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ORIGINAL ARTICLE

The Interplay of Programmed Cell Death Protein-1 (PD-1), Interleukin-25 (IL-25) and Viral Load in Patients with Hepatitis C

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ABSTRACT

Background: The infection instigated by the Hepatitis C virus (HCV) continues to pose a substantial challenge to global health, with the advancement of the disease being profoundly affected by the immune responses of the host organism. Programmed cell death protein-1 (PD-1) and Interleukin-25 (IL-25) are pivotal immunoregulatory entities that have been associated with the phenomena of viral persistence and immune exhaustion. **Aim:** This investigation sought to elucidate the complex interplay among PD-1, IL-25, and viral load in subjects afflicted with HCV. **Patients and Methods:** A total of 110 subjects were enlisted for the purpose of this investigation, encompassing 40 individuals diagnosed with HCV exhibiting viremia, 40 subjects devoid of viremia, and 30 healthy control participants. Demographic attributes were evaluated, while serum concentrations of PD-1 and IL-25 were measured through the application of enzyme-linked immunosorbent assay (ELISA); furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to ascertain the HCV viral load. **Results:** The median serum level of PD-1 in HCV patients with viremia 3.73 ng/ml (range= 1.06-8.43 ng/ml pg/ml) which was higher than non-viremic patients (median= 2.33 ng/ml, range= 1.04-8.32 ng/ml) or controls (median= 2.41 ng/ml, range= 1.04- 3.98 ng/ml), with significant differences. In contrast, non-viremic HCV patients exhibited a markedly elevated mean serum concentration of IL-25 (33.35± 6.59 pg/ml) in comparison to both viremic HCV patients (15.19±3.77 pg/ml) and control subjects (13.64± 4.31 pg/ml), with statistically significant differences observed. Furthermore, PD-1 demonstrated a significant positive correlation with viral load (r= 0.375, p=0.040), as well as a significant negative correlation with IL-25 (r= -0.221, p= 0.043). **Conclusion:** The findings emphasize a multifaceted interplay between PD-1, IL-25, and the viral load of HCV. The augmented expression of PD-1 may promote viral persistence through mechanisms of immune suppression, whereas IL-25 may contribute to the attenuation of disease progression. These immunological indicators could serve as promising therapeutic targets for the modulation of immune responses in the setting of chronic HCV infection.

Keywords: Hepatitis C Virus; Programmed Cell Death-1; Interleukin-25; Viral Load

INTRODUCTION

Chronic infection with the hepatitis C virus (HCV) constitutes a notable public health concern at a global scale, potentially leading to severe hepatic disorders, including cirrhosis and hepatocellular carcinoma (HCC)[1]. A hallmark characteristic of persistent viral infections, encompassing HCV, is the functional depletion of virus-specific T lymphocytes, which is marked by the upregulation of inhibitory receptors, notably programmed cell death protein-1 (PD-1)[2].

PD-1 is recognized as an inhibitory receptor and belongs to the CD28 family of costimulatory receptors, which are

expressed on activated T lymphocytes, B lymphocytes, natural killer T cells, and monocytes[3]. The interaction between PD-1 and its ligands, PD-L1 and PD-L2, conveys inhibitory signals that diminish T-cell activation, proliferation, and effector functions, thereby contributing to immune tolerance and reducing autoimmune responses[4]. In the context of chronic viral infections, such as HCV and hepatitis B virus (HBV), sustained antigen exposure leads to persistent expression of PD-1 on virus-specific CD8+ T cells, culminating in a state of T-cell exhaustion[2, 5]. This state of exhaustion significantly undermines the ability of T cells to effectively clear the virus, thereby promoting viral persistence[3].

