Chronic hepatitis C virus infection triggers spontaneous differential expression of biosignatures associated with T cell exhaustion and apoptosis signaling in peripheral blood mononucleocytes

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Abstract Persistent hepatitis C virus (HCV) infection appears to trigger the onset of immune exhaustion to potentially assist viral persistence in the host, eventually leading to hepatocellular carcinoma. The role of HCV on the spontaneous expression of markers suggestive of immune exhaustion and spontaneous apoptosis in immune cells of chronic HCV (CHC) disease largely remain elusive. We investigated the peripheral blood mononuclear cells of CHC patients to determine the spontaneous recruitment of cellular reactive oxygen species (cROS), immunoregulatory and exhaustion markers relative to healthy controls. Using a commercial QuantiGenePlex® 2.0 assay, we determined the spontaneous expression profile of 80 different pro- and anti-apoptotic genes in persistent HCV disease. Onset of spontaneous apoptosis significantly correlated with the up-regulation of cROS, indoleamine 2,3-dioxygenase (IDO), cyclooxygenase-2/prostaglandin H synthase (COX-2/PGHS), Foxp3, Dtx1, Blimp1, Lag3 and Cd160. Besides, spontaneous differential surface protein expression suggestive of T cell inhibition viz., TRAIL, TIM-3, PD-1 and BTLA on CD4+ and CD8+ T cells, and CTLA-4 on CD4+ T cells was also evident. Increased up-regulation of Tnf, Tp73, Casp14, Tnfrsf11b, Bik and Birc8 was observed, whereas FasLG, Fas, Ripk2, Casp3, Dapk1, Tnfrsf21, and Cflar were moderately up-regulated in HCV-infected subjects. Our
observation suggests the spontaneous onset of apoptosis signaling and T cell exhaustion in chronic HCV disease.

**Keywords** Apoptosis · Caspase · Hepatitis C · Spontaneous immune exhaustion · TRAIL

**Introduction**

Persistent viral infection (PVI) due to human immunodeficiency virus (HIV), hepatitis B (HBV) and C viruses (HCV), and human papillomavirus (HPV) represents a leading global cause of morbidity and mortality from infectious diseases [1]. Recent estimates show that ~170 million persons are chronically infected with HCV worldwide. HCV is an enveloped, single-stranded, positive-sense RNA virus belonging to the Flaviviridae family [2, 3]. Sequencing analysis of HCV genome indicates the existence of ~11 genotypes, of which genotype 1 is the most prevalent in the United States, whereas genotypes 1 and 3 in Malaysia [4]. A vast majority of acutely infected individuals develop chronic hepatitis C (CHC), and eventually progress to develop cirrhosis and hepatocellular carcinoma (HCC) [5].

The biology of HCV in the host is poorly understood and the most challenging attribute is the mechanism underlying persistence [6]. During host-virus encounter, only ~15% of infected individuals spontaneously clear the virus [7]. Intriguingly, in individuals where HCV is not cleared, the virus appears to promote functional exhaustion of virus-specific T cells, including anergy and deletion owing to sustained up-regulation of death markers, lack of positive costimulation [8, 9], and expansion of regulatory T cells (Tregs) [10]. A recent study suggests a role for elevated immune cell apoptosis with the onset of HCV disease [11]. The increase in immune cell death has also been reported in clinical/chronic HIV, SIV and HCV diseases [12–14].

Apoptosis represents a genetic program key to development and differentiation of normal cellular functions whereby senescent cells undergo programmed death [15, 16]. Immune exhaustion, especially due to PD-1 ligation appears to trigger Fas(CD95)-mediated apoptosis of immune cells. Growing evidence suggest that spontaneous apoptosis of peripheral blood mononuclear cells (PBMCs) could be one mechanism of potential immune impairment, especially by HIV, where the involvement of diverse apoptosis pathways has been proposed [17–20]. Death pathways can be activated in host cells via receptor-mediated (extrinsic) and non-receptor-mediated (intrinsic) pathways [21, 22]. Current studies indicate that PVIs program apoptosis via the FasL–Fas interactions [21] TNF-α, mitochondrial, and Bcl-2 family death pathways potentially leading to viral persistence [23, 24]. A resurgence of interest in HCV has led to investigations prompted at deciphering the role of reactive oxygen species (ROS) released via the intrinsic pathway [25], and spontaneous apoptosis of primed immune cells in PVIs [20, 26, 27]. Despite its importance, far less is known about the role of inhibitory molecules and spontaneous apoptosis in chronic HCV-primed immune cells. Here, we observed evidence of signatures of spontaneous immune exhaustion, and intrinsic and extrinsic apoptosis pathways highlighting their possible association with chronic HCV disease. The data described here represents a survey of mediators associated with spontaneous immune exhaustion and apoptosis, and will aid to better understand HCV immunopathogenesis to be able for developing improved intervention strategies against viral persistence.

