

Interferon therapy shifts natural killer subsets among Egyptian patients with chronic hepatitis C

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ABSTRACT

Natural killer cells can be divided into five subpopulations based on the relative expression of CD16 and CD56 markers. The majority of natural killer cells are CD56^{dim}, which are considered to be the main cytotoxic effectors. A minority of the natural killer cells are CD56^{bright}, and function as an important source of immune-regulatory cytokines. Shifts of these subsets have been reported in patients with chronic hepatitis C virus infection. We sought to investigate the shift of natural killer subsets among Egyptian patients with chronic HCV and to analyze the influence of interferon therapy on this shift. We applied a flow cytometric analysis of peripheral blood natural killer subsets for 12 interferon-untreated and 12 interferon-treated patients with chronic HCV, in comparison to 10 control subjects. Among interferon-untreated patients, there was a significant reduction of CD56¹⁶⁺ (immature natural killer) cells. Among interferon-treated patients, the absolute count of natural killer cells was reduced, with expansion of the CD56^{bright} subset and reduction of the CD56^{dim}16⁺ subset. Natural killer subset counts were not significantly correlated to HCV viral load and were not significantly different among interferon responders and non-responders. In conclusion, HCV infection in Egyptian patients has been observed to be statistically and significantly associated with reduction of the CD56¹⁶⁺NK subset, while a statistically significant expansion of CD56^{bright} and reduction of CD56^{dim}16⁺ subsets were observed after interferon therapy. Further studies are required to delineate the molecular basis of interferon-induced shift of natural killer subsets among patients with HCV.

Keywords: natural killer cells, natural killer subsets, chronic hepatitis C, innate immunity, interferon.

[*Braz J Infect Dis* 2010;14(4):398-405]©Elsevier Editora Ltda.

INTRODUCTION

Natural killer (NK) cells form 10-15% of the peripheral blood lymphocyte population. They are found in several lymphoid and non-lymphoid compartments of organs, such as spleen, liver, and lungs.¹ The identification of NK cells can be based on their morphology, function, or phenotype. Morphologically, NK cells are typed as large granular lymphocytes having a kidney-shaped nucleus, a high cytoplasm to nucleus volume ratio, and containing large intracytoplasmic azurophilic granules.² Functionally, NK cells are thought to have two important roles: target cell killing and cytokine production. With regards to target cell killing, NK cells differ from T cells in that their cytolytic response does not require prior sensitization, and they are capable of destroying MHC I-mismatched haematopoietic and lymphoid cells.³ Regarding

cytokine production, NK cells secrete mainly interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and granulocyte monocyte colony-stimulating factor (GM-CSF), which enable them to regulate the host immune mechanism locally.⁴ Phenotypically, they are distinguished from T and B lymphocytes by the absence of the T-cell receptor/CD3 complex and immunoglobulin receptors on their cell surface.⁵ Besides the lack of certain markers on their surface, the presence of CD56 is used as a marker for NK cells in humans. CD56 has been shown to be an isoform of the neural cellular adhesion molecule.⁶ The expression of CD16, which is involved in the antibody dependent cellular cytotoxicity (ADCC) mediated by NK cells, is also used as a phenotypic marker.⁷ Therefore, NK cells are phenotypically defined as CD56⁺CD16⁺CD3⁻ cells. In human peripheral blood, five NK cell subpopulations can be iden-

Submitted on: 07/28/2009

Approved on: 11/17/2009

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We declare no conflict of interest.

tified on the basis of the relative expression of CD16 and CD56 markers; CD56^{bright} CD16⁻, CD56^{bright} CD16⁺, CD56^{dim} CD16⁺, CD56^{dim} CD16⁻, and CD56⁻ CD16⁺. CD56^{dim} cells are the main mediators of NK cytotoxicity, as they contain high levels of perforin, whilst CD56^{bright} act as an important source of immunoregulatory cytokines.^{8,9}

