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 (PKC*β*), 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 PKC*β*-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 PKC*β* with the cellular secretory machinery represents a novel mechanism for HCV to evade the human immune response and to establish persistent infection. (HEPATOLOGY 2011;53:1846-1853)

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

Abbreviations: ALD, alcoholic liver disease; BP, blocking peptide; ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; HCVcc, hepatitis C virus cell culture system; IFNγ, interferon-γ; IL-2, interleukin-2; MCD, methyl-β-cyclodextrin; mRNA, messenger RNA; PBC, primary biliary cirrhosis; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; PKCβ, protein kinase C beta; TNFα, tumor necrosis factor-α.

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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% detergent Igepal, 0.5% 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 10,000*g* 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 (Vectrastain *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 mm^2 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 1×10^5 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 Volocity-2 processing software (Improvision Inc.) were used for image processing and three-dimensional analyses.

For analysis of lipid rafts, HuT 78 cells (1×10^5 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-PKCβ (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 (Randox 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 PKCβ 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)[15] 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.

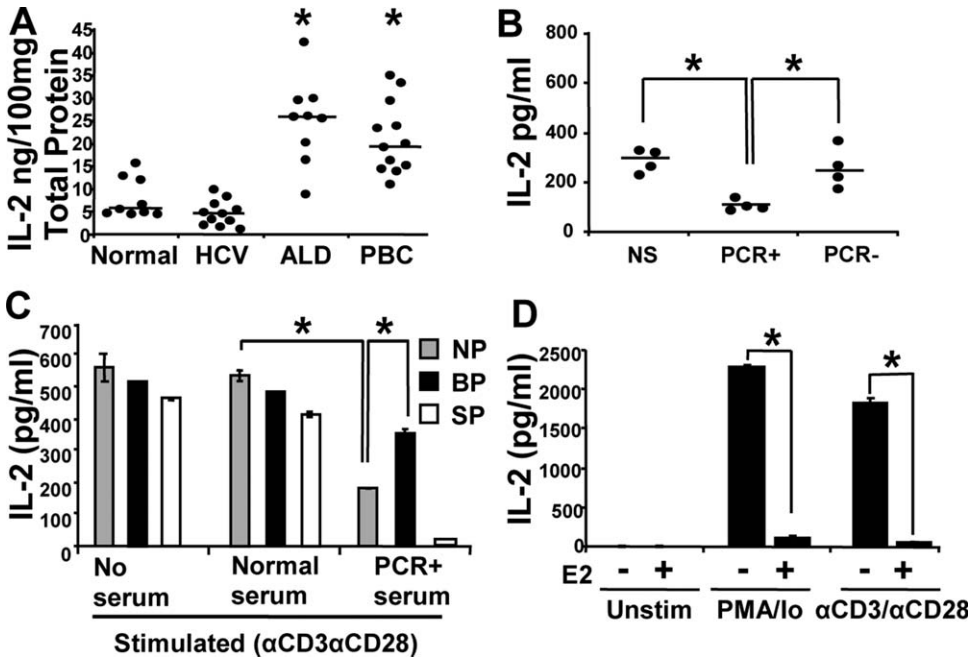


Fig. 1. IL-2 levels in HCV-infected liver and effects of HCV+ serum and HCV E2 on T cell IL-2 production. (A) Hepatic IL-2 protein was quantified by way of ELISA. Levels of IL-2 in end-stage HCV-infected livers are similar to those detected in normal donor livers, whereas IL-2 is significantly increased in ALD and PBC liver tissue compared with both normal control and HCV-infected tissue ($P = 0.0006$ in all cases). Circles represent individual patients, and horizontal lines show median values. (B) PBMCs from normal donors were stimulated with plate-bound anti-CD3 for 24 hours in the presence of normal serum (NS), chronic HCV-infected serum (PCR^+), or serum from subjects who had spontaneously resolved HCV infection (PCR^-). The production of IL-2 by T cells was quantified using ELISA. Serum was added to the cultures 1 hour before addition to plates. (C) PBMCs obtained from healthy donors were untreated (gray bars) or pretreated with a peptide that inhibits HCV E2–CD81 interaction (BP) (black bars) before incubating with either normal or HCV^+ PCR^+ serum followed by stimulation with anti-CD3/anti-CD28 antibodies (αCD3/αCD28). A scrambled version of the BP was used as a peptide control (white bars). BPs or scrambled peptides, either alone or in combination, did not affect basal levels of IL-2 from nonactivated PBMCs. Data are presented as the mean ± SEM of one representative experiment from five independent experiments with similar results. (D) HCV E2 (1 μg/mL) inhibits PBMC IL-2 secretion. HCV E2–mediated inhibition of IL-2 secretion is significant when stimulated either with PMA/ionomycin (PMA/Io versus E2/PMA/Io, $P = 0.002$) or with anti-CD3/anti-CD28 (αCD3/αCD28 versus E2/αCD3/αCD28, $P = 0.02$). IL-2 levels in pg/mL: PMA/Io 2,297.5 ± 3.7 versus E2/PMA/Io 115.2 ± 10.9, $P = 0.002$; anti-CD3/anti-CD28 1825.3 ± 58.3 versus E2/anti-CD3/anti-CD28 53 ± 1.5, $P = 0.02$ (resting cells 27.4 ± 1.4). Data are presented as the mean ± SEM of one representative experiment from three experiments with similar results. In resting and E2-treated cells, the IL-2 concentration was beneath the limit of detection of the ELISA.

