Hepatitis C Virus Sensing by Human Trophoblasts Induces Innate Immune Responses and Recruitment of Maternal NK Cells: Potential Implications for Limiting Vertical Transmission

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Hepatitis C virus (HCV) is the most common blood-borne viral infection for which there is no vaccine. The rates of vertical transmission range between 3 and 6% with odds 90% higher in the presence of HIV coinfection. Prevention of vertical transmission is not possible because of lack of an approved therapy for use in pregnancy or an effective vaccine. Recently, HCV has been identified as an independent risk factor for preterm delivery, perinatal mortality, and other complications. In this study, we characterized the immune responses that contribute to the control of viral infection at the maternal–fetal interface (MFI) in the early gestational stages. In this study, we show that primary human trophoblast cells and an extravillous trophoblast cell line (HTR8), from first and second trimester of pregnancy, express receptors relevant for HCV binding/entry and are permissive for HCV uptake. We found that HCV–RNA sensing by human trophoblast cells induces robust upregulation of type I/III IFNs and secretion of multiple chemokines that elicit recruitment and activation of decidual NK cells. Furthermore, we observed that HCV–RNA transfection induces a proapoptotic response within HTR8 that could affect the morphology of the placenta. To our knowledge, for the first time, we demonstrate that HCV–RNA sensing by human trophoblast cells elicits a strong antiviral response that alters the recruitment and activation of innate immune cells at the MFI. This work provides a paradigm shift in our understanding of HCV-specific immunity at the MFI as well as novel insights into mechanisms that limit vertical transmission but may paradoxically lead to virus-related pregnancy complications.

H. Virois Hepatitis C Virus Sensing by Human Trophoblasts Induces Innate Immune Responses and Recruitment of Maternal NK Cells: Potential Implications for Limiting Vertical Transmission

Silvia Giugliano,* Margaret G. Petroff,†1 Bryce D. Warren,‡ Susmita Jasti,‡ Caitlin Linscheid,† Ashley Ward,‡ Anita Kramer,‡ Evgenia Dobrinskikh,§ Melissa A. Sheiko,‖ Michael Gale, Jr.,‖ Lucy Golden-Mason,* Virginia D. Winn,§ and Hugo R. Rosen*‡‡‡

HCV is the world’s most common blood-borne viral infection for which there is no vaccine. The rates of vertical transmission range between 3 and 6% with odds 90% higher in the presence of HIV coinfection. Prevention of vertical transmission is not possible because of lack of an approved therapy for use in pregnancy or an effective vaccine. Recently, HCV has been identified as an independent risk factor for preterm delivery, perinatal mortality, and other complications. In this study, we characterized the immune responses that contribute to the control of viral infection at the maternal–fetal interface (MFI) in the early gestational stages. In this study, we show that primary human trophoblast cells and an extravillous trophoblast cell line (HTR8), from first and second trimester of pregnancy, express receptors relevant for HCV binding/entry and are permissive for HCV uptake. We found that HCV–RNA sensing by human trophoblast cells induces robust upregulation of type I/III IFNs and secretion of multiple chemokines that elicit recruitment and activation of decidual NK cells. Furthermore, we observed that HCV–RNA transfection induces a proapoptotic response within HTR8 that could affect the morphology of the placenta. To our knowledge, for the first time, we demonstrate that HCV–RNA sensing by human trophoblast cells elicits a strong antiviral response that alters the recruitment and activation of innate immune cells at the MFI. This work provides a paradigm shift in our understanding of HCV-specific immunity at the MFI as well as novel insights into mechanisms that limit vertical transmission but may paradoxically lead to virus-related pregnancy complications.

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provide a potential barrier against maternal–fetal transmission. However, this placental barrier is not completely protective and most viruses (including HCV and hepatitis B virus) can be transmitted to the fetus through the placenta (12). The placenta mediates exchange of nutrients and waste between the maternal and fetal blood supplies via passage across the trophoblast and endothelial cell layers (13). The two primary areas where placental trophoblasts come in contact with the maternal blood and immune system are the villous syncytiotrophoblast, which lines the surface of the placenta, and the extravillous trophoblast (EVT) cells, which migrate out from the placenta and invade the endometrium of the pregnant uterus (decidua). The multinucleate syncytiotrophoblast layer originates from fusion of progenitor cytotrophoblast cells and is bathed by maternal blood delivered by the spiral arteries into the intervillous space. EVTs help form a physical anchor from the placenta to the uterus and are in direct contact with maternal immune and decidua cells as well as blood passing through the maternal spiral arteries (14).

