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CD8+T Cell Activation Associated with Viral Replication in Chronic Infection.

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Abstract

Background: PD-1 expression is controlled during T-cell activation. PD-1 has an important role in regulating immune response as well as tolerance. During chronic hepatitis C virus (HCV) infection there is high level of PD-1 expression on exhausted CD8+T cells. There is also reduced expression of T-bet. T-bet is identified as a transcriptional repressor of Pdcd1.

Aim of the work: The study will attempt to find out the level of expression of PD-1 on peripheral CD8 + T-cells, associated with chronic HCV infection.

Patients and methods: Fifty patients with chronic hepatitis C virus infection (CHCV), whose age ranged between (16-59) years, were selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, before Interferon and ribavirin therapy, and fifteen healthy individuals were included to serve as controls. All the patients and controls were subjected to the following: history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigations. CBCs and analysis of the expression of surface markers on CD8+T cells and PD-1.

Results: Our results suggested that increased expression of PD-1 cells was an additional inhibitory mechanism that contributed to virus-specific CD8 + T cell exhaustion in chronic hepatitis C virus (CHCV) infected patients.

Conclusion: Our study concluded that there's significant increase in PD-1 expression of circulating HCV-specific CD8 + T cells in CHCV patients. **Recommendations:** The blockade of the inhibitory receptors (PD-1) programmed cell death is considered as a novel strategy for the treatment of chronic HCV infection.

Key Words: Hepatitis C virus, CD8 T cells, T cell dysfunction, PD-1.

Introduction

HCV infection is a considerable public health problem and an important cause of liver disease.[1] The estimated worldwide prevalence of HCV infection is 2 % to 3 % which translates to an estimated 170 million infected individuals. [2] HCV is highly persistent human pathogen that infects the liver and causes significant morbidity and mortality due to chronic liver disease.[3] Control of HCV replication may depend on effective Th lymphocyte. [4,5] There is also an enhanced Th2 response during CHCV and high anti-HCV antibodies production which may partly be responsible for the persistence of HCV infection.

Patients with CHCV infection harbor dysfunctional antiviral T cells with increased PD-1 expression in circulating blood. PD-1 blockade can restore their antigen-specific effector function in vitro. [6-10] However, HCV-specific CD8 T cells in the liver (the site of HCV replication) display markedly increased PD-1 expression compared to peripheral blood [6,9,11] and profound functional impairment that is refractory to PD-1 blockade alone. [9] Similarly, highly activated circulating HCV-specific CD8 T cells in acute evolving hepatitis C show markedly increased PD-1 expression with a deep functional impairment that is unresponsive to PD-1 blockade.

The PD-1 molecules is expressed on lymphocytes, especially on T and B cells, and is an inducible inhibitory regulator of T cell activation. [12-13] Dysfunctions of viral specific CD8 + T cell immune responses are closely associated with HCV replication.[14-16] It has been proposed that unbalanced distribution of

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circulating CD8 + T cell subsets and impairment of their homing capacity and effector function are closely associated with HCV / HIV-1-secific immune tolerance and viral persistence. [17-21] Once virus was cleared, most activated CD8+T cells experienced apoptosis and finally a minority of survived effector cells become CD8 + T cell memory (TCM) central memory T cells. [22-23]

The PD-1, programmed cell death 1 surface marker, belongs to the CD28 family and is expressed on T cells, natural killer T cells, B cells and myeloid cells. [24] PD-1 interacts with two B7 family ligands, PD-L1 and PD-L2, PD-L1 is widely expressed on antigen presenting cells (APCs) and hematopoietic cells, whereas PD-L2 is only expressed on macrophages and dendritic cells. [25] The interaction between PD-1 and one of its ligand, expressed on T cell receptor (TCR) or B cell receptor (BCR) activates an inhibitory pathway that leads to abrogation of cellular response. [25,26] PD-1: PD ligand interaction can lead to CD8 + T-cell exhaustion during chronic viral infection, such as LCMV, HIV, and HCV. [27-31]

Patients and methods

Fifty patients with CHCV, whose age ranged between (16-59) years, selected from the National Hepatology and Tropical medicine Research institute were included in this study, before interferon and ribavirin therapy, and fifteen healthy individuals were included to serve as controls. All the patients and controls were subjected to the following: History, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigations: CBCs and the expression of PD-1 and other surface markers on CD8+ T cells analysis.