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-

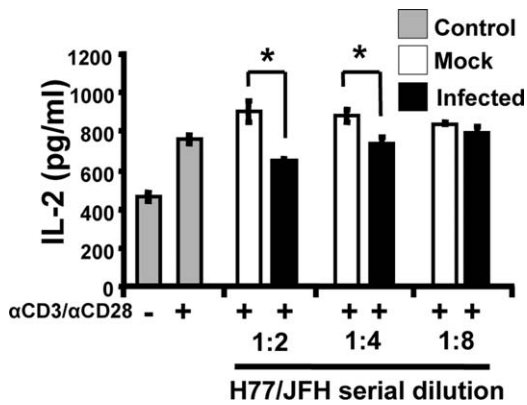


Fig. 2. HCVcc inhibit T cell IL-2 secretion. PBMCs were incubated at 37°C overnight in the presence of HCVcc (black) or mock Huh-7.5 cell–conditioned medium containing no virus (white bars). PBMCs were stimulated with immobilized anti-CD3/anti-CD28, and the supernatants were harvested 24 hours later. IL-2 content was measured by way of ELISA. *$P < 0.05$.

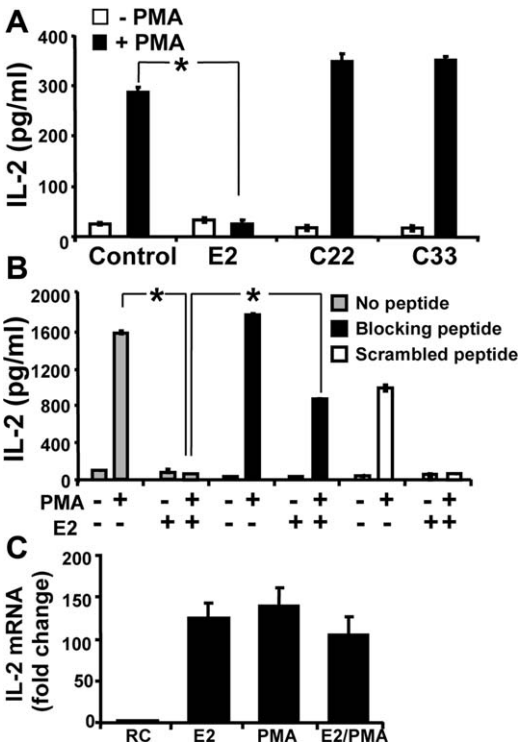


Fig. 3. HCV E2 reduces T cell secretion of IL-2 in a CD81-dependent manner. (A) HuT 78 T cells were incubated with each of the three HCV peptides (E2, C22, or C33 [1 μg/mL]) overnight and stimulated with PMA for 24 hours (black bars). White bars represent unstimulated cells. In HuT 78 cells pretreated with HCV E2, significant reduction in IL-2 secretion is observed (25.3 $\pm$ 6.5 versus 284.9 $\pm$ 14.3 pg/mL (P = 0.009). Data are presented as the mean $\pm$ SEM of one representative experiment from three independent experiments with similar results. (B) A BP that inhibits HCV E2-CD81 association can rescue IL-2 secretion. HuT 78 cells preincubated with BP (black bars) and then treated with HCV E2 prior to PMA stimulation secreted significantly higher levels of IL-2 versus those pretreated with HCV E2 but not exposed to BP (P = 0.0104). IL-2 levels in pg/mL: E2/PMA 60.5 $\pm$ 0.4 versus BP/E2/PMA 866.6 $\pm$ 13.5, P = 0.01; PMA 1,558.5 $\pm$ 22.5; resting cells 28.8 $\pm$ 1.5. Data are presented as the mean $\pm$ SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by asterisks. As a control, a scrambled version of the BP was used (white bars). (C) Effect of HCV E2 on IL-2 mRNA expression in HuT 78 cells. Quantitative real-time PCR data were obtained using the comparative C_T method. The housekeeping gene GAPDH was used as an endogenous control.

duction,[23] consistent with our data showing that anti-CD81 cross-linking cotemporaneously with an anti-CD3 stimulus promoted IL-2 production. However, ligation of CD81 with HCV E2 alone or soluble anti-CD81 prior to T cell stimulation inhibited IL-2 secretion (Supporting Information Fig. 5).

To ascertain whether this inhibition was at 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 = 0.02) but this was not inhibited by HCV E2. Immunofluorescent analysis revealed cytosolic IL-2 protein in HCV E2 pretreated cells stimulated with PMA that was not released externally (Supporting Information Figs. 6 and 7).