Decidualization is the process in early pregnancy whereby the endometrium transforms into the decidua in preparation for development of the placenta (15). During decidualization, maternal leukocytes traffic to the uterus where the fetus-derived placenta has implanted (16), and they ultimately compose ∼40% of total cells in the decidua (17). Early in pregnancy, ∼70% of decidual immune cells consist of CD56brightCD16neg NK cells (18). The origin of these decidual NK cells is unknown, but it has been speculated that a subpopulation of peripheral NK cells with a similar phenotype traffic to the uterus and subsequently proliferate and differentiate (18, 19). The mechanism by which immune cells are recruited to the uterus is not well understood, but chemokines are known to modulate cell trafficking to sites of inflammation (22) and secondary lymphoid organs (23). EVTs express multiple chemokine receptors and their cognate ligands of inflammation (22) and secondary lymphoid organs (23). EVTs express multiple chemokine receptors and their cognate ligands including the one for CXCR3 and CXCR4 (IP-10, I-TAC, and PD-10, I-TAC, and PD-1) and express multiple chemokine receptors and their cognate ligands of inflammation (22) and secondary lymphoid organs (23). EVTs express multiple chemokine receptors and their cognate ligands including the one for CXCR3 and CXCR4 (IP-10, I-TAC, and SDF-1). Peripheral CD56brightCD16dim NK cells are known to express CXCR3 and CXCR4; therefore, it has been postulated that EVTs might facilitate the migration of this placental immune cell subset into decidual tissue (21, 24). The ability to cross the placental barrier is one key determinant of invasive viruses and pathogens. Little is known about the ability of decidual NK (dNK) cells to provide protective immunity against these agents (25).

The aim of this study was to identify antiviral responses triggered by HCV infection in the human placenta at the time of early intrauterine infection. We found that primary trophoblast isolated from normal pregnancies and an EVT cell line (HTR8) expressed receptors important for HCV viral binding and entry. We also detected the nonstructural protein NS5A, involved in HCV viral replication, within primary term syncytiotrophoblast cells and HTR8 infected with full-length HCV Japanese fulminant hepatitis-1 (JFH-1) virus. Villous explants responded to various forms of HCV, including founder/transmitted virus RNA, sucrose-purified JFH-1 virus particles, full-length JFH-1 RNA, and the HCV pathogen-associated molecular pattern (PAMP), by marked upregulation of type I and III IFNs in villous explants and induced apoptosis in HTR8. In villous explants and HTR8, HCV–RNA transfection also elicited secretion of chemokine chemokines involved in the recruitment of NK cells. Finally, we found that conditioned media from HCV–RNA–transfected HTR8 cells induced recruitment and activation of decidua NK cells. Collectively, these data suggest, to our knowledge for the first time that the placenta functions as a highly active immune organ that coordinates the responses to HCV at the maternal–fetal interface (MFI).

**Materials and Methods**

**Ethics statement**

These studies were approved by the University of Colorado at Denver (UCD) Institutional Review Board (protocol numbers 06-1098, 08-0653, and 08-0364). All adult patients signed the informed consent before samples were taken. A parent or guardian of any child participant signed the informed consent on their behalf.

**Tissue collection**

These studies were approved by the University of Colorado at Denver (UCD) Institutional Review Board (protocol numbers 06-1098 and 08-0653). Placental and decidual samples were collected from normal term deliveries and elective terminations between 10- and 20-wk gestation. Tissue was washed extensively in cold 1× PBS (Ambion, Life Technologies, Grand Island, NY) and stored in cytotwash (DMEM/HighGlucose [Thermo Fisher Scientific, Waltham MA] with 2.5% FBS, 1% penicillin/streptomycin, 0.1% gentamicin, and 1% glutamine [Life Technologies]) for transport on ice. All the samples were processed within 1 h.

**Cytotrophoblast isolation**

Cytotrophoblasts were isolated from first or second trimester (26) and term placentas (27) as described previously. Briefly, placental tissues were cut into small pieces (≥2–3 mm), carefully removing any obvious blood vessels, membranes, and decidual tissue. The fragments of chorionic villi were subjected to a series of enzymatic digestions, which removed the overlying stromal cores of the chorionic villi. Detached cytotrophoblasts were isolated on a Percoll gradient. The final purity of the cells was 90–95% positive for cytokeratin 7, with CD45− cell contamination <10%, as assessed by flow cytometry.

**Villus explant culture**

Placental tissues, stored in cytotwash, were cut into small pieces (≥2–3 mm), carefully removing any obvious blood vessels, membranes, and decidual tissues. The anchoring chorionic villous ends were plated on 8-μm poly-carbonate membrane (Ambion, Life Technologies, Grand Island, NY) and incubated in RPMI 1640 medium with 1% penicillin/streptomycin and 0.1% fungizone (Life Technologies) at 37°C for 24 h. The medium was removed, and the samples were subjected to a series of enzymatic digestions, which removed the overlying stromal cores of the chorionic villi. Detached cytotrophoblasts were isolated on a Percoll gradient. The final purity of the cells was 90–95% positive for cytokeratin 7, with CD45− cell contamination <10%, as assessed by flow cytometry.

**Extraction of decidual mononuclear cells**

Decidual tissue was separated from the placenta and placed in a tube filled with cytotwash medium. The tube was placed on ice, and the sample was prepared within 1 h. The decidua was washed with RPMI 1640 (Invitrogen, Carlsbad, CA; with 10% FBS, 1% L-Glut, 10% penicillin/streptomycin [Life Technologies], 10% antibiotic-antimycotic [Invitrogen] and 3.4 ml IL-2 [Adesleukin, Novartis, Emeryville, CA]), placed on a large petri dish, and cut into small pieces using two scalps. Once the tissue was adequately minced into 3–5-mm pieces, it was placed in a 50 ml conical tube containing digestion medium (HBSS [Invitrogen] with 10% FBS, 250 mg collagenase, and 100 mg DNAse [Sigma-Aldrich, St. Louis, MO]) and placed on a plate rocker at 37°C for 1 h. After the incubation, the tissue was passed through a 70-μm filter, and the cell suspension was centrifuged for 10 min at 335 × g. The supernatant was discarded, and the pellet was resuspended in 30 ml RPMI 1640 medium and spun again as described above. The cells were then frozen at 10 × 10^6/ml in freezing medium (200 ml FBS, 10% RPMI 1640 medium with 1% penicillin/streptomycin [Life Technologies] and 10% human serum) until further analyses.