A multitude of investigations have delineated a direct association between PD-1 expression on peripheral CD8+ T lymphocytes and HCV viral load in individuals suffering from chronic hepatitis C[6, 7]. The heightened levels of PD-1 expression are correlated with elevated viral loads and the progression of the disease. More specifically, the degree of PD-1 expression on peripheral CD8+ T cells demonstrates a positive correlation with HCV RNA load, thereby highlighting its function in immune evasion[6]. In addition, CD8+CD28-PD1+ regulatory T cells, known for their production of interleukin 10 (IL-10), are significantly linked to biomarkers of chronic HCV infection and exert an influence on therapeutic responses[8]. The inhibition of the PD-1 signaling pathway has been shown to reinstate the proliferative capacity and antiviral functionality of HCV-specific T lymphocytes *in vitro*; moreover, certain *in vivo* models have indicated a reduction in HCV viremia consequent to this therapeutic intervention[5].

Interleukin-25 (IL-25), which is also referred to as IL-17E, constitutes a cytokine belonging to the IL-17 family and is recognized for its diverse functions in the regulation of the immune system, often linked to type 2 immune responses. Its role in infectious diseases is complex, at times promoting protective immunity while at other instances exacerbating pathological conditions[9]. In relation to hepatitis C virus (HCV) infection, IL-25, alongside IL-33 and IL-17, has been associated with the advancement of the disease, especially concerning the progression from chronic HCV to hepatocellular carcinoma (HCC). Elevated serum levels of IL-25 have been documented in HCV patients, with these levels being significantly higher in individuals who have progressed to HCC compared to those with chronic hepatitis or cirrhosis[1]. This observation suggests that IL-25, notwithstanding its bifunctional roles in infectious pathologies, may intensify chronic inflammation and immune dysregulation that leads to significant hepatic complications in patients afflicted with HCV[9].

The interaction among PD-1, IL-25, and viral load within the framework of chronic HCV infection is an integral aspect of a more extensive continuum of immune dysregulation[10]. The significant intrahost genetic heterogeneity of HCV is also pivotal in immune evasion by facilitating epitope escape, which can lead to progressive phenotypic modifications in CD8+ T cells, encompassing the heightened expression of inhibitory molecules such as PD-1[2]. Therefore, elucidating the complex interplay between inhibitory pathways like PD-1, pro-inflammatory and regulatory cytokines such as IL-25, and viral factors is imperative for the development of effective immunotherapeutic approaches against HCV.

The present study aimed to explore the intricate relationship between PD-1, IL-25, and viral load in individuals infected with HCV, with a comparative analysis between patients exhibiting active viremia, those without viremia, and healthy control subjects.

PATIENTS AND METHODS

The Study Population

This nested case control study included a total of 80 patients attending Baghdad Teaching Hospital, during December 2024 to June 2025. Those patients had a confirmed infection with HCV (positive for anti-HCV Ab in the serum through enzyme-linked immunosorbent assay testing). Other 30 age- and sex-matched apparently healthy subjects were recruited to represent the control group. Patients with under immunosuppressive or immunomodulatory therapy; decompensated liver disease; active malignancy, a history of cancer, pregnancy or lactation; severe cardiovascular or renal disease and those with hepatitis B virus (HBV) infection were excluded from the study.

Demographic data including age, sex, body mass index (BMI), smoking status, residence, and occupation were collected through direct interview with each participant. A consent form explaining the aims of the study was obtained from each participant enrolled in the study before sample collection.

Serum level of PD-1 and IL-25

Peripheral blood samples were collected from each participant under sterile conditions and allowed to clot at room temperature. Serum was separated by centrifugation at 3000 rpm for 10 minutes and stored at −80°C until analysis. The concentrations of PD-1 and IL-25 in serum were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Sunlong/China), according to the manufacturer's protocols. The optical density was measured using a microplate reader at the appropriate wavelength. Concentrations were calculated based on standard curves generated for each biomarker.

Viral load of HCV

The quantification of hepatitis C virus (HCV) RNA levels, also referred to as viral load, was executed employing a real-time polymerase chain reaction (RT-PCR) methodology. Serum specimens were procured and preserved at −80°C until further analysis was conducted. Viral RNA was isolated utilizing a commercially available viral RNA extraction kit (QIAamp viral RNA mini kit from Qiagen, Germany), followed by amplification and quantification via a one-step quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (Coyote, China). The protocol was conducted in accordance with the manufacturer's specifications, and the outcomes were reported in international units per milliliter (IU/mL).