To investigate whether this inhibition of secretion was IL-2–specific or associated with general targeting of the secretory machinery, we examined HCV E2 effects on secretion of other cytokines in PMA-treated HuT 78 cells (Fig. 4) and anti-CD3/anti-CD28–stimulated PBMCs (Supporting Information Fig. 8). HCV E2 inhibited the secretion of interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), and IL-10 from both activated HuT 78 cells and PBMCs (Fig. 4A-C, Supporting Information Fig. 8), suggesting that E2 targets a secretory process. In contrast, E2 had minimal effect on IFNγ mRNA levels in HuT 78 cells (Fig. 4D), although there was a modest decrease in both IL-2 and IFNγ mRNA in PBMCs pretreated with E2 (Supporting Information Fig. 8). Treatment of HuT 78 cells and PBMCs with E2 prior to activation attenuated stimulated levels of both TNFα and IL-10 mRNA (Fig. 4E-F, Supporting Information Fig. 8), suggesting that HCV E2 can target transcriptional activation of these cytokines in T cells. Overall, the data demonstrate that HCV E2 targets the T cell secretory machinery and can inhibit secretion of IL-2 and IFNγ, cytokines that are normally secreted directionally through the centrosome.[24]

E2 Targets PKCβ to Lipid Rafts. We have reported previously that PKCβ is necessary for IL-2 export from PMA-stimulated HuT 78 cells.[16] In resting HuT 78 cells, PKCβ displays a cytosolic distribution (Fig. 5A); however, after incubation with HCV E2, PKCβ localized to lipid rafts, colocalizing with GM-1 (Fig. 5B,C). (Ligation of CD81 with a monoclonal antibody also directed PKCβ to associate with lipid rafts and inhibited PMA-stimulated IL-2 secretion by HuT 78 cells (Supporting Information Fig. 9). The lipid raft disrupting agent, methyl-β-cyclodextrin (MCD), reversed E2-mediated inhibition of IL-2 secretion (Fig. 6A). After treatment with the PKCβ-selective inhibitor Go6976, MCD could no longer reverse the inhibitory effect of E2 on IL-2 secretion (Fig. 6B). In summary, these data suggest that HCV E2 diversion of PKCβ to lipid rafts offers a novel mechanism for HCV to perturb cytokine secretion and to indirectly modulate host immune responses.