**Cell lines**

HTR-8/SVneo immortalized first trimester human trophoblast cells (28) (provided by Dr. V. D. Winn, Department of Obstetrics and Gynecology Stanford University Palo Alto, CA) were cultured in growth medium (provided by Dr. V. D. Winn, Department of Obstetrics and Gynecology Stanford University Palo Alto, CA) were cultured in growth medium (28) (provided by Dr. V. D. Winn, Department of Obstetrics and Gynecology Stanford University Palo Alto, CA) were cultured in growth medium (HBSS [Invitrogen] with 10% FBS, 250 mg collagenase, and 100 mg DNAse [Sigma-Aldrich, St. Louis, MO]) and placed on a plate rocker at 37°C for 1 h. After the incubation, the tissue was passed through a 70-μm filter, and the cell suspension was centrifuged for 10 min at 335 × g. The supernatant was discarded, and the pellet was resuspended in 30 ml RPMI 1640 medium and spun again as described above. The cells were then frozen at 10 × 10^6/ml in freezing medium (200 ml FBS, 10% RPMI 1640 medium with 1% penicillin/streptomycin [Life Technologies] and 10% human serum) until further analyses.

**Flow cytometric analysis of Ag expression**

Multiparameter flow cytometry (FACS) was performed using a BD FACSCanto II instrument (BD Biosciences) compensated with single fluorochromes and analyzed using Diva software (BD Biosciences). Primary and immortalized trophoblast cells were identified by their characteristic forward scatter/side scatter properties. Anti–CD45-FITC (clone HI30),
anti-CD45-PE-PE-Cy7 (clone HI30), anti-CD3-V500 (clone UCHT1), anti-CD56-allophycocyanin (clone B159), anti-CD54-V450 (clone B159), anti-TLR9-allophycocyanin (clone eB27-1665), and anti-IFN-γ-V500 (clone B27) were purchased from BD Biosciences. Anti–TLR3-PE (clone TLR3.7) and anti–TLR4-allophycocyanin (clone HTA125) were purchased from eBioscience (San Diego, CA). Anti-low-density lipoprotein receptor (LDL-R)-allophycocyanin (clone 472418), anti–TLR7-PerCP-Cy5.5 (clone 535707), and anti–CD1-1-allophycocyanin (clone 421203) were obtained from R&D systems (Minneapolis, MN). MNCs from PBMCs (clone hLA33) was purchased from BioLegend (San Diego, CA). Rabbit mAb anti-Occulin (clone EPR8202; Abcam, Cambridge, MA) and polyclonal rabbit anti-human scavenger receptor class B type 1 (SR-BI; Novus Biologicals, Littleton, CO) were detected with anti–rabbit-Alexa Fluor 488 Ab (Invitrogen). Anti-CD45 and anti–Cytcokine-7-FITC (clone LP5K; Millipore, Billerica MA) were used to identify primary cytrophoblasts.

One million cells were stained for surface Ag expression at 4°C in the dark for 30 min and then washed in 2 ml PBS containing 1% BSA and 0.01% sodium azide (FACS Wash). The pellet was resuspended in 100 μl 2% paraformaldehyde (PFA; Sigma-Aldrich) and incubated for 20 min at 4°C. For intracellular Ags, 1 ml BD PermBuffer III (BD Biosciences) was added overnight. Cells were then washed and incubated for 30 min in the presence of mAbs in the dark at 4°C and washed in 2 ml FACS Wash. Isotype-matched control Abs were used to determine background levels of staining.

Immunohistochemistry

First trimester (gestation age, 5–8 wk) were obtained from elective pregnancy termination (n = 3–4/Ab) and fixed in 4% PFA overnight, dehydrated through a series of increasing concentrations of ethanol, and embedded in paraffin. Sections (5-μm thick) were placed onto slides and subjected to Ag retrieval using a commercially available citric acid solution (BioCare Medical, Walnut Creek, CA). Alternatively, samples were fixed for 4 h in 1% PFA, transferred to 18% sucrose, and embedded in OCT medium (Ted Pella, Redding, CA). Then, 10-μm-thick sections were placed onto slides. All samples were blocked for nonspecific Ig binding in 10% normal goat, horse, or rabbit serum. Primary Abs against cytokertan 7 (5 μg/ml mouse clone CK-7; Abcam), CD61 (0.8 μg/ml mouse clone B-11; Santa Cruz Biotechnology, Dallas, TX), Claudin-1 (3 μg/ml mouse clone C5-D9; Abnova, Walnut, CA), CD81 (0.8 μg/ml rabbit polyclonal; Sigma-Aldrich), retinoic acid–inducible gene-I (RIG-I) (5 μg/ml goat polyclonal; LSBio, Seattle, WA), SR-BI (5 μg/ml rabbit polyclonal; Thermo Fisher Scientific), TLR3 (4 μg/ml rabbit polyclonal; LSBio), TLR8 (4 μg/ml rabbit polyclonal; LSBio), or their controls were added to the tissues, which were then incubated overnight at 4°C, except in the case of TLR7 (10 μg/ml rabbit clone EPR20882 (2); LSBio) Ab, which was added for 1 h at room temperature. After addition of the appropriate secondary Ab, the samples were depleted of endogenous peroxidases by incubating in 0.5% H2O2/methanol. Ab binding was detected using the avidin–biotin-peroxidase and aminoethyl carbazole reagent sets (Invitrogen), and images were captured on a Nikon Eclipse 80i microscope. Images were viewed microscopically as reddish-brown coloration, and images were captured on a Nikon Eclipse 80i microscope.

