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Analysis of Factors Associated with Th1/Th2 Cytokine Responses to Non-Structural HCV Peptides NS3-NS4 and NS5 in Treatment-Naïve Chronic Hepatitis C Patients

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ABSTRACT

There are multiple factors modulating differential response to hepatitis C viral (HCV) infections, which include both viral and host factors. Interplay between host and viral factors affect outcomes of HCV infections including effect on type-1 helper T cell (Th1) and Th2 cytokines response. The aim of this study was to analyze the effect of host factors on cytokine profiles after stimulation with genotype 4 HCV peptides. Peripheral blood mononuclear cells (PBMCs) from 18 treatment-naïve Egyptian chronic HCV patients were stimulated with HCV non-structural (NS)-3-NS4a and NS5b proteins. Supernatant levels of gamma-interferon (IFN-γ), interleukin (IL)-2, IL-12, IL-4 and IL-10 were measured using Luminex x MAP technology. HCV PCR viral load, ALT and AST levels were measured in sera and PBMCs were tested for the negative strand of the virus and carboxy-fluorescein-succinimidyl ester (CFSE) proliferation assay as an indicator of HCV active replication and exposure respectively. Overall analysis showed that IL-10 levels showed a significant difference in response to NS5b vs. NS3-NS4a. Correlation analysis revealed that upon stimulation with NS3/NS4a, ALT levels correlated with Th1 IL-2 levels. While NS5b stimulated PBMCs, showed a negative correlation between viral load and IFN-γ levels and positive correlation of viral load with IL-10 levels. In conclusion, host liver functions, and viral load significantly affected Th1/Th2 balance underscoring the complexity of this infectious disease. Future studies on cytokines responses elicited by combinations of viral proteins and its significance in eliciting broad immune responses should prove beneficial.

Key words: Hepatitis C virus, (HCV), NS3-NS4a, NS5b, Th1, Th2, proliferation assay, chronic HCV, IL-2, IL-4, IL-10, IL-12, IFN-γ.

Introduction

Chronic hepatitis C virus infection is currently the second most common viral infection worldwide, with approximately 170-200 million people infected (Takedai *et al.,* 2008). The World Health Organization (WHO) estimates about 3% of the world's population is infected with HCV (Lavanchy, 2011). Although the majority of the cases occur in Asia and Africa, the incidence has also been rising in the developed world. In the United States, the incidence has tripled over the last three decades (Dhanasekaran *et al.,* 2012). Egypt has high prevalence of HCC. It is the second most common cancer among males and seventh among females (Abdelgawad *et al.,* 2013). Th1 cells produce IFN- γ, IL-2 and direct immune responses to cell-mediated killing of viruses and intracellular pathogens. Thus, Th1 are essential in virus–directed immune responses as they also activate macrophages and natural killer cells (NK) (Sarih *et al.,* 2000) Th2 cells, which produce IL4, IL10 are essential for the regulation of humoral immunity and influence B-cell and their rising levels are often associated with persistent infections cells (Sarih *et al.,* 2000). The secretion of IFN- γ is usually stimulated in response to IL-2 and IL-12. Production of IFN-γ is inhibited by IL-4 and IL-10 (Schroder *et al.,* 2004; Gattoni *et al.,* 2006). Conventional dendritic cells (cDCs) produce high

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amounts of cytokines, such as IL-12, which has been shown to play an important role in stimulating IFN-γ production from activated T cells, inducing the development of Th1 protective immune response (Buonaguro, 2012). DCs in chronic HCV patients, display impaired function, proliferative T cell responses and reduced Th1 cytokine concentrations with HCV NS proteins. Particularly NS4, it changes the DC phenotype and reduces antigen-specific T cell stimulatory function (Takaki *et al.,* 2010). Th1 and Th2 cells cross regulate their own development (Guidotti and Chisari, 2006; Matsui *et al.,* 2008). Although high levels of Th1 cytokines are found in the serum of chronic HCV patients, Th1 response is related to viral clearance. Meanwhile Th2 response leads to chronicity (Ruiz-Extremera *et al.,* 2013). Memory CD4 and CD8 cells are less frequent, functionally impaired and target less viral proteins in chronic HCV than in patients with resolved infection (Zeisel *et al.,* 2009).