Discussion

In resolving HCV infection, a potent CD4+ (Th1-oriented) response precedes the maturation of a protective memory CD8+ T cell response.[25] In contrast, the proliferative capacity of both HCV-specific CD4+ and

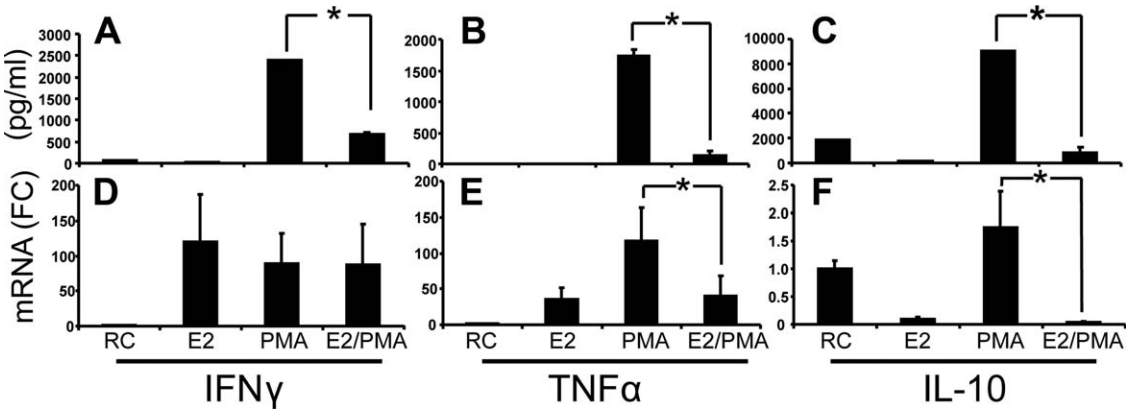
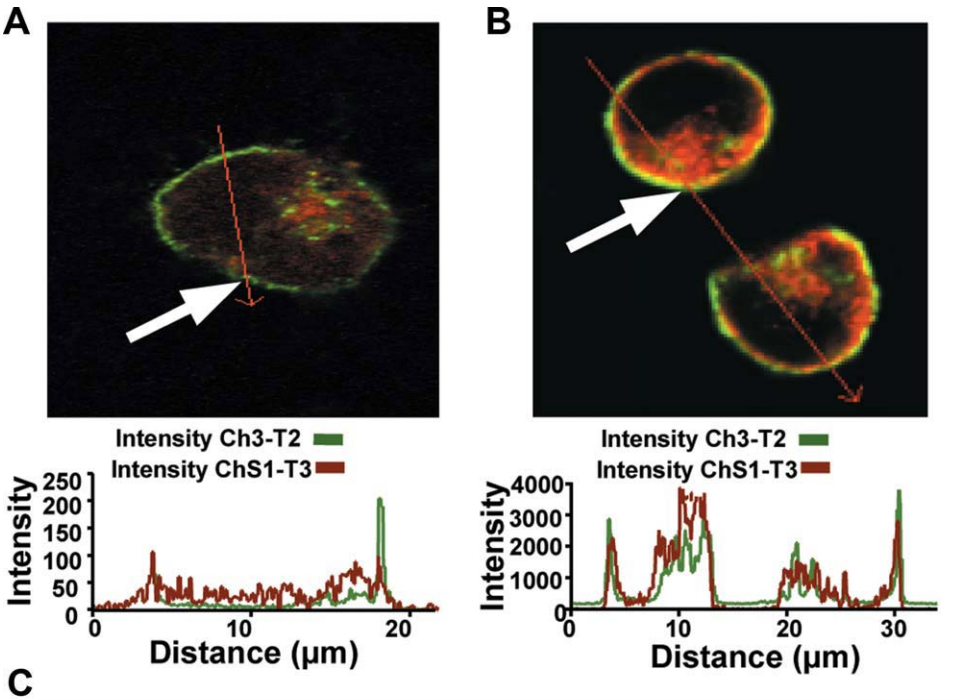


Fig. 4. HCV E2 inhibits T cell secretion of IFNγ, TNFα, and IL-10 from activated HuT 78 cells. HuT 78 cells were activated with PMA after preincubation overnight with HCV E2. (A-C) Cytokines were measured using the Evidence Investigator multiplex analysis. (D-F) mRNA levels were quantified in HuT 78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

CD8+ effector T cells is weak or absent during persistent infection.[26] Molecular mechanisms that may contribute to this reduced T cell response include the presence of IL-10, increased expression of the inhibitory molecule, programmed death-1, and loss of costimulatory molecules such as CD86.[27-29] 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.[30-32] Semmo et al.[33] reported on the reduced proliferation of



C

Pearson's Correlation Coefficient - Resting Cells	Rr = 0.4472
Pearson's Correlation Coefficient - E2 treated Cells	Rr = 0.6035
T test	p = 0.0055

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).

