



Research Article

Hepatitis C Virus Core Protein Increases Expression of DR4 and DR5 in Peripheral Blood Mononuclear Cells

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Abstract

A robust innate and adaptive immune response is essential to viral clearance. Hepatitis C virus (HCV) infection typically leads to alteration of the innate and adaptive immune response, which is caused by interaction of HCV core protein with various host factors. It is important to investigate the alterations to the immune response during the transition from acute HCV to chronic HCV infection to develop better therapeutic methods for HCV infection. In this work, to determine whether HCV viral persistence occurs via tumor necrosis apoptosis-inducing ligand (TRAIL)-mediated apoptosis, we stimulated peripheral blood mononuclear cells (PBMCs) with recombinant HCV core protein within 12 h to measure the relative expression of death receptors 4 and 5 (DR4 and DR5) in PBMCs. We show that recombinant HCV core protein causes increased DR4 and DR5 expression in PBMCs. We also show that TRAIL interacts with DR4 and DR5 after cleavage of membrane-bound TRAIL yielding soluble TRAIL. Our results show that HCV core protein increases PBMC susceptibility to apoptosis and may cause increased TRAIL pathway activity within 12 h of infection. In addition, we observed that increased death receptor expression may contribute to HCV pathogenesis, as typically observed in chronically HCV-infected individuals.

Keywords: Hepatitis; Innate immunity; Viral pathogenesis; Host response; Apoptosis

Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is a major pathogen, affecting an estimated 170 million people worldwide [1, 2]. Major targets for HCV infection include hepatocytes and B and T lymphocytes, which typically result in impairment of host immunity [3]. Although some HCV-infected individuals can naturally clear the virus, most individuals with HCV infection progress to chronic hepatitis [4]. Among those with chronic

infection, an estimated 10-20% develop cirrhosis over 30 years [1]. HCV also accounts for 25% of those with hepatocellular carcinoma (HCC) [4-6]. Direct-acting antivirals (DAAs) have significantly aided in the achievement of a sustained virologic response (SVR) in many HCV genotype I-infected individuals, but the high costs of DAAs remain a limiting factor in their use for an effective means of treating HCV [7-10]. Also, the efficacy of DAAs in reinfection with HCV remain unclear, particularly when chronic HCV prevalence and reinfection is high, such as, in people

who inject drugs (PWID).

There are several pathways that mediate apoptosis, including the extrinsic, the intrinsic or mitochondrial, and the execution pathways [11]. Specifically, the extrinsic pathway involves transmembrane receptor-mediated interactions of members of the TNF-receptor gene superfamily [12]. Moreover, TRAIL has five known receptors, which include DR4 and DR5. DR4 and DR5 contain cytoplasmic “death domains” that induce apoptosis of infected cells that exhibit over-expression of these receptors [11-13]. Contrary to CD95 and TNF-receptor-1, DR4 and DR5 only induce programmed death of infected cells [13]. This interaction induces the recruitment of the death-inducing signaling complex (DISC), Fas-associated death domain (FADD), and TNFR1 associated death domain (TRADD), which further results in activation of caspases-8 and 3 and stimulation of apoptosis [11-13].

Notably, it was shown that NK cells in HCV-infected individuals can induce apoptosis of human hepatic stellate cells, which was dependent on up-regulated TRAIL expression [14]. Several other viruses, including hepatitis B virus and human immunodeficiency virus have also demonstrated increased activation of the TRAIL pathway [15-16]. Because TRAIL is known to induce apoptosis in infected cells and transformed cells only as well as HCV core’s immunomodulatory effects, we wanted to determine how HCV core protein impacts the TRAIL pathway in the total peripheral blood mononuclear

cell (PBMC) population. We hypothesize that increased death receptor expression in the PBMC population increases apoptosis of critical immune cells, contributing to HCV pathogenesis. Therefore, the objective of this study was to determine the level of death receptor expression in the total PBMCs after stimulation with HCV core protein within 12 h of exposure (Fig. 1). As control samples, we used PBMCs without antigen as well as PBMCs stimulated with lipopolysaccharide (LPS) to compare death receptor responses with PBMCs stimulated with HCV core protein. We report that recombinant HCV core protein caused increased expression of DR4 and DR5 in PBMCs. We also report that HCV interacts with soluble TRAIL after cleavage of membrane-bound TRAIL.

Experimental

PBMCs were purchased from Interstate Blood Bank (Memphis, TN), which were collected in acid-citrate dextrose (ACD) from healthy donors and were negative for HCV, hepatitis B virus (HBV), and human immunodeficiency virus (HIV), approved by Morgan State University’s Institutional Review Board for Human Subjects.

Peripheral blood mononuclear cells (PBMCs) culture

Cryopreserved PBMCs were thawed rapidly in a 37 °C water bath for approximately 2 min. The

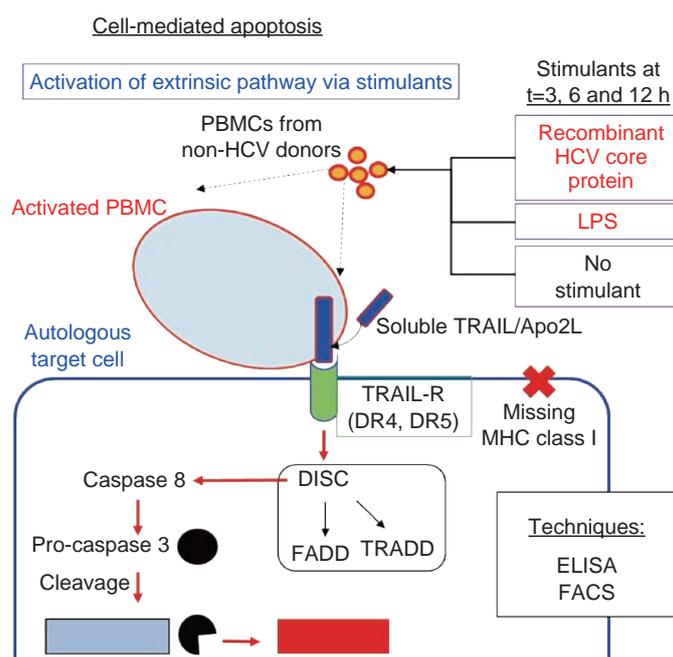


Fig. 1 Diagram displaying initiation of TRAIL-mediated apoptosis of autologous PBMC target cells.

cryovial was transferred aseptically to the biosafety cabinet level 2 and placed on ice. The contents of the cryovial were transferred to a 15 mL conical tube. The remaining contents of the cryovial were rinsed with 1 mL ice-cold thawing media (Hank's Balanced Salt Solution, supplemented with 10% FBS), and the 1 mL was transferred to the 15 mL conical tube. Thereafter, 10 mL ice-cold thawing media was added to the conical tube. An aliquot of the cell suspension was removed to perform the Trypan Blue Exclusion assay to determine cell viability. The conical tube containing the cells was centrifuged at $500 \times g$ for 12 min at 4 °C to pellet the cells. The supernatant was aspirated, and the cells were resuspended in 20 mL complete media (RPMI 1640, supplemented with 10% FBS). The resuspended cells were placed in a T-25 cm² flasks at 37 °C with 5% CO₂ for overnight recovery.

Stimulation of PBMCs

After overnight recovery, PBMCs were seeded in 24-well plates at a cell density of 2×10^5 cells/mL. The cells were stimulated with either lipopolysaccharide (LPS) from *Salmonella enterica* serotype Minnesota or recombinant HCV core protein at a final concentration of 10 µg/mL for various incubation periods (i.e., 3, 6, or 12 h). PBMCs without any antigen served as control PBMCs. We also examined protein expression directly after overnight recovery in unstimulated PBMCs for a time control ($t = 0$), which we refer to as a baseline.

