotypes.

Notably, at 8 hours, only Type I IFN induced remarkably high transcription of prototypical ISGs (**Figure 5B and C**), in particular, *RSAD2* [394041]. At 24 hours, Type I IFN maintained broad induction of ISGs, whereas, of the genes examined, only *RSAD2* was significantly up-regulated by Type III IFNs (**Figure 5D and E**). The distinct set of ISGs with different kinetics of induction supports divergent signaling pathways following receptor

engagement[33]. In order to further explore autocrine/paracrine effects specifically following HCV-RNA sensing, we added supernatants from HCV-PAMP-transfected primary HLSECs to newly-cultured HLSECs; this resulted in induction of *IFNB*, IFNLs and ISG mRNAs within HLSECs (**Supplemental Figure 7**). Thus, our data support the roles of HLSECs in the amplification of IFN effects; addition of exogenous Type I/III IFNs broadly induced IFNs, particularly *IFNL3*, and ISG responses, contrasting with the recent observation that IFNs are not induced in either primary nor immortalized human hepatocytes following the same stimulation [33].

LSECs Demonstrate Enhanced Antiviral Pathways Compared to other Vascular Endothelial Cells (ECs), Control HCV Replication and Induce an Antiviral State in Primary Human Hepatocytes

To further elucidate the antiviral potential of HLSECs compared to other ECs, we performed a microarray analysis on TMNK-1 cells versus human microvascular endothelial cells (HMEC-1)[18]. Of the 703 differentially expressed transcripts (false discovery rate 0.01), 100 were associated with viral infection and were differentially regulated to yield a global decrease in viral infection (activation z-score=-1.374, p-value=4.71×10^{-7}) (**Supplemental Table 1**). Pathway analysis of upstream regulators revealed significant activation of Type I/III interferon signaling (**Figure 6A**; **Supplemental Table 1**) suggesting TMNK-1 cells are constitutively primed for an anti-viral response. Microarray results were confirmed by qPCR for relevant ISGs (**Figure 6B**). Similar transcriptional trends were observed in our primary HLSECs (**data not shown**).

In order to be effective, most IFN-induced proteins need to be dispatched to the site of pathogen replication[36]. Considering the close proximity of LSECs to hepatocytes, we tested their ability to control infection by co-culturing HCV-infected Huh7.5.1 cells with either TMNK-1 cells or purified $CD8^+$ T cells transduced with a tyrosinase receptor. TMNK-1 cells induce significant greater antiviral control (**Figure 6A**) and increased transcription of ISGs (**Figure 6B**). As a complementary approach, supernatants from primary HCV-PAMP-transfected HLSECs also induced control of HCV replication (**Supplemental Figure 8A**). Next, using mechanical digestion and sort purification, we found that $CD31^+CD45^-$ cells from whole livers of HCV-positive patients demonstrated increased *ex vivo* expression of *IFNB* and ISGs (**Figure 6E**) compared to healthy donor livers.

Because of the remarkable expression of IFNs by HLSECs in the current study, we sought to elucidate additional mechanisms whereby IFNs might confer replicative control. The addition of supernatants from IFN-α (washed after 6 hours) or IFNLs-stimulated TMNK-1 cells (washed after 24 hours) to Huh7.5.1 cells markedly inhibited HCV replication after 5 days of infection (**Supplemental Figure 8B**). Type I IFN-stimulated liver non-parenchymal cells are known to secrete exosomes containing ISG products with broad antiviral properties[17]. Supernatants derived from IFN-α-stimulated TMNK-1 cells treated with the exosomal release inhibitor nSMase2-inhibitor spiroepoxide (5μM)[17] throughout duration of culture were harvested, pre-incubated with the type I IFN blocking antibody B18R and cultured with JFH-1 infected Huh7.5.1 cells for 5 days. Blocking exosomal release significantly increased HCV replication (**Supplemental Figure 8C**) within infected

hepatocytes. To further elucidate the contribution of exosomes in control of HCV viral replication We stimulated TMNK-1 cells with IFN-α or a cocktail of IFNLs, isolated exosomes (**Supplemental Figure 8D**), and tested their ability to control HCV replication in Huh 7.5.1 cells. Exosomes derived from Type I/III IFNs-treated TMNK-1 cells demonstrated viral control in a dose-dependent manner (**Figure 6F**).

Although the cell line Huh 7.5.1 produces workable titers of culture-derived virus, these cells are defective in RIG-I and do not express TLR3 [42], thus lack the innate immune signaling of primary human hepatocytes (PHH) [4333]. We isolated PHH and established a micro-patterned culture as described previously[44]; PHHs were subjected to supernatants from either resting or IFN-α-treated TMNK-1 cells (IFN-α was washed 6 hours after stimulation, and the supernatants collected at 6 and 24 hours). *RSAD2* and *ISG56* were significantly up-regulated in the PHH (**Supplemental Figure 8E**). Collectively, these data indicate HLSECs confer antiviral responses in neighboring hepatocytes that restrict HCV infection.