Host and viral factors from Studies showed that disease progression and outcomes differences are due to both host and viral factors. Race, age, gender, ALT levels as a host factors affected immune responses (Irshad *et al.,* 2008; Barnaba, 2010; Abdel-Hakeem and Shoukry, 2014). Genotype of HCV, plasma HCV-RNA level and early viral kinetics as a viral factors modulating differential response (Samreen *et al.,* 2012; Duffy *et al.*, 2014). Therefore, the present study aims to detect and investigate the role of selected Th1 and Th2 cytokines in chronic HCV immune response upon stimulation of the peripheral blood mononuclear cells (PBMCs) by different HCV peptides including (NS3-NS4 and NS5) and analyzing possible correlations with their age, gender, viral load and active replication of virus.

Subjects and Methods

Subjects

In the current study, 18 treatment-naïve chronic HCV patients were selected from outpatient clinics of the tropical and gastroenterology department at Ain Shams University hospitals. Criteria for inclusion included age ≥ 18 years, transaminases less than three times of the upper limit of normal, positive HCV antibody and positive HCV-RNA for more than 6 months. All patients were treatment-naïve chronic HCV patients and recently diagnosed.

All experiments were conducted with the understanding and the consent of participants who were provided with written informed consents. Study was conducted in accordance with the Declaration of Helsinki (1964). Approval for the study was obtained from Research Ethics Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt MI 1069.

Study design

PBMCs were separated from venous blood using Ficoll-Hypaque (GE healthcare Life Sciences, Germany) gradient method. PBMCs were used for HCV RT-PCR negative strand and CFSE proliferation assay. In addition, 2 ml of blood was drawn into a vacutainer plain tube for serum separation. Serum was stored at -80ºC and later used for testing for HCV RNA PCR, HCV antibody and liver enzymes. Transaminases such as AST and ALT levels were assessed in the clinical pathology laboratory of Ain Shams University Hospitals using Elitech (Synchron CX9, Beckman Coulter Inc., Nyon, Switzerland).

HCV detection and viral load quantification

Serum was tested for the presence of HCV-specific antibodies using the commercially available Ortho version 3.0 ELISA (Ortho Clinical Diagnostics, New Jersey, USA) and HCV RNA assay was performed using real time PCR (Qiagen, Germany). HCV infection was detected by utilization of previously isolated PBMCs by testing for HCV RT-PCR negative strand detection by One step RT-PCR with duplex detection utilizing AgPath-ID One-Step RT-PCR Kit (Applied Biosystems®).

Proliferation assay

Peripheral blood mononuclear cells (PBMCs) were stimulated by HCV peptides in a CFSE proliferation assay (Lyons and Parish, 1994) for 7 days culture. The peptide pools used as stimuli in

the CFSE assay representing sequence of HCV genotype 4 were, NS3-NS4a (10 peptides) and NS5b (8 peptides). These sequence regions were prepared by Proimmune (Oxford, UK) as shown in table (1).

Table 1: Type of HCV peptides and their amino acid sequences.

Peptide type and Position	Amino acid sequence
NS3-NS4a peptide	
1610-1619	PPSWDTMWKC
1620-1629	LIRLKPTLHG
1630-1639	PTPLLYRLGS
1640-1649	VQNEVVLTHP
1650-1659	ITKYIMACMS
1660-1669	ADLEVVTSTW
1670-1679	VLVGGVLAAL
1680-1689	AAYCLSVGSV
1690-1699	VIVGRVVLSG
1700-1709	VIVGRVVLSG
NS5b peptide	
2830-2839	PVNSWLGNII
2840-2849	VYAPTIWVRM
2850-2859	ILMTHFFSIL
2860-2869	QSQEALEKAL
2870-2879	DFDMYGVTYS
2880-2889	ITPLDLPAII
2890-2899	QRLHGLSAFT
2900-2909	LHGYSPHELN

Cytokine assays

Supernatants of stimulated PBMCs were collected and stored at -80°C until analyzed. We used fluorescence bead immunoassay for measurment of Th1 and Th2 cytokines (IL-2, IL-4, IL-10, IL-12 and IFN-γ) Luminex xMAP technology *(R and D diagnostics USA),* a *Luminex100™,* based Analyzer (Luminex Corporation).