Statistical Analysis

All statistical analyses were conducted utilizing the Statistical Package for Social Sciences (SPSS) software (Version 25). Continuous variables were assessed for normality through the implementation of the Shapiro-Wilk test. Variables that conformed to a normal distribution were reported as mean ± standard deviation (SD) and underwent an analysis of variance (ANOVA) for evaluation. Conversely, variables that exhibited a non-normal distribution were represented as median and range and were analyzed employing a non-parametric Kruskal-Wallis test. Categorical variables were delineated in terms of frequency and percentages and analyzed employing the Chi-square test. Spearman's correlation coefficient was employed to explore the possible correlation between PD-1 and IL-25 with other continuous variables in the patient cohort. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Demographic characteristics of the study population

Table. 1 summarizes the demographic characteristics of the three study groups. The mean age and BMI of participants was similar across the three groups, with no statistically significant differences.

In terms of sex distribution, males represented 57.5% of the viremic group, 47.5% of the non-viremic group, and 53.33% of the controls, with no significant difference observed (p = 0.667).

Likewise, residence, occupation and smoking were comparable across the groups with no significant differences.

Table 1: Demographic characteristics of the study population

Variables	HCV patients with viremia (n=40)	HCV patients without viremia (n=40)	Controls (n=30)	P- value
Age, years				
Mean±SD	42.93±13.47	40.13±11.78	42.03±12.83	0.606
Range	18.0-76.0	23.0-76.0	23.0-74.0	
BMI (kg/m^2)				
Mean±SD	26.62±32.26	27.17±3.2	25.64±3.33	0.101
Range	22.3-32.4	22.4-33.1	20.5-33.4	
Sex				
Male	23(57.5%)	19(47.5%)	16(53.33%)	0.667
Female	17(42.5%)	21(52.5%)	14(46.67%)	
Residence				
Rural	29(72.5%)	27(67.5%)	21(70%)	0.888
Urban	11(27.5%)	13(32.5%)	9(30%)	

Variables	HCV patients with viremia (n=40)	HCV patients without viremia (n=40)	Controls (n=30)	P- value
Occupation				
Employee	17(42.5%)	17(42.5%)	13(43.33%)	0.707
Housewife	6(15%)	5(12.5%)	4(13.33%)	
Free job	11(27.5%)	15(37.5%)	12(40%)	
Retired	6(15%)	3(7.5%)	1(3.33%)	
Smoking				
Never	31(77.5%)	34(85%)	26(86.67%)	0.539
Ex/current	9(22.%%)	6(15%)	4(13.33%)	

Serum level of PD-1 and IL-25

Data regarding serum level of PD-1 were found to be non-normally distributed. Accordingly, these data were expressed as median and range, and analyzed with non-parametric Kuskal Wallis test. The median serum level of PD-1 in HCV patients with viremia 3.73 ng/ml (range= 1.06-8.43 ng/ml pg/ml) which was higher than non-viremic patients (median= 2.33 ng/ml, range= 1.04-8.32 ng/ml) or controls (median= 2.41 ng/ml, range= 1.04- 3.98 ng/ml), with significant differences Fig. 1.