Discussion

Despite the fact LSECs are the first cells in contact with blood flow in hepatic sinusoids and account for the largest proportion of non-parenchymal cells in the liver[45], little is known about how these cells recognize hepatitis C. Given their strategic anatomic location[46] we reasoned that human LSECs (HLSECs) could play a central antiviral role. We show that HLSECs express many of the requisite receptors for antiviral recognition, including TLRs and retinoic acid inducible gene-I (RIG-I), the cytoplasmic sensor of HCV. HLSECs are permissive for HCV entry and viral translation; moreover, intracellular HCV-RNA sensing triggers robust antiviral pathways that regulate HCV infection in hepatocytes. Transmission of highly diverse viruses such as HCV or HIV across mucosal barriers can be inefficient and is most likely mediated by a single founder (T/F) virus[47]; we found that exogenous addition of an HCV-specific T/F vRNA to cultured HLSECs induced Type I/III IFN responses. Chloroquine, which inhibits endocytic TLR signaling, significantly attenuated T/F vRNA-induced responses within an HLSECs cell line (TMNK-1). Our results point to both TLR7 (endosomal receptor known to recognize single-stranded RNA and signal through MyD88 [48]) and RIG-I pathways[49] as critical to mediating IFN messages following HCV sensing by HLSECs. The fact that HLSECs demonstrate distinct transcriptional responses to HCV-RNA, Poly I:C, and LPS indicate they have evolved the ability to differentiate between different types of TLR and RLR stimulation.

Although Type I/III IFNs utilize different receptor complexes, they signal through overlapping JAK-STAT intracellular pathways and up-regulate the transcription of ISGs required to control viral infection [50]. Stimulation of HLSECs with Type I/III IFNs consistently induced transcription of *IFNL3*, known to have the greatest *in vitro* antiviral potency against HCV replication among the IFNLs[3338]. Type I IFN induces immediate and vigorous transcription of prototypical ISGs within HLSECs. In hepatocytes, IFNLs have been shown to induce prolonged STAT1 activation and ISG expression compared to Type I IFNs whose effects peak early and decline rapidly [3851]. Our kinetics analyses indicate that Type I IFN affects HLSECs by rapidly inducing *ISG15, OAS1,* and *RIGI* (*DDX58*) with a

decrease over time, whereas *RSAD2* is further increased at 24 hours. HLSECs exposed to IFNLs demonstrated an ISG expression profile that is relatively delayed and narrow (limited to *RSAD2* or Viperin). Viperin has broad-spectrum antiviral effects[52] and is particularly interesting because it can restrict HCV replication by physically associating with the HCV-NS5A protein [39]. As further corroboration, supernatants from IFN-α-stimulated HLSECs increased ISGs in PHHs to a greater extent than supernatants from resting HLSECs (**Supplemental Figure 8C**) suggesting that HLSECs might "prime" adjacent hepatocytes to combat HCV infection. The strong antiviral effector responses by HLSECs provide a plausible mechanism to limit HCV replication, extending the concept of "viral repulsion"[53] to the liver microenvironment. Accordingly, we found that HLECs pre-treated with IFN-α and then exposed to HCVcc express less HCV-NS5A protein (**Supplemental Figure 9**), suggesting that antiviral responses might prevent uptake and sensing of HCV by adjacent HLSECs. Moreover, we found that HLSEC-derived supernatants also up-regulated hepatocyte transcription of suppressor of cytokine signaling-1 (*SOCS1*) (**Supplemental Figure 10**), known to bind to the JAKs and inhibit catalytic activity. This might prove negative regulation of IFNs [54].

Several limitations of our study are worth addressing. We used isolated primary liver endothelial cells described recently [17], *ex vivo* purified LSECs, as well as an immortalized HLSEC line, TMNK-1 [205556]. Prior reports indicate that $CD31^{+}LYVE\text{-}1^{+}$ liver endothelial cells lose their fenestrae in culture [57], but nonetheless, when transplanted into mice, exhibit fenestrae *in vivo*, suggesting that in the appropriate liver microenvironment, these cells resemble true LESCs [45]. We considered using VEGF-A in order to induce fenestrations[5859], but decided against that approach because it could potentially complicate analyses on HCV replication[1560].