**Materials and methods**

**Human subjects**

Chronically HCV infected patients (n = 7) and healthy controls (n = 7) were recruited for this investigation. The study was carried out following approval of the protocols by the Medical Ethics Committee (MEC) of University of Malaya Medical Center, for ethical issues. Written, informed consent was obtained from all the participants before study enrolment. The study was carried out in compliance with good clinical practice, including the International Conference on Harmonization Guidelines and the Declaration of Helsinki. All chronic HCV-infected patients were positive for HCV antibodies and HCV RNA, and negative for HIV antibodies and hepatitis B surface antigen (HBsAg). At the time of recruitment, none of the patients were receiving or had received any pegylated interferon-α or immunosuppressive therapy within 6 months. Additional exclusion criteria were recent illness and/or vaccination within 4 weeks prior to phlebotomy, diabetes mellitus, hypertension/cardiovascular disease, pregnancy and treatment for any form of inflammatory manifestations. The demographics and clinical characteristics of participants are summarized in Table 1.

Liver transaminases and HCV plasma viral load

Plasma aspartate transaminase (AST) levels were estimated using a commercial ELISA (IBL America, Minneapolis,
MN), according to manufacturer’s instructions. Plasma alanine transaminase (ALT) levels were determined with a Hitachi7050 Automatic Analyzer (Hitachi Corp, Tokyo, Japan) using a commercial ALT assay kit (Wako Pure Chemicals, Osaka, Japan). The cut-off values were set at 20 ng/ml. HCV plasma viral loads (PVLs) were measured using a commercial COBAS AMPLICOR HCV test, version 2.0 (Roche Molecular Systems, Branchburg, NJ, USA). The analytical sensitivity (95 % threshold) of the COBAS AMPLICOR HCV 2.0 was 60 and 100 HCV IU/ml with EDTA plasma and serum, respectively. The total lymphocyte counts (TLCs) for each HCV positive participant were determined by flow cytometry.

Peripheral blood mononuclear cells

Isolation of PBMCs from buffy coats was carried out as previously described [28]. Briefly, 9 ml of whole blood was obtained per patient or control in a BD Vacutainer® heparin lithium tubes (BD Biosciences, Franklin Lakes, NJ, USA), and PBMCs were prepared by density-gradient centrifugation over Ficoll-Paque™ (Amersham Pharmacia, Piscataway, NJ, USA). The cells were resuspended in a freezing media (10 % DMSO in 90 % fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and cryopreserved in liquid nitrogen until use in the experiments.

Primary cell culture

Culture of PBMCs was made in RPMI1640 medium supplemented with HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 µg/ml), sodium pyruvate (1 mM), gentamicin (5 µg/ml) (all procured from Life Technologies, Victoria, Australia), and 10 % of FBS (Gibco). Cells were cultured at 37 °C in a 5 % CO2 incubator.

Table 1 Demographic and clinical characteristics of chronic hepatitis C-infected individuals

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy controls (n = 7)</th>
<th>Chronic HCV-infected (n = 7)</th>
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<tbody>
<tr>
<td>Sex (M/F)</td>
<td>5/2</td>
<td>3/4</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>11.20 ± 3.43</td>
<td>52.6 ± 9.31</td>
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<tr>
<td>AST (IU/l)</td>
<td>13.00 ± 3.54</td>
<td>57.43 ± 28.8</td>
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<tr>
<td>PVL (105 IU/ml)</td>
<td>N/A</td>
<td>3.2</td>
</tr>
<tr>
<td>Patient no.</td>
<td>ALT (IU/l)</td>
<td>AST (IU/l)</td>
</tr>
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Indoleamine 2,3-dioxygenase (IDO) assay

Serum Indoleamine 2,3-dioxygenase (IDO) was measured using a commercial Sandwich ELISA (Uscn Life Science Inc, Wuhan, Hubei, PRC) according to the manufacturer’s instructions with a detection range of 1.563–100 ng/ml. This assay has a sensitivity of 0.61 ng/ml for detection of IDO from serum, plasma, tissue homogenates and other body fluids. Briefly, blood from CHC-infected patients and healthy controls were centrifuged at 3,000 rpm for 10 min and dispensed into microtiter wells immobilized with a biotin-conjugated anti-IDO. For IDO evaluation, the enzyme-substrate reaction mixture was terminated by adding 20 % (w/v) trichloroacetic acid. IDO concentration was estimated by comparing the optical density of samples with the standard curve.