Hepatitis C virus (HCV) infects over 170 million people worldwide.¹⁰ A minority of infected persons may resolve the acute stage of infection and clear the virus, but most patients develop a life-long infection, making HCV a leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma.¹¹ The host immune response to HCV antigens is thought to determine whether viral clearance or chronic infection occurs. Hepatotropic viruses induce production of type I IFN by hepatocytes and other cells in the liver, which, in turn, promotes infiltration of NK cells in virus infected livers.¹² The production of type I IFN and other cytokines (including IL-12, IL-15, and IL-18) by hepatocytes activates NK cells and induces IFN- γ production by them. The IFN- γ produced by NK cells recruits activated T cells to the liver. It is noteworthy that IFN- γ produced by NK cells plays a major role in liver infiltration of CD4 and CD8 T cells. It has been shown in animal models that deliberate depletion of NK cells before a hepatotropic viral infection leads to inhibition of a virus-specific T cell response, as well as inhibition of liver injury.¹³ Human NK cells co-cultured with HCV replicon inhibit the replicon expression at protein and RNA levels by secreting antiviral factors, including IFN- γ .¹⁴ Thus, NK cells could also potentially contribute towards control of HCV replication. It is possible that an adequate NK cell response may control hepatotropic viruses such as HCV, even in the absence of virus-specific immune responses. This notion is supported by the observation that, like humans, a certain percentage of HCV-infected chimpanzees can clear the virus spontaneously, and this clearance does not correlate with the appearance of acquired immunity.¹⁵ However, the potential importance of NK cells in the control of HCV was disputed in a study that demonstrated that depletion of CD8 T cells by mAb aggravates HCV infections in animal models.¹⁶ It is noteworthy that about one third of human and chimpanzee NK cells express CD8, and depletion of CD8 T cells by anti-CD8 antibodies would also deplete CD8 NK cells. Therefore, the results from such studies should be interpreted with care. Meier *et al.*¹⁷ identified significantly reduced numbers of total NK cells and a striking shift in NK subsets, with a marked decrease in the CD56^{dim} cell fraction compared to CD56^{bright} cells in HCV infections. This shift influenced the phenotype and functional capacity (IFN- γ production and killing) of the total NK pool. The aim of this work was to assess the influence of interferon therapy and HCV infection on NK subsets among Egyptian patients.

MATERIAL AND METHODS

Study design

This is a cross-sectional analytical study, as we are measuring exposure (\pm interferon) and outcome (NK subsets) at the same time.

Study subjects

Twenty four patients, attending either Suez Canal University Hospital or Kasr El-Aini Hospital, were divided into two groups. Patients with chronic HCV who had never received interferon at any time during their course of illness comprised an interferon-untreated group (n = 12). Patients who suffered from chronic HCV infection and had been receiving standard interferon therapy for 24 weeks comprised an interferon-treated group (n = 12). The standard treatment protocol included: interferon (interferon-alpha 2a), three million units, three times per week for 48 weeks. Patients were eligible for inclusion in this study after 24 weeks of therapy. Patients co-infected with HIV or HBV were excluded from this study. A normal control group was comprised by healthy HCV negative blood donors (n = 10). Written informed consent was obtained from all subjects.

Clinical history and examination

A detailed history and clinical examination were carried out for all groups. They were diagnosed as chronic hepatitis if the following criteria were present after a six month follow-up: a) significant and persistent symptoms, b) fluctuating or persistently elevated ALT and AST (> 1.5 fold of normal levels), normal serum albumin and prothrombin time, c) ultrasonography revealed an enlarged bright texture \pm portal tract thickening and \pm normal spleen. Liver biopsy was done for the untreated group. Hepatic injury was assessed using the histological activity index (HAI) as modified by Ishak.¹⁸ This consisted of a necro-inflammatory grading score (range 0 to 18; 0 = no activity, 18 = severe activity) and a fibrosis staging score (range 0 to 6; 0 = no activity, 6 = cirrhosis).

Laboratory investigations

Venous blood samples (10 mL in EDTA and plain tubes) were drawn from each subject. Serum samples were obtained after clotting of the blood. For PCR testing, serum samples were stored at -80 °C until the time of assay. All samples were subjected to the following laboratory investigations:

- 1 – Complete blood count using an automated cell counter (Cell Dyne, Abbott Diagnostics).
- 2 – Liver function tests:
 - a) Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, direct bilirubin, serum albumin, and alkaline phosphatase (Cobas Integra Auto Analyzer, Roche).
 - b) α -fetoprotein was analysed by VIDAS autoanalyzer (Biomerieux, France).

3 – Viral hepatitis markers: HCV antibodies (HCV-EIA, Abbott Laboratories and HBsAg (Eleclys1010, Roche Diagnostic GmbH) were assessed.