Taken together, our compelling data lead to conceptual insights and a novel paradigm identifying HLSECs as central to HCV recognition and immunity (**Figure 7**). These results raise a number of intriguing questions, for example, how the use of exogenous IFN to treat viral hepatitis could be expected to induce additional, previously unrecognized antiviral mechanisms involving HLSECs. Further work is warranted to understand why despite these innate immune responses, HCV is able to establish persistence and fail eradication with IFN-based antiviral therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HCV	hepatitis C virus
HSC	hepatic stellate cells
JFH-1	Japanese Fulminant Hepatitis
KC	kupffer cell
IFN	interferon
ISG	interferon-stimulated genes
LSEC	liver sinusoidal endothelial cell
PAMP	pathogen-associated molecular pattern
PHH	primary human hepatocytes
RIG-I	retinoic acid inducible gene-I
TLR	toll-like receptor

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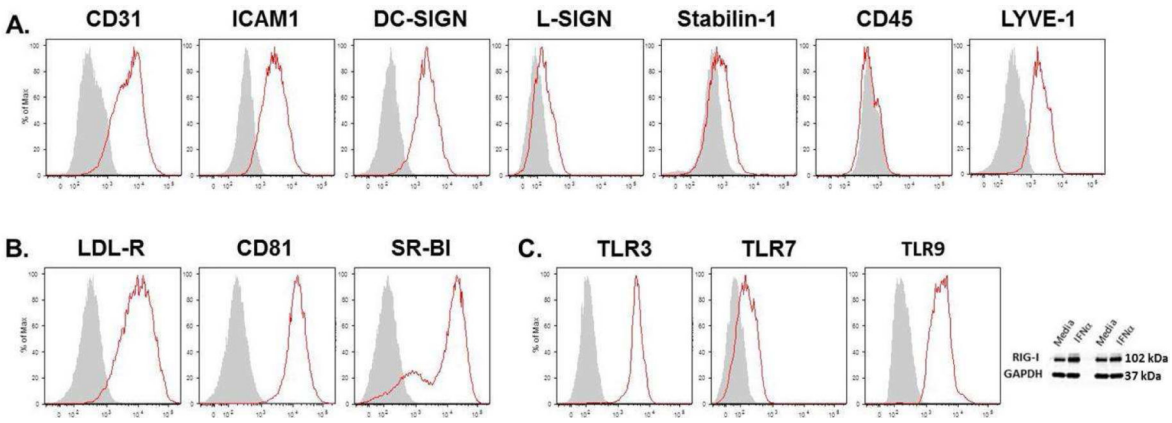

Figure 1. Characterization of primary HLSECs

FACS analysis demonstrates that primary HLSECs are negative for the pan-leukocyte antigen CD45 but do express classic endothelial-associated antigens **(A)**. Several receptors, involved in HCV binding and entry, are also expressed **(B)**. HLSECs are positive for certain Toll-like receptors (TLRs) and RIG-I (further induced by IFN-α stimulation Western blotting) **(C)**. Shaded histograms represent isotype controls.