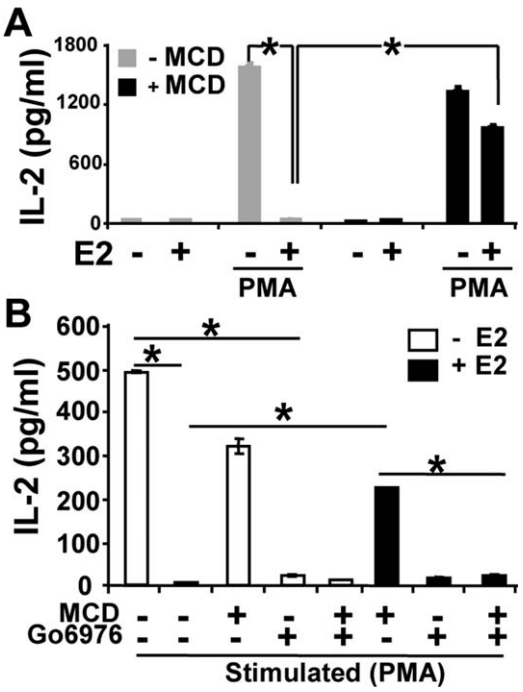


Fig. 6. Disruption of lipid rafts recovers the inhibitory effect of HCV E2 on IL-2 secretion. (A) Cells were pretreated with HCV E2 overnight and incubated with or without MCD for 1 hour before PMA stimulation. Cells treated with MCD (black bars) secreted significantly higher levels of IL-2 compared with those treated with HCV E2 alone ($P = 0.02$). Data are presented as the mean ± SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by asterisks. (B) The PKCα/β selective inhibitor Go6976 inhibits MCD-induced rescue of IL-2 secretion in cells pretreated with E2 (black bars). Data are presented as the mean ± SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by asterisks.

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.[18] 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.[34] 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)[24] *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.[35] 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,[15] 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.[36]

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,[37] *Herpesvirus saimiri* tip,[38] and measles virus,[39] 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,[40,41] 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

