# Natural killer cell populations in Egyptians infected with hepatitis C virus

*M. Rafik,[1] G. Sidhom,[2] R. Mamdouh,[1] D. Ellebedy[2] and M. Mohamed[2]*

مجموعات الخلايا الفاتكة الطبيعية لدى المصريين المصابين بالتهاب الكبد الفيروسي "سي"

منى رفيق، جلوريا سيدهم، رشا ممدوح، داليا اللبيدى، مى محمد

الخلاصــة: تؤدي الخلايا الفاتكة الطبيعية دوراً رئيسياً في الاستجابة المناعية للفيروسات. وتتحرّى هذه الدراسة أثر التهاب الكبد الفيروسي "سي" على تكرار الخلايا الفاتكة الطبيعية وعلى مجموعاتها الفرعية لدى الأشخاص المصابين بحصائل سريرية مختلفة؛ وشملت الدراسة 20 شخصاً إيجابياً لمضادات فيروس التهاب الكبد "سي" ولرنا فيروس التهاب الكبد "سي" (التهاب الكبد المزمن بالفيروس "سي") و20 شخصاً إيجابياً لمضادات فيروس التهاب الكبد "سي" ولكنهم سلبيون لرنا فيروس التهاب الكبد سي (شفاء تلقائي)، و20 من الشواهد الأصحاء الخالين من فيروس التهاب الكبد "سي" وكان هناك نقص ملحوظ في تواتر مجمل الخلايا الفاتكة الطبيعية لدى مجموعة المرضى المزمنين مقارنة بمجموعة الشواهد (0.001 = P) أو بمجموعة الذين شفوا من التهاب الكبد "سي" (0.01 = P). واتضح أيضاً أن النسبة المئوية للخلايا CD56 (المشرقة) كان أعلى بقدر يعتد به (0.04 = P) مما هو لدى مجموعة الشواهد، وأن النسب المئوية للخلايا CD56 (القاتمة) وتعبيرها CD16 أخفض لدى مجموعة المرضى المزمنين دون أن يكون لذلك اعتداد إحصائي. كما كان تكرار الخلايا T –CD56+ CD3 أخفض بمقدار يُعْتَدُّ به لدى كل من مجموعتَيْ المرضى المزمنين والذين شفوا من التهاب الكبد "سي" بالمقارنة مع المجموعة الشاهدة (0.04 = P). ويرى الباحثون أن النتائج التي حصلوا عليها تؤكّد الدور المحتمل للخلايا الفاتكة الطبيعية ومجموعاتها الفرعية المختلفة في إمراض العدوى بفيروس التهاب الكبد "سي".

ABSTRACT Natural killer (NK) cells are key players in the immune response to viruses. This study examined the effect of hepatitis C virus (HCV) on the frequency of NK cells and their subsets in individuals with different clinical outcomes; 20 positive for anti-HCV and HCV-RNA (chronic hepatitis C), 20 positive for anti-HCV but negative for HCV-RNA (spontaneously resolved) and 20 healthy controls free of HCV. There was a significant reduction in the frequency of total NK cells in the chronic group compared to the control ($P = 0.001$) or resolved ($P = 0.01$) groups. The percentage of CD56(bright) cells was significantly higher than the control group ($P = 0.04$). While the percentages of CD56 (dim) cells and their CD16 expression were lower in the chronic group, this was not statistically significant. The frequency of CD3+CD56– T cells was significantly lower in both the chronic and resolved groups compared to the control group ($P = 0.04$). Our results confirm a potential role of NK cells and the different subsets in the pathogenesis of chronic HCV infection.