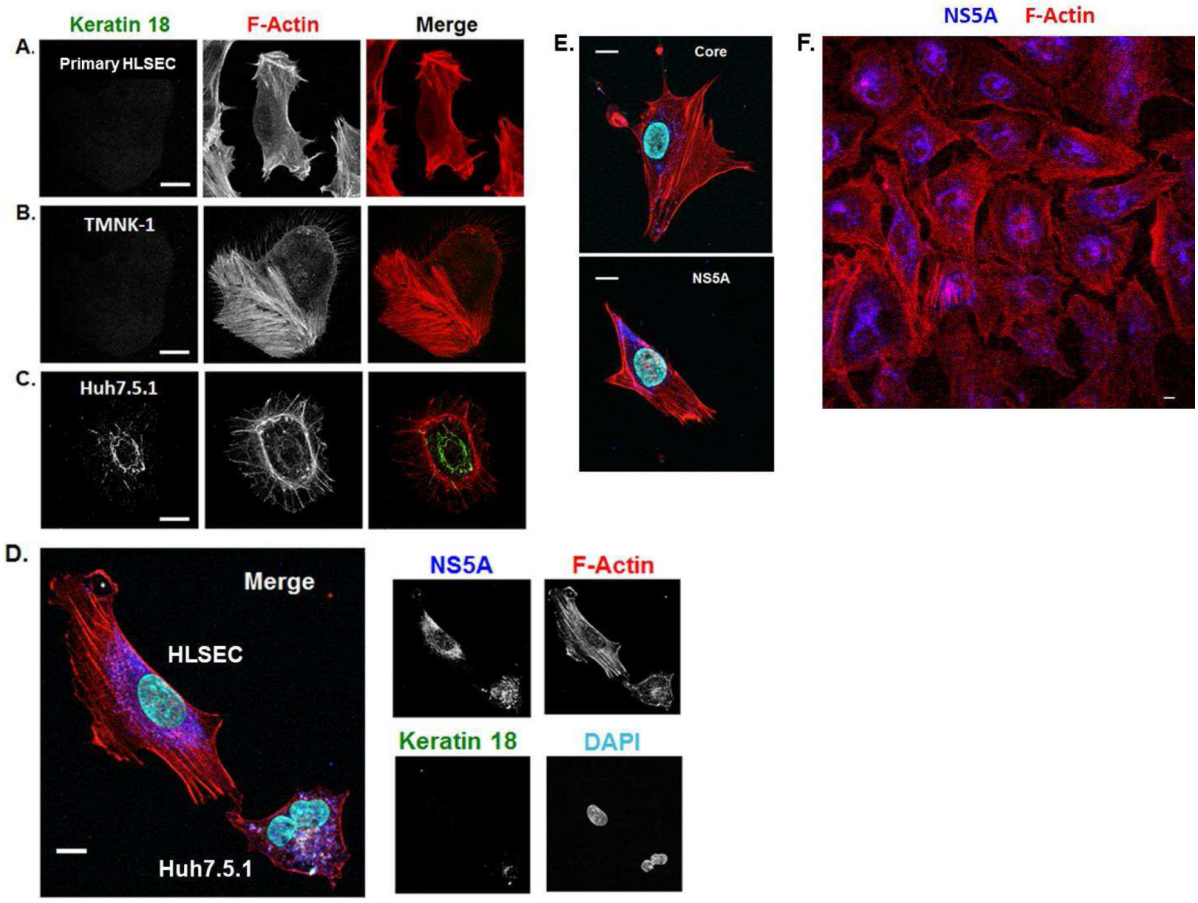

Figure 2. HCV is taken up by HLECs

Primary HLSECs **(A)**, TMNK-1 **(B)** and Huh7.5.1 cells **(C)** were plated for 24 hours and stained for Keratin 18 (K18, green) and F-actin (red). Endothelial cells contrary to hepatocytes do not express K18 (magnification 40X). Co-culture experiment with primary HLECs and Huh7.5.1 was performed as described in the Methods. Cells were stained for NS5A (blue), F-actin (red), K18 (green) and DAPI. HCV-NS5A is detected in both cell types, confirming that HLSECs can uptake HCV (magnification 40X) **(D)**. Primary HLSECs were plated for 24 hours with supernatant of JFH-1-infected Huh7.5.1. Cells were stained for F-actin (red) and core or NS5A (blue) (magnification 40X) **(E)**. The vast majority of TMNK-1 cells exposed to cell-free supernatant from JFH-1-infected Huh7.5.1 cells demonstrated HCV-NS5A protein **(F)** (magnification 25X). Cells were visualized using confocal microscopy as described in the Methods. Bar represents 10 μm.

