Hepatitis C Virus Targets the T Cell Secretory Machinery as a Mechanism of Immune Evasion

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T cell activation and the resultant production of interleukin (IL-2) is a central response of the adaptive immune system to pathogens, such as hepatitis C virus (HCV). HCV uses several mechanisms to evade both the innate and adaptive arms of the immune response. Here we demonstrate that liver biopsy specimens from individuals infected with HCV had significantly lower levels of IL-2 compared with those with other inflammatory liver diseases. Cell culture–grown HCV particles inhibited the production of IL-2 by normal peripheral blood mononuclear cells, as did serum from HCV-infected patients. This process was mediated by the interaction of HCV envelope protein E2 with tetraspanin CD81 coreceptor. HCV E2 attenuated IL-2 production at the level of secretion and not transcription by targeting the translocation of protein kinase C beta (PKCβ), which is essential for IL-2 secretion, to lipid raft microdomains. The lipid raft disruptor methyl-β-cyclodextrin reversed HCV E2-mediated inhibition of IL-2 secretion, but not in the presence of a PKCβ-selective inhibitor. HCV E2 further inhibited the secretion of other cytokines, including interferon-γ. Conclusion: These data suggest that HCV E2–mediated disruption of the association of PKCβ with the cellular secretory machinery represents a novel mechanism for HCV to evade the human immune response and to establish persistent infection. (HEPATOL OGY 2011;00:000-000)

The hepatitis C virus (HCV), a member of the flavivirus family phylogenetically classified into seven genotypes, is an enveloped, icosahedral particle harboring a positive-strand RNA.1-3 Binding of HCV to the host cell involves an initial interaction between its envelope protein (E1/E2) and the receptors required for viral entry, potentially including CD81, scavenger receptor B type I low density lipoprotein receptor (LDL-R), and claudin-1 (CLDN1) (reviewed in Stamataki et al.4). The CD81 molecule, a member of the tetraspanin superfamily, binds HCV E2 with high affinity through its large extracellular loop.5-6 Up to 80% of HCV cases result in chronic hepatitis associated with liver fibrosis, cirrhosis, hepatocellular carcinoma, and in some cases non-Hodgkin lymphoma.7,8 HCV evades the host immune response through a combination of both viral genetic mutation and interference with both innate and adaptive arms of the host immune response.9-11 T cell–mediated immunity is important for prevention of persistent infection with impaired T cell proliferative responses and changes in effector function associated with chronic infection.12-14 We previously demonstrated that a recombinant soluble form of HCV E2 interacts with CD81 to inhibit T lymphocyte migration through...
relocalization of signaling molecules to the lipid raft compartment.15

We and others have shown that expression of protein kinase C beta (PKCβ) is necessary for secretion of the cytokine interleukin-2 (IL-2) in T cells.16,17 Chronically infected HCV patients frequently demonstrate a failure of their CD4+ T helper cells to secrete IL-2,18 and reduced CD4+ T cell proliferative capacity during acute infection is reported to contribute to viral persistence.19 We hypothesize that HCV E2/CD81–dependent sequestration of PKCβ into lipid raft compartments could reduce IL-2 secretion and contribute to HCV persistence. Using the recently developed HCV cell culture system (HCVcc)20 and recombinant HCV E2, we demonstrate that HCV E2 engagement of CD81 sequesters critical components of the T cell secretory machinery (including PKCβ) in the lipid raft compartment with resultant inhibition of cytokine secretion.

Materials and Methods

Reagents. HCV recombinant E2 protein, core protein C22, and protein from NS3 region C33 (purity >98%), were a kind gift from Sergio Abrignani, Chiron Corporation, Emeryville, CA. A blocking peptide (BP), previously proven to inhibit CD81–E2 interaction,21 sequence CSPQYWTGPAC [OH], and control scrambled peptide CPWSAGYTQPC [OH] were prepared by the Organic Synthesis Core, Royal College of Surgeons, Ireland (purity >98%).

Cell Culture. HuT 78 cells (American Type Culture Collection, Rockville, MD) were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, Paisley, UK) containing supplements.16 Peripheral blood mononuclear cells (PBMCs), obtained from healthy volunteers, were separated on Ficoll-Histopaque density gradient (Fresenius Kabi Norge AS, Oslo, Norway). The effect of HCV infectious serum on IL-2 production was tested using PBMCs from normal donors stimulated with plate-bound anti-CD3 or anti-CD3 and anti-CD28 (Pharmingen, San Diego, CA). For these experiments, normal/PCR+/PCR− serum (100 μL in a final volume of 500 μL serum-free medium) was incubated with cells for 1 hour prior to stimulation.

