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Research paper

CD8+ T cells of chronic HCV-infected patients express multiple negative immune checkpoints following stimulation with HCV peptides

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ABSTRACT

Hepatitis C virus (HCV)-specific CD4+ and CD8+ T cells are key to successful viral clearance in HCV disease. Accumulation of exhausted HCV-specific T cells during chronic infection results in considerable loss of protective functional immune responses. The role of T-cell exhaustion in chronic HCV disease remains poorly understood. Here, we studied the frequency of HCV peptide-stimulated T cells expressing negative immune checkpoints (PD-1, CTLA-4, TRAIL, TIM-3 and BTLA) by flow cytometry, and measured the levels of Th1/Th2/Th17 cytokines secreted by T cells by a commercial Multi-Analyte ELISArray™ following *in vitro* stimulation of T cells using HCV peptides and phytohemagglutinin (PHA). HCV peptide-stimulated CD4+ and CD8+ T cells of chronic HCV (CHC) patients showed significant increase of CTLA-4. Furthermore, HCV peptide-stimulated CD4+ T cells of CHC patients also displayed relatively higher levels of PD-1 and TRAIL, whereas TIM-3 was up-regulated on HCV peptide-stimulated CD8+ T cells. Whereas the levels of IL-10 and TGF-β1 were significantly increased, the levels of pro-inflammatory cytokines IL-2, TNF-α, IL-17A and IL-6 were markedly decreased in the T cell cultures of CHC patients. Chronic HCV infection results in functional exhaustion of CD4+ and CD8+ T cells likely contributing to viral persistence.

1. Introduction

Hepatitis C is a chronic liver disease caused by hepatitis C virus (HCV), an enveloped virus containing a single-stranded RNA genome of 9600 base pairs [1]. Of the 200 million individuals infected with HCV worldwide,~170 million are chronically infected, and are at risk for progression to hepatocellular carcinoma (HCC) [2]. The risk of HCV disease progressing to terminal liver disease increases with extremes of age, obesity, alcohol consumption and human immunodeficiency virus (HIV) co-infection [2]. Virus-specific cellular immune responses play an effective role in viral clearance and progression to chronic HCV disease [3]. Evidence suggests that chronically-infecting viruses suppress certain immunological mechanisms in the host leading to sub-optimal virus-specific T-cell responses [4]. Hence, approaches targeting to alleviate the magnitude of T-cell exhaustion and eventual impairment of functional T-cell responses [5] are key to sustenance of protective immune responses in chronic viral infections (CVIs).

Individuals with certain CVIs are reported to show increased frequencies of exhausted T cell phenotypes expressing multiple negative immune checkpoints (or co-inhibitory receptors), namely programmed death-1 (PD-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), T-cell immunoglobulin mucin-3 (TIM-3), killer-cell lectin-like receptor G1 (KLRG1) and 2B4 (CD244), which reportedly lead to functional exhaustion of virus-specific T-cell responses [5,6]. T-cell exhaustion and dysfunction are

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characterized by poor cytolytic abilities and abnormal cytokine-secreting potentials [7,8]. Exhaustion is not only limited to CD8+ T-cell responses as CD4+ T cells have also been shown to display functional unresponsiveness (clonal anergy). This is partly because CD4+ T-cell responses are equally essential for the maintenance of effective CD8+ T-cell responses in CVIs [9]. Multiple lines of evidence indicate that expression of immune inhibitory checkpoints and secretion of immunoregulatory cytokines by virus-specific T cells in chronic hepatitis B virus (HBV)[9], lymphocytic choriomeningitis virus (LCMV)[10], and HIV infections[11] suggesting that co-inhibitory molecules might facilitate the activation of sub-optimal T-cell responses in these chronic infections. The likely association of co-inhibitory receptors and immunoregulatory cytokines with sub-optimal HCV peptide-specific T cells responses in chronically HCV-infected individuals remains poorly understood. Recent literature suggests that T-cell exhaustion could still be a part of the natural history of chronic HCV disease [12,13]. Here, we studied the phenotypes of virus-specific T cells expressing co-inhibitory receptors (PD-1, CTLA-4, TNF-related apoptosis inducing ligand (TRAIL), TIM-3 and B- and T-lymphocyte activator (BTLA)) by flow cytometry, and measured the levels of Th1/Th2/Th17 cytokines secreted by T cells by a commercial Multi-Analyte ELISArray™ (Qiagen, USA) following *in vitro* stimulation of T cells using HCV peptides and phytohemagglutinin (PHA) in patients with chronic HCV infection and compared the differences with healthy controls (HCs).