1. Wang C, Sarnow P, Siddiqui A. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J Virol 1993;67:3338-3344.
2. Brazzoli M, Crotta S, Bianchi A, Bagnoli F, Monaghan P, Wileman T, et al. Intracellular accumulation of hepatitis C virus proteins in a human hepatoma cell line. J Hepatol 2007;46:53-59.
3. Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. HEPATOLOGY 2005;42:962-973.
4. Stamataki Z, Grove J, Balfe P, McKeating JA. Hepatitis C virus entry and neutralization. Clin Liver Dis 2008;12:693-712.
5. Petracca R, Falugi F, Galli G, Norais N, Rosa D, Campagnoli S, et al. Structure-function analysis of hepatitis C virus envelope-CD81 binding. J Virol 2000;74:4824-4830.
6. Levy S, Todd SC, Maecker HT. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. Annu Rev Immunol 1998;16:89-109.
7. McHutchison JG. Understanding hepatitis C. Am J Manag Care 2004; 10:S21-S29.
8. Mazzaro C, Tirelli U, Pozzato G. Hepatitis C virus and non-Hodgkin's lymphoma 10 years later. Dig Liver Dis 2005;37:219-226.
9. Gale M Jr, Foy EM. Evasion of intracellular host defence by hepatitis C virus. Nature 2005;436:939-945.
10. Burke KP, Cox AL. Hepatitis C virus evasion of adaptive immune responses: a model for viral persistence. Immunol Res 2010;47:216-227.
11. Horner SM, Gale M Jr. Intracellular innate immune cascades and interferon defenses that control hepatitis C virus. J Interferon Cytokine Res 2009;29:489-498.
12. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J Virol 1994;68:8056-8063.
13. Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, et al. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. Science 1997;278:1447-1450.
14. Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med 2001;194:1395-1406.
15. Volkov Y, Long A, Freeley M, Golden-Mason L, O'Farrelly C, Murphy A, et al. The hepatitis C envelope 2 protein inhibits LFA-1-transduced protein kinase C signaling for T-lymphocyte migration. Gastroenterology 2006;130:482-492.
16. Long A, Kelleher D, Lynch S, Volkov Y. Cutting edge: protein kinase C beta expression is critical for export of Il-2 from T cells. J Immunol 2001;167:636-640.
17. Dreikhausen UE, Gorf K, Resch K, Szamel M. Protein kinase Cbeta1, a major regulator of TCR-CD28-activated signal transduction leading to IL-2 gene transcription and secretion. Int Immunol 2003;15:1089-1098.
18. Semmo N, Day CL, Ward SM, Lucas M, Harcourt G, Loughry A, et al. Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection. HEPATOLOGY 2005;41:1019-1028.
19. Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R, et al. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. Gastroenterology 1999;117:933-941.
20. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
21. Cao J, Zhao P, Miao XH, Zhao LJ, Xue LJ, Qi Zt Z. Phage display selection on whole cells yields a small peptide specific for HCV receptor human CD81. Cell Res 2003;13:473-479.
22. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
23. Wack A, Soldaini E, Tseng C, Nuti S, Klimpel G, Abrignani S. Binding of the hepatitis C virus envelope protein E2 to CD81 provides a co-stimulatory signal for human T cells. Eur J Immunol 2001;31:166-175.
24. Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. Nat Immunol 2006;7:247-255.
25. Urbani S, Amadei B, Fisicaro P, Tola D, Orlandini A, Sacchelli L, et al. Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. HEPATOLOGY 2006;44:126-139.
26. Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, et al. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J Immunol 2002;169:3447-3458.
27. Radziewicz H, Ibegbu CC, Hon H, Bedard N, Bruneau J, Workowski KA, et al. Transient CD86 expression on hepatitis C virus-specific CD8+ T cells in acute infection is linked to sufficient IL-2 signaling. J Immunol 2010;184:2410-2422.
28. Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MB. Interleukin-10 determines viral clearance or persistence in vivo. Nat Med 2006;12:1301-1309.
29. Golden-Mason L, Palmer B, Klarquist J, Mengshol JA, Castelblanco N, Rosen HR. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. J Virol 2007;81:9249-9258.
30. Fuller MJ, Hildeman DA, Sabbaj S, Gaddis DE, Tebo AE, Shang L, et al. Cutting edge: emergence of CD127high functionally competent memory T cells is compromised by high viral loads and inadequate T cell help. J Immunol 2005;174:5926-5930.
31. Fuller MJ, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. J Immunol 2003;170:477-486.
32. Iyasere C, Tilton JC, Johnson AJ, Younes S, Yassine-Diab B, Sekaly RP, et al. Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. J Virol 2003;77: 10900-10909.
33. Semmo N, Krashias G, Willberg C, Klenerman P. Analysis of the relationship between cytokine secretion and proliferative capacity in hepatitis C virus infection. J Viral Hepat 2007;14:492-502.
34. Rajendran L, Simons K. Lipid rafts and membrane dynamics. J Cell Sci 2005;118:1099-1102.
35. Crotta S, Brazzoli M, Piccioli D, Valiante NM, Wack A. Hepatitis C virions subvert natural killer cell activation to generate a cytokine environment permissive for infection. J Hepatol 2010;52:183-190.
36. Yu HY, Bement WM. Control of local actin assembly by membrane fusion-dependent compartment mixing. Nat Cell Biol 2007;9:149-159.
37. Djordjevic JT, Schibeci SD, Stewart GJ, Williamson P. HIV type 1 Nef increases the association of T cell receptor (TCR)-signaling molecules with T cell rafts and promotes activation-induced raft fusion. AIDS Res Hum Retroviruses 2004;20:547-555.
38. Cho NH, Kingston D, Chang H, Kwon EK, Kim JM, Lee JH, et al. Association of herpesvirus saimiri tip with lipid raft is essential for downregulation of T-cell receptor and CD4 coreceptor. J Virol 2006;80:108-118.
39. Avota E, Muller N, Klett M, Schneider-Schaulies S. Measles virus interacts with and alters signal transduction in T-cell lipid rafts. J Virol 2004;78:9552-9559.
40. Alric L, Thebault S, Peron JM, Balard P, Metivier S, Pipy B, et al. Pilot study of interferon-alpha-ribavirin-interleukin-2 for treatment of non-responder patients with severe liver disease infected by hepatitis C virus genotype 1. J Viral Hepat 2006;13:139-144.
41. Glesby MJ, Bassett R, Alston-Smith B, Fichtenbaum C, Jacobson EL, Brass C, et al. Pilot study of low-dose interleukin-2, pegylated interferon-alpha 2b, and ribavirin for the treatment of hepatitis C virus infection in patients with HIV infection. J Infect Dis 2005;191:686-693.