4 – Flow cytometry analysis: NK cell subsets were assessed by flow cytometry using FACS Calibur (BD Bio-sciences, San José, CA, USA). Cells were labeled with mAb against CD3 APC (UCHT1), CD16 FITC (B-E16), and CD56 PE (MOC-1). Monoclonal antibodies against CD3, CD16 and CD56 were purchased from IQProduct (Groningen, Netherlands). A sample of 20 µL of each monoclonal reagent was added to 100 µL of whole EDTA blood in 12×75 test tubes and incubated at room temperature for 15 min in the dark. Red blood cells were lysed using FACS Lyse reagent (BD, USA) and centrifuged at 2000 rpm for 5 min. The supernatants were discarded and the cells were washed twice with phosphate-buffered saline (PBS), re-suspended in 300 µL of PBS. Data were analyzed using FACS Calibur and Flowjo software (TreeStar, Ashland, OR, USA). The typical forward and side scatters were used for lymphocytes gating. Gated lymphocytes were further gated by selecting CD3⁻ population. CD3⁻ cells were divided according to the expression of CD56 and/or CD16. Accordingly, NK cells were subgrouped into CD56^{bright} or CD56^{dim} and further defined by CD16 expression.

5 – PCR: Quantitative HCV real-time PCR was performed for untreated patients before the start of interferon treatment, using a Light Cycler-RNA Master Hybridization Probe system in accordance with the manufacturers' instruction (Roche diagnostic, Mannheim, Germany). The lower detection limit is < 1000 IU/mL. Qualitative PCR was performed for treated patients at 24 weeks of treatment. HCV-

RNA was isolated by the QIAmp RNA viral kit (Qiagen, Hildene, Germany). HCV-RNA was reverse transcribed into cDNA, amplified by PCR (Qiagen), and detected by electrophoresis in 2% agarose gel stained with ethidium bromide. The PCR product (270 bp) was detected using an ultraviolet trans-illuminator.

Statistical analysis

Statistical analysis was done using SPSS program version 11. Data were presented as mean ± standard deviation. The Kruskal-Wallis test for analysis of non parametric data was used to compare between groups followed by Dunnett's post-hoc test. Pearson correlation analysis was used when appropriate. Statistical significance was considered at the 5% level of probability ($p < 0.05$).

RESULTS

The interferon-untreated patients ($n = 12$) had ages ranging from 29 to 53 (42.7 ± 8) years. Seven were female and five were male, and their duration of hepatic illness ranged from 12 to 50 (28 ± 16) months. The interferon-treated patients ($n = 12$) had ages ranging from 35 to 58 (45.5 ± 7) years. Three were female and nine were male, and the duration of their hepatic illness ranged from 18 to 53 (31 ± 15) months. Control subjects ($n = 10$) had ages ranging from 38 to 50 (44.7 ± 5) years. Seven were male and three were female. The study subjects were matched for age and sex.

Clinical, histological and virological features of INF-untreated and treated patients are listed in Table 1, and the routine laboratory data of the studied groups are listed in Table 2.

Table 1. Clinical, histological and virological features of IFN-untreated and IFN-treated patients

	IFN-untreated n = 12	IFN-treated n = 12
Risk factor		
Anti-Bilhazial injection	2	3
Blood transfusion	2	0
Tooth extraction	5	6
Tattooing	2	2
Surgical intervention	5	2
Histology		
Grading	6.9 ± 1.8	ND
Staging	2.9 ± 1.3	ND
Viral load		
Quantitative HCV - RNA(IU/mL×10 ³)	296 ± 349	ND
Qualitative PCR	ND	7 cases negative HCV-RNA 5 cases positive HCV-RNA

Values are expressed as mean ± standard deviation. ND: not determined

The proportion of total NK and NK subsets, as well as the absolute numbers of various NK cells in interferon-untreated, interferon treated chronic HCV patients, and healthy controls are presented in Table 3. Figure 1 (a,b,c)

shows the flow cytometry analysis for a subject from each group. The relative and absolute counts of CD56⁺CD16⁺ subset were decreased in interferon-untreated and interferon treated patients when compared to controls (Fig. 2a),