**Populations de cellules tueuses naturelles chez des Égyptiens infectés par le virus de l'hépatite C**

RÉSUMÉ Les cellules tueuses naturelles jouent un rôle majeur dans la réponse immunitaire aux virus. La présente étude a examiné l'effet du virus de l'hépatite C (VHC) sur la fréquence des cellules tueuses naturelles et de leurs sous-groupes chez des personnes présentant des issues cliniques différentes : 20 patients positifs pour les anticorps anti-VHC et pour son ARN (hépatite C chronique) ; 20 personnes positives pour les anticorps anti-VHC mais négatives pour son ARN (résolution spontanée) ; et 20 témoins en bonne santé ne présentant pas d'infection par le virus de l'hépatite C. Une réduction importante de la fréquence des cellules tueuses naturelles totales dans le groupe des patients porteurs d'une infection chronique a été observée par rapport au groupe des témoins ($P = 0,001$) ou au groupe des patients dont l'infection a connu une résolution spontanée ($P = 0,01$). Le pourcentage des cellules CD56 (bright) était significativement supérieur ($P = 0,04$) à celui du groupe témoin. Si le pourcentage des cellules CD56 (dim) et de leur expression de CD16 était inférieur dans le groupe de patients infectés chroniquement, la différence n'était pas statistiquement significative. La fréquence des cellules T CD3+CD56 était très inférieure à la fois dans le groupe de patients infectés chroniquement et dans le groupe ayant bénéficié d'une résolution spontanée par rapport au groupe témoin ($P = 0,04$). Nos résultats confirment le rôle potentiel des cellules tueuses naturelles et de ses différents sous-groupes dans la pathogénèse de l'infection chronique par le virus de l'hépatite C.

[1]Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt. [2]Clinical and Chemical Pathology Department, Medical Division, National Research Center, Cairo, Egypt (Correspondence to G. Sidhom: gloriasidhom@yahoo.com).

## Introduction

Hepatitis C virus (HCV) infection has the ability to establish chronic infection in approximately 75%–80% of cases [1–4]. The elimination or persistence of the infection depends on a balance between the immune response and the virus replication rate [5] and play a crucial role in the pathogenesis of chronic hepatitis C [6].

Natural killer (NK) cells are potent antiviral cells which eliminate the virus through direct killing and cytokine production, and are the key players in immune responses to viruses [7]. Like many other viruses, HCV has evolved strategies to evade detection and elimination by NK cells and these have been proposed as a mechanism of virus persistence and chronic infection [8].

According to CD56 expression, NK cells are divided into 2 subsets, dim and bright [9]. Dim NK cells are mainly cytolytic and comprise more than 90% of NK cells in peripheral blood, whereas bright NK cells are immune-regulatory mainly through cytokine production, and display homing receptors required for migration to 2ry lymph nodes [10,11].

CD56 together with natural T (NT) cells comprise about 5%–15% of the peripheral T cell pool and up to 50% of T cells within the liver microenvironment, which is the primary site of HCV replication [12]. These cells have been reported to be impaired in the blood and/or liver of chronic HCV patients [12–16].

HCV may evade the immune response either by inhibition of NK cell activation receptor genes or by decreasing the activity of these cells by reducing their number and function in chronically infected individuals [17,18]. Also, the inheritance of particular inhibitory killer immunoglobulin-like receptors genes, which control NK cell activity, may play a role in predisposing to chronicity [19].

Recently, altered NK cell frequency has been suggested to contribute to the impaired cellular immune response and virus persistence [8]. Some T cells are known to express NK cells receptors (NKRs) and mediate functions of both T cells and NK cells [13]. Studies suggest that HCV inhibits receptor genes in the activation of NK cells [20], and the phenotypes and/or functional activities of various populations of NKR+ T cells have been reported to be impaired in chronic hepatitis C patients [16,21].

In order to study the possible role that NK cells might play in the pathogenesis of the different clinical outcomes of HCV infection in our population, we aimed in this study to assess the impact of HCV on the frequency and subset distribution of circulating NK cells, including CD56 dim and bright populations and CD56 + NT cells in different clinical outcomes of HCV infection.

## Methods

### Participants

The study included 60 participants: 40 health care workers (HCWs), who were a part of a screening programme for HCV infection among HCWs at the internal medicine hospital of Ain-Shams University, and 20 age- and sex-matched healthy volunteers recruited from the same hospital, who were negative for both HCV antibody and HCV RNA, who served as a control group. Exclusion criteria for all groups included interferon or ribavirin therapy and dialysis. Written informed consent was obtained from all the participants and the study was approved by the ethical committee of the National Research Centre in Cairo, Egypt.

### Methodology

All participants were screened for HCV antibodies (HCV-Ab) by a 3rd generation enzyme-linked immunosorbent assay (Diasorin SPA, Italy) and HCV-RNA by qualitative real-time polymerase chain reaction (Stratagene Mx3000P). The percentage of total circulating NK cells, NK subsets (including both CD56 dim and bright, with their CD16 expression), total T cells and CD56+NT cells was characterized by flow cytometry using anti CD3, CD56 and CD16 monoclonal antibodies.