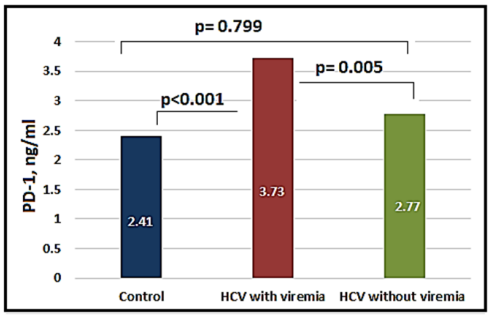

Fig. 1: Median serum level of PD-1 in patients with HCV and controls

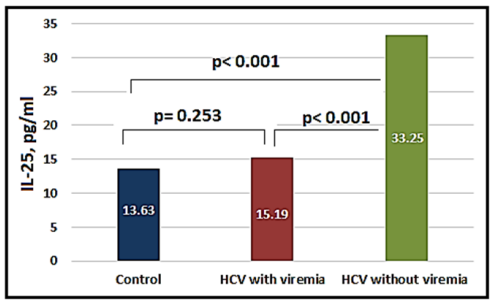

Fig. 2: Mean serum level of IL-25 in patients with HCV and controls

In contrast, non-viremic HCV patients displayed higher mean serum level of IL-25 (33.35± 6.59 pg/ml) than either virmic HCV patients (15.19±3.77 pg/ml) or controls (13.64± 4.31 pg/ml) with significant differences Fig. 2.

Viral load in patients with viremic HCV

Data regarding viral load were found to be non-normally distributed and were expressed as median and range. The median viral load was 430064.5 IU/ml (range: 13355-48515978 IU/ml) as shown in Table. 2.

Table 2: Antigen, Anti-HCV antibody and viral load in patients

Viral load, copy/ml	Value
Mean±SD	6294834.9±12897596.7
Median	4300640.5
Range	13355-48515978

Correlation of PD-1 and IL-25 with other variables

Spearman's correlation was used to explore the possible correlation between PD-1 and IL-25 with other continuous variable Table. 3.

Table 3: Spearman's correlation between sCTLA-4 with age, BMI and viral load in patients with HCV infection

Variables	PD-1, ng/ml		IL-25, pg/ml	
	Coefficient	p-value	Coefficient	p-value
Age, years	0.181	0.058	-0.151	0.138
BMI, kg/m^2	-0.061	0.528	0.086	0.399
Viral load, IU/ml	**0.375**	**0.040**	-0.221	0.240
IL-25, pg/ml	**-0.221**	**0.043**		

PD-1 displayed a significant positive correlation with viral load (r= 0.375, p=0.040) Fig. 3, and significant negative correlation with IL-25 (r= -0221, p= 0.043) Fig. 4.

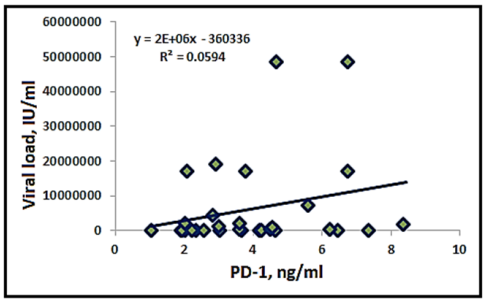

Fig. 3: Scatter plot and regression line between PD-1 and viral load in patients with viremic HCV

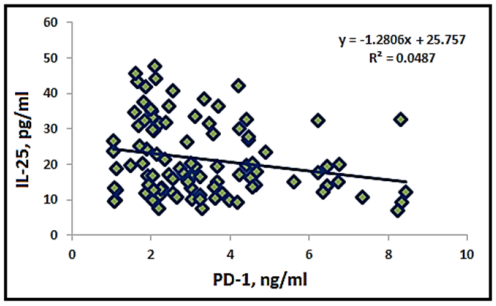

Fig. 4: Scatter plot and regression line between PD-1 and IL-25

DISCUSSION

The present study aimed at the intricate relationship between PD-1, IL-25, and viral load in individuals infected with HCV. According to the result of the study, HCV patients with viremia 3 had significantly higher PD-1than non-viremic patients or controls, with significant differences. In contrast, non-viremic HCV patients displayed higher mean serum level of IL-25 than either viremic HCV patients or controls with significant differences. Most previous studies did not compare viremic and non-viremic patients. For Joda *et al.*[11] measured PD-1 and PD-L1 in 30 Iraqi patients with chronic HCV under antiviral treatment and other 30 patients with no treatment. Treated patients showed significantly higher PD-1 and PD-L1 than either non-treated or healthy control.

Cabral *et al.*, have shown that IL-25 was produced significantly by peripheral blood mononuclear cells stimulated by HCV antigens and did not correlate to HCV viremia[12]. Li *et al.*, showed that IL-25 serum levels were significantly raised in HCC patients; however, no direct correlation between IL-25 and the development of HCC cells has been observed[13].