Table 2. The laboratory data of IFN-untreated, IFN-treated and controls

Laboratory variables	IFN-untreated n = 12	IFN-treated n = 12	Controls n = 10
Albumin (mg/L)	4.4 ± 0.3	4.4 ± 0.36	4.3 ± 0.3
Alkaline phosphatase (u/L)	129 ± 66 [†]	118 ± 70*	71 ± 13
AST (u/L)	44.5 ± 17.4 [§]	28.9 ± 8.9 [†]	18.3 ± 6.1
ALT (u/L)	63.5 ± 29 [§]	30.9 ± 10*	22.1 ± 5.6
T.Bilirubin (mg/dL)	1.39 ± 2.0*	2.1 ± 4.3*	0.53 ± 0.20
D.Bilirubin (mg/dL)	0.45 ± 0.34*	1.5 ± 3.9 [†]	0.17 ± 0.18
AFP (ng/mL)	9.3 ± 8.3	17.9 ± 25.1	5.2 ± 2.1
Hemoglobin (g/dL)	14.0 ± 0.72*	11.2 ± 1.73 [§]	14.7 ± 0.60
Leukocytes count (cells/uL)	6483 ± 1625	3625 ± 2092 [†]	6440 ± 1649
Platelets count ×10 ³	168 ± 55 [†]	136 ± 73 [†]	250 ± 38

Values are expressed as mean ± standard deviation.

* Significant p < 0.05 compared with controls.

[†] Significant p < 0.01 compared with controls.

[§] Significant p < 0.001 compared with controls.

Table 3. Lymphocytes, NK and NK subsets in IFN-untreated, IFN-treated and controls

	IFN-untreated n = 12	IFN-treated n = 12	Controls n = 10
Percentage of lymphocytes	32.1 ± 8.0	31.7 ± 7.8	40.7 ± 13.6
CD3 ⁺	35.6 ± 11.1	39.2 ± 11.7	42.8 ± 10.7
NK	43.7 ± 15.2	53.6 ± 14.4	44.3 ± 14
CD56 ^{bright}	5.4 ± 5.2	20.2 ± 9.9 [§]	3.3 ± 1.72
CD56 ^{dim} CD16 ⁺	84.4 ± 10.5	68.3 ± 10.7 [†]	80.9 ± 5.3
CD56 ^{dim} CD16 ⁻	5.9 ± 4.8	7.0 ± 3.1	7.0 ± 4.3
CD56 ⁻ CD16 ⁺	3.9 ± 3.2 [†]	4.2 ± 2.3 [†]	8.4 ± 2.7
Absolute counts of lymphocytes	2632 ± 413	1085 ± 559 [†]	2526 ± 784
CD3 ⁺	920 ± 269	623 ± 780 [†]	1096 ± 457
NK	392 ± 155	204 ± 76 [†]	471 ± 207
CD56 ^{bright}	19 ± 19	44 ± 29 [†]	13 ± 8
CD56 ^{dim} CD16 ⁺	335 ± 150	127 ± 64 [†]	388 ± 187
CD56 ^{dim} CD16 ⁻	21 ± 18	20 ± 22	27 ± 14
CD56 ⁻ CD16 ⁺	15 ± 12 [†]	7 ± 3 [§]	40 ± 23

CD3⁺ is expressed as % of gated lymphocytes, total NK is expressed as % of CD3⁺ and NK subsets are expressed as % of NK. The calculated absolute numbers are expressed as cells/μL. Values are expressed as M ± SD.

* Significant p < 0.05 compared with controls.

[†] Significant p < 0.01 compared with controls.

[§] Significant p < 0.001 compared with controls.

Figure 1: Flow cytometry analysis of NK cells and NK subsets in untreated chronic hepatitis C subject.
 a) Representative dot plots of NK and NK subsets in untreated chronic hepatitis C subject.
 b) Representative dot plots of NK and NK subsets in interferon-treated subject.
 c) Representative dot plots of NK and NK subsets in normal healthy subject.

