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Natural Killer Cell Subsets Distribution in Spontaneously Resolved and Chronic Persistent Hepatitis C Virus Infection

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Abstract

Altered frequency and distribution of natural killer cell subsets have been reported in hepatitis C virus (HCV) infection.We investigated the frequency of NK cells and inhibitory receptor CD158 in a sample of Egyptian patients with spontaneously resolved (SR) and chronic persistent hepatitis C virus (CPHC) infection, and correlated data with other clinical and diagnostic parameters. The study was conducted on 48 patients divided into 3 groups. Group I; 16 CPHC patients, Group II; 16 SR individuals and Group III; 16 healthy controls. Chronic persistent HCV patients and SR individual's data were reported from patients ' reports. Healthy controls serum antibodies against HCV were measured using ELISA technique. The three studied groups fresh peripheral blood samples were analyzed by flow cytometry to determine total NK cells, their subsets and $CD158b^+$ cells percentages. Total NK cells and $CD56^{+dim}$ $CD16^+$ NK cells were significantly decreased in CPHC patients and SR individuals in comparison to healthy controls($P<0.001$).In contrast, $CD56^{+bright}$ $CD16^-$ NK cells were significantly increased in CPHC patients and reduced in SR individuals in comparison with healthy controls ($P<0.001$). Significant elevation of CD158b inhibitory receptor frequency in CPHC patients in comparison with healthy controls ($P<0.001$) and it was positively correlated with stage of cirrhosis, unresponsiveness to IFN, WBCs and lymphocytes counts and AST and ALT levels. In conclusion, during the chronic HCV infection stage, the frequency of NK cells is significantly depressed and CD158b+ inhibitory receptor might be represent this impairment. On the other hand, in SR individuals, total NK cells were significantly decreased. Also, $CD56^{+dim}$ $CD16^+$ NK cells and $CD56^{+bright}$ CD16− NK cells percentages were significantly decreased ($P<0.001$) although preserving nearly the same ratio of healthy controls. Also, there was no significant elevation in $CD158b^+$ cells frequency ($P>0.05$).

Keywords: Global Fund's support; HIV/AIDS; Thailand; Transition

Introduction

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide. The long-term impact of chronic persistent HCV infection is highly variable, ranging from minimal histological changes to extensive fibrosis and cirrhosis with or without hepatocellular carcinoma [1].

Natural killer (NK) cells are large granular lymphocytes that account for the majority of innate immune cells in the human liver. They play an important role in the control of viral infections. Their functions are mediated by a diverse array of inhibitory and activating cell-surface receptors [2].

In human peripheral blood, the CD3-NK cells are divided into five subpopulations which can be defined on the basis of the relative expression of the markers CD16 (Fc γ RIII) and CD56:CD56 dim $CD16^+$, CD56 dim CD16-, CD56 bright $CD16^-$, CD56 bright $CD16^+$, and $CD56^-CD16^+$[3].

$CD56^{+dim}$ $CD16^+$ NK cells normally account approximately 90% of peripheral NK cells. They usually express CD16 which is the Fc receptor for IgG, killer cell immunoglobulin-like receptors (KIRs) and homing markers for inflamed peripheral sites. They carry perforin, and are the main mediators of NK cytotoxicity [4].

$CD56^{+bright}$ $CD16^-$NK cells account nearly 10% of peripheral NK cells. They express homing markers for secondary lymphoid tissues where they accumulate. They do not express KIRs, contain low levels of perforin, and are only weakly cytotoxic. However, they are important secretors of cytokines including IFN-gamma, TNF-α, granulocyte-macrophage colony-stimulating factor (GMCSF), interleukin 10 (IL10) and IL13 [5].

Most prominent among NK receptors are the killer cell immunoglobulin-like receptors (KIRs). CD158b is one of KIRs which confers inhibitory signals to NK cells leading to their suppression [6].