Preparation of whole cell lysates

PBMCs were harvested by centrifugation at $500 \times g$ for 12 min. The supernatants were removed and placed into a fresh microcentrifuge tube for storage at -80 °C for further analysis on soluble factors. The cell pellets were washed twice with ice-cold phosphate buffered saline (PBS) and centrifuged at $500 \times g$ for 8 min. After centrifugation, the PBS was removed, and the cells were re-suspended in cold NETN lysis buffer containing protease inhibitors. The cells were agitated on ice for 30 min. The cell lysates were clarified by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatants were removed and placed into fresh microcentrifuge tubes for storage at -80 °C until further analysis.

Protein quantification

Cell culture supernatants and whole cell lysates were quantified by the Bradford Assay. Briefly, 20 µL of standard or sample were mixed with 1 mL Bradford Reagent. The solutions were incubated at room

temperature (RT) for 30 min. The absorbance of each sample and standard was measured at 595 nm. Each standard and sample were performed in duplicates. The unknown sample protein concentrations were determined using a quadratic regression fit for the standard curve.

Detection of TRAIL and death receptors using indirect ELISA

Supernatants and whole cell lysates were diluted to 20 µg/mL with PBS. Microplates were coated with 50 µL sample for 2 h at RT. Samples were removed, and wells were washed three times with 200 µL wash buffer (PBS with 0.5% Tween-20 [PBST]). The wells were blocked with 200 µL blocking buffer (1% BSA in PBS) for 1 h. After several washes with PBST, wells were incubated with (1:200) primary antibody for 2 h at RT (i.e., anti-TRAIL, anti-DR4, and anti-DR5 [Santa Cruz Biotechnology]). The wells were washed thoroughly with PBST, and 200 µL goat anti-mouse-HRP (Santa Cruz Biotechnology) (1:5000) was added to each well to incubate for 2 h at RT. The secondary antibody was removed. The wells were washed with PBST, and 50 µL OPD (o-phenylenediamine dihydrochloride) substrate solution (26 mg OPD, 9 mL H₂O, 1 mL H₂O₂) was added to each well. We incubated the wells with substrate solution for 30 min in the dark at RT. Thereafter, 50 µL stop solution (2.5 M H₂SO₂) was added to each well to stop the color reaction. The yellow product was measured at 490 nm with a Cary 60 UV/Vis spectrophotometer.

FACS analysis of PBMCs

PBMCs were harvested by centrifugation at $500 \times g$ for 12 min after stimulation with either LPS from *Salmonella enterica* serotype Minnesota or recombinant HCV core protein. Cells were washed once with PBS, and then washed twice with FACS buffer (PBS, supplemented with 10% FBS). The cells were surface-stained at RT for 30 min in the dark with monoclonal anti-DR4 PE, anti-DR5 PE, and anti-TRAIL PE (BioLegend). After staining, the cells were washed four times with FACS buffer before fixation in 0.1% paraformaldehyde (PFA) in PBS. The cells were stored in the dark at 4 °C until analysis. Cells were acquired on a BDFACS Canto II at the University of Maryland School of Medicine Center for Innovative Biomedical Resources (Flow Cytometry Core Facility) and analyzed using FlowJo (version 10).

Statistical analysis

We examined the relative difference in both soluble,

membrane-bound, and cytosolic protein expression using Sigma Plot, version 12.5. Error bars display the standard deviation. The relative percent change between incubation periods was also determined by the following equation:

$$\left[\frac{\text{Absorbance (stimulated PBMC)} - \text{Absorbance (unstimulated PBMC)}}{\frac{\text{Absorbance (stimulated PBMC)} + \text{Absorbance (unstimulated PBMC)}}{2}} \right] \times 100$$

Unstimulated PBMCs (i.e., PBMCs without any antigenic stimulation) were used as the reference, relative percent = 1. We examined the differences in the average protein level using an analysis of variance (ANOVA) test. To determine which groups differed, we applied a multiple pairwise comparison using the Holm-Sidak method. A p value <0.05 was considered statistically significant.

Results and Discussion

Determine relative expression of soluble death receptors in total PBMCs using ELISA

PBMCs were stimulated with either LPS or

recombinant HCV core protein for 0 (time control), 3, 6, and 12 h. Unstimulated PBMCs were used for control samples. The expression of the death receptors capable of inducing apoptosis (i.e., DR4 or DR5) were examined using an indirect ELISA and fluorescent-activated cell sorting (FACS).

We began our investigation by performing an indirect ELISA on cell culture supernatants to measure the relative expression of soluble DR4 or DR5 in PBMC samples. Fig. 2 displays the colorimetric responses measured at 490 nm and 492 nm from the indirect ELISA. After 3 h of incubation, DR4 and DR5 protein levels of both PBMCs stimulated with LPS and recombinant HCV core protein were approximately eight percent lower than protein levels of unstimulated PBMCs. DR4 expression in all PBMCs (i.e., unstimulated PBMCs, PBMCs stimulated with LPS, and PBMCs stimulated with recombinant HCV core protein) was higher (~8%) than DR4 levels at time 0 (baseline). However, baseline DR5 protein levels were 6% higher compared to DR5 levels in unstimulated