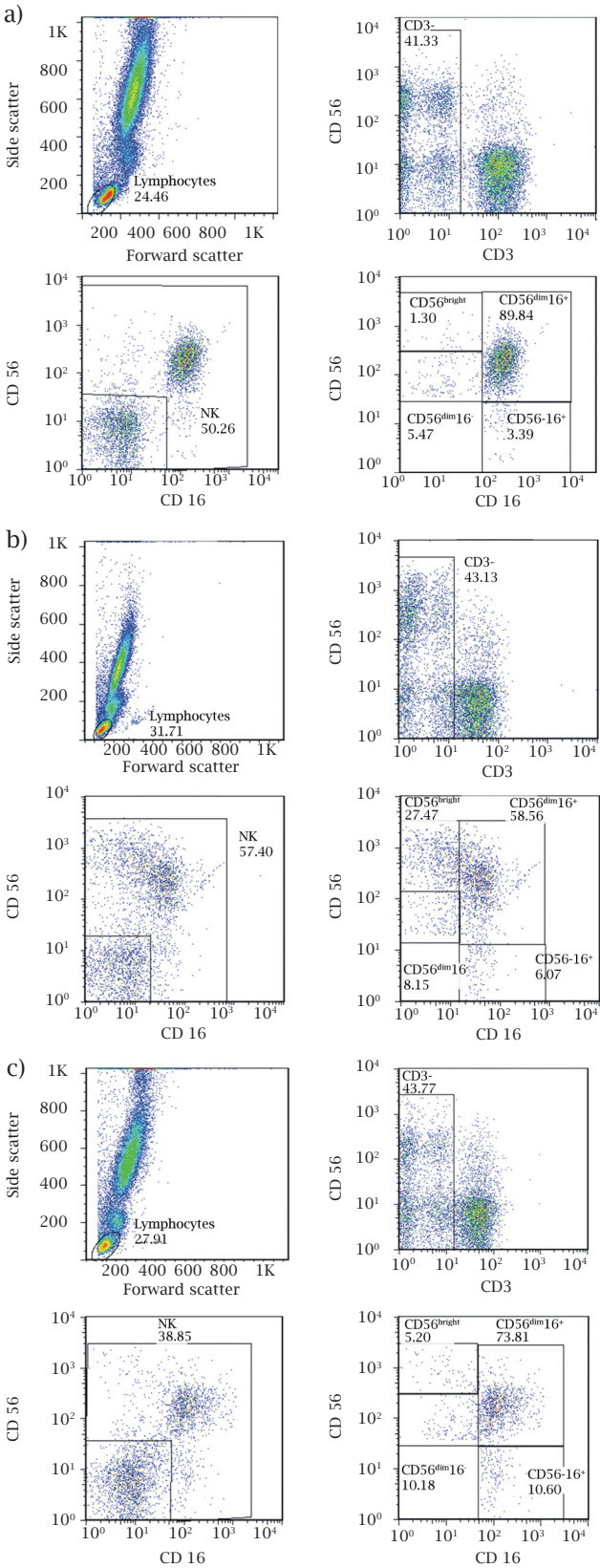
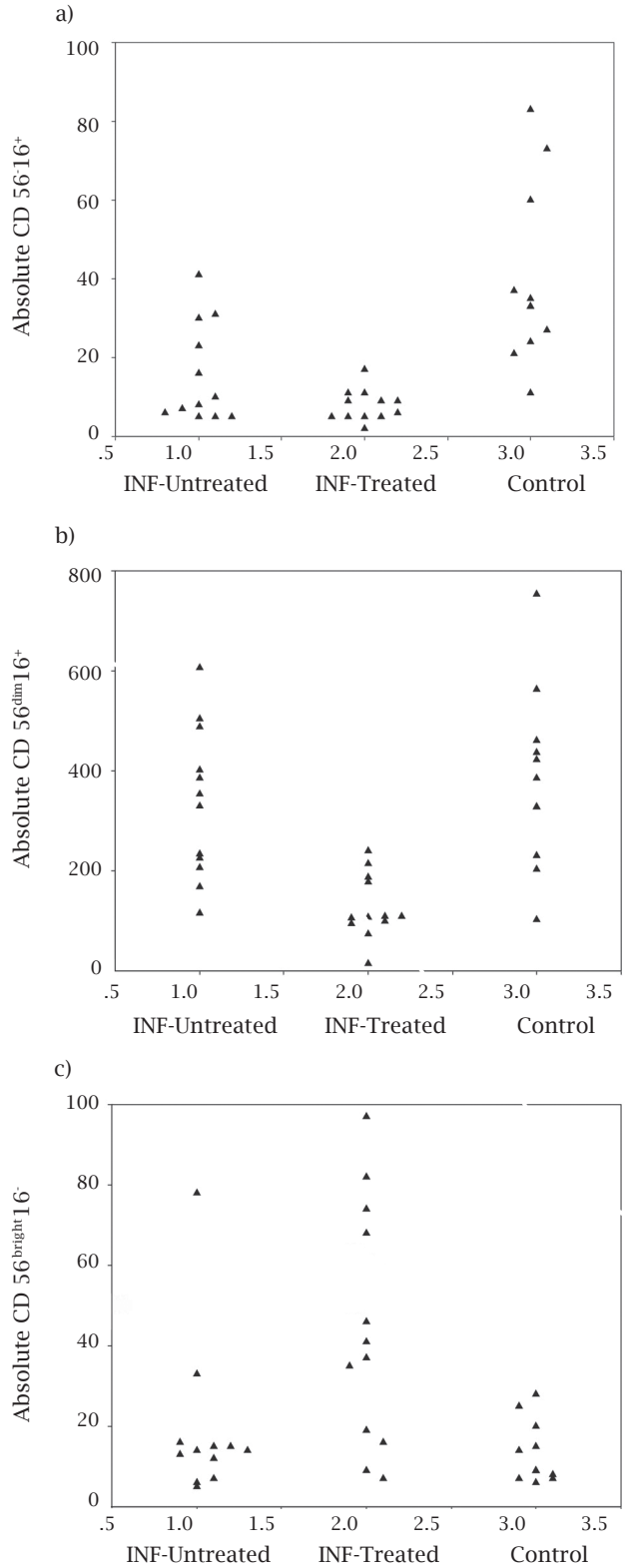