In chronic persistent HCV infection, NK cells display alterations in their phenotype and function, and cytolytic NK cells seem to be impaired through high expression of inhibitory receptors including CD 158b [7].

On the other hand, CD158b low frequency seems to predict resolution of chronic persistent HCV infection. Also, CD158b may correlate with some diagnostic parameters in chronic HCV patients [8]. On contrary, in spontaneously resolved HCV infection, the activated NK cells' responses suggest an important contribution to resolution of the infection, as there is a good cytolytic function of NK cell and normal expression of inhibitory cell surface receptors [9].

Material and Methods

The aim of this work was to study the frequency of the NK cell and the distribution of its subsets in spontaneously resolved and chronic persistent hepatitis C virus infection. In addition, to study the frequency of inhibitory receptor CD158b in these clinical outcomes and to correlate its frequency with certain clinical and diagnostic parameters.

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Study design

This case control study was conducted in Medical Microbiology and Immunology Department and Clinical pathology Department, Faculty of medicine, Zagazig University during the period from June 2014 to December 2015.

Ethical considerations

Approval for performing this study was obtained from Microbiology and Immunology Department and Clinical Pathology Department after taking Institutional Review Board (IRB) and ethical committee approval. Also, written informed consent was obtained from each participant.

Subjects

The study included 48 subjects from both sexes.

Inclusion criteria

Group (I): They were 16 chronic persistent HCV infection patients.

They were anti-HCV antibody positive and had detectable serum level of HCV RNA by PCR for at least 6 months [10].

Group (II): It included 16 spontaneously resolved (SR) individuals. Those individuals were attending the outpatient clinic of Tropical Medicine Department, Zagazig University Hospitals. They were anti-HCV antibody positive and had detectable serum level of HCV RNA by PCR. They discovered HCV clearance accidentally while performing pre-treatment investigations including PCR which revealed negative result. They were retested again for serum HCV RNA by PCR after 12 weeks for confirmation of resolution and exclusion from treatment regimen [11].

Group (III): They were 16 apparently healthy individuals, proved to be negative for HCV Abs by ELISA and had no history of liver diseases.

Exclusion criteria for the three groups

Individuals with history of or had current HBV or HIV infections were excluded. In addition, individuals who had autoimmune hepatitis, metabolic liver disease, history of exposure to hepatotoxin or immuno-suppressive therapy were also excluded.

Data collection

1. Age and sex for all groups.
2. Other data

Clinical data including ascitis, jaundice and cirrhosis of variable degrees were reported from patients' files. Recent history of interferon treatment, results:

For group (I): Clinical data including ascitis, jaundice and cirrhosis of variable degrees were reported from patients' files. Recent history of interferon treatment, results of HCV Ab by ELISA and HCV RNA by PCR were also recorded.

For group (II): Previous and recent results of HCV Ab by ELISA, HCV RNA by PCR as well as routine laboratory investigations done in Clinical Pathology Department were taken from patients' files.

Sampling

Blood sample (3mL) was taken from each healthy control. Serum was separated, divided in two tubes. One tube was used for routine laboratory investigations and the other one was stored at -20°C for ELISA assay (Axiom, Worm (WO), Germany). On the other hand, fresh blood sample (2ml) form each subject included in the study was collected. Each sample was taken in a sterilized tube containing EDTA as the reagent's performance may be affected by the use of other anticoagulant [12]. Samples were transported immediately to laboratory for flow cytometric analysis. Samples with hemolysis, clots or suspended cell aggregates were discarded.

Sample preparation and flow cytometric analysis: Peripheral blood mononuclear cells (PBMCs) were purified using whole blood lysis technique [12]. Cell surface staining was performed using mouse anti-human monoclonal antibodies (mAbs); anti-CD3 fluorescein isothiocyanate (FITC) conjugate and anti-CD3 phycoerythrin (PE) conjugate, anti-CD16 peridinin chlorophyll protein (PerCP) conjugate, anti-CD56 phycoerythrin (PE) conjugate and anti-CD158b fluorescein isothiocyanate (FITC) conjugate (BD Biosciences, San Diego, USA).