Summary ELISA: Protein expression in total PBMCs

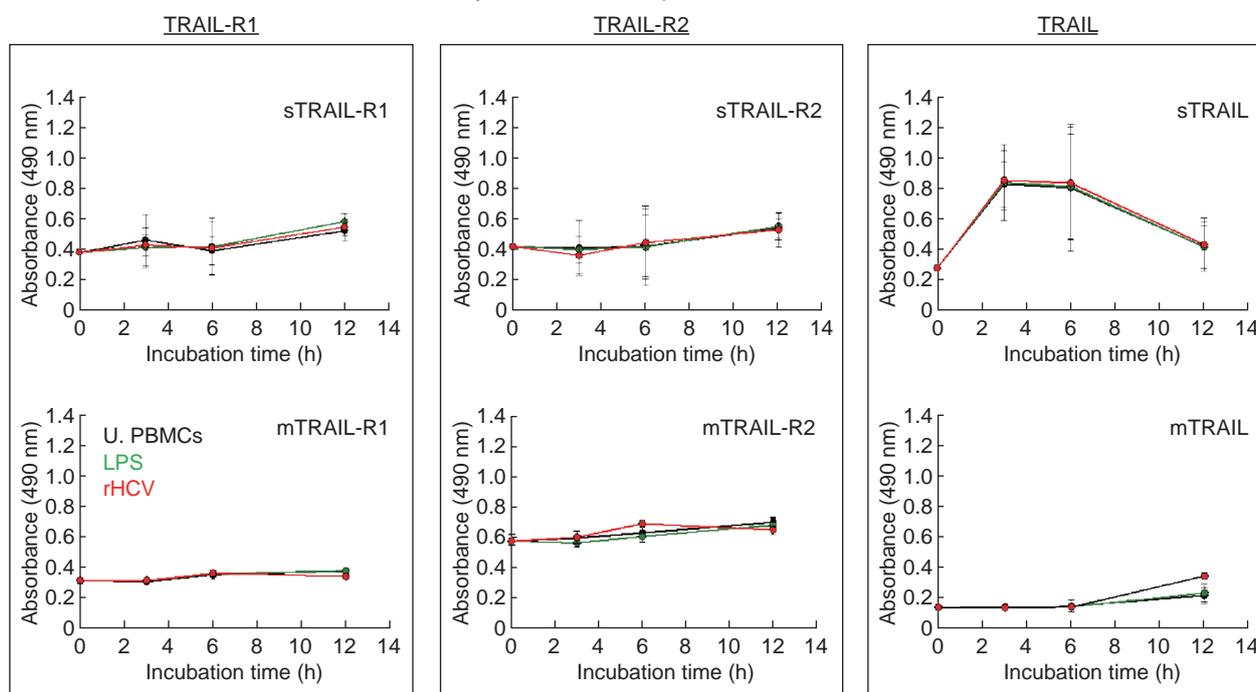


Fig. 2 Time-trend of TRAIL-R1, TRAIL-R2, and TRAIL expression in total PBMCs obtained by indirect ELISA. Total PBMCs were stimulated with either LPS from *Salmonella enterica* serotype Minnesota or recombinant HCV core protein for various incubation periods (i.e., 3, 6, or 12 h). Absorbance of *o*-phenylenediamine dihydrochloride (OPD) product was measured at 490 nm or 492 nm. Abbreviations: sTRAIL-R1, soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; mTRAIL-R1, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; sTRAIL-R2, soluble tumor necrosis factor apoptosis inducing ligand-receptor 2; mTRAIL-R2, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand-receptor 2; sTRAIL, soluble tumor necrosis factor apoptosis inducing ligand; mTRAIL, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand; U.PBMCs, unstimulated peripheral blood mononuclear cells; LPS, Lipopolysaccharide; rHCV, recombinant HCV core protein.

ELISA: Differences in TRAIL-R1 and TRAIL-R2 levels between 3, 6, and 12 h

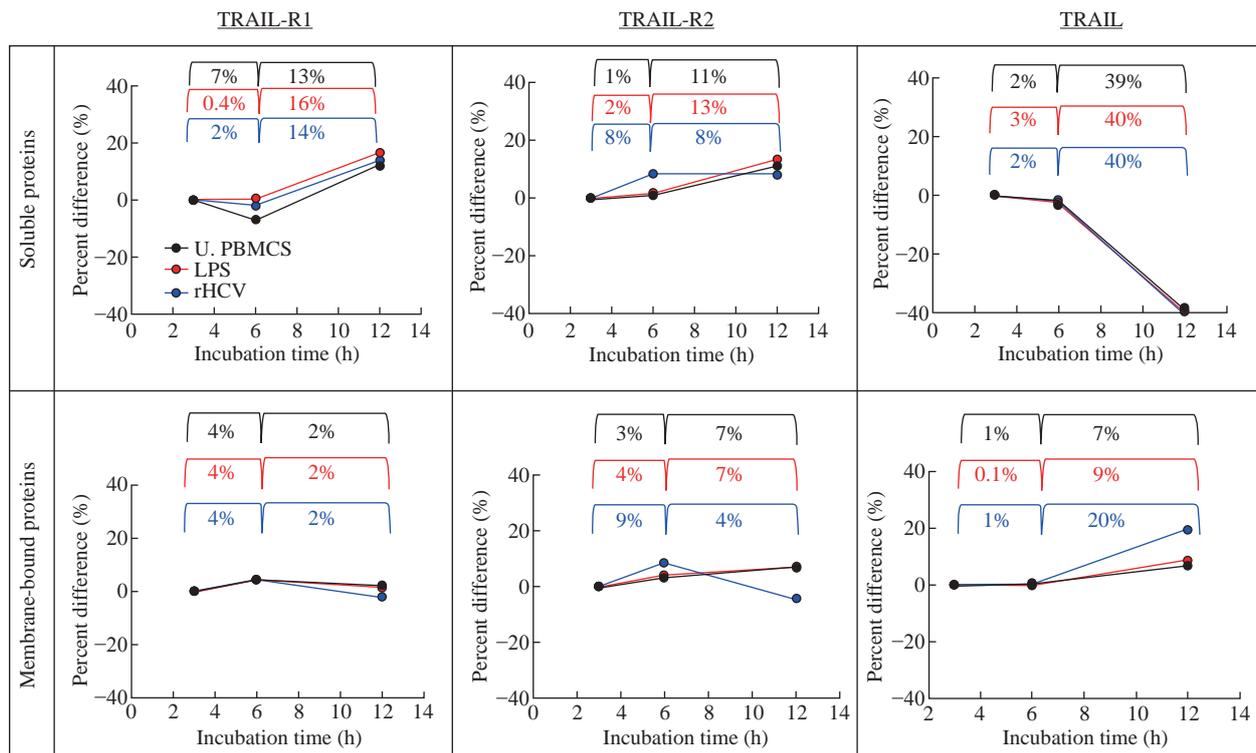


Fig. 3 Percent difference in levels of soluble and membrane-bound forms of TRAIL-R1, TRAIL-R2, and TRAIL between various incubation periods (i.e., 3, 6, and 12 h) of incubation. Unstimulated PBMCs were used for control PBMCs. Abbreviations: sTRAIL-R1, soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; mTRAIL-R1, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; sTRAIL-R2, soluble tumor necrosis factor apoptosis inducing ligand-receptor 2; mTRAIL-R2, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand-receptor 2; sTRAIL, soluble tumor necrosis factor apoptosis inducing ligand; mTRAIL, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand; U.PBMCs, unstimulated peripheral blood mononuclear cells; LPS, Lipopolysaccharide; rHCV, recombinant HCV core protein.

PBMCs, PBMCs stimulated with LPS, and PBMCs stimulated with recombinant HCV core protein-stimulated PBMCs.

Interestingly after 6 h of incubation, there was a decrease (~7%) in soluble DR4 protein levels in unstimulated PBMCs (Fig. 3). Soluble DR5 levels in unstimulated PBMCs were slightly higher (~1%) after 6 h of incubation compared to levels observed after 3 h of incubation. Both PBMCs stimulated with LPS and recombinant HCV core protein displayed higher DR4 protein levels than unstimulated PBMCs after 6 h of incubation. Soluble DR4 protein levels of PBMCs incubated with recombinant HCV core protein increased by two percent, and DR5 levels increased by eight percent after 6 h of incubation (Fig. 3). PBMCs stimulated with LPS exhibited a slight increase from 3 to 6 h in both soluble DR4 and DR5 levels, 0.2% and 2%, respectively. Nevertheless, unstimulated PBMCs, PBMCs stimulated with LPS, and PBMCs stimulated with recombinant HCV core protein displayed higher soluble DR4 and DR5 protein levels compared to

baseline.

Moreover, there was approximately a 15% increase and 10% increase in both DR4 and DR5 levels, respectively in all cell culture supernatants after 12 h of incubation. Although unstimulated PBMCs displayed an increase in both soluble DR4 and DR5 levels, soluble DR4 and DR5 levels were higher in PBMCs stimulated with either LPS or recombinant HCV core protein. Fig. 3 shows the percent difference in soluble DR4 and DR5 between 3, 6, and 12 h of incubation. Supporting Information 1 displays bar graphs of soluble TRAIL-R1 and TRAIL-R2 in total PBMCs.