Statistical analysis

Frequency and percentage of non-numerical data were performed, and repeated measure ANOVA tests statistical significance of the difference among multiple related parametric variable using SPSS 15.0.1 for Windows (SPSS Statistics Inc., USA). Friedman procedure tests were employed for measuring statistical significance of the difference among multiple related non-parametric variable. In addition, correlation analyses were performed to assess the strength of association between two quantitative variables. The correlation coefficient denoted symbolically "r" defines the strength and direction of the linear relationship between two variables. Finally, multiple linear regression analysis was used to test and estimate the dependence of a quantitative variable based on its relationship with a set of independent variables. $P < 0.05$ was considered significant and $P<0.01$ was considered highly significant.

Results

The current study focused on examining the effect of patients' age, gender, ALT, AST, and viral load on different cytokines in response to stimulation by NS3-NS4 and NS5 HCV peptides in eighteen chronic HCV patients. Overall analysis showed that IL-10 levels showed significant difference in response to NS5 vs. NS3-NS4 ($P \leq 0.05$) (table 2).

Table 2: Overall summary of cytokines profiles in response to different HCV peptides.

Cytokine (pg/ml)	NS3-NS4			NS5		
	Mean ± SEM	Median	Range	Mean ± SEM	Median	Range
IL-2	3.84 ± 0.33	3.7	0 – 6.7	4.59 ± 0.56	4.0	0 – 11.0
IFN-γ	15.07 ± 0.61	15.1	6.8 – 17.6	14.29 ± 0.62	15.0	6.7 – 17.6
IL-12	2.58 ± 0.17	2.3	2.3 – 4.6	2.58 ± 0.17	2.3	2.3 – 4.6
IL-10	1.68 ± 0.13*	1.3	1.0 – 2.4	2.29 ± 0.26*	2.3	1.0 – 6.0
IL-4	9.11 ± 0.75	9.0	0 – 15.5	9.41 ± 0.43	9.0	6.8 – 14.0

** Significant difference between IL-10 levels in response to NS5 vs. NS3-NS4 based on non-parametric Student T-test P value ≤ 0.05*

Correlation analysis to study the effect of each of HCV peptides on differential cytokines responses and correlation with age, ALT, AST, PCR viral load in chronic HCV patients

Host and viral factors were analyzed to see if there is any hidden effect of viral peptides, gender via correlation and regression analyses. Correlation analysis of various cofactors modulated the levels of cytokines specific to the HCV peptide used in stimulation of patients PBMCs. Upon stimulation with NS3-NS4 peptide, ALT was the only cofactor significantly correlating with Th1 cytokine, IL-2 (table 3). Using NS5 peptide in stimulation of patients PBMCs showed that viral load affected the levels of Th1 cytokine IFN-γ (table 3). While ALT and viral load significantly correlated with changes in levels of Th2 cytokine IL-10 (table 3).

Table 3: Correlations between each of age, ALT, AST, PCR viral load and cytokines in response to HCV peptides in chronic HCV patients.

Host and Viral Factors	Correlation Coefficient (r) and P value	Cytokine (pg/ml)									
		IL-10		IL-2		IL-4		IFN-γ		IL-12	
		NS3-NS4	NS5	NS3-NS4	NS5	NS3-NS4	NS5	NS3-NS4	NS5	NS3-NS4	NS5
Age	r	0.02	0.13	-0.001	-0.15	0.05	-0.46	0.20	-0.14-	0.26	0.004
	P	0.92	0.61	0.99	0.55	0.85	0.05	0.43	0.57	0.30	0.99
ALT	r	0.14	0.51*	0.49*	0.20	-0.08	0.02	-0.04	-0.11	-0.16	0.09
	P	0.57	0.03	0.04	0.42	0.74	0.92	0.86	0.67	0.51	0.72
AST	r	0.21	0.24	0.31	-0.37	0.10	-0.10	-0.04	-0.005	-0.10	0.34
	P	0.40	0.32	0.21	0.13	0.70	0.68	0.86	0.98	0.68	0.16
PCR viral load	r	0.40	0.65**	0.20	0.31	-0.20	0.21	-0.21	-0.53*	0.08	0.13
	P	0.10	0.004	0.43	0.20	0.42	0.396	0.41	0.02	0.74	0.61

*r Pearson product-moment correlation coefficient, * P value ≤ 0.05, ** P value ≤ 0.001*

Correlation analysis to understand the effect of patient gender on cytokine levels stimulated with different HCV peptides

In response to NS3-NS4 peptide, patient gender showed no significant effect on different cytokines at different patient age, ALT, AST and PCR viral load (table 4). In response to stimulation of PBMCs with NS5 peptide, there was a positive correlation between IL-10 levels and both ALT and PCR viral load in male patients (table 5). In addition, female patients' IL-2 levels had a positive correlation with PCR viral load in response to NS5 peptide (table 5).