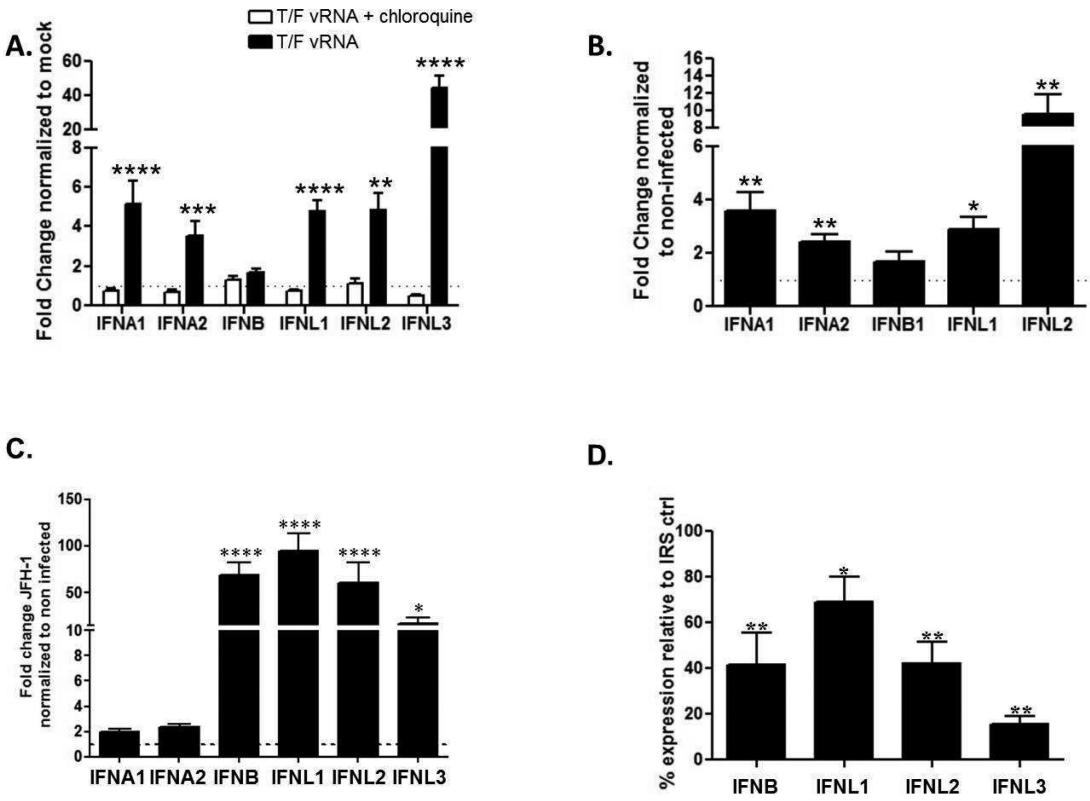

Figure 3. HLSECs respond to various forms of HCV full-length virus

Full-length genotype 1a HCV T/F vRNA was added exogenously to TMNK-1 cells for 24 hours (normalized to mock) in presence (white bars) or absence of (black bars) chloroquine (100 μM) (n=3). Chloroquine significantly inhibits up-regulation of Type I/III IFN genes in response to exogenous T/F vRNA. Mann-Whitney test, two-tailed **(A)**. TMNK-1 cells were treated with sucrose-purified JFH-1 virus for 48 hours at MOI=0.08 (n=3, normalized to non-infected). Wilcoxon Signed rank Test **(B)**. Full length JFH-1-RNA was transfected into TMNK-1 cells for 24 hours (normalized to non-infected) (n=5). Wilcoxon signed-rank test **(C)**. Full length JFH-1-RNA was transfected into TMNK-1 cells for 24 hours in the presence of the IRS661 (TLR7 specific antagonist) or the control (IRS-Ctrl) (n=3). Fold increase of the mRNA was normalized to the IRS-Ctrl considered 100%. *IFNA1* and *IFNA2* were not included because they were not up-regulated by transfected JFH-1. Wilcoxon signed-rank test **(D)**. For every experiment gene up-regulation was assessed by real-time RT-PCR. Bars represent mean plus SEM, * $p<0.05$, ** $p<0.01$, *** $p< 0.001$, **** $p< 0.0001$.