Immunofluorescence

Images were acquired on a Zeiss LSM 510 confocal microscope (Zeiss NLO 510 with META; Zeiss Plan–Phakoromat 63/1.4NA oil; Thornwood, NY). Imaging settings were defined empirically to maximize the signal-to-noise ratio and to avoid saturation. In comparative imaging, all settings were kept constant between samples. The illumination was provided by 30 mW argon (488 nm), 5 mW HeNe (633 nm), and 1 mW HeNe (543 nm) lasers. Image processing was performed using Zeiss ZEN 2009 software. Pictures were mounted using Adobe Photoshop CS4 (Adobe Systems).

In the experimental setup, cells were seeded onto 35-mm glass bottom dishes (MatTek, Ashland, MA) and infected on days 1 and 7 with JFH-1 at multiplicity of infection of 10 (multiplicity of infection of 10). After 14 d of infection, cells were fixed in 4% PFA in PBS. Cells were then blocked with 10% normal serum, labeled with primary polyclonal Abs against HCV-NS5A (clone 9E10, 1:100, provided by C. Rice, Rockefeller University) and early endosomal Ag 1 (EEA1) (rabbit polyclonal 1:200; Abcam), and washed and labeled with the appropriate secondary Ab that was conjugated to Alexa Fluor 633 or Alexa Fluor 568 (Invitrogen). F-Actin was concurrently stained with Alexa-Phalloidin 56 (Invitrogen). Images were acquired as described above.

HCV-PAMP preparation

pUUC and X-region plasmids were provided by Dr. M. Gale, Jr. (29). The plasmids were amplified using PCR (X-region forward 5′-TAATACGACTCACTATAGGGTCGTCATCCATCTAGCCATCTA-3′; X-region reverse 5′-ACTGTAATCAGAGAGGACCCAGTAGAT-3′; HCV pUUC forward 5′-TAATACGACTCACTATAGGTCGTCATCCATCTAGCCATCTA-3′; HCV pUUC reverse 5′-AAAGGAAAGAAGAGAAAGAGG-3′) with a high-fidelity polymerase (Invitrogen). The PCR products were separated by electrophoresis on an (1.5%) agarose gel. The bands of interest were extracted from the gel (Gel extraction kit; Qiagen, Valencia, CA) and transcribed in vitro (Applied Biosystems, San Francisco, CA). The final product was quantified using a Nanodrop microspectrometer (Thermo Fisher Scientific).

HCV-PAMP stimulation

Villus explants were isolated from electively terminated in the first and second trimester and grown on Matrigel overnight at 37°C in 5% CO2 in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and 10 mM HEPES. HTR8 cells were plated in a 12-well low-adherence plate at a concentration of 1 × 105 cells in 1 ml/well. The next day, 1 μg pUUC RNA (PAMP) or X-region RNA (control) was transfected into the cells (coinubcation with Muris 2250; Muris Bio, Madison, WI) for 6 or 24 h at 37°C in 5% CO2. Cellular RNA was isolated using RNeasy Mini kit (Qiagen), quantified using a Nanodrop microspectrometer, and 1 μg RNA was used to transcribe cDNA using Quantitect RT kit (Qiagen). Quantitative RT-PCR was performed using SYBR Green primers and master mix from Qiagen and run on a StepOnePlus quantitative PCR machine (Applied Biosystems). Data were analyzed using the ΔΔCT method. All primers used in the quantitative RT-PCR assays were purchased from Qiagen.

To assess apoptosis, HTR8 were plated overnight, and the next day HCV-PAMP (or X-region control) transfection was performed as described above. Cells were harvested after 24 h of culture and stained, as described above with antiapoptotic caspase-2-AF647 and Annexin V (BD Biosciences) following manufacturer’s instructions.

Chemotaxis assay

NK cells were isolated using CD56 magnetic–labeled beads (Miltenyi Biotec, San Diego, CA) from PBMCs of healthy donors (patients were recruited and consented using UCS Institutional Review Board approved protocol number 08-0364) and decidual mononuclear cells (DMNCs) of elective terminations (10- and 20-wk gestation). Purity of isolated NK cells was assessed by flow cytometry (>80%). Cells were loaded (100 μl) onto Transwell filters (5-μm pore size, 24-well cell clusters; Corning) and were incubated in wells containing 600 μl conditioned medium of PAMP-transfected, X-region-transfected, JFH-1-transfected, mock-transfected or mock control media for 3 h at 37°C and 5% CO2. The upper chambers were then removed and cells in the bottom chamber were collected and counted by flow cytometry. The migration index (MI) was calculated using the formula: MI = M/M0, where M0 is the migration area with PAMP and X-region–transfected or JFH-1 and mock-transfected conditioned medium, and M is the mean migration area of control cultures.