Table 4: Correlations between each of age, ALT, AST, PCR viral load and cytokines in response to NS3-NS4 peptide among males and females in chronic HCV patients

Host and Viral Factors	Correlation Coefficient (r) and P value	Cytokine (pg/ml)									
		IL-10		IL-2		IL-4		IFN-γ		IL-12	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Age	r	0.337	0.294-	-0.149-	-0.198-	0.041	0.327	0.179	0.288	0.047	0.411
	P	0.311	0.523	0.662	0.670	0.904	0.474	0.598	0.531	0.891	0.359
ALT	r	-0.082-	0.436	0.499	0.429	0.018-	-0.414-	-0.133-	0.532	0.014	-0.556-
	P	0.810	0.328	0.118	0.337	0.957	0.355	0.697	0.219	0.967	0.195
AST	r	0.178	0.291	0.499	0.500	0.078	-0.180-	-0.138-	0.118	-0.080-	-0.148-
	P	0.600	0.527	0.118	0.253	0.821	0.699	0.687	0.801	0.815	0.751
PCR viral load	r	0.178	0.655	0.101	0.393	-0.233-	-0.306-	-0.395-	0.394	-0.113-	-0.037-
	P	0.600	0.111	0.768	0.383	0.491	0.504	0.230	0.382	0.741	0.937

Table 5: Correlations of cytokines response to NS5 peptide with each of age, ALT, AST, PCR viral load among males and females in chronic HCV patients

Host and Viral Factors	Correlation Coefficient (r) and P value	Cytokine (pg/ml)									
		IL-10		IL-2		IL-4		IFN-γ		IL-12	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Age	r	-0.10	0.17	-0.30	-0.04	-0.30	-0.34	0.02	-0.28	-0.297-	0.82*
	P	0.77	0.71	0.37	0.92	0.36	0.46	0.95	0.54	0.37	0.02
ALT	r	0.61*	0.34	-0.08	0.58	0.11	-0.16	-0.28	0.15	0.10	-0.41
	P	0.04	0.45	0.81	0.17	0.76	0.73	0.40	0.75	0.76	0.36
AST	r	0.39	0.59	-0.25	-0.22	-0.15	-0.02	-0.15	0.04	0.21	-0.15
	P	0.23	0.16	0.45	0.64	0.67	0.97	0.67	0.94	0.53	0.75
PCR viral load	r	0.70*	0.70	0.00	0.92**	0.07	0.27	-0.57	-0.74	0.53	0.33
	P	0.02	0.08	1.00	0.003	0.83	0.56	0.07	0.06	0.09	0.46

*r Pearson product-moment correlation coefficient, * P value ≤ 0.05, ** P value ≤ 0.001*

Correlation analysis to understand the effect of active replication of virus (negative strand, positive cases) on cytokine levels stimulated with different HCV peptides

HCV negative strand positive cases indicate active HCV viral replication in chronic patients, and from the recruited chronic HCV patients in this study, four PCR negative strand positive cases (22%) were identified. Different peptides triggered different immune responses among the negative strand positive cases. Correlation analysis of Th1 and Th2 cytokines levels with various cofactors after stimulation with NS3-NS4 peptides showed that in negative strand positive cases, IL-2 levels were significantly altered. Meanwhile, patients' age was inversely correlated with IL-10 an IL-4 levels and ALT levels were inversely correlated with IFN- γ levels (table 6). Upon stimulation with NS5 peptide, IL-10 and IFN- γ levels were directly correlated with negative strand positive cases while patients' ALT levels were inversely correlated with IL-12 levels (table 7).