Cyclooxygenase-2 (COX-2)/prostaglandin H synthase (PGHS-2) assay

Serum COX-2/PGHS-2 was measured using a commercial ELISA (R&D Systems, Abingdon, United Kingdom). Blood samples from CHC-infected individuals and controls were centrifuged at 3,000 rpm for 10 min and dispersed into microtiter wells immobilized with a biotin-conjugated anti-IDO. For IDO evaluation, the enzyme-substrate reaction mixture was immediately added to 100 μl of 8 % formaldehyde in phosphate buffered saline (PBS) solution (v/v), and the mixture was read at 450 nm.

Multicolor flow cytometry

PBMCs were stained for spontaneous expression of surface protein markers associated with immune exhaustion and death ligand, TRAIL (ApoL2). Cells were stained with peridinin chlorophyll protein-cyanin-5.5 (PerCP-Cy5.5)-conjugated antibody to CD3 (clone SK7), phycoerythrin-
cyanin7 (PE-Cy7)-conjugated antibody to CD4 (clone RPA-T4), allophycocyanin-H7 (APC-H7)-conjugated antibody to CD8 (clone SK-1) (all from BD Pharmingen™), APC-conjugated antibody to TIM-3 (clone 344823, R & D Systems), PE-conjugated antibody to BTLA (clone J168-540, BD Pharmingen™), PE-conjugated antibody to TRAIL (clone RIK-2), fluorescein isothiocyanate (FITC)-conjugated antibody to PD-1 (clone MIH-4), and Brilliant Violet™ 421 (BV421)-conjugated antibody to PD-1, mouse IgG2a FITC (MAB349051), mouse IgM κ FITC (MAB555583), PE-labeled mouse IgG1 κ PE-CYT™ mouse IgG1 κ (PMG557872), mouse IgM κ Alexa 647 (PMG560806), IgG1 κ APC-H7 (PMG561067), PerCP-CY™5.5 mouse IgG1, κ (PMG552834), APC mouse IgG2b κ (PMG555745), mouse IgG2b κ FITC (MAB55742) and normal goat IgG fluorescein control (GZ-IC108F). All isotype controls were procured from R & D Systems, USA. Data were acquired on a 7-color FACS Canto II™ (BD Immunocytometry Systems, San Jose, CA, USA) using the FCAP array (BD Biosciences) and analyzed using FlowJo software (Tree-Star, Ashland, OR, USA).

Measurement of cellular reactive oxygen species (cROS)

cROS formation was measured using a commercial oxidation-sensitive fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) ROS detection assay kit (Molecular Probes, Eugene, OR, USA). The kit employs a fluorogenic dye 2′,7′-dichlorofluorescein diacetate (DCFDA), which measures hydroxyl, peroxyl and any other intracellular ROS. Following diffusion, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is subsequently oxidized by intracellular ROS to form a highly fluorescent 2′,7′-dichlorofluorescin (DCF), detectable by fluorescence spectroscopy. Briefly, the cells were seeded in 100 μl culture medium overnight in a 96-well plate in the dark. On the day of experiment, cells were washed with PBS and incubated with 25 μM DCFH-DA for 45 min in the dark. Later, the cells were washed with PBS to remove residual DCFH-DA. Cellular fluorescence was measured at 485 nm (excitation) and 535 nm (emission) using a Varioskan Flash microplate reader (Thermo-Scientific, Walther, MA, USA).

mRNA isolation and one-step quantitative real-time PCR

Total RNA was extracted from PBMCs of CHC-infected patients and healthy controls using a commercial Spin technology (RNase Mini Kit, Qiagen, Solna, Sweden) according to the manufacturer’s protocol. DNase treatment was carried out to remove any contaminating DNA using an RNase-Free DNase Set (Qiagen). RNA concentration was determined using a NanoDrop™ spectrophotometer. qRT-PCR was performed in a total volume of 20 μl including 2 μl of RNA, forward and reverse primers (0.2 mM each) and 25 μl of SYBR®Green PCR mix (SYBR® Green: Applied Biosystems, Stockholm, Sweden). Reactions were run on a q5 Thermal cycler (BioRad, Hercules, CA, USA) using the universal thermal cycling parameters. Results were obtained using the sequence detection software iq5 thermal cycler and analyzed using MS Excel software. For all samples, melting curves were acquired for quality control purposes. For gene expression quantification, we used the comparative Ct method. Initially, gene expression levels for each sample were normalized against the average Ct values of two endogenous controls (β-actin and tubulin), according to manufacturer’s protocol.