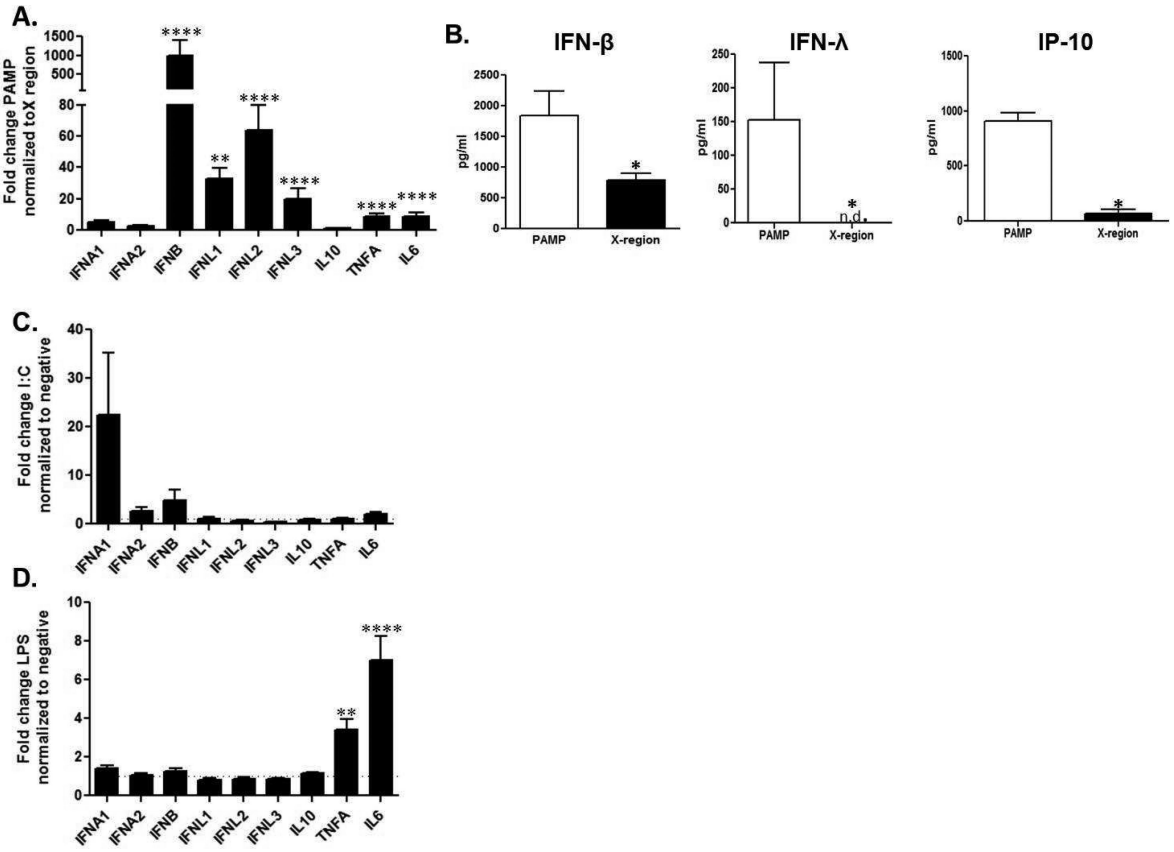

Figure 4. HLSECs sense HCV-RNA and demonstrate differential response to TLR3 and TLR4 ligation

Primary HLSECs were transfected with the HCV-PAMP for 8 hours (normalized to X-region control) [31] (n=8) **(A)**. Protein secretion was higher in supernatants of HCV-PAMP-transfected primary HLSECs compared to control as assessed by ELISA (representing 3 to 7 experiments) **(B).** Primary HLSECs stimulated with Poly I:C (TLR3) **(C)** or LPS (TLR4) **(D)** (normalized to mock) showed different transcriptional profiles (n=4). Gene up-regulation was assessed by real-time RT-PCR. Bars represent mean plus SEM, Wilcoxon signed-rank test, * $p<0.05$, ** $p<0.01$, **** $p< 0.0001$.