HCVcc Genesis. HCVcc was generated as described.22 Briefly, RNA was transcribed in vitro from full-length genomes using the Megascript T7 kit (Ambion, Austin, TX) and electroporated into Huh-7.5 cells. High-titer stocks were generated by 2 serial passages through naïve Huh-7.5 cells. Supernatants were collected at 72 and 96 hours after infection, pooled, concentrated, and stored at -80°C.

Tissue Specimens. Permission was received from the Ethics Committees of both St Vincent’s and St James’s Hospitals, Dublin, for all work on human tissue. Informed consent was obtained from all subjects. Normal liver wedge biopsies were obtained from donor organs. HCV-infected liver was obtained at time of transplantation for end-stage liver disease. Liver samples were immediately washed three times in Hank’s balanced salt solution and snap-frozen in liquid nitrogen, powdered using the Braun Mikrodismembrator II (Braun Apparate, Melsungen, Germany). Protein was extracted from ≈100 mg powdered tissue using 300 μL of lysis buffer (1% deoxycholic acid, 0.1% sodium dodecyl sulfate in phosphate-buffered saline) containing protease inhibitors. The extract was passaged several times through a 21-gauge needle (Beckton Dickinson) in lysis buffer, incubated on ice for 30 minutes, and centrifuged at 10,000g for 10 minutes at 4°C. Supernatant was harvested and total protein was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Immunohistochemical Staining and T Cell Counts. Formalin-fixed paraffin-embedded explant liver sections were immunostained with anti-CD3 or isotype-matched immunoglobulin G (DAKO) using the avidin-biotin complex immunoperoxidase method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA). Sections were microwaved for antigen retrieval in a 0.1 M sodium citrate buffer for 12 minutes prior to staining. Sections were evaluated for the presence of CD3+ cells using an Olympus light microscope by two independent observers. Positive cells in both portal tract and parenchyma were counted within a fixed-area graticule at a magnification of ×400. The number of each cell type per mm2 of portal tract and parenchyma was calculated by counting positive cells in 10 portal tracts and in every tenth field of parenchyma, respectively.

Immunofluorescence Analysis. Immunofluorescence assays were performed in LabTek 8-well Permanox chamber slides (Nalge Nunc International, Rochester, NY) coated with poly-l-lysine hydrobromide. For IL-2 detection, wells were coated with IL-2 capture antibodies (7 μg/mL). HuT 78 cells were added at a concentration of 1 × 10^7 per mL and left at 37°C to adhere. Following treatment, cells were fixed in 3% paraformaldehyde and where necessary permeabilized with 0.5% Triton X-100 detergent (Sigma Aldrich). IL-2 (secreted or intracellular) was detected using an Alexafluor 488–conjugated rat anti-human antibody. Confocal microscopy was performed using a 100× oil immersion objective on a Nikon TE2000-U inverted microscope using a PerkinElmer LSI confocal system, equipped with an Ar/Kr laser (488 nm). Ultraview
image acquisition system (Perkin Elmer) and Velocity-2 processing software (Improvision Inc.) were used for image processing and three-dimensional analyses.

For analysis of lipid rafts, HuT 78 cells (1 x 10^5 per mL) were left at 37°C to adhere and then either left resting or treated with 1 μg/mL of E2 for 24 hours. Cells were fixed in 1% paraformaldehyde and lipid rafts were stained using a Vybrant Labeling Kit. Cells were then labeled with Alexafluor 568–conjugated anti-PKCB (Molecular Probes, Inc.). Confocal microscopy was performed using a 63× oil immersion objective on a Zeiss 510 Meta Confocal Laser Scanning Microscope (laser excitation 488 nm and 561 nm).

RNA Extraction and Real-Time Polymerase Chain Reaction of IL-2, Interferon-γ, IL-10, and Tumor Necrosis Factor α Messenger RNA. Polymerase chain reactions (PCRs) were performed with a TaqMan Master Mix kit (Applied Biosystems, UK) and a mix of primers and fluorescently labeled TaqMan MGB probes (Applied Biosystems, UK) was used for the target gene; the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. Quantitative real-time PCR data were obtained using the comparative C_T method.

Quantification of Cytokines. For cell culture supernatants, a human IL-2 DuoSet enzyme-linked immunosorbent assay (ELISA) development kit (R&D Systems, Oxon, UK) was used according to the manufacturer’s instructions. For tissue samples, ELISA antibody pairs for the detection of cytokine proteins were obtained from R&D Systems. For multiplex analysis, a Biochip Array Technology system, the Evidence Investigator (Randomx Laboratories Ltd., UK), was used to measure multiple cytokines in cell culture supernatants.