ELISAs

IFNA (VeriKine Human IFN-α [PBL IFN Source, Piscataway, NJ]), IFNL (VeriKine-DY Human IFN A [IL-28B/29/28A]), and the Multi-Analyte ELISArray (Qiagen) were performed according to the manufacturer’s instructions. All supernatants were harvested at 24 or 48 h after PAMP or X-region stimulation. Conditioned medium of JFH-1 and mock-transfected villous explants were harvested after 48 h and analyzed by Multi Analyte ELISArray (Qiagen), according to manufacturer’s instructions.

Statistical analysis

Results are expressed as mean ± SEM. The Wilcoxon signed-rank test was used to compare fold increases of stimulated conditions with control conditions. The Mann–Whitney U test and t test were used to compare differences between groups. Calculations were performed using Graphpad Prism software. Statistical significance was defined as p < 0.05.

Results

Human trophoblast cells express cellular receptors relevant for HCV uptake and sensing

To investigate the expression of cell surface receptors important for HCV binding and entry, we stained primary human villous cytrophoblast cells, isolated from first or second trimester elective pregnancy terminations (30, 31), for the SR-BI, the tight junction components Claudin-1 and Occludin, the LDL-R and the tetraspanin CD81. Primary trophoblast cells expressed high levels of Occludin, Claudin-1, and LDL-R, which are necessary for HCV uptake.
binding and entry (Fig. 1A). Immunohistochemical staining for HCV binding and entry receptors confirmed in situ expression of these proteins in trophoblast cells (Fig. 1B). First trimester syncytiotrophoblast and villous cytotrophoblast cells, both of which can be identified by cytokeratin 7 immunoreactivity, strongly and uniformly expressed SR-BI, Claudin-1, and LDL-R. Notably, syncytiotrophoblast expressed SR-BI most strongly at the apical brush border, which directly interfaces with maternal blood. Fetal fibroblasts and macrophages were also positive for SR-BI, Claudin-1, LDL-R, and CD81. EVT cells that are associated with cell columns also strongly expressed SR-BI, Claudin-1, and LDL-R; again, these cells were negative for CD81 (data not shown). Taken together, flow cytometric analysis and immunohistochemical staining unequivocally show that human villous cytotrophoblast cells and syncytiotrophoblast express multiple receptors necessary for HCV entry.

We next asked whether trophoblast cells express the pattern recognition receptors (PRRs) necessary for conferring responsiveness to infection by HCV. Primary cytotrophoblast cells and villous explants express multiple PRRs implicated in innate antiviral immunity including Toll-like receptors (32, 33). Flow cytometry analyses of primary villous cytotrophoblast cells (isolated from first or second trimester placentas) showed expression of TLR3, TLR7, and TLR9 (Fig. 1C). Immunohistochemical staining of first-trimester placentas revealed strong immunoreactivity of cytotrophoblast, syncytiotrophoblast, and EVT cells for the RIG-I, the cytosolic receptor for HCV (34), as well as TLR3; weaker, but positive immunoreactivity was observed for TLR7 (Fig. 1D). Syncytiotrophoblast and EVTs were also positive for TLR8; weaker and more variable staining of cytotrophoblast was observed. Further, fetal macrophages were strongly positive for all four pattern recognition receptors. The immortalized first trimester EVT cell line (HTR8) was also phenotyped by flow cytometry (Supplemental Fig. 1), showing a profile similar to the primary cytotrophoblasts.

Human trophoblasts are permissive for HCV uptake

To determine whether HCV is taken up by human trophoblasts, we cultured primary term cytotrophoblast (Fig. 2A, 2B) and HTR8 cells (Fig. 2C, 2D) with full-length JFH-1. After 14 d of culture with JFH-1–infected conditioned medium, primary syncytiotrophoblasts demonstrated HCV viral protein, in keeping with and extending a recent report (35). Colocalization of NS5A with the EEA1 suggests that endocytosis is involved in HCV uptake (Fig. 2E) by human trophoblast cells.