2. Patients and methods

2.1. Clinical specimens

A total of 20 individuals comprising of chronic HCV infection (CHC, n = 10) and healthy controls (HCs, n = 10) were recruited for a cross-sectional analysis. Peripheral blood was obtained from participants at the Hepatology and Gastroenterology Unit of the University of Malaya Medical Centre (UMMC), Malaysia, between 2014 and 2015. CHC-infected subjects were defined as persons with plasma viral load (PVL) ranging from 198 to 1,310,000 RNA copies/mL. HCs were individuals free from viral and bacterial infections, and received no immunization in the last 6 months. The study protocols were approved by the Medical Ethics Committee (MEC) of UMMC (MEC201311-0496), and the work was carried out in accordance with the principles of the International Conference on Harmonization Guidelines and the 1962 Declaration of Helsinki. Written informed consents were obtained from all participants or their legal representatives as a voluntary participation before recruitment into the study. Descriptive (sex) and clinical data viz., HCV viral load, serum aspartate (AST) and alanine transaminase (ALT) levels, total lymphocyte counts and HCV genotype details are presented in Table 1.

2.2. Liver transaminases

Serum AST levels were estimated using a commercial ELISA (IBL America, Minneapolis, MN), and serum ALT levels by a Hitachi 7050 Automatic Analyzer (Hitachi Corp, Tokyo, Japan) using a commercial ALT assay kit (Wako Pure Chemicals, Osaka, Japan) according to manufacturer's instructions. The cut-off values were set at 20 ng/mL.

2.3. Plasma viral load

HCV PVLs were measured using a commercial COBAS® AmpliPrep/COBAS® AMPLICOR HCV ver. 2.0 (Roche Molecular Systems, Branchburg, NJ, USA) with an analytical sensitivity (95% threshold) of 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.

2.4. Peripheral blood mononuclear cells

Ten milliliters of venous blood was collected by venipuncture from each of the participant at the phlebotomy unit in lithium heparin BD Vacutainer® (BD Biosciences, Franklin Lakes, USA) tubes. Peripheral blood mononuclear cells (PBMCs) were extracted from blood samples by density-gradient centrifugation using Ficoll® Paque Plus (Sigma-Aldrich, USA) reagent overlay method within 3 h of collection. Cell viability was assessed by 0.4% trypan blue vital staining method. All cells were cryopreserved in fetal bovine serum (FBS) containing 10% dimethylsulphoxide (DMSO) and stored in liquid nitrogen until use in the experiments.

2.5. T-cell stimulation

PBMCs (6×10^5 cells per 2 mL) were stimulated in wells of a flat-bottomed 6-well plates (BD Falcon, Stockholm, Sweden) in a 5% carbon dioxide (CO_2) incubator for 48 h at 37 °C using a cocktail of lyophilized peptides (5ug/mL) derived from HCV consisting of 15-mer sequences with 11 amino acid overlap, covering the sequences of HCV genotype 1b core protein. Negative control cells were incubated with 10 U/mL of recombinant human IL-2 (rhIL-2) only, and non-specific stimulation was carried out using PHA and

Table 1
Clinical and demographic characteristics of study participants.

Group	Age (Range)	Total Lymphocyte Count (%)	HCV Plasma Viral Load (RNA copies/mL)	Serum AST (IU/L)	Serum ALT (IU/L)	
HC (n = 10)	26 (24–33)	76 (60–85)	NA	NA	NA	
CHC (n = 10)	40 (29–55)	37 (31–58)	258729 (198–1,310,000)	76.3 (26–118)	109.8 (28–223)	
Patient No	Sex	ALT	AST	Viral load (IU/mL)	Lymphocyte (%)	Genotype
1	M	198	73	180,000	36.2	3
2	F	28	26	156,000	32.3	3
3	M	184	86	202,000	31	3
4	M	33	45	198	58	1
5	F	102	118	7214	34.8	3
6	M	69	67	377,860	32.8	3
7	M	223	94	287	50	1
8	M	82	105	26,700	41.9	3
9	F	68	62	86,740	20.6	1
10	F	111	87	1,310,000	40.9	3

Abbreviations: HC, healthy controls; CHC, chronic hepatitis C-infected; AST, serum alanine aminotransferase; ALT, serum aspartate transaminase; IU, international units.