The primers were designed using the NCBI Primer-BLAST (Basic Local Alignment Search Tool) online software at http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINKLOC=BlastHome. The primers used against the corresponding genes were synthesized by First Base Labs (First Base, Kuala Lumpur, Malaysia), and are as follows: Blimp1, forward 5′-CAGCTCAGCCACCTGCGAGAA-3′ and reverse 5′-GCCGCACGCAGTCCCTCTT-3′; Btlα, forward 5′-TGCTTGTTTCTTCTTCCAGGC-3′ and reverse 5′-TGGGTCACTACCCTGTTGCTGC-3′; Cd160, forward 5′-GCGCTGGCCTTACACTGAGC-3′ and reverse 5′-TCCCCGTGCTCCTGTTGTG-3′; Cia4, forward 5′-GGGCATAGCAACGGAACCACA-3′ and reverse 5′-GGGGGATATTCACATAAGCCCCTG-3′; Foxp3, forward 5′-CAGCACATTCCCAGAGTGCACG-3′ and reverse 5′-CCGGTGCAACAGTTGCTGC-3′; Lag3, forward 5′-CTAGCCGAGTGCCCACACGGC-3′ and reverse 5′-GCCCTGGAGGCTGTAATCCCG-3′; Pdl1, forward 5′-CTCACAGTTAGGAGGAGAG-3′ and reverse 5′-GACCAAAACACACAGGTGTT-3′; Tim3, forward 5′-AGGGGACTAGGCACAGAAG-3′ and reverse 5′-GCCAGGCAGACAGATCCC-3′; Trail, forward 5′-CTTTACCAACGAGCTG-3′ and reverse 5′-
QuantiGene Plex® 2.0 assay

Total RNA from PBMCs of participants was processed by means of QuantiGene Plex® 2.0 assay (Affymetrix, Santa Clara, CA, USA) according manufacturer’s instructions. The QuantiGene Plex® 2.0 is a hybridization-based assay using the xMAP® Luminex® magnetic beads and performed on 96-well plates designed to quantify 80 different RNA targets of the extrinsic and intrinsic apoptosis pathways. The assay directly quantifies the RNA targets using xMAP Luminex beads for multiplexing of 3–80 RNA targets and branched DNA (bDNA) signal amplification technology. Briefly, a working bead mix was prepared containing lysis mixture, blocking reagent, capture beads, and 2.0 probe set. The bead mix was dispensed into the hybridization plate, and 20 μl of total RNA was added to each well. For background control wells, 20 μl of sterile nuclease-free water was added to the bead mix. The hybridization plate was placed in a shaking incubator (600 rpm) for 22 h at 54 °C. After hybridization, the wash solution, pre-amplifier, amplifier, label probe, and streptavidin–phycoerythrin (SAPE) solutions were prepared. Later, the hybridized samples were transferred to a magnetic separation plate. Samples were washed and incubated for 1 h each (50 °C at 600 rpm) with the pre-made amplifier solutions. Later, the SAPE solution was added and the samples were incubated at 25 °C for 30 min at 600 rpm in the dark. The unbound SAPE was washed, and 130 μl of SAPE wash buffer was added to each RNA sample. The plate was shaken at 25 °C for 3 min at 800 rpm to resuspend the beads. The plate was read immediately using a Bio-Plex® 200 system (BioRad, Hercules, CA, USA). Fluorescent readings from blank wells were subtracted from fluorescent values for each mRNA of interest. The values were normalized against the geometric mean expression of two internal control genes, Ppib (peptidylpropyl isomerase B) and Hprt1 (hypoxanthine–guanine phosphoribosyltransferase) for each sample that was run in triplicates.

Statistical analysis

Statistical significance was analysed using MS Excel and GraphPad Prism 6.0 softwares (GraphPad, La Jolla, CA, USA). The levels of significance for comparisons between two or more independent samples were determined using a two-tailed unpaired Student’s t test. Data are presented as mean ± SD, where n refers to the number of independent experiments. For QuantiGene Plex® 2.0 assays, expression values for each gene were multiplied by a constant, and compared between CHC-infected patients and healthy controls. The values were normalized against the geometric mean expression of two internal control genes Ppib and Hprt1 for each sample, and expressed as fold change >2-fold, highly up-regulated; 1.5- to 2-fold, moderately up-regulated; 1- to 1.5-fold, mildly up-regulated. For expression of immune exhaustion molecules by FACS analysis, the data were transformed and normalized, and a two-tailed non-parametric paired t test was used for revealing significance. The level of significance was considered at P < 0.05, and measures represented by *P < 0.05, **P < 0.005, ***P < 0.001 and ****P < 0.0001.