To determine if there were significant differences in DR4 or DR5 levels according to incubation time or type of antigenic stimulation, we performed a two-way ANOVA. The ANOVA test showed that there were significant differences in DR4 and DR5 between the different incubation periods, $p = 0.007$ and 0.002 , respectively (Table 1). Soluble death receptors did not significantly vary by the types of antigenic stimuli. Furthermore, we used the Holm-Sidak method to

perform post-hoc multiple pairwise comparisons between the incubation periods. We observed that DR4 and DR5 levels in 3 h differed significantly from 12 h ($p = 0.015$ and 0.003 , respectively). DR4 and DR5 levels in 6 h also differed significantly from levels observed after 12 h of incubation. Table 2 shows the post-hoc multiple comparisons for death receptor expression levels between the different incubation periods. Supporting Information 2 shows stacked proportion graphs indicating the relative percent change in soluble TRAIL-R1 and TRAIL-R2 protein levels in various incubation periods.

Table 1 is the two-way analysis of variance (ANOVA) examining if protein levels differ according to incubation time or type of antigenic stimulation. Abbreviations: sTRAIL-R1, soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; mTRAIL-R1, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; sTRAIL-R2, soluble tumor necrosis factor apoptosis inducing ligand-receptor 2; mTRAIL-R2, membrane-bound soluble tumor necrosis factor apoptosis inducing

Table 1 Two-way ANOVA: Soluble and membrane-bound TRAIL and death receptors

Proteins	Time	Type of antigen
sTRAIL-R1	0.007	0.853
sTRAIL-R2	0.002	0.759
sTRAIL	<0.001	0.009
mTRAIL-R1	0.021	0.665
mTRAIL-R2	0.071	0.539
mTRAIL	0.034	0.510

** P value < 0.05 considered statistically significant. Bold print indicates significant P value.

Table 2 Two-way ANOVA: Pairwise comparisons

Time			Type of antigenic stimulation	
3 h v. 6 h	3 h v. 12 h	6 h v. 12 h	LPS v. U.PBMCs	rHCV v. U.PBMCs
0.286	0.015	0.010	NT	NT
0.089	0.003	0.006	NT	NT
0.006	<0.001	<0.001	0.182	0.012
0.037	0.032	0.540	NT	NT
NT	NT	NT	NT	NT
0.908	0.061	0.046	NT	NT

** P value < 0.05 considered statistically significant. Bold print indicates significant P value.

ligand-receptor 2, sTRAIL, soluble tumor necrosis factor apoptosis inducing ligand; mTRAIL, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand; U.PBMCs, unstimulated peripheral blood mononuclear cells; LPS, Lipopolysaccharide; rHCV, recombinant HCV core protein.

Table 2 is the post-hoc multiple pairwise comparisons after two-way ANOVA, using the Holm-Sidak method. Abbreviations: NT, Not tested. Insignificant p values for the two-way ANOVA were not tested for multiple pairwise comparisons.

Determine relative expression of membrane-bound death receptors in total PBMCs using ELISA

Because membrane-bound death receptors are shown to be more biologically effective in inducing TRAIL-mediated apoptosis [17], we determined the relative expression of membrane-bound DR4 and DR5 in total PBMCs whole cell lysates. In 3-h PBMCs, all PBMCs displayed similar levels of DR4 on their cell surface compared with baseline levels (Fig. 2). Membrane-bound DR5 expression was considerably higher than DR4 expression in all PBMCs. Moreover, we observed a slight increase in DR4 and DR5 expression after 6 h of incubation in unstimulated PBMCs, PBMCs incubated with LPS, and PBMCs incubated with recombinant HCV core protein. However, PBMCs stimulated with recombinant HCV core protein displayed higher levels of both DR4 and DR5 protein levels compared to unstimulated PBMCs after 6 h of incubation. However, DR5 levels were considerably higher than DR4 levels relative to unstimulated PBMC protein levels. DR5 levels of PBMCs incubated with LPS remained lower than both unstimulated PBMCs. However, membrane-bound DR4 expression was similar in PBMCs incubated with LPS compared to unstimulated PBMCs (Fig. 2).

Unlike soluble death receptors, membrane-bound DR4 and DR5 expression did not show a consistent pattern after 12 h of incubation. DR4 and DR5 expression in both unstimulated PBMCs and PBMCs incubated with LPS showed a slight increase from 6 to 12 h of incubation (Fig. 3). Contrarily, we observed a decrease in both membrane-bound DR4 and DR5 PBMCs stimulated with recombinant HCV core protein after 12 h of incubation (Fig. 3). Fig. 4 also shows a heatmap representation of both soluble and membrane-bound forms of soluble DR4 and DR5 in all incubation periods. Supporting Information 3 displays bar graphs

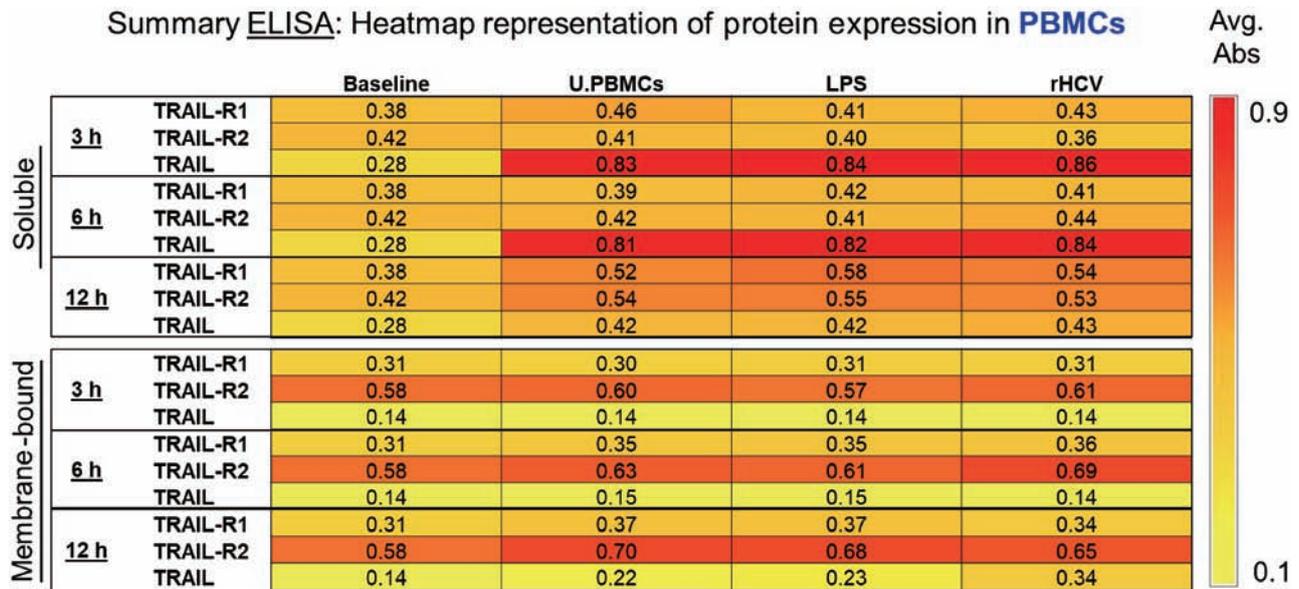


Fig. 4 Heatmap representation of soluble and membrane-bound protein expression of TRAIL-R1, TRAIL-R2, and TRAIL in total PBMCs. Each color matrix represents the average absorbance of OPD product formation at 490 or 494 nm from three replicates in 3, 6, or 12 h. Baseline protein levels were used as a time control, directly after overnight recovery from cryopreservation. Unstimulated PBMCs were used as control PBMCs. sTRAIL-R1, soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; mTRAIL-R1, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand-receptor 1; sTRAIL-R2, soluble tumor necrosis factor apoptosis inducing ligand-receptor 2; mTRAIL-R2, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand-receptor 2; sTRAIL, soluble tumor necrosis factor apoptosis inducing ligand; mTRAIL, membrane-bound soluble tumor necrosis factor apoptosis inducing ligand; U.PBMCs, unstimulated peripheral blood mononuclear cells; LPS, lipopolysaccharide; rHCV, recombinant HCV core protein.

of membrane-bound TRAIL-R1 and TRAIL-R2 in total PBMCs.