Figure 2: A scatter diagram compares the absolute counts of NK subsets in the studied groups
 a) Representative of absolute CD56⁺16⁻.
 b) Representative of absolute CD56^{dim}16⁺.
 c) Representative of absolute CD56^{bright}16⁻.



whereas no significant difference was detected in these subsets between interferon-untreated and treated patients (data not shown). Similarly, the proportion of total NK and NK subsets, as well as the absolute numbers of various NK cells in interferon responder patients versus non responders, did not show any significant difference (data not shown). The absolute numbers of total NK cells and both the relative and absolute numbers of CD56^{dim}-CD16⁺ cell subsets were decreased in interferon-treated patients when compared to interferon-untreated patients and controls (Figure 2b). Conversely, the relative and absolute counts of CD56^{bright} cell subsets were increased in interferon-treated patients when compared to interferon-untreated patients and controls (Figure 2c).

The relative and absolute numbers of CD3⁺ T cells and CD3⁺/CD56⁺ NKT in interferon-untreated, interferon-treated chronic HCV patients and healthy controls are presented in Table 4. There was a statistically significant decrease in the absolute counts of CD3⁺ T cells and CD3⁺/CD56⁺NKT of interferon-treated patients compared with interferon-untreated patients and controls.

No significant correlations between NK subsets and HCV viremia were seen. However, a significant positive correlation between CD56^{bright} counts and hepatic injury, based on HAI, was detected ($r = 0.63$, $p = 0.025$). A significant negative correlation between CD56^{dim}CD16⁺ counts and hepatic fibrosis was also detected ($r = -0.61$, $p = 0.03$).

DISCUSSION

NK cells constitute the first line of host defense against invading pathogens. They eliminate virus-infected cells by direct and indirect killing methods. Direct killing of target cells is achieved through the release of cytotoxic molecules, such as perforin and granzymes. Alternatively, apoptosis may be induced by over expression of Fas/FasL, TNF- α , and TNF-related apoptosis-inducing ligand (TRAIL)/death receptor4 (DR-4) and DR-5 interactions.¹⁹⁻²¹ Indirect killing

of target cells may also be achieved by secretion of soluble factors such as IFN- γ and TNF- α . In addition, NK cells play a role in inhibiting virus replication by the induction of an antiviral state in host cells.²² Furthermore, NK cells play important immunoregulatory roles through their interaction with T cells, B cells and antigen-presenting cells.