Table 6: Correlations between each of age, ALT, AST, PCR positive and cytokine after NS3-NS4 peptide among PCR negative strand positive case

Host and Viral factors	Correlation coefficient (r) and P value	Cytokine (pg./ml)				
		IL-10	IL-2	IL-4	IFN- γ	IL-12
Age	r	-1.000	0.316	-0.949	-0.105	-0.105
	P	0.001**	0.684	0.051*	0.895	0.895
ALT	r	-0.105	0.600	-0.400	-1.000	0.800
	P	0.895	0.400	0.600	0.001**	0.200
AST	r	-0.738	-0.400	-0.600	0.400	-0.200
	P	0.262	0.600	0.400	0.600	0.800
PCR positive	r	-0.316	1.000	-0.400	-0.600	0.0001
	P	0.684	0.001**	0.600	0.400	1.000

*r Pearson product-moment correlation coefficient, *P value ≤ 0.05, **P value ≤ 0.001*

Table 7: Correlations between each of age, ALT, AST, PCR positive and cytokine after NS5 peptide among PCR negative strand positive case

Host and Viral factors	Correlation coefficient (r) and P value	Cytokine (pg./ml)				
		IL-10	IL-2	IL-4	IFN- γ	IL-12
Age	r	0.316	0.211	-0.632	-0.316	-0.105
	P	0.684	0.789	0.368	0.684	0.895
ALT	r	0.600	0.400	0.400	-0.600	-1.000
	P	0.400	0.600	0.600	0.400	0.001**
AST	r	-0.400	0.400	-0.400	0.400	0.400
	P	0.600	0.600	0.600	0.600	0.600
PCR positive	r	1.000	-0.400	-0.400	-1.000	-0.600
	P	0.001**	0.600	0.600	0.001**	0.400

*r Pearson product-moment correlation coefficient, *P value ≤ 0.05, **P value ≤ 0.001*

Multivariate linear regression analysis of independent cofactors affecting different cytokines in response to each HCV peptide in chronic HCV patients

In response to NS3-NS4 peptide there was no significant effect on different cytokines (table 8). Upon the use of NS5 peptide as a stimulator of patients PBMCs, we found a significant negative correlation between both Th1 IFN-γ and Th2 IL-10 with PCR viral load (table 9). As for the second Th2 cytokine, IL-4 had a significant positive correlation in case of age, and AST (table 9).

Table 8: Multivariate linear regression analysis to study independent factors affecting different cytokines in response to NS3-NS4 peptide in chronic HCV patients.

Cytokine	Regression Analysis	Age	Sex (female)	PCR viral load	ALT	AST
IL-10	Regression Coefficients	0.01	0.34	-0.02	0.008	0.003
	P	0.71	0.34	0.80	0.36	0.69
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.07-0.10	-0.42-1.11	-0.29-0.98	-0.01-0.02	-0.01-0.02
IL-2	Regression Coefficients	0.06	0.06	-0.007	0.02	-0.008
	P	0.53	0.94	0.33	0.37	0.67
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.13-0.25	-1.63-1.75	-0.001-0.98	-0.02-0.05	-.05-0.03
IL-4	Regression Coefficients	0.30	3.25	-0.24	0.04	-0.04-
	P	0.10	0.12	0.50	0.42	0.35
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.18-0.78	-1.02-7.53	-0.98-0.64	-0.06-0.13	-0.14-0.05
IFN-γ	Regression Coefficients	0.19	2.21	-0.02	0.04	-0.03-
	P	0.33	0.21	0.94	0.28	0.45
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.22-0.60	-1.43-5.85	-0.10-0.09	-0.04-0.12	-0.11-0.05
IL-12	Regression Coefficients	-0.05-	0.07	-0.10	0.00	0.01
	P	0.31	0.87	0.38	0.98	0.17
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.16-0.06	-0.90-1.04	-0.01-0.66	-0.02-0.02	-0.007-0.04

Table 9: Multivariate linear regression analysis to study independent factors affecting different cytokines in response to NS5 peptide in chronic HCV patients.

Cytokine	Regression Analysis	Age	Sex (female)	PCR viral load	ALT	AST
IL-10	Regression Coefficients	0.02	-0.33	-0.002	0.008	-0.005
	P	0.48	0.28	0.0001**	0.21	0.49
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.05–0.097	-0.98–0.31	-0.02– -0.37	-0.007– 0.02	-0.02–0.01
IL-2	Regression Coefficients	-0.19	-1.79	-0.03	0.006	0.02
	P	0.14	0.19	0.22	0.096	0.82
	95.0% CI for Regression Coefficients (Lower– upper bound)	-4.18–25.23	-0.48–0.11	-1.76–6.70	0.00–0.00	-0.05–0.07
IL-4	Regression Coefficients	-0.34	-0.81	-0.001	-0.015	0.050
	P	0.004**	0.35	0.65	0.45	0.021*
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.54– -0.13	-2.63–1.004	-0.01–0.007	-0.06–0.03	0.009–0.09
IFN-γ	Regression Coefficients	-0.01	1.61	-0.006	0.012	0.014
	P	0.91	0.017*	0.007**	0.64	0.58
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.28–0.26	-0.79–4.01	-0.017– -0.26	-0.04–0.07	-0.04–0.07
IL-12	Regression Coefficients	-0.02	-0.28	-0.001	-0.02	0.004
	P	0.61	0.52	0.39	0.10	0.65
	95.0% CI for Regression Coefficients (Lower– upper bound)	-0.13–0.08	-1.22–0.65	-0.06–0.001	-0.04–0.004	-0.02–0.03