Results

Demographic analysis

Our study consisted of 14 participants that included seven CHC-infected individuals and seven healthy controls. Table 1 shows the mean age of participants, TLCs, HCV PVLs, and serum ALT and AST liver enzyme levels of CHC-infected individuals. Of the seven CHC subjects, three were males and four were females with a mean PVL of 3.2 × 10⁵ HCV IU/ml of blood. In contrast, the healthy controls included five males and two females. We observed that the TLCs decreased with increasing frequency of spontaneous apoptosis.

Serum indoleamine 2,3-dioxygenase levels were elevated in chronic HCV-infected individuals

IDO is an intracellular enzyme that deprives immune cells of tryptophan, an amino acid essential for T cell proliferation [30], facilitating T cell apoptosis aided by inhibitory molecules and induction of oxidative stress [31]. IDO activity increases with disease severity in patients with PVIs, especially HIV disease [32]. COX-2/PGHS-2 represents an inflammatory metabolite occurring in most tissues [33] usually at extremely low or undetectable levels, and could be associated with tumorigenesis [34, 35]. Here, we assessed the serum levels of IDO and COX-2 and their association with spontaneous apoptosis in PBMCs derived from CHC-infected individuals. Our investigations showed that serum IDO (Fig. 1a) and COX-2/PGHS-2 levels (Fig. 1b) were significantly increased among CHC-infected individuals relative to healthy controls (Table 2) although there was no association between PVL and IDO levels (data not shown). We speculated that the elevated serum levels of IDO and COX-2/PGHS-2 could likely influence spontaneous apoptosis of immune cells in chronic HCV-infected individuals.
Cellular reactive oxygen species levels were increased in chronic HCV-infected subjects. Next, we determined the cROS levels in PBMCs of CHC-infected subjects and healthy controls as the level of cROS reflects ongoing oxidative stress and apoptosis via the caspase cascades [36, 37], and most likely the onset of intrinsic apoptosis pathways. COX-2/PGHS-2 indicates an ongoing inflammation and ROS levels contribute to oxidative stress in HCV infection [38]. Our findings showed a significant increase in cROS levels in CHC-infected individuals compared to healthy controls (Fig. 1c; Table 2). The observed increase in cROS indicates increased intracellular oxidative stress that could likely be the mediators of ongoing apoptosis in CHC disease.

Immune cells of chronic HCV-infected subjects showed up-regulated expression of TRAIL and certain other biosignatures of immune exhaustion.

PVIs appears to facilitate immune exhaustion in CD8+ T cells [39] and impairment of immune cells [40]. For instance, increased intrahepatic expression of Trail-induced apoptosis in primed immune cells [41]. The association between increased levels of cROS and Trail prompted us to investigate the possible role of molecules associated with immune exhaustion in CHC disease. We initiated this by studying the expression levels of mRNA of immune inhibitory molecules by qRT-PCR. Our investigations showed that the gene expression of inhibitory molecules in CHC-infected patients was marginally increased as compared to healthy controls. We observed relatively increased gene expressions of Tim3, Pd1, Cd160, Cta4, Trail, Btla, and Lag3 in CHC-infected individuals (Fig. 2a, b) (Table 3). Conversely, the increased expression of inhibitory molecules in CHC-infected individuals suggest a role for immune exhaustion molecules, especially Trail (14.7-fold) with accentuated apoptosis in CHC cases.

CD4+ T cells of chronic HCV-infected individuals displayed increased surface expression of TRAIL and differential expression of immune exhaustion molecules.

Next, we investigated the surface expression levels of selected immune inhibitory molecules on CD4+ and CD8+ T cells in PBMCs from CHC-infected subjects by flow cytometry (Fig. 3a–f; Table 4). Our findings indicated that the surface expression of TRAIL, TIM-3, PD-1, and CTLA-4 in CD4+ T cells of CHC-infected subjects were up-regulated relative to healthy controls. On CD8+ T cells, we observed an up-regulation in TRAIL, TIM-3, and PD-1 in CHC-infected individuals relative to healthy controls (CD8+ CTLA-4+...
We found that BTLA expression was significantly decreased in CHC-infected subjects in CD4+ and CD8+ T cells (Fig. 3f; Table 4). Of note, we found that CD4+ T cells of chronic HCV-infected individuals displayed increased surface expression of TRAIL and differential expression of other immune exhaustion molecules.

Increased expression of inhibitory molecules was consistent with up-regulation of Blimp-1, Dtx1 and FoxP3 in immune cells of chronic HCV disease.