Staining was performed by adding 20 µL of each mAb to 100 µL of blood, followed by incubation for 30 minutes in the dark at 4ºC. The tubes were washed twice with FACs buffer. Erythrocytes were lysed using BD FACS lysing Solution (BD Biosciences, San Diego, USA). Finally, 0.5 mL of phosphate buffered saline was added to the washed cells prior to measurement of (CD3−CD16+and/or CD56+) cells .We used appropriate isotype controls of anti-human mAbs to prevent nonspecific Fc receptor staining. They were PE Mouse IgG2a, PERCP Mouse IgG2a and FITC Mouse IgG1.

Before analyzing samples by the flow cytometer (FACS caliber, Becton Dickinson, San Diego, USA), the threshold or discriminator was adjusted to minimize debris and ensure that populations of interest were included. Cytometer was correctly aligned and standardized for light dispersion and fluorescent intensity. Compensation was set following cytometer manufacturer instructions to avoid erroneous results if laser is misaligned or if gates are incorrectly set.

A minimum of 10.000 events were acquired in the gating region and analyzed using FACs Cell Quest software (BD Biosciences, San Diego, USA). Lymphocytes present in the sample were determined by flow cytometry based on its typical pattern of FSC/SSC (size/granularity or complexity). The NK cell population was then identified as ($CD3^{-}CD16^{+}$and/or $CD56^{+}$) cells and further categorized into different NK cell subsets depending on the expression of CD16 and CD56 molecules as regulatory ($CD3^{-}CD56^{+bright}$ $CD16^{-}$) and cytotoxic ($CD3^{-}CD56^{+dim}CD16^{+}$). $CD3^{-}CD56^{+bright}CD16^{-}$ NK cells and $CD3^{-}CD56^{+dim}CD16^{+}$ NK cells were distinguished by CD56 fluorescence density. CD3− $CD56^{+bright}$ CD16− NK cell had a mean fluorescence intensity of ≥613 and CD3− $CD56^{+dim}$CD16+ NK cells had a mean fluorescence intensity of (612-303). CD158b frequency on NK cells was also determined [13].

Statistical Analysis: The collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 18.0. Qualitative data were represented as frequencies and relative percentages. Chi square test was used to calculate difference between qualitative variables. Quantitative data were expressed as mean ± SD (Standard deviation). Independent T test was used to calculate difference between quantitative variables in two groups. ANOVA F-test test was used to calculate difference between quantitative variables in more than two groups.KruskalWallistest (K) was used to calculate difference between quantitative variables in more than 2 groups in not normally distributed data. Pearson correlation coefficient used to calculate correlation between quantitative variables. The significance

Level for all above mentioned statistical tests done. The threshold of significance is fixed at 5% level (P-value). P value of >0.05 indicates non-significant results while that of <0.05 indicates significant results.

Results

This study was conducted on 3 groups; group (I): It included 16 chronic persistent HCV infection patients. They were 9 males and 7 females, the ages ranged from 18 to 65 years old (Mean ± SD: 38.88 ± 14.89), group (II): It consisted of 16 SR individuals. They were 9 males and 7 females, their ages ranged from 18 to 65 years old (Mean ± SD: 38.63 ± 14.22) and group (III): It included 16 healthy controls. They were 13 males and 3 females, their ages ranged from 19 to 55 years old (Mean ± SD: 37.06 ± 11.69).There were no statistical significance differences between the three studied groups as regards age (P>0.05) and sex (P>0.05) distributions (data not shown).