Innate responses induced by HCV sensing in human trophoblasts

To elucidate the immune responses generated by HCV, we transfected human trophoblasts with a PAMP specifically expressed by HCV (polyuridine motif of the HCV genome 3′-nontranslated...
region), previously shown to function as the substrate of RIG-I, the cytosolic PRR for HCV (29, 36, 37). Extensive analysis and characterization of the HCV genomic RNA has identified that the pU/UC tract in the 3′-untranslated region (3′-UTR) has the greatest capacity to stimulate IFN-β (IFNB) production in hepatocytes (29). As a negative control, trophoblast cells were transfected with the adjacent highly-conserved X-region (3′-UTR), which is non-immunogenic in hepatocytes (29). HCV-PAMP transfection of HTR8 cells (Fig. 3A) and also of villous explants (Fig. 3B) for 6 h induced robust upregulation of several key antiviral genes. Of particular interest is the induction of type III IFN mRNAs (IFNL1 [IL29], IFNL2 [IL28A], and IFNL3 [IL28B]), recently identified as critical for the host defense against HCV (1, 38, 39). Moreover, supernatants from HCV-PAMP–stimulated placental explants showed extremely high levels of IFNλ (IFNL) (>5000 pg/ml) at 24 h (Fig. 3C) compared to control (29, 40). IFN-α (IFNA) production was also 2.3-fold higher (58.9 pg/ml) in PAMP-transfected villous explants compared with our specificity control (data not shown). Next, we transfected villous explants for 6 h with full JFH-1 RNA. Also, in this case we detected significant upregulation of type I and type III IFN genes, similar to the immune response we observed after HCV-PAMP RNA transfection (Fig. 3D). Moreover, when villous explants were cultured with supernatant containing sucrose-purified JFH-1 viral particles (Fig. 3E) (41) and viral RNA of a full-length infectious molecular clone of HCV known to efficiently transmit infection (transmitted founder [T/F] virus (42, 43)) (Supplemental Fig. 2) for 48 h, we found significant transcription of multiple type I and type III IFN genes. These data indicate that HCV is taken up directly by human trophoblasts and induces innate immune responses.

**HCV sensing induces apoptosis in human EVT cells**

Next, we determined the impact of HCV sensing on the viability of human EVT cell line HTR8 to define whether HCV infection could alter normal placental development during the early gestational stages. HCV-PAMP transfection for 24 h induced a proapoptotic response within HTR8 cells, as evidenced by a significant increase in Annexin V staining (Fig. 4A, 4B) and caspase-3 activation (Fig. 4C, 4D) compared with HCV X-region stimulated control. Induction of apoptosis was also investigated after JFH-1 transfection. HTR8 transfected for 24 h with JFH-1 RNA induced significant increase of capsase-3 activation compared with mock-transfected cells (Supplemental Fig. 3).

**HCV sensing by human trophoblasts induces secretion of chemotactic molecules**

To elucidate whether HCV sensing by trophoblast cells induces upregulation of chemokines involved in the recruitment of immune cells, we transfected villous explants from first and second trimester of pregnancy with HCV-PAMP versus X-region control for 24 h and examined chemokine gene upregulation. PAMP-transfected villous explants induced strong upregulation of several chemokine genes that are known to play a central role in NK cell recruitment (Fig. 5A). Specifically, CCL5 (RANTES), CXCL10 (IP-10), and CXCL11 (I-TAC) were upregulated. A similar response was observed after transfection of villous explants with JFH-1 RNA (Fig. 5B). Chemokines (e.g., CXCL10) that are known to play key roles in the recruitment of dNK cells (44) were also confirmed at the protein level by ELISA after 48 h of transfection with PAMP and JFH-1 RNA (Fig. 5C, 5D). Interestingly, CXCL11 gene upregulation observed in both experimental
settings was not confirmed at the protein level. When villous explants were cultured with sucrose-purified viral particles for 48 h, we detected significant upregulation of the CXCL11 (I-TAC) and CXCL12 (SDF-1) genes (Fig. 5E). Exposure of villous explants to T/F viral RNA also induced significant CXCL10 (IP-10) and CXCL11 (I-TAC) gene upregulation (Supplemental Fig. 2). These results indicate that HCV sensing by human trophoblasts induces release of chemotactic mediators that might elicit recruitment of dNK cells.

We also addressed whether the EVT cells, which invade the maternal decidua and are in direct contact with maternal immune leukocytes, release chemokines after sensing HCV-RNA. Considering the technical difficulty in isolating purified EVTs for in vitro studies (18), we performed the experiments using the first-trimester immortalized cell line HTR8. We transfected HTR8 cells with HCV-PAMP for 48 h and examined gene upregulation and protein secretion of chemotactic chemokines involved in NK cell recruitment. PAMP transfection of HTR8 induced significant upregulation of multiple chemokines at the gene (Fig. 6A) and protein level (Fig. 6B), including CCL3 (MIP-1α), CCL5 (RANTES), and CXCL8 (IL-8). These chemokines have established roles in the recruitment of NK cells to the decidua (20, 45).

**HCV sensing by human trophoblasts elicits recruitment and activation of dNK cells**

To assess whether HCV sensing by HTR8 could induce recruitment of peripheral NK (pNK) cells, we performed a chemotaxis assay. We cultured CD56 bead–purified pNK cells from three healthy individuals on Transwell filters plated in wells containing conditioned media from PAMP and X-region–transfected HTR8 (Fig. 7A). After 3 h of culture, we detected a significantly increased cell MI of pNK cultured with PAMP-transfected conditioned medium. To assess whether migration of dNK cells was altered in a similar way by HCV-PAMP–conditioned medium, we repeated the experiment using dNK cells from three early
termination pregnancies (first and second trimester). Similarly, dNK cells cultured with PAMP-transfected conditioned medium showed significantly higher migration index compared with dNK cultured with X-region–transfected medium (Fig. 7B). When dNK cells were cultured with supernatant of JFH-1–transfected HTR8, we observed significantly higher migration index compared with dNK cells cultured with mock-transfected medium (Fig. 7C). These results suggest that HCV sensing by EVTs elicits release of chemotactic molecules able to recruit both pNK and dNK cells to the MFI.