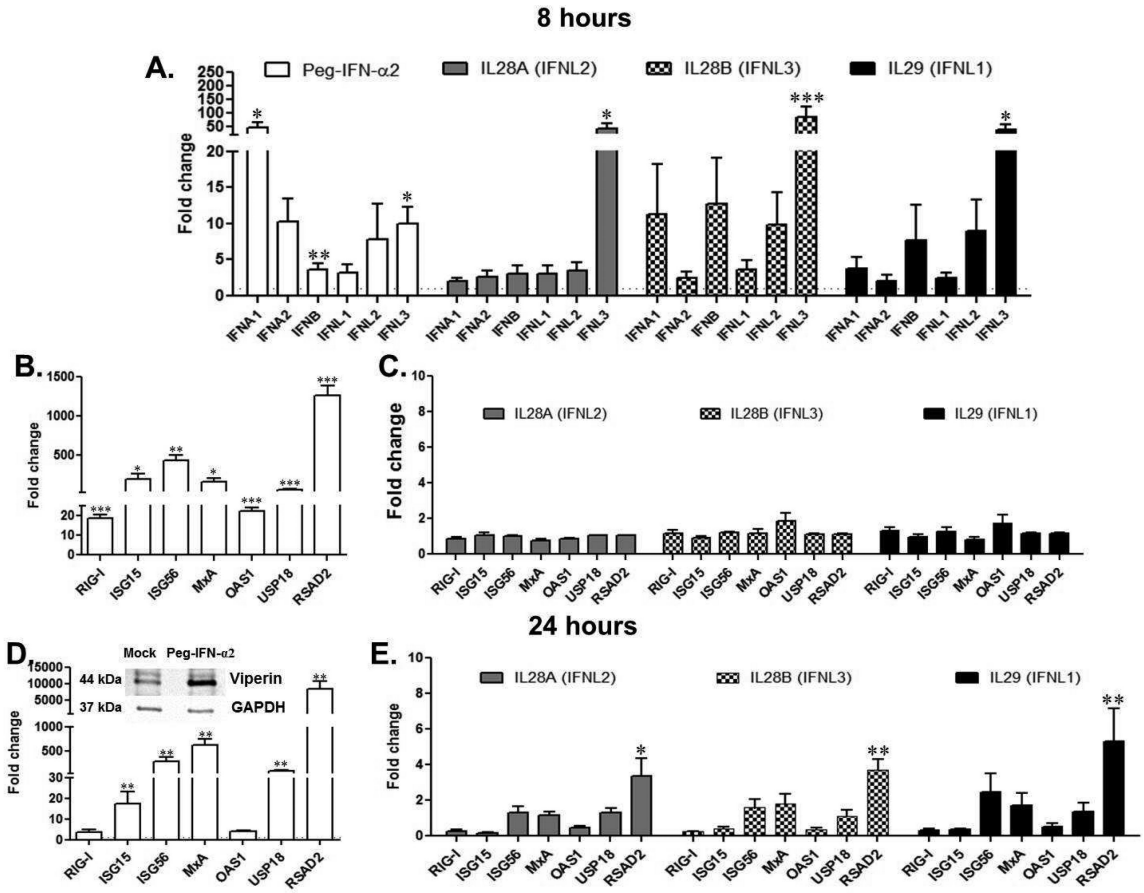

Figure 5. IFN stimulation of HLSECs induces IFN and ISG responses

Primary HLSECs were stimulated with pegylated-IFN-α2 (n=3) or Type III IFNs (n=4) for 8 hours. Pegylated-IFN-α2, IFNL2, IFNL3 and IFNL1 stimulation induced significant up-regulation of *IFNL3* (normalized to mock) **(A)**. Primary HLSECs stimulated with pegylated-IFN-α2 (n=3) for 8 hours induced up-regulation of multiple ISGs **(B)** not observed in primary HLSECs simulated with Type III IFNs (n=4) **(C)**. Stimulation with pegylated-IFN-α2 for 24 hours (n=3) up-regulated ISGs, particularly *RSAD2* (confirmed by Western blot analysis as shown in the inset) **(D)**. All of the Type III IFNs induced significant up-regulation of *RSAD2* after 24 hours stimulation **(E)**. Gene up-regulation was assessed by real-time RT-PCR. Bars represent mean plus SEM, Wilcoxon signed-rank test, * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

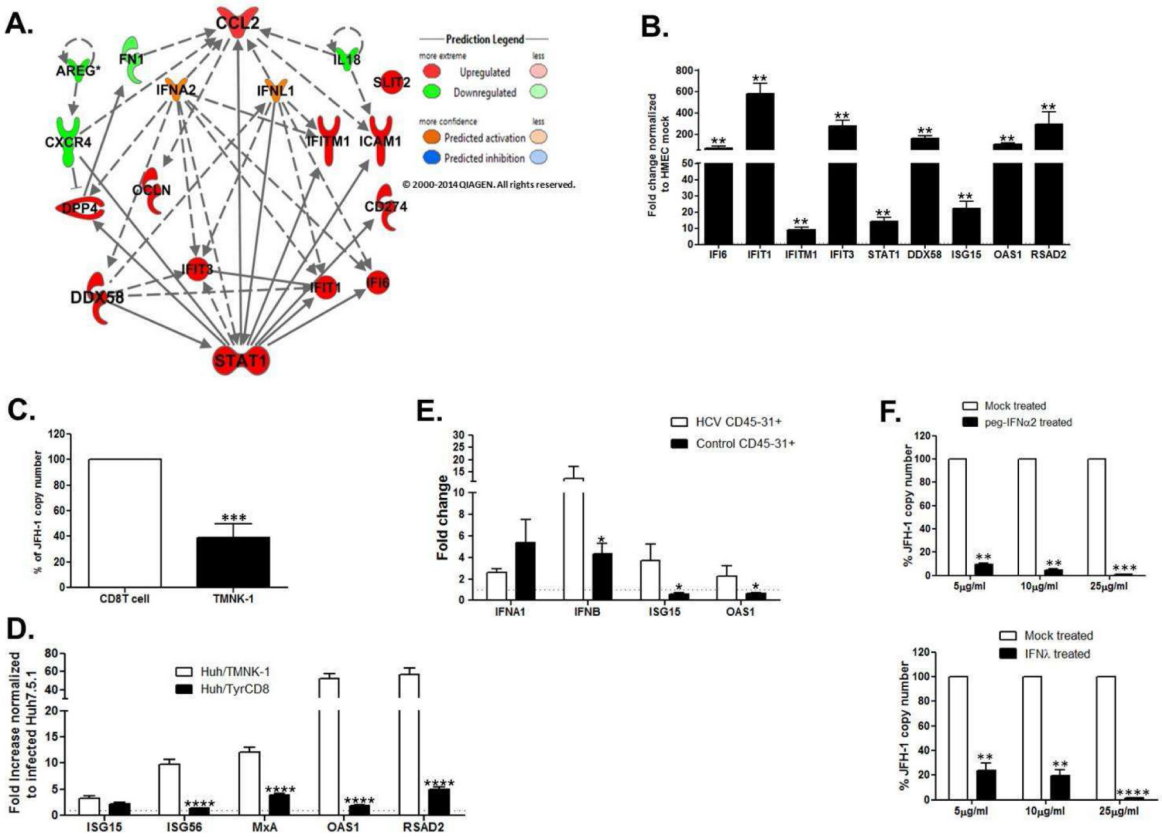

Figure 6. HLSECs demonstrate increased antiviral potential compared to non-liver vascular ECs, up-regulate antiviral genes in HCV-infected livers, and control HCV replication *in vitro*