Transcription factors, Dtx1, Foxp3 and Blimp1 reportedly limit T cell survival and functionality in PVIs [42]. Decreased Blimp1 expression results in improved CD8+ T cell survival owing to reduced expression of Pd1, Lag3, and Cd160 on virus-specific CD8+ T cells in PVIs [13]. Moreover, Foxp3 has recently been identified as a pro-apoptotic protein [23]. Hence, we broadly surveyed the spontaneous expression of transcriptional factors in PBMCs of the study population. Our results showed marginal up-regulation of Foxp3, and Dtx1, and Blimp1 in CHC-infected individuals relative to healthy controls (Fig. 4) (Table 3), suggesting a likely role of these transcription factors with the increase of co-inhibitory molecules in chronic HCV disease.

Extrinsic and mitochondrial pathways regulated the spontaneous onset of expression of apoptosis markers in immune cells of chronic HCV-infected subjects.

Given the increased expression of TRAIL both at the gene and protein levels in PBMCs hinting the likely role of apoptosis in persistent HCV disease, next we asked if the genes encoding the extrinsic and intrinsic apoptosis pathways played any role in the CHC disease. Spontaneous apoptosis is induced simultaneously by both extrinsic and intrinsic pathways, and therefore we surveyed the expression of an array of apoptotic genes, using a commercial QuantiGene Plex® 2.0 assay. Our results clearly showed that CHC infection modulates the spontaneous expression of a large array of apoptotic genes in immune cells. High up-regulation of Tnf (24.1-fold), Bcl2a1, Tp73, Bcl2l10, Casp14, Tnfrsf11b, Bik and Birc8 was observed in CHC-infected cases (Fig. 5). The profile of expression of apoptosis-related genes has been shown in Table 5. Together, we demonstrated that spontaneous onset of apoptosis in PBMCs of CHC-infected individuals was likely co-regulated by both extrinsic and intrinsic pathways.

**Discussion**

The cell death mechanisms operational in HCV-pre-primed immune cell phenotypes remain ambiguous despite that apoptosis of hepatocytes has been well-described in clinical HCV disease [43]. Cell death characteristically follows one of two patterns: apoptosis (ATP-
dependent) or necrosis (ATP-independent) [44, 45]. Several hypotheses have been proposed to explain PVI-induced cell death [46]. A growing body of literature suggests that HBV- [47, 48], SIV- [13] and HIV- [49] infected PBMCs undergo apoptosis. Nevertheless, the contribution of spontaneous T cell exhaustion and apoptosis of PBMCs during PVIs remain unclear. Here, we show evidence of spontaneous T cell exhaustion and apoptosis in immune cells of CHC-infected subjects. Our current findings are in accordance with previous findings of markers of oxidative stress in HCV infection [43]. Interestingly, significant expression of inhibitory molecules, transcription factors and immunoregulatory enzymes were clearly evident among CHC-infected individuals indicating the likely association between immune exhaustion and immune cell apoptosis.

IDO is a T cell proliferation-limiting enzyme [30], and increased IDO activity has been linked to disease severity.

Fig. 3 Persistent HCV disease results in differential surface protein expression of TRAIL, TIM-3, PD-1, CTLA-4 and BTLA on CD4+ and CD8+ T cells. a Depiction of the gating strategy used throughout: lymphocytes were selected on the basis of forward- and side-scatter characteristics, dead cells excluded, then CD3+, CD4 and CD8+ T cells selected. Surface expression of b TRAIL, c TIM-3, d PD-1, e CTLA-4 and f BTLA on CD4+ and CD8+ T cells of CHC-infected subjects and healthy controls (Top panel, healthy controls; Bottom panel, chronic HCV infection). PBMCs were stained with the following antibody combinations: TRAIL: anti-PerCP-Cy5.5-CD3, PE-Cy7-CD4, APC-H7-CD8, and PE-TRAIL; TIM-3: anti-PerCP-Cy5.5-CD3, PE-Cy7-CD4, APC-H7-CD8, and PE-TRAIL; PD-1: anti-PerCP-Cy5.5-CD3, PE-Cy7-CD4, APC-H7-CD8, and FITC-PD-1; CTLA-4: anti-PerCP-Cy5.5-CD3, PE-Cy7-CD4, APC-H7-CD8, and FITC-PD-1; and BTLA: anti-PerCP-Cy5.5-CD3, PE-Cy7-CD4, APC-H7-CD8, and PE-BTLA mAbs, and acquired on a 7-color FACS Canto II™ and analyzed using FlowJo™ software. Data were transformed and normalized, and paired t tests were used for revealing statistical significance. Data are presented as mean ± SD, where n refers to the number of independent experiments. Bars represent mean values ± SEM. *P < 0.05, **P < 0.005, ***P < 0.001 and ****P < 0.0001.
in PVIs, especially HIV infection [32]. The other attribute in our research is the elevation of serum COX-2 in CHC-infected subjects. COX-2 catalyzes the conversion of arachidonic acid to prostaglandins and thromboxanes [33]. Under normal conditions COX-2 is low or undetectable, but can be readily induced in response to cellular activation by physiologic stimuli. Up-regulation of COX-2 leads to chronic inflammation, limiting lymphocyte expansion [34, 35]. HCV is known to up-regulate ROS via inducible nitric oxide synthase (iNOS) and COX-2 in chronic HCV infection [43]. Oxidative stress involves generation of superoxide anions, H$_2$O$_2$, singlet oxygen, and hydroxylperoxy radicals triggering caspase activation [50]. Our finding of increased cROS levels in CHC indicates activation of the intrinsic pathway [51]. Although, there were a few anti-apoptotic genes that were elevated, this likely could be to sustain cellular homeostasis [52, 53] and requires additional investigation.