Immunophenotyping analysis by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from anticoagulated whole blood samples by Ficoll-Histopaque (Sigma Chemical Co., St Louis, MO) density centrifugation. Cells were stained by fluorescent antibodies per manufacturer's instructions and analyzed with Flow cytometer (Tree Star Inc., San Carlos, CA) 17, PD-1 positivity was determined by an isotype control-defined cutoff (99.5%). [32]

Detection of PD-1 gene expression using real time PCR (RT–PCR)

RNA extraction:

Total RNA was isolated from whole blood using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to manufacturer instruction . The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically, concentrations of the RNA were assessed using the OD 260/280 ratio. The integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide .

cDNA synthesis:

First-strand cDNA synthesis was performed with cDNA synthesis kit supplied by Qiagene. Briefly, 2 µg total RNA was mixed with 0.5 µg of oligo (dT) 12-18 primer in a total volume of 12 µL. After the mixture was heated at 70°C for 10 min, a solution containing 50 mmol/L Tris. Hcl (PH 8.3), 75 mmol/ L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 0.5 µL RNase inhibitor, and 200 U Superscript Reverse Transcriptase was added, resulting in a total volume of 20.5 µL. This mixture was incubated at 42°C for 1 h.

Real-time quantitative polymerase chain reaction (PCR):

For real-time quantitative PCR, 5 µL of first-strand cDNA was used in a total volume of 25 µL, containing 12.5 µL 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer (see table 1) PCR reaction cycle consisting of 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles), were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems). Data were analyzed with the ABI Prism 7500 sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the beta actin genes. [33]

Table 1. Primer sequences used for RT-PCR

Primer	Sequence
PD-1	F: 5'-CCACAGTAAACTATAGCCCTGAAAA -3 ' R: 5'GTTTGGAGGTTCCACTTCT3T-3'
Beta actin	Forward 5' TCT GGC ACC ACA CCT TCT ACA ATG 3' Reverse 5' AGC ACA GCC TGG ATA GCA ACG 3'

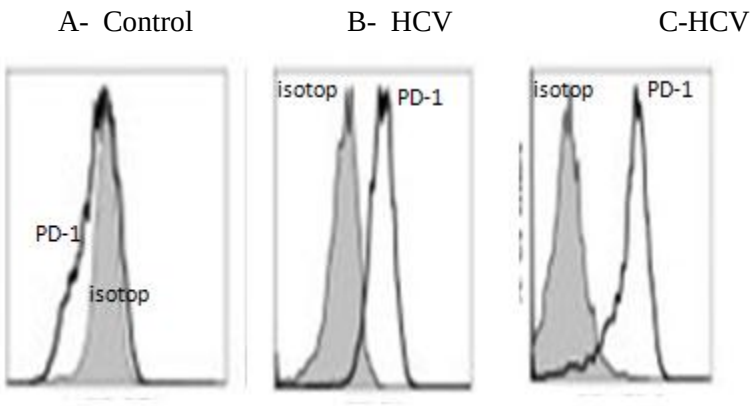


Figure 1. Increased PD-1 expression in circulating HCV-specific CD8 T-cells from patients with hepatitis C in (B&C) compared to control (A)
the black shadow is control isotop used for flow cytometry analysis and black line for PD-1

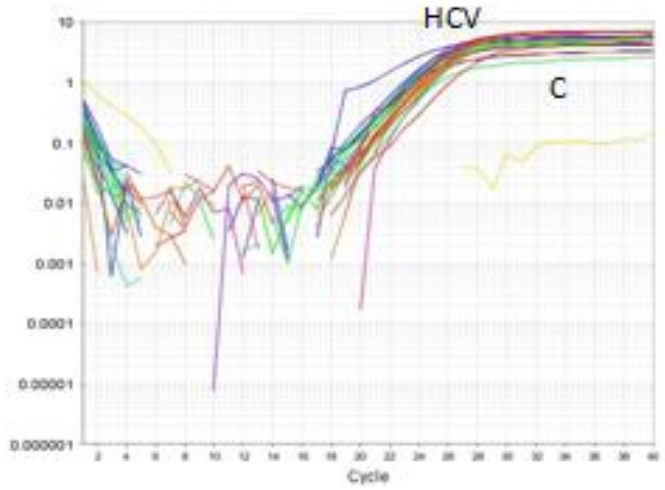


Figure 2: An amplication curve of quantitative real time –PCR

An amplification curve of real time PCR of PD-1 in HCV and control group

Ethical consideration: Informed consent was obtained from each patient at the time of drawing blood samples. The Research Ethical Committee of the General Organization for Teaching Hospitals and Institutes approved the study protocol.