Pathway analysis demonstrating over-representation of Type I (IFNA2, activation z-score 4.459, p-value=2.89×10^{-15}) and Type III (IFNL1, activation z-score 3.973, p-value=4.30×10^{-11}) interferon upstream regulators in TMNK-1 cells (**A**). Quantitative PCR confirmed relevant ISGs (**B**), fold changes relative to mock HMEC. Wilcoxon sign-ranked test used to determine significance (**p<0.005). On day 4, $CD8^+$ T (white bar) or TMNK-1 cells (black bar), were added to infected Huh7.5.1 for 24 hours (n=3). HCV viral copy number **(C),** and gene up-regulation **(D)** were assessed as described in the Methods. Results were normalized to $CD8^+$ T cells/Huh7.5.1 co-culture considered 100%. Mann-Whitney test, two-tailed. Liver sinusoidal endothelial cells (CD45-CD31+) isolated form HCV-infected individuals significantly up-regulate *IFNB, ISG15* and *OAS1* compared to healthy controls (n=3) **(E)**. Exosomes derived from mock or IFN-treated TMNK-1 cells were added to Huh7.5.1 at the time of infection in increasing doses (5-10-25 μg/ml) for 5 days **(F)**. Exosomes from Peg-IFNα2-treated TMNK-1 were isolated after 24 hours (n=1) and 48 hours (n=1) with pooled data displayed. Exosomes from IFN-λ1/2/3-treated TMNK were isolated after 48 hours (n=3). Results were normalized to Huh7.5.1/mock-treated exosomes culture considered 100%. One-sample t test. Bars represent mean plus SEM, *p<0.05** p<0.01, ***p< 0.001, ****p< 0.0001.

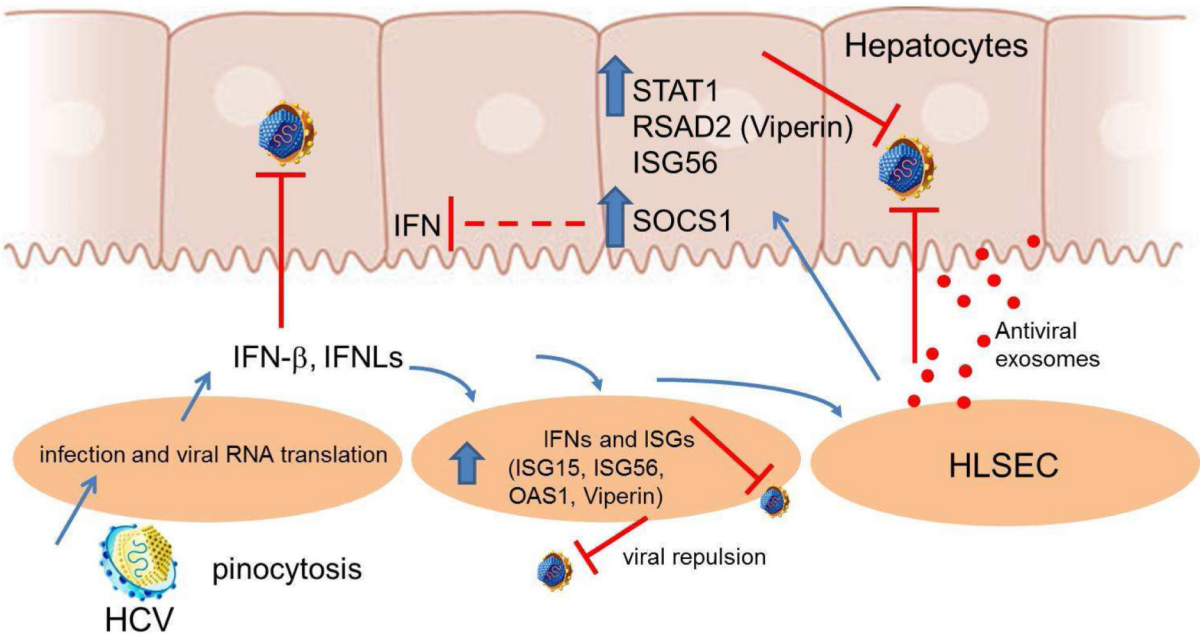

Figure 7. Paradigm for roles of HLSECs in HCV entry, uptake, recognition, and replication

HCV is pinocytosed and viral RNA is translated within HLSECs. HLSECs respond with production of Type I/III IFNs and ISGs activation. This enhanced antiviral state contributes to prevention of new infection within HLSECs, i.e., "viral repulsion" and inhibition of viral replication within hepatocytes. HLSECs supernatants, in addition to stimulating *STAT1* induce up-regulation of *SOCS1* that may counter-regulate the antiviral response in hepatocytes. Exosomes produced by HLSECs following stimulation with either Type I/III IFNs have antiviral properties.