Next, we wanted to define whether dNK cells cultured with PAMP-transfected conditioned medium for 24 h were more cytotoxic compared with cells cultured with X-region–transfected conditioned medium and media control. It is known that dNK cells contain high levels of perforin and granzyme (comparable to CD56dim pNK cells); however, they display a poor ability to kill classical NK targets (18, 46). Correspondingly, CD56 bead–purified dNK cells cultured for 24 h with PAMP-transfected medium showed a significantly higher degranulation rate compared with dNK cells cultured with mock-transfected medium (Fig. 7D). Cells cultured with X-region–transfected conditioned medium did not show a significant higher activation state compared with cells cultured with control medium. The same experiment was performed using DMNCs, and again, in this case we observed higher degranulation rate (data not shown) and significantly higher IFN-γ secretion (Fig. 7E) in dNK cells cultured with PAMP-transfected HTR8. When DMNCs were cultured with supernatant of JFH-1–transfected HTR8, we observed significantly higher CD16 expression in dNK cells compared to control cells (Fig. 7F). Taken together, our data suggest that HCV-transfected conditioned media contains soluble components that activate dNK cells through mechanisms that might play a role in limiting the spread of HCV to fetal tissue.

Even though maternal HCV infection is an independent risk factor for pregnancy complications, its effect on pregnancy is not devastating. Therefore, we investigated whether a compensatory mechanism limiting dNK activation at the MFI could be induced by HCV sensing. There is evidence that increased pNK cells and activated NK cells are associated with increased risk of miscarriage and failed in vitro fertilization treatment (47, 48). The healthy trophoblast does not express classical HLA-A and HLA-B products; however, trophoblasts do express the nonclassical MHC class I molecule HLA-E, the ligand for the inhibitory CD94/NKG2A and activating CD94/NKG2C receptors (49). Increased expression of HLA-E within the livers of HCV-infected patients has been implicated as an immune evasion strategy because it suppresses innate immune cell function (50). Therefore, we explored whether HCV sensing by trophoblasts affected HLA-E expression. FACS phenotyping reveals that HLA-E was constitutively expressed on trophoblasts, and it was further upregulated by HCV-PAMP and JFH-1 transfection compared with control transfection (Fig. 8A, 8B). To investigate the effect that HLA-E upregulation induced by HCV-sensing might have on dNK, we transfected HTR8 with JFH-1 for 24 h and cocultured them with DMNCs additionally for 24 h. dNK cells cocultured with JFH-1–transfected HTR8 showed significantly higher IFN-γ and TNF-α production.
secretion but no significant reduction in CD107a (Fig. 8C–E). No differences were observed in NKG2A and NKG2C expression levels (data not shown). Collectively, our results suggest that HCV sensing by EVT cells activates dNK cells that might contribute to viral control but also be responsible for immune-mediated pathology triggered by HCV.

Discussion

The pregnant state represents a paradoxical challenge whereby tolerance to the allogeneic fetus is balanced with host defense against pathogens (51). Immune responses against microorganisms at the MFI may have a significant impact on pregnancy outcomes. Multiple components of the immune system can survey, recognize, and eliminate invading microorganisms with variable success rates (32, 52–54). For example, whereas 70% of children born to mothers positive for hepatitis B e Ag become chronically infected (55), the rate of mother-to-child transmission of HCV is significantly lower (9, 56). In this study, we tested the hypothesis that fetal trophoblasts recognize and respond to HCV infection as a mechanism to explain decreased transmission.

Both human villous and EVT cells express many of the receptors involved in HCV entry and innate signaling. Accordingly, by immunofluorescence, we detected HCV NS5A protein after a 2-wk culture period colocalized with the early endosomal marker EEA1, suggesting endocytosis is likely involved in the uptake of HCV by human trophoblast cells. These results expand an electron microscopic study demonstrating HCV-like particles within cytotrophoblasts near the rough endoplasmic reticulum (35). Further work is warranted to examine different steps in the viral life cycle within trophoblast cells. Using sucrose-purified HCV without potential contaminants from infectious cell culture–derived HCV (57) and the exogenous HCV-specific T/F viral RNA, we demonstrated that HCV directly triggers type I and III IFN responses from primary villous trophoblasts. Furthermore, HCV-PAMP