Statistical analysis : Analysis of data of all patients was done by IBM computer using SPSS (statistical Program for social science version 12) as follows: Description of quantitative variables as mean, SD, and range. Description of qualitative variables as number and percentage. Unpaired t-test

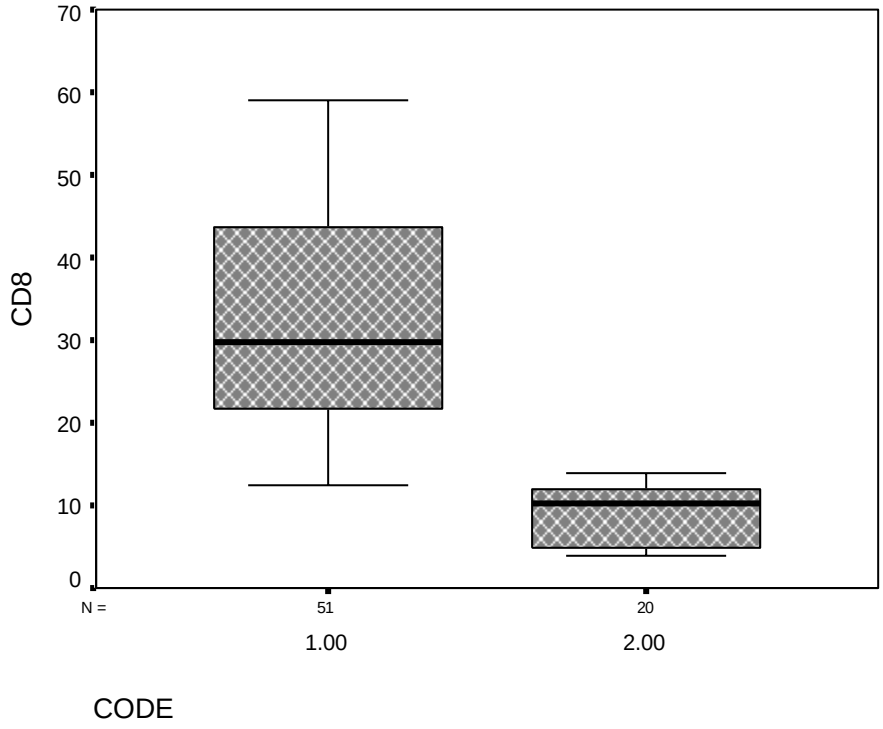
was used to compare two groups as regard quantitative variable. Correlation coefficient test was used to rank different variables positively or inversely versus each other. [34]

Table (2) Comparison between both groups as regard lab data

Variables	Cases N=51	Controls N=20	T	P
PD1% expression in CD8	**32.7±12**	**9±3.5**	**8**	**<0.001** HS
PD1 gene	**0.65±0.3**	**0.09±0.02**	**6**	**<0.001** HS
Viral load	**9,101,35±179566**			

This table shows statistically significant difference between both groups as regard PD gene and expression by using unpaired t-test

Graph (1): Quantitative Expression level of CD8 + T cell in PBMCs from patients with CHCV in comparison to the healthy controls (Code: 1.00 = patients & 2.00 = controls).



CD8

Graph (2): Quntitative expression level of PD-1 % in PBMCs from patients with CHCV in comparison to the healthy controls (Code: 1.00 = patients & 2.00 = controls).