There were statistically significant reduction in the total NK cells among chronic HCV patients and SR patients compared to healthy controls, (P<0.001). Although there were no statistically significant differences between the two patient groups, (P>0.05). On the other hand, there were statistical significance reduction in CD56^{+dim} CD16^{+} NK cells percentages among chronic HCV cases compared to SR individuals and healthy controls, (P<0.001). As regards the CD56$^{+bright}$ CD16^{-} NK cells percentage, it was significantly expanded in chronic HCV cases compared with healthy controls, (P<0.001) while it was significantly reduced in the SR group compared to the control group, (P <0.001) (Tables 1) and (Figures 1 and 2).

Concerning CD158b inhibitory receptor, there were significant elevations in its frequency among chronic persistent HCV patients compared to SR group and healthy controls (P<0.001), while no

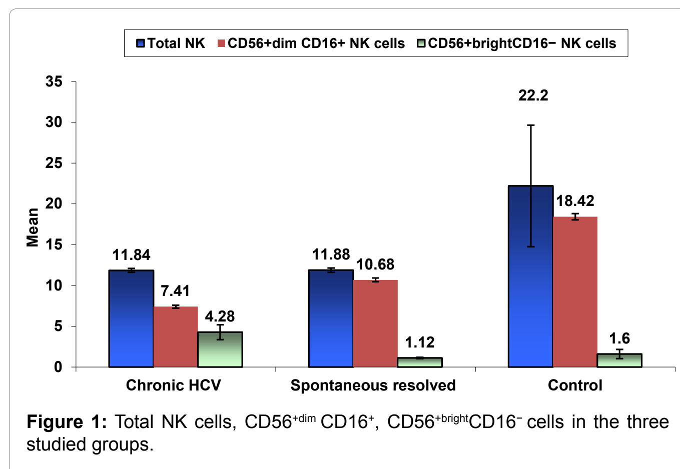

Figure 1: Total NK cells, CD56^{+dim} CD16^{+}, CD56$^{+bright}$CD16^{-} cells in the three studied groups.

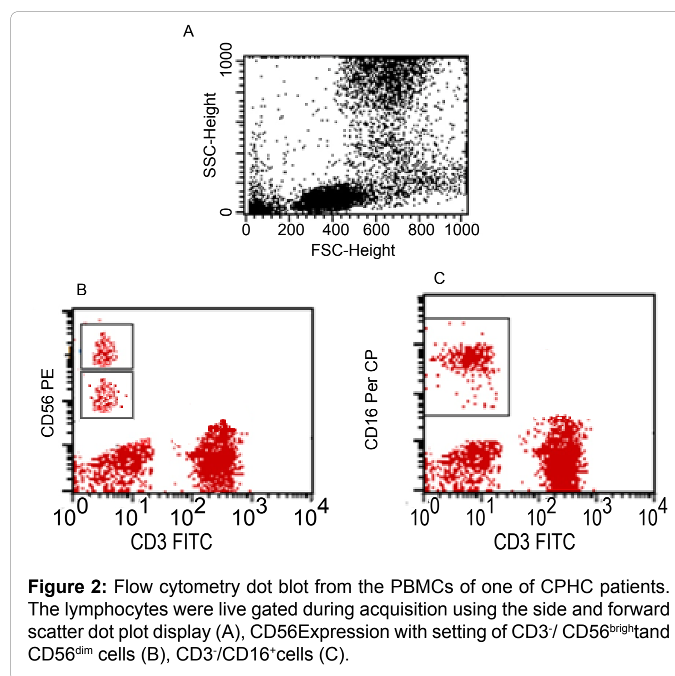

Figure 2: Flow cytometry dot blot from the PBMCs of one of CPHC patients. The lymphocytes were live gated during acquisition using the side and forward scatter dot plot display (A), CD56Expression with setting of CD3^{-}/ CD56^{bright}and CD56^{dim} cells (B), CD3^{-}/CD16^{+}cells (C).