In this study, we found that CD3⁺CD56⁺CD16⁺ cell counts were decreased in HCV infected patients. In contrast, Gonzalez *et al.*²³ and Zarife *et al.*²⁴ reported increased frequency of CD3⁺CD56⁺CD16⁺ NK cells in patients with chronic HCV/HIV, or in blood donors with HCV among European and Brazilian populations, respectively. This difference may be attributed to either host or viral heterogeneity, as our study was done on Egyptian populations, in which HCV genotype 4 is the most prevalent genotype.²⁵

A significant reduction of circulating NK cells and expansion of CD56^{bright}, and reduction of CD56^{dim}CD16⁺ subsets were seen in patients with chronic HCV after interferon treatment. These results agreed with the data reported by Gonzalez *et al.*,²³ who indicated that the CD56^{bright} immunoregulatory NK cells subset temporarily expanded in response to interferon treatment. This also agreed with the data reported by Saraste *et al.*,²⁶ who observed an expansion of CD56^{bright} NK cells with a concomitant decrease of CD56^{dim} cells in multiple sclerosis patients after 12 months of treatment with IFN- β . In contrast to our findings, Meier *et al.*¹⁷ and Golden-Mason *et al.*²⁷ reported a significant reduction of total NK cells count and a striking shift in NK subsets, with a marked decrease in the CD56^{dim} cell fraction compared to CD56^{bright} cells in HCV infection. This can be explained by the observation that Meier *et al.*¹⁷ did not exclude patients with history of interferon therapy and included patients who were not on current or recent (within the last six months) IFN- α treatment.

CD56^{bright} NK cells are very likely precursor cells of the CD56^{dim} subset. Indeed, CD56^{dim} NK cells display shorter telomeres than CD56^{bright} NK cells from peripheral blood, which implies that the latter are less mature than the former.

Table 4. CD3⁺T cells and CD3⁺/CD56⁺ NKT in IFN-untreated, IFN-treated and controls

	IFN-untreated n = 12	IFN-treated n = 12	Controls n = 10
CD3 ⁺ T%	53.4 \pm 8.9	51.8 \pm 12.5	48.4 \pm 9.1
CD3 ⁺ /CD56 ⁺ NKT %	9.4 \pm 6.1	8.6 \pm 3.6	8.5 \pm 3.7
Absolute counts of CD3 ⁺ T	1418 \pm 364	584 \pm 405 [†]	1208 \pm 407
CD3 ⁺ /CD56 ⁺ NKT	258 \pm 198	102 \pm 101*	212 \pm 119

* Significant $p < 0.05$ compared with untreated patients and controls.

† Significant $p < 0.01$ compared with untreated patients and controls.

Expansion of CD56^{bright} subsets was reported in patients who are treated daily with a low dose of IL-2.²⁷ Since the mechanism of interferon-induced NK cells shift is not fully understood, we suggest that interferon may influence the differentiation of CD56^{bright} to CD56^{dim}. On the other hand, the CD56^{dim}16⁻ subset was not altered either by HCV infection or interferon therapy; the main function of this subset is largely unknown.

In this study, no significant difference in total NK cells and NK subsets among interferon responders were seen when compared to non-responders. Different results were reported by Panasiuk *et al.*,²⁸ who found a slight increase in NK cell counts in patients who did not clear HCV. Also, Khakoo *et al.*,²⁹ showed that genes encoding the NK cell receptor directly influence resolution of HCV infection. However, in our study, we did not assess the function of NK cells, as the immunodeficient NK cells may not be able to kill virus-infected cells. Further studies in this area, to correlate NK function with interferon response, are required.

In this study, a significant positive correlation between the relative counts of CD56^{bright} subsets with HAI was found. Lin *et al.*,³⁰ reported that increasing activated bright NK cells and increasing activated apoptotic bright NK cells were both significantly associated with increasing hepatic necro-inflammatory grade. This finding may be explained by the fact that CD56^{bright} subsets have immunoregulatory functions and produce potent pro-inflammatory cytokines, like INF- γ and TNF- α .³¹ Absence of correlation between NK or its subsets with HCV viral load agrees with similar data reported by Pernollet *et al.*,³² who found that NK populations did not correlate with any biochemical or viral parameters.

In conclusion, this study has shown that, in Egyptian patients, HCV infection is associated with reduction of CD56⁻16⁺ NK subsets, and interferon-alpha therapy is associated with expansion of CD56^{bright} and reduction of CD56^{dim}16⁺. Further studies are required to delineate the molecular basis of interferon-induced shift of NK subsets among patients with HCV. As this study was conducted on two distinct groups of chronic HCV patients before and after interferon treatment, a further longitudinal study is recommended to address the limitations which are inherent in cross sectional studies of this type.

ACKNOWLEDGEMENTS

We thank Dr Omar Fathy for technical assistance.

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