To determine if there were significant differences in membrane-bound DR4 or DR5 levels according to incubation time or type of antigenic stimulation, we performed a two-way ANOVA. Remarkably, membrane-bound DR4 and DR5 did not show similar ANOVA results relative to soluble DR4 and DR5. Notably, only membrane-bound DR4 displayed statistical differences between each incubation period ($p = 0.021$). Pairwise multiple comparisons showed that there were significant differences between 3 and 6 h of incubation and between 3 and 12 h of incubation (Table 2). Supporting Information 4 shows stacked proportion graphs indicating the relative percent change in membrane-bound TRAIL-R1 and TRAIL-R2 protein levels in various incubation periods.

Determine relative expression of death receptors in total PBMCs using FACS

We also determined the relative expression of DR4 and DR5 in the total PBMC population using FACS. Of note, DR4 expression was increased (~0.14%) in PBMCs stimulated with recombinant HCV core protein from baseline DR4 levels after 3 h of incubation.

PBMCs incubated with LPS were slightly lower than baseline levels after 3 h of incubation (Fig. 5). DR5 expression in PBMCs incubated with recombinant HCV core protein displayed a substantial increase (~0.55%) in DR5 expression after 3 h of incubation relative to baseline levels and unstimulated PBMCs. Fig. 6 displays a representative zebra plot of DR4 and DR5 expression in PBMCs after 3 h of incubation using FACS. Supporting Information 7 and 8 display representative zebra plots of multicolor FACS of TRAIL-R1 and TRAIL-R2 in total PBMCs after 6 and 12 h, respectively.

Furthermore, DR4 expression in both unstimulated PBMCs and PBMCs stimulated with recombinant HCV core protein decreased by approximately 0.27% between 3 h and 6 h of incubation. Nonetheless, DR4 expression in PBMCs incubated with recombinant HCV core protein was still increased (~0.10%) in comparison to unstimulated PBMC levels after 6 h of incubation. We observed an increase (~0.14%) in DR4 expression in PBMCs stimulated with LPS in comparison to Baseline and unstimulated PBMCs. Likewise, DR5 expression for all PBMCs decreased significantly after 6 h of incubation. Nonetheless, recombinant HCV core protein-stimulated PBMCs

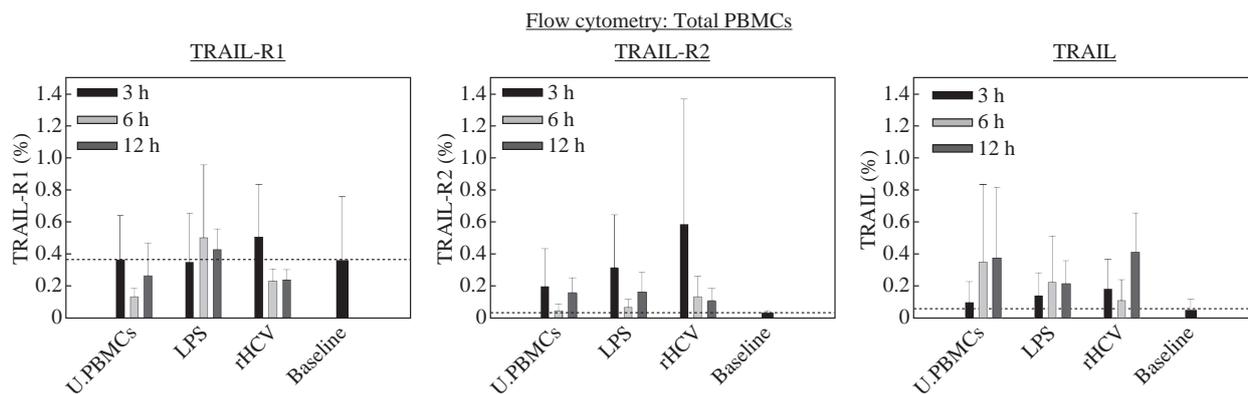


Fig. 5 FACS analysis examining TRAIL-R1, TRAIL-R2, and TRAIL protein expression in total PBMCs after 3, 6, and 12 h of incubation. Each bar denotes the average percentage for each replicate. Error bars represent the standard deviation SD. Abbreviations: TRAIL-R1, tumor necrosis factor apoptosis inducing ligand-receptor 1; TRAIL, tumor necrosis factor apoptosis inducing ligand; U.PBMCs, unstimulated peripheral blood mononuclear cells; LPS, lipopolysaccharide; rHCV, recombinant HCV core protein.

displayed a higher level ($\sim 0.10\%$) of DR5 expression compared to unstimulated PBMCs and PBMCs stimulated with LPS (Fig. 5).

We did not observe a trend in either DR4 or DR5 expression between unstimulated PBMCs, PBMCs stimulated with LPS, or PBMCs stimulated with recombinant HCV core protein after 12 h of incubation. We observed a slight increase ($\sim 0.13\%$) in DR4 levels in unstimulated PBMCs after 12 h of incubation. This observation was opposite of that displayed in PBMCs incubated with LPS, whereby PBMCs incubated with LPS displayed a slight decrease ($\sim 0.07\%$) in DR4 expression. DR4 expression in PBMCs stimulated with recombinant HCV core protein were similar after 12 h of incubation to levels observed after 6 h of incubation. DR4 expression in all PBMCs except PBMCs stimulated with LPS were lower than baseline levels (Fig. 5).

Likewise, DR5 expression displayed a similar trend to DR4, whereby there was a gradual increase ($\sim 0.10\%$) in the number of cells expressing DR5 after 12 h of incubation. We observed this trend for all PBMCs, except PBMCs stimulated with recombinant HCV core protein, which decreased by 0.08% after 12 h of incubation. To add, we observed similar levels in DR5 expression in both unstimulated PBMCs and PBMCs stimulated with LPS after 12 h of incubation. Also, DR5 levels in all PBMCs were higher after 12 h of incubation compared to Baseline levels (Fig. 5). Fig. 7 also shows a heatmap representation of DR4 and DR5 expression in PBMCs in all incubation periods. Although there were differences in the level of DR4 and DR5 expression between unstimulated and stimulated PBMCs, two-way ANOVA showed that

Table 3 Two-way ANOVA: Flow cytometry TRAIL and death receptors

Protein	Time	Type of antigenic stimulation
TRAIL-R1	0.532	0.347
TRAIL-R2	0.083	0.407
TRAIL	0.186	0.664

these levels were not statistically significant (Table 3).

Table 3 is the two-way ANOVA for membrane-bound TRAIL-R1, TRAIL-R2, and TRAIL.