For flow cytometric analysis, 3 mL whole blood were withdrawn from each participant on ethylenediaminetetraacetic acid (EDTA), and layered over Ficoll-Hypaque (density 1.077 g/L) (Amersham Biosciences) and centrifuged at 2000× g for 20 min at 24 °C. The mononuclear layer was carefully separated, washed 3 times with phosphate buffer saline (PBS), and the cell pellet was resuspended in 1 mL FACS buffer (500 mL PBS + 5 mL pooled human serum + 5 mL fetal bovine serum + 1 mL sodium azide). Then, the mononuclear layer was stained with CD16-FITC, CD56-PE and CD3-PE-Cy5 monoclonal antibodies (Sigma), according to the manufacturer's instructions. Briefly, 5 µL of each monoclonal antibody were added to 100 µL of the resuspended cells and incubated for 20 min at 4 °C in the dark. Then 1 mL of FACS buffer was added followed by centrifugation for 5 min at 1500× g. Finally, 500 µL of supernatant were discarded and samples were ready for processing on the flow cytometer.

Flow cytometric analysis was done using the EPICS-XL Coulter (Coulter Diagnostics, Hialeah, Florida, United States of America), with standard filter setting using 488 nm wavelength laser excitation. The following parameters were used: linear forward scatter, side scatter (log), log fluorescence 1 for detection of CD16-FITC, log fluorescence 2 for detection of CD56-PE and log fluorescence 4 for CD3-PE-Cy5.

### Statistical analysis

Data were analysed using *SPSS*, version 15.0. Qualitative data are presented as frequency and percentage, while

quantitative data are presented as mean, standard deviation (SD) and range. The chi-squared test was used to compare qualitative variables, and the Student *t*-test was used to compare quantitative variables. A *P*-value < 0.05 was considered statistically significant; *P* < 0.01 was considered highly significant.

## Results

According to their HCV-Abs and HCV-RNA results, our patients were divided into a "spontaneously resolved patient group" which included 20 patients who were positive for anti-HCV and negative for HCV-RNA, and a "chronic hepatitis C patient group" included 20 patients who were positive for both anti-HCV and HCV-RNA. The mean percentage of the cell populations in the different groups are shown and compared in Table 1.

On comparing the percentage of different cell populations in the different groups, the percentage of the total circulating NK cell population was significantly lower in chronic hepatitis C patients [mean 3.3 (SD 1.9)] compared to either the spontaneously resolved group [9.3 (SD 6.3)] (*P* = 0.01) or the healthy control group [9.5 (SD 4.5)] (*P* = 0.001) (Table 1).

As regards NK subsets, the CD56 (bright) NK cell population was significantly higher in chronic hepatitis C patients than in the healthy control group [mean 12.2 (SD 7.1) vs 5.9 (SD 5.5), *P* = 0.04]. In the spontaneously resolved group the CD56 (bright) NK cell population was also higher but this was not statistically significant [mean 12.2 (SD 7.1) vs 10.6 (SD 9.40), *P* = 0.4]. Although the percentage of CD56 (dim) NK cells was lower in chronic hepatitis C patients than in the control group, this was not was statistically significant (*P* = 0.05) (Table 1). CD16 expression was lower on CD56 (dim) NK cells of chronic hepatitis C patients than healthy controls or spontaneously resolved patients but not significantly so (*P* = 0.13 and *P* = 0.2 respectively).

The percentage of non-CD56 expressing T cells (CD3+CD56–T) was significantly lower in both chronic hepatitis C patients and spontaneously resolved patients compared to healthy controls (*P* = 0.04). The percentage of CD56+ NT cells showed no statistically significant difference between the different groups.

## Discussion

In our study, we examined the frequency of circulating NK cell populations (total and subsets) as well as total T cells and CD56+NT cells in peripheral blood of both chronic hepatitis C patients and spontaneous resolvers compared to a control group of healthy volunteers using flow cytometry.

Our results revealed that the total circulating NK cells were highly significantly different in the different studied groups. A significant reduction in the frequency of circulating total NK cells in chronic hepatitis C patients compared to either healthy controls or spontaneous resolvers was found. Total NK cell frequency and/or cytotoxic activity have been reported to be low in chronically infected patients in several studies [8,22].