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** EVT cells sensing HCV secrete chemokines. (A) HTR8 cells were transfected for 48 h with HCV-PAMP or X-region control. Gene upregulation was assessed by real-time RT-PCR. Bars represent mean ± SEM, Wilcoxon signed-rank test (n = 3). (B) Supernatants from PAMP-transfected HTR8 has a significantly higher CCL3 (MIP-1α), CCL5 (RANTES) and CXCL10 (IP-10) protein concentration compared with the control (X-region–transfected cells) as assessed by ELISA. Bars represent mean ± SEM, Mann–Whitney U test (n = 3). *p < 0.05, **p < 0.01.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** EVT cells sensing HCV elicit recruitment and activation of NK cells. Supernatant of PAMP-transfected HTR8 cultured with CD56 bead-purified pNK (A) and dNK (B) cells for 3 h induced a significantly higher migration index of pNK and dNK compared with the control. (C) CD56 bead-purified dNK cells showed significantly higher migration index also when cultured with supernatant of JFH-1–transfected HTR8. (D) The percentage of degranulating (CD107a+) CD56 bead–purified dNK cells cultured with PAMP-transfected, X-region–transfected or control medium is displayed. Compared with control medium, PAMP-transfected medium enhanced cytotoxicity of dNK. (E) DMNCs were cultured for 24 h with supernatant of PAMP or X-region–transfected HTR8. dNK cells (CD3neg, CD56high) showed significant higher IFN-γ secretion when cultured with PAMP-conditioned medium. (F) The same experimental setting was performed using conditioned medium of JFH-1–transfected HTR8. In this case, dNK cells showed significant higher CD16 expression when cultured with JFH-1–transfected supernatant. DMNCs and dNK cells were isolated from decidua of early terminated pregnancy between 10- and 20-wk gestation. Lines represent mean, t test. *p < 0.05, **p < 0.01, ***p < 0.001.
and JFH-1 transfection of placental explants consistently upregulated mRNA. Gene upregulation of IFNA and IFNL was also confirmed at the protein level in PAMP-transfected explants. Of interest, a recent study shows a greater spontaneous recovery rate among infants infected with HCV who have the IFNL3 (IL-28B) CC rs12979860 polymorphism (58).

Another important antiviral mechanism in tissue involves the migration of innate immune cells. The first trimester of pregnancy is characterized by high numbers of dNK cells within the decidua, perhaps accounting for the low rate of congenital CMV infection (25). It has been shown that NK cells that infiltrate during the first trimester can switch their phenotype after sensing infection and that dNK cells can be found in the vicinity of infected cells within floating chorionic villi (59). Thus, modulation of their migration phenotype might be a response to invading pathogens. To our knowledge, for the first time, we present data that HCV-RNA sensing by villous explants and EVTs induced remarkably broad and robust expression of chemokines, including CXCL10 (IP-10), CCL3 (MIP-1α), and CCL5 (RANTES) (20, 44, 45), that are known to elicit recruitment of NK cells. Hepatitis C viral sensing by EVTs, which invade deep into the uterus wall (60) and are in direct contact with maternal immune cells (14), leads to recruitment of pNK and dNK cells. Furthermore, dNK cells showed significantly higher expression of CD16, IFN-γ secretion and increased degranulation when cultured with conditioned medium of HCV-transfected EVT cells. Interestingly, we also detected a higher HLA-E expression in EVT cells transfected with HCV-PAMP and JFH-1 RNA. HLA-E is the ligand for the inhibitory receptor CD94/NKG2A expressed at high levels in all dNK cells and for the activating receptor CD94/NKG2C (18). DMNCs cocultured with JFH-1–transfected HTR8 showed significant higher IFN-γ and TNF-α secretion but a nonsignificant reduction in CD107a expression. DMNCs were isolated from decidua of early terminated pregnancy between 10- and 20-wk gestation. Lines represent mean, t test. *p < 0.05, **p < 0.01.

The fact that HCV-PAMP–transfected EVT cells demonstrated increased apoptosis, in keeping with prior studies that demonstrated that viral ssRNA induces apoptosis in first-trimester trophoblasts through the activation of an inflammatory mechanism (33), points to an additional regulatory mechanism. These results underscore the delicate balance between immune response that controls the virus and an overzealous response that damages the MFI (32, 52–54, 61). An

![FIGURE 8](http://www.jimmunol.org/)

**FIGURE 8.** HCV sensing by EVT cells induces HLA-E upregulation and cytokine production by dNK cells. (A) HLA-E expression was upregulated in HTR8 transfected with HCV-PAMP (compared with the control X-region) for 24 h. (B) The same upregulation was observed in HTR8 transfected with JFH-1 RNA compared with mock-transfected cells. One representative experiment of n = 3 is shown. (C-E) DMNCs were added to JFH-1–transfected (or mock-transfected) HTR8 after 24 h culture. dNK cells (CD3negCD56high) cocultured for 24 h with JFH-1–transfected HTR8 showed significant higher IFN-γ and TNF-α secretion but a nonsignificant reduction in CD107a expression. DMNCs were isolated from decidua of early terminated pregnancy between 10- and 20-wk gestation. Lines represent mean, t test. *p < 0.05, **p < 0.01.

![FIGURE 9](http://www.jimmunol.org/)

**FIGURE 9.** Working model of the role of the placenta in the context of HCV infection. Primary trophoblasts express receptors involved in HCV binding and entry. Trophoblasts internalize HCV and respond to HCV-RNA sensing with production of IFNs and induction of chemokines responses that recruit and activate dNK cells at the MFI. Furthermore, HCV sensing upregulates HLA-E expression and triggers an apoptotic response in human EVT cells that may contribute to pregnancy-related complications.
excessive immune response may account for the higher complication rate in HCV-infected mothers. Collectively, these data provide conceptual insights and a novel paradigm for innate immune signaling by fetal-derived trophoblasts (Fig. 9), which contributes to local control of HCV and attenuated transmission. These results have broader implications for understanding protection against vertical transmission of infections.

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References
Corrections


The authors revised the grant footnote to include additional funding information. The corrected footnote is shown below:

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