Determine relative expression of soluble TRAIL in total PBMCs using ELISA

We determined the expression of soluble TRAIL in PBMC culture supernatants to determine if the level of soluble TRAIL expression correlated with the time-dependent trend observed in death receptor expression. Fig. 2 displays the relative expression of soluble TRAIL in unstimulated PBMCs, PBMCs stimulated with LPS, and PBMCs stimulated with recombinant HCV core protein. In all PBMC samples, there was a comparable trend in the level of soluble TRAIL expression, in which TRAIL levels were approximately 3% different from 3 to 6 h and approximately 40% different between 6 and 12 h. However, TRAIL levels in unstimulated PBMCs, PBMCs stimulated with LPS and PBMCs stimulated with recombinant HCV core protein were approximately 50% higher than soluble TRAIL levels measured at baseline. After 6 h of incubation, there was a slight decrease ($\sim 3\%$) in soluble TRAIL expression in all PBMCs. Notably, we observed a significant decrease ($\sim 40\%$) in soluble TRAIL expression in unstimulated PBMCs and stimulated PBMCs after 12 h of incubation. PBMCs

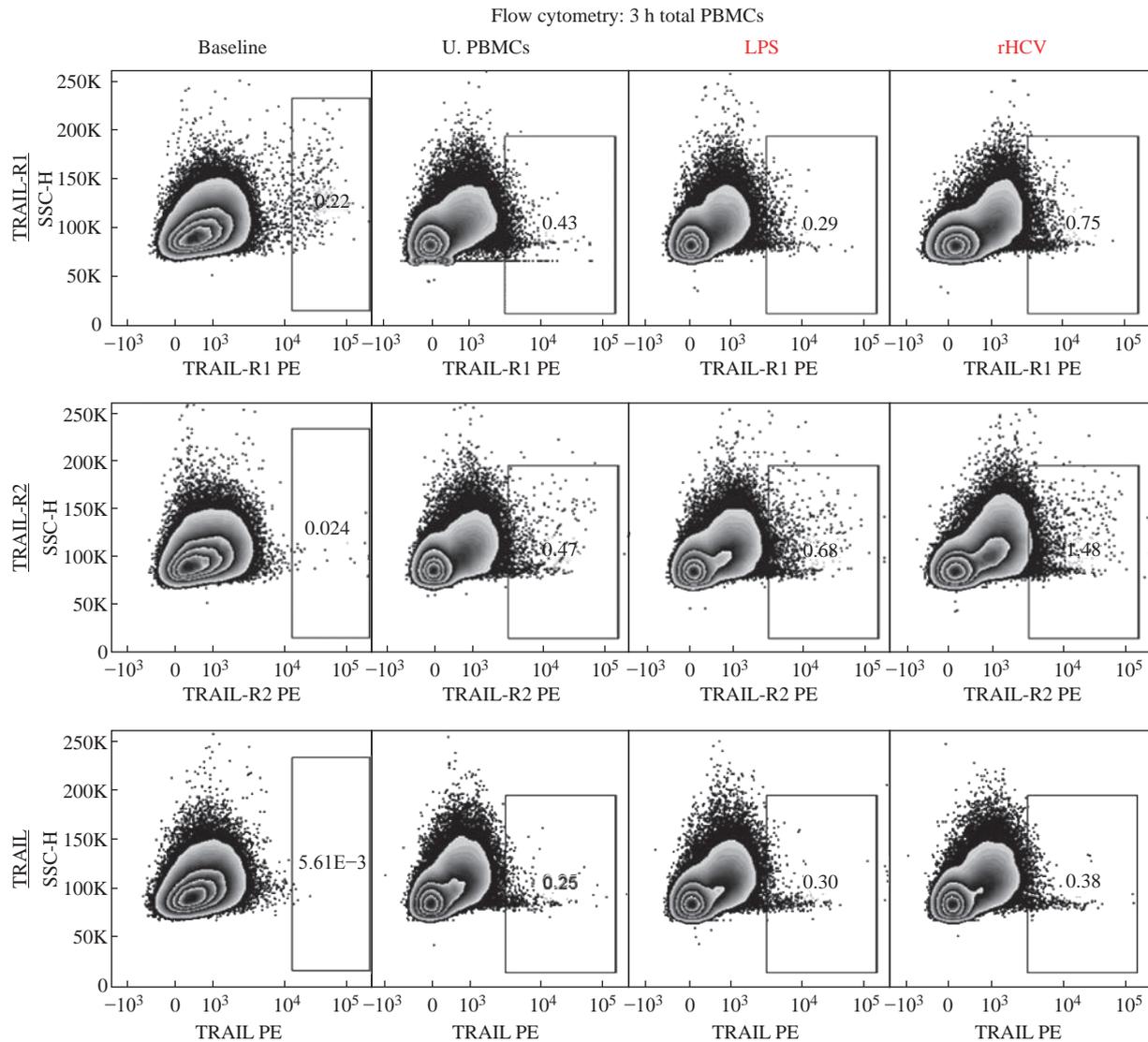


Fig. 6 Representative zebra plots of multicolor FACS analysis of TRAIL-R1, TRAIL-R2, and TRAIL in peripheral blood mononuclear cells after 3 h of incubation from one replicate. Baseline was the time control ($t = 0$). The numbers in each box represent the percentage of positive cells. Abbreviations: TRAIL-R1, tumor necrosis factor apoptosis inducing ligand-receptor 1; TRAIL-R2, tumor necrosis factor apoptosis inducing ligand-receptor 2; TRAIL, tumor necrosis factor apoptosis inducing ligand; U.PBMCs, unstimulated peripheral blood mononuclear cells; LPS, lipopolysaccharide; rHCV, recombinant HCV core protein.

stimulated with LPS showed a slight ($\sim 1\%$) decrease in soluble TRAIL expression relative to unstimulated PBMCs after 12 h of incubation. Contrarily, PBMCs stimulated with recombinant HCV core protein displayed a slight ($\sim 1\%$) increase in soluble TRAIL expression relative to unstimulated PBMCs (Fig. 3). Supporting Information 5 displays a bar graph and a stacked proportion graph indicating the relative percent change in soluble TRAIL protein levels in various incubation periods.

We performed a two-way ANOVA to determine if the average level of soluble TRAIL varied according to incubation time or the type of antigenic stimulation. There was a statistically significant difference in

soluble TRAIL protein expression between the various incubation periods as well as the type of antigenic stimulation ($p < 0.001$ and 0.009 , respectively). Therefore, we performed post-hoc multiple pairwise comparisons using the Holm-Sidak method. All incubation periods showed statistically significant differences in the average expression of soluble TRAIL (Table 2). There were also statistically significant differences in soluble TRAIL expression between unstimulated PBMCs and PBMCs stimulated with recombinant HCV core protein ($p = 0.012$). PBMCs stimulated with LPS did not show statistically significant differences in soluble TRAIL in comparison to unstimulated PBMCs ($p = 0.182$).