Statistical Analysis. The results are expressed as the mean ± SEM. The data were analyzed using Microsoft Excel statistical software using the Student t test. The levels of IL-2 in HCV, alcoholic liver disease (ALD), and primary biliary cirrhosis (PBC) livers are expressed as the median, and data were analyzed using the Mann-Whitney U test. P < 0.05 was considered statistically significant.

A quantitative assessment of the level of colocalization between PKCB and lipid raft was performed using Pearson’s correlation coefficient. Relevant images were imported to Image Pro Plus version 4.5 for colocalization analysis.

Results

Low Levels of IL-2 in HCV-Infected Liver. IL-2 levels were measured in protein extracts of normal, HCV-infected, cirrhotic ALD and end-stage PBC liver biopsy tissue. Levels in HCV-induced cirrhosis (n = 9) were significantly lower (median 4.72 ng/100 mg total protein [range 1.3-10.03]) compared with those in cirrhotic tissue from both ALD (n = 9; median 26.01 ng/100 mg total protein [range 9.01-42.53]; P = 0.0006) and PBC (n = 12; median 19.69 ng/100 mg total protein [range 11.09-35.20]; P = 0.0006) and were comparable to those observed in normal (donor) tissue (median, 5.65 ng/100 mg total protein [range, 4.5-15.75]) (Fig. 1A). Furthermore, the number of CD3+ T cells both in the portal tract and parenchyma were comparable in HCV, ALD, and PBC liver samples (Supporting Information Fig. 1).

HCV Inhibits T Cell Production of IL-2. We next investigated the effect(s) of 1 hour preincubation of serum from control uninfected and HCV-infected patients on anti-CD3–stimulated T cell production of IL-2 (Fig. 1B). Preincubation with HCV+ serum before anti-CD3 stimulation significantly reduced IL-2 production (P = 0.0076), whereas serum from uninfected subjects or spontaneously resolved patients had no effect. The observed inhibitory effect of HCV+ serum was dose-dependent (Supporting Information Fig. 2). Preincubation of donor cells with a peptide reported to inhibit HCV E2–CD81 interaction (Cao et al.21 and Supporting Information Fig. 3) before exposure to HCV+ serum reversed inhibition of stimulated IL-2 secretion (Fig. 1C, P = 0.0008; E2-mediated inhibition of IL-2 secretion, P = 0.005), suggesting a role for this interaction (see also Supporting Information Fig. 2; patient information is provided in Supporting Information Table 1). Rescue of IL-2 secretion was not observed in the presence of a scrambled control peptide.

To examine whether recombinant HCV E2 inhibits IL-2 secretion, PBMCs from healthy donors were incubated with E2 (1 μg/mL) overnight and stimulated with either PMA/ionomycin or anti-CD3/anti-CD28 antibodies. HCV E2 preincubation induced a 20-fold reduction in IL-2 secretion in response to PMA/ionomycin and a >30-fold reduction in anti-CD3/anti-CD28–stimulated PBMCs (Fig. 1D). This HCV E2–mediated inhibition of IL-2 secretion is concentration-dependent (Supporting Information Fig. 4). We attempted to measure levels of E2 in the virus preparations (HCVcc) used for our experiments and found that they were beneath the cutoff of the E2 ELISA used in our laboratory (E2 ELISA cutoff is in the order of 10-50 ng [data not shown]). Therefore, the observation that HCVcc (<50 ng of E2) has an effect on lymphocyte cytokine secretion suggests that the virus is more effective than HCV E2 to modulate cytokine secretion.
We then used a cell culture model of HCV (HCVcc), representing whole virus of defined genotype, to investigate the effect of HCV particles on T cell IL-2 production. Serial dilutions of HCVcc (H77/JFH genotype 1a/2a chimera) inhibited anti-CD3/CD28–stimulated IL-2 production in a dose-dependent manner (Fig. 2).

**HCV E2 Inhibits T Cell Secretion of IL-2.** The HuT 78 T cell line secretes IL-2 following stimulation with the phorbol ester PMA. We used this model system to investigate the mechanism of HCV E2–mediated effects on reduced IL-2 production. HCV E2 significantly reduced PMA-stimulated Hut 78 cell IL-2 release compared with untreated or recombinant core (C22 or C33)-treated cells (Fig. 3A). To confirm that this effect of HCV E2 was CD81-mediated, we confirmed that the BP could reverse the effect (Fig. 3B). Previous reports have demonstrated that CD81 is costimulatory for IL-2 pro-
IL-2 levels were observed in proteins from HCV-infected cells (Fig. 3). To investigate whether this inhibition was a transcriptional level, we quantified IL-2 messenger RNA (mRNA) levels using real-time PCR (Fig. 3C). IL-2 mRNA levels in PMA-stimulated cells increased 126-fold over resting cells (P = 0.01). The housekeeping gene GAPDH was used as an endogenous control.