Variable	Chronic HCV(n=16)	Spontaneous resolved (n=16)	Control(n=16)	Test	p	LSD
Total NK						<0.001*1
Mean ± SD	11.84 ± 0.24	11.88 ± 0.26	22.2 ±7.45	30.72	<0.001**	<0.001*2
Range (%)	11.5-12.3	11.5-12.3	19.8-50.05			0.98 3
CD56+dim CD16+						<0.001*1
NK cells:						
Mean ± SD	7.41 ± 0.18	10.68 ± 0.23	18.42 ±0.39	646.2	<0.001*	<0.001*2
Range (%)*	7.07-7.75	10.23-11	17.86-18.9			<0.001*3
CD56$^{+bright}$CD16−						<0.001*1
NK cells:						
Mean ± SD	4.28 ± 0.91	1.12 ± 0.08	1.6 ± 0.56	396.5	<0.001*	<0.001*2
Range (%)*	3.9-4.6	1-1.23	1.03-2.8			<0.001*3

Table 1: Flow cytometeric analysis of NK cell subsets in the three studied groups; Control versus Chronic HCV, Control versus Spontaneous resolved, Chronic HCV versus spontaneous resolved. (%): percentage of total NK cell count to the total lymphocytic count. (%)*: percentage of CD56^{+dim} CD16^{+}NK cells and CD56$^{+bright}$CD16^{-}NK cells count to the total NK cell count.

	Chronic	Spontaneous	Control	Test F	P	LSD
	HCV (n=16)	resolved (n=16)	(n=16)			
CD 158 b:						<0.001*[1]
Mean ± SD	7.32 ± 1.67	0.02 ± 0.02	0.02 ± 0.02	K	<0.001**	0.99[2]
Range (%)	5-9.3	0-0.05	0-0.06	31.75		<0.001*[3]

Table 2: CD158b frequency in the three studied groups. [1]Control versus Chronic HCV, [2]Control versus spontaneous resolved, [3]Chronic HCV versus spontaneous resolved.

significant difference was found in spontaneously resolved individuals compared to the control group (P>0.05) (Table 2 and Figure 3). We found no statistical significant correlation between CD158b level among the three groups and total NK cell, $CD56^{+dim}$ $CD16^{+}$ cells nor $CD56^{+bright}$ $CD16^{-}$ cells percentages (data not shown).

There was a statistical significance correlation between CD158b and severity of cirrhosis and response to interferon (P<0.001) with increase of its level among severe cirrhotic cases and non-responder cases. Furthermore, a significant positive correlation was found between it versus WBCs and lymphocytes counts and AST and ALT levels in chronic HCV patients. However, there was negative correlation between it and total protein. Also, there was no correlation between it and viral load in chronic HCV patients (Tables 3 and 4).

Discussion

In our study, the percentage of total NK cells was shown to be decreased significantly in chronic HCV patients and spontaneously resolved individuals compared to their levels in healthy controls. However, no significant differences were found between their levels between spontaneously resolved individuals and chronic HCV patients. This observation was consistent with Morishima et al. [13], Bonorino et al. [14] and Dessouki [15].

This could be explained by Meier [16] who suggested that the deficit in interleukin 15 (IL-15) might be responsible for this change. It is thought that IL15 regulates the function of NK cells and promotes their survival.

On contrary, others like Nattermann and co-workers [17] noticed no differences in NK total cell count between HCV infected individuals and healthy controls. On the other hand, Dessouki [15] reported that spontaneous resolvers and healthy controls showed the same total NK cell frequency.

The diversity of observations in the above-mentioned studies regarding NK frequency is likely to be due to heterogeneity of patients,

Variable	N	CD 158 b mean ± SD	Test	P
Cirrhosis:				
No	7	5.57 ± 0.47	T 12.21	<0.001**
Severe	9	8.68 ± 0.54		
Ascites:				
No	12	7.36 ± 1.62		
				0.56
Mild	1	9.1 ± 0.00	F 0.72	NS
Moderate	2	7.08 ± 2.5		
Severe	1	5.56 ± 0.00		
Jaundice:				
No	11	7.86 ± 1.47	t 2.11	0.06
Yes	5	6.14 ± 1.59		NS
Response to interferon:				
No clearance	5	8.9 ± 0.61		
Partial clearance	4	8.51 ± 0.46	F 77.03	<0.001**
Complete clearance	7	5.57 ± 0.47		
Viral load (PCR):				
Mild viremia	4	7.26 ± 1.72		
			F 0.27	0.77
Moderate viremia	7	7.65 ± 1.87		NS
Severe viremia	5	6.90 ± 1.62		