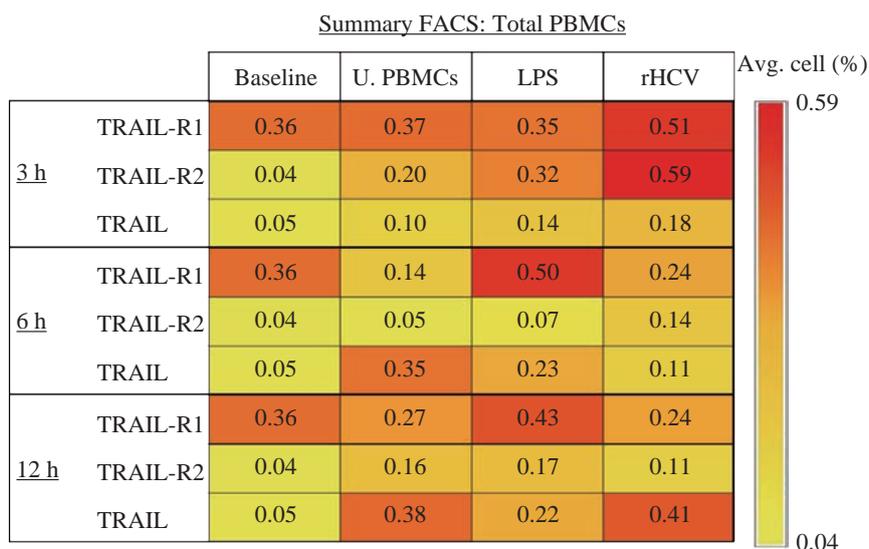


Fig. 7 Heatmap representation of the expression of TRAIL-R1, TRAIL-R2, and TRAIL in total PBMCs by FACS analysis. Unstimulated PBMCs were used as control PBMCs. Baseline is the time control ($t = 0$). Each color matrix represents the average percentage of cells expressing either TRAIL-R1 or TRAIL-R2 from three replicates after 3, 6, and 12 h of incubation. Abbreviations: TRAIL-R1, tumor necrosis factor apoptosis inducing ligand-receptor 1; TRAIL-R2, tumor necrosis factor apoptosis inducing ligand-receptor 2; TRAIL, tumor necrosis factor apoptosis inducing ligand; U.PBMCs, unstimulated peripheral blood mononuclear cells; LPS, lipopolysaccharide; rHCV, recombinant HCV core protein.

Determine relative expression of membrane-bound TRAIL in total PBMCs using ELISA

We investigated if the expression of membrane-bound TRAIL correlated with the expression of soluble TRAIL released into cell culture supernatants. Whole cell lysates were examined for the relative expression of TRAIL. Fig. 2 displays the relative amount of surface-bound TRAIL expressed in unstimulated and stimulated PBMCs. The trend in the expression of membrane-bound TRAIL was similar in all PBMCs. Membrane-bound TRAIL levels were similar in all PBMCs after 3 h of incubation. These levels were also comparable to membrane-bound TRAIL measured at baseline. Furthermore, there was a slight (~1%) increase in the expression of TRAIL after 6 h of incubation in both unstimulated and stimulated PBMCs. Still, TRAIL expression for all PBMCs was similar after 6 h of incubation (Fig. 3). Interestingly, there was a substantial increase in the expression of membrane-bound TRAIL after 12 h of incubation, particularly for PBMCs stimulated with recombinant HCV core protein (~20%) (Fig. 3). Both PBMCs stimulated with LPS and PBMCs stimulated with recombinant HCV core protein displayed higher levels of membrane-bound TRAIL, approximately 5% and 32%, respectively (Fig. 3). Nevertheless, PBMCs stimulated with recombinant HCV core protein exhibited the highest expression of membrane-bound TRAIL after 12 h of incubation. Supporting

Information 6 displays a bar graph and a stacked proportion graph indicating the relative percent change in membrane-bound TRAIL protein levels in various incubation periods.

Determine relative expression of TRAIL in total PBMCs using FACS

We also examined the relative expression of TRAIL in the total PBMC population using FACS. After 3 h of incubation, TRAIL expression in unstimulated PBMCs and stimulated PBMCs was increased relative to TRAIL levels at baseline (Fig. 5). Fig. 6 displays a representative zebra plot of TRAIL expression in PBMCs after 3 h of incubation using FACS. Supporting Information 7 and 8 display representative zebra plots of multicolor FACS of TRAIL in total PBMCs after 6 and 12 h, respectively. TRAIL expression in PBMCs stimulated with LPS and PBMCs stimulated with recombinant HCV core protein were slightly higher (less than 1%) than unstimulated PBMCs, with PBMCs incubated with recombinant HCV core protein displaying the highest level of TRAIL expression after 3 h of incubation. 6 h-incubated PBMCs showed the following trend: TRAIL expression in unstimulated PBMCs increased (~0.25%) from 3 h to 6 h. Moreover, there was only a slight increase (0.09%) in TRAIL expression in PBMCs stimulated with LPS after 6 h of incubation (Fig. 5). In contrast, PBMCs stimulated with recombinant HCV core protein showed a decrease (~0.07%) in TRAIL expression after 6 h of incubation.

Also, all PBMCs, excluding PBMCs stimulated with LPS, displayed a slight increase, approximately 0.03% for unstimulated PBMCs and approximately 0.30% for PBMCs stimulated with recombinant HCV core protein, in TRAIL expression after 12 h of incubation (Fig. 5). The number of cells expressing TRAIL was similar after 12 h of incubation to 6 h of incubation in PBMCs stimulated with LPS (Fig. 3). Two-Way ANOVA did not show any statistically significant differences in TRAIL expression relative to incubation time or type of antigenic stimulation (Table 3). Fig. 7 also shows a heatmap representation of TRAIL expression in PBMCs in all incubation periods.

In summary, we observed an estimated 20% increase in both soluble and membrane-bound forms of DR4 and DR5 in PBMCs stimulated with recombinant HCV core protein. Soluble DR4 and DR5 was higher in PBMCs stimulated with recombinant HCV core protein after 6 h compared to both baseline and unstimulated PBMC death receptor levels.

Although we did find increased expression of death markers in PBMCs stimulated with recombinant HCV core protein, FACS did not show the same time-dependent trend compared to ELISA for membrane-bound death receptor expression. With the ELISA technique, we observed that PBMCs stimulated with recombinant HCV core protein had the highest level (~11%) of DR4 and DR5 expression after 6 h, whereas with FACS, PBMCs stimulated with recombinant HCV core protein displayed the highest number (~0.39%) of cells expressing either DR4 or DR5 compared to unstimulated PBMCs after 3 h of incubation.

The differences in the trend observed for DR4 expression determined by ELISA and FACS could be due to the difference in technique. For example, ELISA measures how much antibody-conjugate there is in the entire protein sample. Whereas, the readout for flow cytometry is mean intensity for the marker of interest per cell. Thus, the increase in DR4 expression for both unstimulated and stimulated PBMCs observed after 6 h of incubation could be attributed to the specificity of FACS. We observed a statistically significant difference in the level of DR4 protein expression between incubation periods using the ELISA method. However, we did not observe any statistically significant differences in DR4 expression

between each incubation period using FACS. However, the trend in DR5 expression was similar for both ELISA and FACS, whereby DR5 levels decreased between 3 h and 6 h then increased between 6 and 12 h of incubation. Moreover, though statistically insignificant, increased death receptor expression observed in PBMCs stimulated with HCV core protein imply that total PBMCs are susceptible to apoptosis even after short exposure to HCV core protein. This susceptibility might partially explain the impaired innate immune response observed in those with chronic HCV infection. The differences in the trend in membrane-bound death receptors between ELISA and FACS is shown in Fig. 8.

Both soluble and membrane-bound forms of TRAIL displayed a similar trend with the ELISA technique. Interestingly, soluble TRAIL was remarkably high (~40%) in unstimulated PBMCs, PBMCs stimulated with LPS, and PBMCs stimulated with recombinant HCV core protein compared to death receptor expression after 3 h and 6 h of incubation. This high level of TRAIL expression was also drastically different (~56%) than baseline soluble TRAIL levels, which may imply that there is a high density of TRAIL expression on PBMCs in culture conditions. A comparison in the trend in TRAIL expression observed in PBMCs using ELISA and FACS is displayed in Fig. 9.