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**Discussion**

In resolving HCV infection, a potent CD4+ (Th1-oriented) response precedes the maturation of a protective memory CD8+ T cell response. In contrast, the proliferative capacity of both HCV-specific CD4+ and
CD8+ effector T cells is weak or absent during persist-ent infection. Molecular mechanisms that may con-tribute to this reduced T cell response include the pres-ence of IL-10, increased expression of the inhibitory molecule, programmed death-1, and loss of costimula-}

tory molecules such as CD86. Studies in humans and mice have reported that the reduced proliferative capacity of CD4+ T cells during viral infection is accompanied by decreased levels of IL-2 secretion. Semmo et al. reported on the reduced proliferation of

![Image](image1)

**Fig. 4.** HCV E2 inhibits T cell secretion of IFNγ, TNFα, and IL-10 from activated HuT 78 cells. HuT 78 cells were activated with PMA after preincubation overnight with HCV E2. (A-C) Cytokines were measured using the Evidence Investigator multiplex analysis. (D-F) mRNA levels were quantified in HuT 78 in response to activation (with or without prior exposure to E2).

![Image](image2)

**Fig. 5.** HCV E2–CD81 interaction induces the translocation of PKCβ from a cytosolic distribution to associate with lipid raft microdomains. Subcellular localization of PKCβ (red) and lipid raft/ganglioside GM-1 (green) in (A) resting cells and (B) E2-treated cells. A significant difference (P = 0.005) in the level of colocalization was observed when Pearson's correlation coefficients (means) were calculated for both resting and E2-treated cells (C).
HCV-specific CD4+ T cells isolated from patients with chronic disease in concert with reduced IL-2 secretion. These investigators had previously demonstrated loss of IL-2 secreting CD4+ T helper cells in chronic HCV infection. In this study, we demonstrate that both serum from HCV-infected patients and HCVcc reduced T cell IL-2 release and that this inhibitory effect was mediated via E2-CD81 ligation.

HCV-presented E1/E2 glycoproteins are multivalent and likely to cross-link CD81. However, the virus is unlikely to saturate all available CD81 receptors. We found the effect of anti-CD81 on IL-2 expression to be dose-dependent. The degree of cross-linking may also be influenced by the size of viral particles (45-60 nm) relative to lymphocytes (6-8 μm), which together with the affinity and avidity of E2 for CD81 will be factors in how ligation of CD81 (alone or in combination with CD3) can modulate levels of IL-2 expression/secretion.

HCV E2-CD81 interaction stimulates the translocation of PKCβ from the cytosol to lipid raft subdomains of the cell membrane. Lipid rafts are specific membrane compartments composed of cholesterol, glycolipids, and protein, which host receptors and signaling molecules involved in different cellular events, including cell signaling, pathogen invasion, and immune responses. PKCβ sequestration in lipid rafts prevented its association with the centrosome and the cellular secretory machinery necessary for IL-2 secretion in a process reversible by lipid raft disruption. The observation that HCV E2 can inhibit the secretion of another centrosome/synapse-directed cytokine (IFNγ) in vitro suggests that PKCβ is mechanistically involved more generally in the secretory process. HCVcc also inhibits IL-12–stimulated natural killer cell IFNγ secretion through a CD81-dependent pathway. Interestingly, we found levels of IFNγ to be significantly elevated in livers of HCV-infected patients (Supporting Information Fig. 10). This finding highlights the presence of an additional source of IFNγ-producing cells in response to HCV present in the liver. Even though HCV E2 can inhibit the ability of T cells to secrete IL-2 and IFNγ, this protein may have different effects on other cell types (as will the whole virus) in mixed cell populations present in an organ such as the liver.

In targeting PKCβ, HCV interferes with two microtubule-associated processes, secretion and migration, both of which are critical to the T cell–mediated immune response. More recently, it has been demonstrated that PKCβ is required for activation of Cdc42, driving fusion-dependent compartment mixing and exocytosis in a Xenopus model system, underscoring the importance of this enzyme in secretion.

In this study, we highlight the importance of CD81 engagement in modulating the quantitative and qualitative composition of lipid rafts and the regulation of signaling molecules such as PKCβ. Other viruses, including the human immunodeficiency virus, Herpesvirus saimiri, and measles virus, are known to modulate diverse T cell functions through lipid raft interaction. The inhibition of IL-2 secretion by both HCVcc and HCV+ human serum suggests that this is a realistic mechanism of viral immune suppression in vivo. Moreover, whereas low-dose IL-2 has not proven to be a successful therapy in HCV, targeting the association of PKCβ with lipid rafts may prove to be more successful in delivering enhanced cytokine secretion at a tissue-specific level.

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References