Table 3: Correlation between CD 158b frequency and certain clinical data, response to interferon and viral load in chronic HCV group.

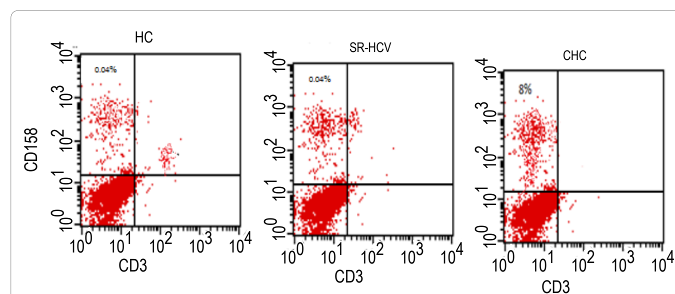

Figure 3: Flow cytometry dot blot of the NK cell population among the three studied groups that was further identified and differentiated into regulatory ($CD3^{-}CD16^{-}CD56^{+}$), cytotoxic ($CD3^{-}CD16^{+}CD56^{+}$) NK cell subsets on the basis of the expression of CD56 and CD16.

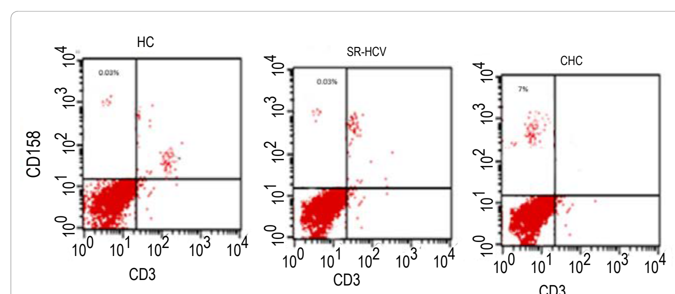

Figure 4: Flow cytometry dot blot analysis for the percentage of CD158b expression on the natural killer cells among the three studied groups. Peripheral blood mononuclear cells were stained for ($CD3^{-}CD16^{+}$and/or$CD56^{+}$) cells of the live gated lymphocytes populations during acquisition. Then CD158b percentage was measured among it.

Variable	CD158b					
	Spontaneous					
	Chronic HCV(n=16)		resolved(n=16)		Control	
	r	P	r	P	r	P
WBCs (x10³/mm³):	0.9	<0.001*	0.28	0.29	-0.51	0.06
Lymphocyte (%):	0.83	<0.001*	0.17	0.54	-0.03	0.91
Total Bilirubin (mg/dL):	-0.23	0.4	0.13	0.11	-0.21	0.31
Direct Bilirubin (mg/dL):	-0.06	0.83	0.32	0.12	-0.22	0.32
AST (IU/L):	0.98	<0.001*	-0.07	0.81	0.003	0.99
ALT (IU/L):	0.93	<0.001*	0.05	0.85	0.08	0.77
Total protein (g/dL):	-0.58	0.02*	-0.24	0.38	0.1	0.71
Albumin (g/dL):	-0.42	0.1	0.41	0.12	-0.22	0.41

Table 4: Correlation between CD158b frequency and certain routine laboratory investigations in the three groups.

diversity of HCV subtypes and viral loads, variations in the duration of HCV infection and the presence of other microbial infections [16].