The elevated levels of PD-1 observed in viremic patients imply an intensified state of T-cell exhaustion, in which the immune system's capacity to eradicate the virus is diminished[2]. In the context of chronic HCV infection, prolonged exposure to viral antigens results in continuous PD-1 expression on CD8+ T cells that are specific to the virus, thereby obstructing their proliferation, cytokine production, and cytolytic capabilities[14]. The condition of immunological anergy significantly enhances the capacity of the virus to evade immune surveillance and facilitate the establishment of a chronic infection, which bears a direct correlation with an elevated viral load[6]. The notable intrahost genetic diversity of HCV additionally contributes to immune evasion by fostering epitope escape, which may subsequently lead to progressive phenotypic modifications in CD8+ T cells, including the heightened expression of inhibitory molecules such as PD-1[2].

The mechanisms that underlie the impact of PD-1 transcend the simplistic notion of T cell inhibition. PD-1 imposes a negative regulatory influence on the expression of interleukin-12 (IL-12) by limiting the phosphorylation of STAT-1 in monocytes and macrophages in the context of chronic HCV infection[15]. IL-12 serves as a critical cytokine for the initiation of effective antiviral immune responses, promoting Th1 responses. Its downregulation further intensifies the prevailing immunosuppressive environment that facilitates viral persistence. This systemic immune dysregulation epitomizes a core feature of chronic HCV infection, enabling the virus to maintain elevated viral loads in spite of the presence of an active immune response[3, 14].

It is plausible that IL-25, through its modulation of the cytokine milieu, indirectly affects the expression or functional dynamics of PD-1 and other inhibitory signaling pathways, consequently aggravating the host's incapacity to eradicate the virus. An elevation in IL-25 concentrations may serve as a marker of a more profound immune dysregulation that synergistically fosters both viral persistence and disease progression, in association with PD-1-mediated T-cell exhaustion[16].

Successful antiviral strategies employing direct-acting antivirals (DAAs) have been shown to diminish PD-1 expression and improve other indicators of T-cell exhaustion, indicating a restoration of immune functionality concurrent with viral eradication[16]. This further reinforces the proposition that the elevated levels of PD-1 detected in viremic individuals are a direct consequence of ongoing viral replication and a critical factor in the mechanism of immune evasion.

In the current investigation, there was a statistically significant positive correlation between PD-1 expression and viral load ($r = 0.375$, $p = 0.040$), alongside a significant negative correlation between PD-1 and Interleukin-25 (IL-25) ($r = -0.221$, $p = 0.043$), within the context of HCV infection. This elucidates essential aspects of immune dysregulation and viral persistence. These observations are congruent with recognized mechanisms of immune exhaustion prevalent in chronic viral infections and imply a multifaceted interaction among inhibitory immune checkpoints, regulatory cytokines, and the dynamics of viral replication[2, 6].

Research has elucidated a robust association between the expression of PD-1 on peripheral CD8+ T lymphocytes and the viral load of Hepatitis C virus (HCV)[6, 7]. For instance, one study revealed that the expression of PD-1 on CD8+ T lymphocytes showed a positive relationship with the concentration of HCV RNA[6].

The established positive association between PD-1 expression and viral load is a well-documented occurrence in the realm of chronic HCV infection. The PD-1 receptor, which functions as an inhibitory molecule, is markedly upregulated on virus-specific T cells in response to sustained antigenic stimulation, a characteristic feature of chronic viral infections[2]. This extended expression leads to T-cell exhaustion, a state defined by functional deterioration in which T cells exhibit reduced proliferative ability, cytokine production, and cytolytic function[17]. In the context of chronic HCV infection, such exhaustion hampers effective viral clearance, thus promoting viral persistence and resulting in heightened viral loads[6].