Immune exhaustion has also been linked to spontaneous apoptosis [40], which is regulated by a balance between anti- and pro-apoptotic molecules via Fas-FasL interactions and/or the mitochondrial pathway [54]. In agreement with this, here we found that CD160, Trail, Lag3, Tim3, Pdl1, and Ctra4 were up-regulated in HCV-primed PBMCs suggesting ongoing immune exhaustion in parallel with immune cell apoptosis in CHC infection. The most intriguing finding is the significantly down-regulated surface expression of BTLA in spite of up-regulated gene expression, which is in line with our recent investigations on HIV-infected T cells in vitro [32], and others also support this finding [54, 55]. Dysregulation of B cells could also be associated with decreased BTLA in HIV viremic individuals [56], and therefore the functional implications of decreased BTLA in CHC disease warrants further evaluation.

Exhausted CD8+ T cells during PVIs specifically increases the expression of Cd160 [57, 58], which is in line with our current finding. Another study showed that PD-1+ CD8+ T cells undergo both spontaneous and Fas-mediated apoptosis [21]. One study showed that Trail triggers
apoptosis in fresh liver explants of patients with viral hepatitis [59]. Further, lack of Lag3 in mice markedly delays T cell apoptosis [34]. Likewise, TIM-3 generates an inhibitory signal leading to reduced secretion of antiviral cytokines [41]. Blimp1 is a transcriptional repressor of memory differentiation in CD8+ T cells, which also appears to intrinsically regulate Pdl1, Lag3, and Cd160 expression in virus-specific CD8+ T cells during PVls [60]. Hence, the expression of these molecules may likely be linked to one another to modulate the functions of each other. HCV infection reportedly elevates the turn-over frequencies of TIM-3+ and PD-1+ HCV-specific T cells during PVls [61]. Besides, HCV-HIV co-infected patients also appear to show increased expression of PD-1 and Fas that consequently has been correlated with stages of HCV liver disease [14]. One recent finding showed that intrahepatic CD8+ T cells of CHC patients expressed increased levels of TIM-3 and PD-1 [62]. Besides, enhanced neutrophil apoptosis via activation of caspase 10 has also been linked to HCV infection although its role with disease progression needs elaborate discussion [63]. Together, the concerted role played by immune exhaustion molecules and immune cell apoptosis still remains a gray area of investigation.

The extrinsic pathway is triggered by ligation of death receptors, such as Fas/CD95 or the TRAIL receptors, TRAIL-R1 and TRAIL-R2 or TNFR-1 or 2 via their cognate ligands FasL or TRAIL or TNF-α respectively, resulting in receptor trimerization, assembly of death domains, and recruitment of adaptor molecules, Fas-associated death domain (FADD) via homophilic contact facilitated by the death domain [64, 65]. FADD recruits caspase-8 to the activated Fas to form the Fas death-inducing signaling complex (DISC). Oligomerization of caspase-8 upon DISC formation drives its activation via self-cleavage. Subsequently, caspase-8 activates downstream effector caspases 3, 6, and 7 (all up-regulated). Eventually, caspase-8 initiates activation of BH3 interacting-domain death agonist (Bid).