We demonstrated that CD56^{+dim} CD16^{+} was lower in HCV-infected patients compared with healthy controls and resolvers. This agreed with Morishima et al. [13] and Lee et al. [18]. Although this disagreed with some studies such as Nattermann et al. [17] and De Maria et al. [19] who reported no significant reductions in NK cytolytic activity in patients with chronic HCV infection. The difference is likely to be due to pathogen-associated factors such as diversity of HCV viral quasispecies and HCV genotype [20]. In our study we noticed an expansion in CD56$^{+bright}$ CD16^{-} NK cells in chronic HCV patients compared to healthy controls and spontaneously resolved individuals. This result was consistent with Ferlazzo et al. [21] and Dessouki et al. [15] and Jost et al. [22].

Jost and co-workers [22] mentioned that altered NK cell subset distributions are unlikely to be due to selective sequestration in the liver because NK cells are not expanded in HCV-infected livers only and their numbers in blood positively correlated with those in liver.

Alternatively, Chan et al. [23] suggested that the altered NK cell subset distributions in persistently infected patients could be the result of decreased rates of differentiation of CD56$^{+bright}$CD16^{-}NK cells into CD56^{+dim} CD16^{+} NK cells.

On contrast to all previous studies, Hui-Fan et al. [8] noticed no significant changes in the CD56^{+}dimCD16^{+} NK cell and CD56$^{+bright}$ CD16^{-} NK cell levels in chronic HCV patients in comparison with healthy controls and spontaneously resolved individuals.

By analyzing the NK cell phenotypes after HCV infection, we found that the high expression levels of CD158b (KIR2DL3) were only present in individuals with chronic HCV infections while its expression in spontaneously resolved individuals was very low similar to expression levels in healthy controls. This data indicated that the increased number of inhibitory receptors present on NK cells suppresses NK cell activation and this agreed with Khakooet al. [24] and Knapp et al. [25].

Furthermore, we analyzed the relationship between CD158b and the degree of cirrhosis. We noticed a positive correlation between CD158b and degree of cirrhosis. This agreed with Hui-Fan et al. [8] and Bin and Svetlana [26] who hypothesized that high expression level of this receptor on NK cells interfered with the development of an immune response and associated with HCV immune escape. These persistently weak immune responses resulted in liver cell destruction and cirrhosis.

On contrary, lee and co-workers, [18] mentioned that low NK cell cytotoxicity by CD158b limits liver damage and fibrosis.

Regarding the response of the chronic HCV patients to interferon (IFN), we observed that patients with high CD158b expression are unresponsive to IFN therapy. This is consistent with Golden-Mason, et al. [27] and Hui-Fan et al. [8].

Also, we found no correlation between CD158b on NK cells and serum HCV RNA loads in chronic HCV patients. This result is consistent with Hui-Fan et al. [8].

We further analyzed the role of altered CD158b expression patterns on NK cells in relation to chronic HCV pathogenesis. Correlation analysis revealed that the percentage of CD158b^{+} NK cells significantly positively correlated with serum AST and ALT levels in chronic HCV patients. This agreed with Vidal-Castineira et al. [10] and Hui-Fan et al. [8].The later explained that the impaired immune response caused by CD158b leads to exaggerated hepatocytes damage by HCV with high liver enzymes.

As regards other clinical data such as ascitis, jaundice and other diagnostic laboratory parameters such as total, direct bilirubin and albumin, there was no significant correlation between them and CD158b in our study. No other reports showing significant correlation were found.

Conclusion

We found that during the chronic HCV infection stage, there were reduction in the total NK and CD56^{+dim} CD16^{+} NK cells. While there was expanded CD158b^{+} population among the chronic HCV group with absence of this finding among the SR group. A positive correlation were found between CD158b^{+} with both AST and ALT levels among the chronic HCV and SR while no correlation was detected between CD158b and viral load. Finally, we concluded that the antiviral activity of NK cells is significantly depressed among the chronic HCV patients and the antiviral activity of NK cell function was significantly depressed with inhibitory signals.

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