In contrast, the pronounced negative correlation between PD-1 expression and IL-25 suggests a multifaceted regulatory paradigm that warrants further investigation. IL-25, which is typically linked to type 2 immune responses, is primarily produced by Th2 cells along with various epithelial cells, functioning as a distress signal that is generated in response to cellular injury or tissue damage to stimulate immune cell activation through its interaction with IL-17RA and IL-17RB receptors. Although IL-25 is acknowledged for its pivotal role in the initiation and maintenance of type 2 immunity, as well as the modulation of other immune cell lineages, its particular role in the context of chronic HCV and its inverse correlation with PD-1 continues to be a matter of significant scholarly interest[16].

The negative correlation observed with PD-1 may suggest several possible interpretations. One theoretical framework proposes that increased viral loads, which correlate with heightened PD-1 expression, may actively impede the production of IL-25 or its associated signaling cascades. Alternatively, IL-25 may function to modulate the immune microenvironment in such a way that counteracts the mechanisms that drive PD-1 upregulation, or it might represent an effort by the immune system to instigate an alternative response paradigm (e.g., type 2 immunity) in instances where the type 1 antiviral response is being inhibited by PD-1. Nonetheless, given that elevated IL-25 levels are similarly associated with the progression towards hepatocellular carcinoma (HCC), it is conceivable that IL-25 promotes a pro-inflammatory and pro-fibrotic environment that, in conjunction with PD-1-induced T-cell exhaustion, collectively contributes to immune dysregulation and disease progression[1]. The identification of an inverse correlation may indicate a shift in immune response phenotypes, wherein the predominance of PD-1-mediated exhaustion (Th1 suppression) could correspond with variations in other cytokine pathways (e.g., Th2 or Th17 responses modulated by IL-25).

Understanding these interrelations is crucial for the development of effective immunotherapeutic strategies. For instance, the modulation of the PD-1 signaling pathway, particularly via PD-1 blockade, has exhibited promise in re-establishing the functional capacities of HCV-specific T lymphocytes and reducing viremia in certain experimental settings[3]. While direct mechanistic links between IL-25 and PD-1 in relation to HCV require further rigorous investigation, the observed inverse relationship adds a layer of intricacy to the immunological paradigm of chronic HCV infection. Ongoing research into the interactions between IL-25 and the PD-1/PD-L1 axis, as well as its implications for viral load, could unveil novel therapeutic targets designed to combat immune exhaustion and facilitate sustained viral clearance[16].

This study is subject to numerous limitations that necessitate acknowledgment during the interpretation of the results. The relatively modest sample size may restrict the generalizability and statistical robustness of the outcomes. Furthermore, the cross-sectional design inherently limits the ability to ascertain causal relationships among PD-1, IL-25, and HCV viral load. The absence of longitudinal follow-up data hinders the evaluation of fluctuations in these biomarkers over time or in response to therapeutic interventions. Although demographic variables were uniformly represented across the cohorts, additional potential confounding factors, including co-morbidities, alcohol consumption, and medication history, were not considered. Moreover, the fact that this study was conducted at a single institution may introduce regional biases, thereby limiting the broader applicability of the findings.

Nonetheless, the inclusion of both viremic and non-viremic HCV patients, in conjunction with healthy controls, establishes a comprehensive framework for investigating the immunological disparities associated with active viral replication. Additionally, the emphasis on

both PD-1 and IL-25 provides a novel lens through which to examine the interaction between immune exhaustion and regulatory cytokines within the context of chronic HCV infection. These insights may facilitate the identification of potential immunological biomarkers or therapeutic targets for the monitoring and management of the disease.

CONCLUSION

In conclusion, this research delineates a significant correlation between immune regulatory markers and the status of HCV infection. The elevated expression of PD-1 in viremic HCV patients suggests a potential role in immune exhaustion and the persistence of the virus, while the reduced levels of IL-25 may indicate a shortfall in protective immune modulation. The established associations between these biomarkers and viral load underscore their potential as indicators of disease activity. These findings provide essential insights into the immunopathogenesis of HCV and encourage further exploration of PD-1 and IL-25 as promising therapeutic targets or prognostic markers in the management of chronic hepatitis C.

DISCLOSURE

Conflict of Interest: None.

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