Fig. 3 continued
Caspase 10 also appears to bind to Fas and CFLAR that were all up-regulated in CHC infection in the current study. While it is also well-known that CD27 can bind to TRAF2 and TRAF3 in addition to TNF (all up-regulated) and mediate T cell apoptosis in CHC-infected subjects, up-regulation of anti-apoptotic genes, \( \text{Bcl-2A1} \) and \( \text{Bcl-2L10} \), and a mild up-regulation of \( \text{Bcl-2L2} \), and \( \text{Mcl-1} \) in CHC patients indicate that HCV preserves some cells (preferably unprimed cells) from undergoing apoptosis. However, since other pro-apoptotic genes were up-regulated in immune cells of CHC patients inducing spontaneous apoptosis, the cROS-induced cell death is likely an alternate death pathway resulting from caspase activation [55]. Our data suggest that elevated cROS levels activate the intrinsic pathway involving the release of mitochondrial intermembrane space proteins, including Cyt c, and AIF into the cytosol. Later, cytosolic Cyt c activates the apoptosome complex, eventually cleaving procaspase-9 and effector caspases 3, 6, and 7 resulting in apoptosis [66, 67]. The onset of spontaneous apoptosis is further fortified by the up-regulation of Bcl-2 family members in the PBMCs of CHC-infected patients. The ‘Bcl-2-regulated pathway’ is a vital trigger of intrinsic and extrinsic apoptosis, which
synergistically regulates the mediators of apoptosis. Here, we also hypothesized that cROS owing to persistent HCV infection initiates specific BH3-only proteins, which then inactivates Bcl-2-like pro-survival molecules [68] as supported by our current findings. Our current findings are also in agreement with the up-regulation of apoptosis pathways in chronic HCV disease and HCV-HIV co-infection [69].

Our investigation has shown that an elevated expression of molecules that induce spontaneous apoptosis and T cell inhibition in immune cells of CHC-infected subjects, although it still remains to be seen if the functional attributes of the immune cells studied here were compromised. Our observations showed evidence of increased levels of oxidative stress and immune exhaustion in CHC-infected patients, triggering the potential recruitment of apoptotic genes. Furthermore, up-regulation of pro-apoptotic factors functioning via the extrinsic and intrinsic pathways suggests their utility for intracellular viral persistence, which however, remains to be investigated. There are also certain limitations in the current investigation, which remain unresolved. Our original aim was to investigate the potential association between immune exhaustion and apoptosis. Nonetheless, we were only partly successful in linking both the spontaneous entities. One of the major

<table>
<thead>
<tr>
<th>Protein expression</th>
<th>Healthy controls (n = 7)</th>
<th>Chronic HCV-infected (n = 7)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIM-3</td>
<td>27.49 ± 2.9</td>
<td>31.43 ± 3.6</td>
<td>P = 0.06</td>
</tr>
<tr>
<td>PD-1</td>
<td>36.74 ± 2.8</td>
<td>42.39 ± 3.0</td>
<td>P = 0.1</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>4.456 ± 0.4</td>
<td>5.241 ± 0.3</td>
<td>P = 0.1</td>
</tr>
<tr>
<td>BTLA</td>
<td>62.31 ± 3.6</td>
<td>43.46 ± 6.9</td>
<td>*P = 0.03</td>
</tr>
<tr>
<td>TRAIL</td>
<td>39.97 ± 1.4</td>
<td>46.36 ± 2.4</td>
<td>*P = 0.006</td>
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<tr>
<td>CD8+ T cells</td>
<td></td>
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<td></td>
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<tr>
<td>TIM-3</td>
<td>49.66 ± 2.6</td>
<td>56.06 ± 3.0</td>
<td>P = 0.1</td>
</tr>
<tr>
<td>PD-1</td>
<td>27.91 ± 3.4</td>
<td>34.80 ± 3.5</td>
<td>P = 0.1</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>62.39 ± 4.7</td>
<td>54.99 ± 3.4</td>
<td>P = 0.2</td>
</tr>
<tr>
<td>TRAIL</td>
<td>21.19 ± 2.5</td>
<td>20.74 ± 2.9</td>
<td>P = 0.3</td>
</tr>
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</table>
limitations is the sample size due to poor turnover of individuals with HCV infection in the setting, and use of cells for other investigations. Further, there was limited clinical data that correlated with markers of apoptosis studied. In addition, it is also not clear if the ongoing inflammation in chronic HCV patients originated from ongoing liver cell damage, and therefore use of controls, for instance, inclusion of subjects with non-alcoholic steatohepatitis (NASH) is warranted. It remains to be seen how the immune cells program the expression of the bio-signatures investigated herein, following stimulation with standard HCV protein antigens to clearly underpin the concrete association of virus-specific immune exhaustion with apoptosis signaling in viral persistence.

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Conflict of interest The authors declare that they have no conflict of interest to disclose.

References

differential responsiveness of human memory T cells to Fas (CD95)-mediated apoptosis. J Immunol 162(7):3851–3858