Discussion

HCV is a public health concern many countries, including Egypt, where it is becoming a burden on society (Hajarizadeh *et al.,* 2013). Prevalence and incidence of HCV in Egypt is the highest worldwide with rates up to 15% (Kamal, 2011; Derbala *et al.,* 2012). Progression of infection can be

associated with either clearance of acute HCV or progressing to chronic HCV that can lead to cirrhosis and in some cases cancer. As any complex disease, multiple factors modulate this differential response to HCV. Viral load and genotype are two of the well-studied factors attributed to the virus and linked to differential outcomes of HCV infections. On the host side, studies have suggested that multiple factors such as age, gender, co-infections and patient's genetics modulate progression of disease and response to therapy. Moreover, control of HCV is heavily dependent on immune responses specifically in acute infections, with coordinated activation of viral-specific Th1 responses. Well-coordinated strong immune response against acute infection, can clear the virus within a short time period underscoring the importance of balanced effective immune responses in modulating severity of infection (Thimme, *et al.,* 2012; Shulla and Randall, 2012). However, there is still more to the immune mechanisms of virus control to be elucidated.

It has been shown that an imbalance between T helper (Th) cells and their associated cytokines is associated with several infectious diseases (Zhang *et al.,* 2014; Yue *et al.,*2013 ; Wang *et al.,*2003 ; Myrmel *et al.,*2009 ; Gad *et al.,*2012; Rafik *et al.,*2013; Claassen *et al.,* 2013). Th1 associated with cell-mediated immune responses and with several cytokines including IFN-γ, IL-2 and IL-12 play a central role in protection against viral infections (Rehermann, 2013). Cytokines, such as IFN-γ, IL-2 and TNF-α have been associated in the immune responses against HCV infection (Mueller and Rouse 2008; Larrubia *et al.,* 2009). Th2 cells associated with humoral immune responses and several cytokines including IL-4, IL-10 were also recently implicated in immune responses against viruses (Tarr *et al.,*2012). A delicate balance between Th1 and Th2 immune responses coordinates a balanced effective immunity; as these two cells cross regulate their own development (Matsui *et al.,*2008).

Studies suggested that in acute HCV infections, a weak response of Th1 cells might be linked to the failure of viral clearance. In addition, a possible suppressive Th2 cell response in chronic HCV might explain the weak cellular immune response (Matsui *et al.,*2008). Persistently exposed healthy family members to their chronically HCV-infected relatives displayed immune memory cells, likely established by a subclinical infection (Al-Sherbiny *et al.,* 2005). Similarly, exposed HCV sero-negative individuals, such as health-care workers or family members, displayed cell-mediated immune responses although no detectable viral RNA (Rafik, *et al.* 2013).

In this study, stimulation of PBMCs from chronic HCV patients with either NS3-NS4 or NS5 showed cytokine responses that are affected by multiple factors. This study results showed a positive correlation between IL-2 and ALT in response to NS3-NS4 and also a negative correlation between IL-4 and age, but in negative strand positive cases, while in response to NS5; there was a significant positive correlation in case of IL-10 and negative in case of IFN-γ, both versus PCR viral load. Moreover, IL-4 had a significant positive correlation in case of ALT, and AST. Response of female HCV patients showed a significant positive correlation with each of IL-2 and IL-12 with PCR viral load and age respectively in response to NS5 peptide. While, in male IL10 positively correlated with ALT and PCR viral load in response to NS5 peptide.

In conclusion, interactions between host elements fine-tune cytokine responses among chronic HCV patients with genotype 4. Thus underscoring the multifaceted nature of this infectious disease. These components ought to be altogether kept in mind when planning suitable preventive measure or therapies to HCV genotype 4. Future studies on cytokines responses to mixtures of viral proteins should be advantageous.

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