Still, PBMCs stimulated with recombinant HCV core protein displayed a statistically significant increase in soluble TRAIL expression in all incubation periods relative to unstimulated PBMCs. This increase occurred in concurrence with an increase in death receptor expression after 6 and 12 h of incubation. To add, we did not observe a significant increase in membrane-bound TRAIL in PBMCs stimulated with recombinant HCV core protein. A possible mechanism that PBMCs may be utilizing after exposure to HCV core protein is cleavage of TRAIL by metalloproteases. This is an interesting observation because membrane-bound ligands that are capable of inducing apoptosis are more efficient at initiating cell death than their soluble forms. Thus, a reason for the significant increase in soluble TRAIL versus membrane-bound TRAIL is the body's needs to exert apoptotic activities locally and systemically to combat the effects of HCV infection. Falcón et al., found that there was evidence of PBMC apoptosis in patients positive for anti-HCV

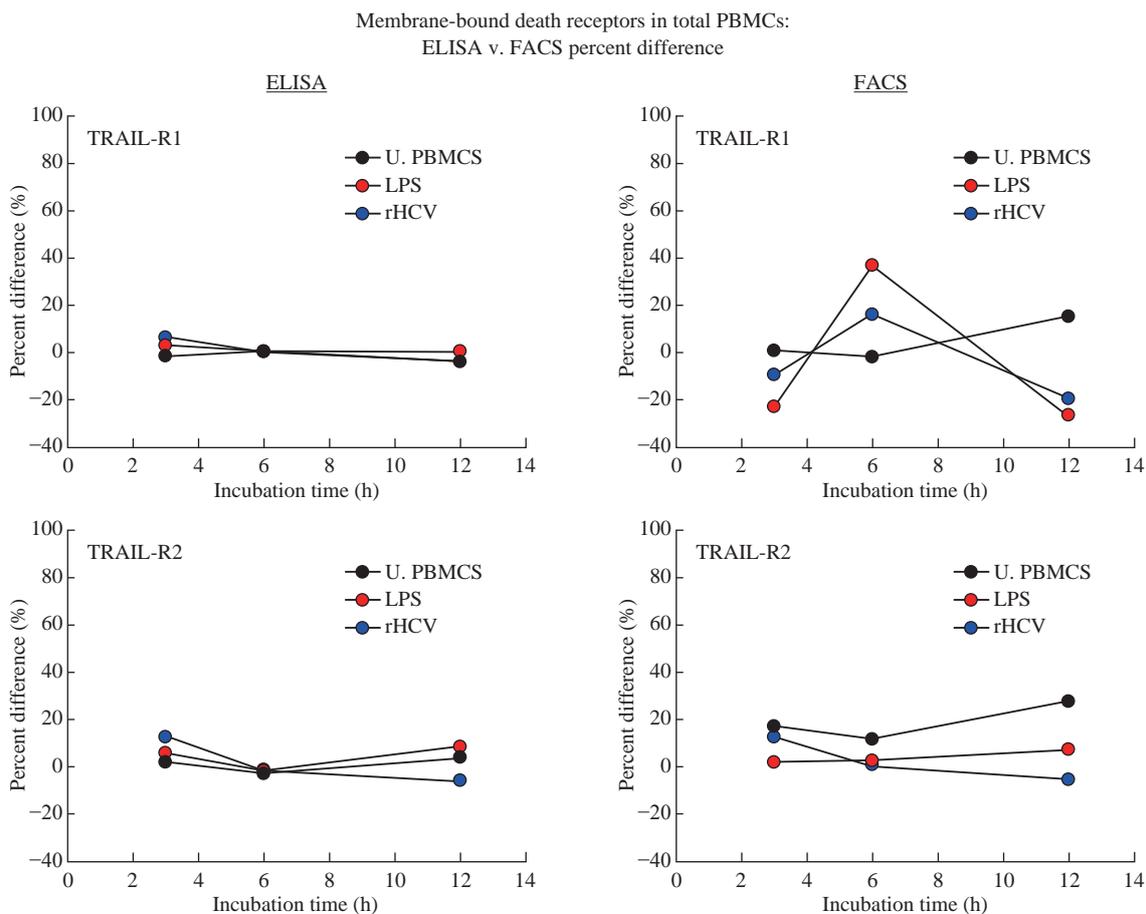


Fig. 8 Percent difference in TRAIL-R1 and TRAIL-R2 between 3, 6, and 12 h of incubation using ELISA and FACS. Each dot represents the average value from three replicates. Abbreviations: TRAIL-R1, tumor necrosis factor apoptosis inducing ligand-receptor 1; TRAIL-R2, tumor necrosis factor apoptosis inducing ligand-receptor 2; U.PBMCS, unstimulated peripheral blood mononuclear cells; LPS, lipopolysaccharide; rHCV, recombinant HCV core protein.

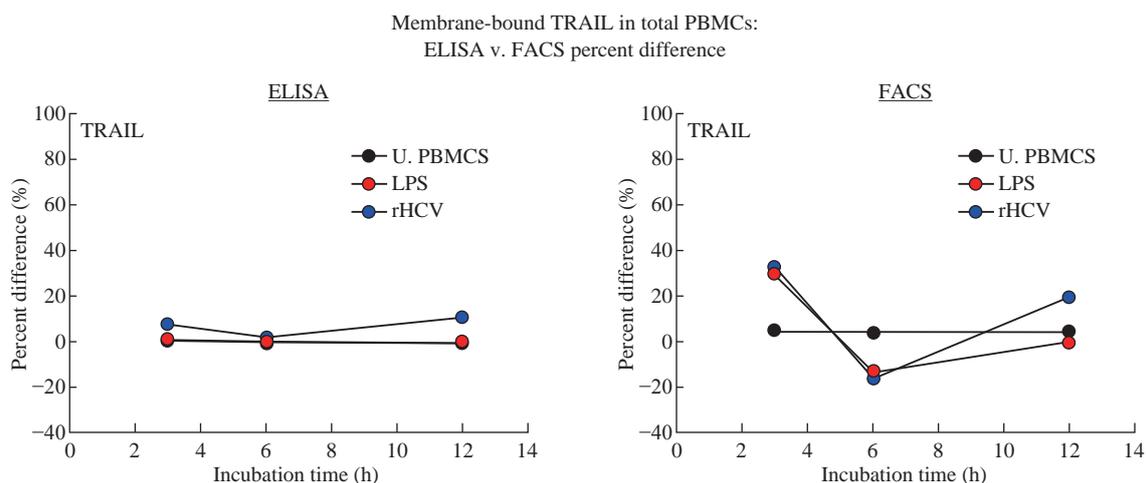


Fig. 9 Percent difference in TRAIL between 3, 6, and 12 h of incubation using ELISA and FACS. Each dot represents the average value from three replicates. Abbreviations: TRAIL, tumor necrosis factor apoptosis inducing ligand; U.PBMCS, unstimulated PBMCs; LPS, lipopolysaccharide; rHCV, recombinant HCV core protein.

antibodies [18]. However, this work suggested that apoptosis may be due to the Fas and tumor necrosis factor pathways of apoptosis. Thus, further work is needed to determine the role of the TRAIL-mediated pathway in apoptosis of PBMCs and specific subsets of

cells collected from HCV-infected patients.

Conclusions

Our results imply that HCV core protein causes increased susceptibility to apoptosis in the total PBMC

population within 12 h. We also found that cleavage of TRAIL by metalloproteases may be the mechanism of action utilized by PBMCs after exposure to HCV core protein. This virus-cell interaction may contribute to the impaired immune response often seen in individuals who develop chronic HCV infection. On the one hand, death receptor-expressing PBMCs could prevent the spread or the elimination of infection if the pool of noninfected PBMCs mediated an effective immune response. In the absence of such a pool, death receptor-expressing PBMCs could cause widespread cell death leading to viral persistence, contributing to HCV chronic infection.

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Conflicts of Interests

The authors declare that no competing interest exists.

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