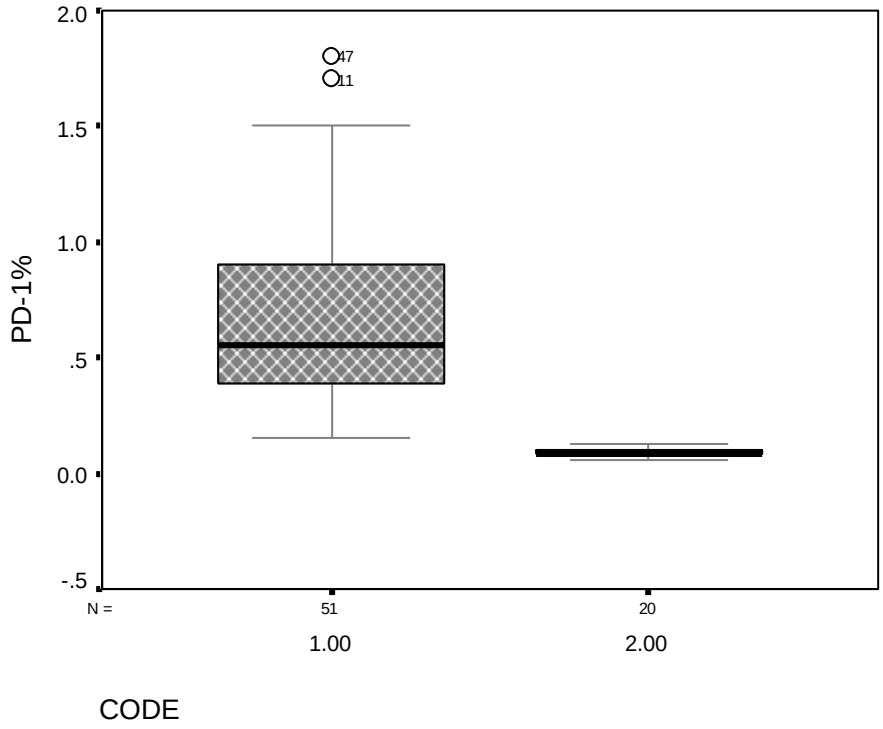


Table (3) Correlation between PD gene versus viral load

Variables	Viral load r	P
PD1% expression in CD8	-0.04	>0.05
PD1 gene	-0.15	>0.05

This table shows no statistically significant correlation between viral load versus gene expression by using correlation co-efficient test

Results

The Real time-PCR expression of activation by HCV level of CD8 + T cell in PBMCs of CHCV patients was higher level as compared to the healthy controls with statistically highly significant difference ($p < 0.001$) by using unpaired t-test (table 2), Fig (1&2), and Graph (1). The Real time-PCR expression level of PD-1, CD8 + T cells in PBMCs of CHCV patients was higher compared to the healthy

controls with statistically highly significant difference ($p < 0.001$) by using unpaired t-test table (1,2), Fig (1,2) and Graph (1,2).

There was no statistically significant correlation between viral load in CHCV

patients versus PD-1 Gene expression in CD8 + T cells by using correlatin co-efficient test (table 3).

Discussion

Programmed cell death 1 (PD-1) protein marker on CD8 + CT cells is an inhibitory receptor involved in T-cell activation, tolerance and exhaustion. [35] Understanding how PD-1 expression is controlled during T-cell activation is of great interest as PD-1 has an important role in regulating immune response as well as tolerance.[25,26,36-38]

In the present study, we found that CHCV patients had a higher expression levels of PD-1 in circulating HCV specific CD8+T cells than the healthy control.

Kao *et al*. [39] observed that during chronic infection, the high level of PD-1 expression on exhausted CD8 + T cells is due to the reduced expression of T-bet gene identifying T-bet gene as a transcriptional repressor of Pdcd1. Oestreich *et al.* [40] postulated that, a better understanding of the molecular events controlling PD-1 expression levels is essential to control T-cell response. During T-cell activation, the TCR-induced transcription factor NFATc1 binds to the Pdcd1 (PD-1) promoter and contributes to Pdcd1 transcription . Furthermore, interferon-α synergizes with the TCR signal to promote and sustain Pdcd1 transcription, via direct binding of IRF9 to the Pdcd1 promoter. [41]

In the present work the expression levels of CD8 + T-cells from CHCV patients are significantly higher in comparison to the healthy controls.

Phares *et al.* [42] discovered that, anti-viral CD8 T-cell activity is enhanced and prolonged by CD4 T-cell-mediated help, but negatively regulated by inhibitory B7-H1 interactions. The magnitude, quality and longevity of CD8 T-cell effector function is positively regulated by CD4 T cells, and negatively regulated by various T-cell inhibitory molecules. CD4 T cells augment CD8 T cell activation and expansion, directly through the production of cytokines or indirectly by activation of dendritic cells (CDs) resident in draining lymph nodes. [43-44] Moreover, CD4 T cells can further enhance the primary anti-viral responses of CD8 T cells and promote their survival in the target organ. [45-50]This function is especially crucial in sustaining CD8 T-cell activity during prolonged and chronic infections.

Lachman *et al*. [26], Nishimura [36], Ansari[37], and Fife *et al.* [38], discovered that high level of PD-1 expression by antigen (Ag)-specific T cells correlates with high viral load, and blocking PD-1:PD-L1 interaction restores CD8 + T-cell proliferation and effector functions. Our study results shows no statistically significant correlation between viral load versus PD-1 % expression in CD8 T-cells, nor PD-1 gene level.

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