As regards NK cell subsets, the percentage of CD56(bright) NK cells was significantly higher in chronic hepatitis C patients than in the resolved or control groups, while the percentage of CD56(dim) NK cells was lower in chronic hepatitis C patients than in the other groups. This concurs with several studies [8,16,23,24]; Morishima et al. found that the percentage of CD56(dim) cytolytic NK cells was 30% lower in HCV-infected patients compared with uninfected subjects and this was paralleled by a decrease in circulating NK cells [16]. Meier et al. suggested that the deficit in interleukin 15 (IL-15) might be responsible for

**Table 1 Frequency (%) of the different cell populations in different hepatitis C virus (HCV) infection outcomes**

| Cell population | Controls | Chronic hepatitis C[a] | Spontaneously resolved[b] | *P*-value (control vs chronic) | *P*-value (control vs resolved) | *P*-value (chronic vs resolved) |
|---|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | Mean (SD) | | | |
| Total T cells | 64.1 (10.4) | 50.2 (16.9) | 58.6 (10.9) | 0.08 | 0.12 | 0.3 |
| CD56+NT | 3.3 (1.3) | 4.7 (4.8) | 4.7 (3.5) | 0.8 | 0.4 | 0.7 |
| CD3+CD56–T | 60.8 (10.7) | 45.5 (15.8) | 53.9 (12.2) | 0.04 | 0.04 | 0.2 |
| Total NK | 9.5 (4.5) | 3.3 (1.9) | 9.3 (6.3) | 0.001 | 0.5 | 0.01 |
| NK(dim) | 94.1 (5.5) | 87.8 (9.6) | 89.4 (9.4) | 0.05 | 0.1 | 0.3 |
| NK(dim) CD16+ | 92.2 (6.2) | 86.0 (8.9) | 90.7 (6.03) | 0.13 | 0.5 | 0.2 |
| NK(bright) | 5.9 (5.5) | 12.2 (7.1) | 10.6 (9.4) | 0.04 | 0.1 | 0.4 |
| NK(bright) CD16+ | 38.9 (13.3) | 41.1 (9.5) | 47.9 (18.1) | 0.6 | 0.14 | 0.5 |

[a]*Positive for both anti-HCV and HCV-RNA.*
[b]*Positive for anti-HCV and negative for HCV-RNA.*
*SD = standard deviation; NT = natural T cells; NK = natural killer cells.*

this change [25]. IL-15 is thought to regulate the function of NK cells and to promote their survival *ex vivo* [26], and abnormalities in the production of this cytokine contribute to a reduction in NK cell number and a decrease in the CD56(dim) population in HCV infection.

The frequency of the CD56(dim)16+ subset was low in our chronic hepatitis C patients compared to the resolved or healthy control groups, which is in agreement with Dessouki et al. [8], suggesting the expansion of NK cells is to produce interferon-gamma, while dim cells decrease to minimize cytotoxicity and limit liver damage [27].

In agreement with Corado et al. [28], there was no statistically significant difference in the percentage of total T cells between the different studied groups. However, the percentage of CD3+CD56– cells was significantly lower in chronic hepatitis C patients and spontaneous resolvers than in healthy controls. This might be due to a decrease in other T cell populations including CD4+ and/or CD8+ T cells [29].

Collectively, the results of our study confirm a potential role of NK cells and the different subsets in chronic HCV infection. More focused research on this cell population with its different stimulatory, co-stimulatory and inhibitory receptors might answer questions concerning the pathogenesis of chronicity in HCV infection. This knowledge could help in the design of more effective therapeutic approaches for HCV infection and contribute to the improvement of antiviral therapy and the eradication of the virus.

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***Prevention and control of viral hepatitis infection: framework for global action***

Around 500 000 000 people are chronically infected with hepatitis B virus (HBV) or hepatitis C virus (HCV). Approximately 1 000 000 people die each year (about 2.7% of all deaths) from causes related to viral hepatitis, most commonly liver disease, including liver cancer. An estimated 57% of cases of liver cirrhosis and 78% of cases of primary liver cancer result from HBV or HCV infection.

Despite affecting such a huge number of people worldwide, these diseases remain largely unknown, undiagnosed and untreated. WHO's *Prevention and control of viral hepatitis infection: framework for global action* describes the 4 areas of work to prevent, treat and save the lives of people infected with hepatitis through targeted regional and country-specific strategies. The 4 strategic axes are: i) Raising awareness, promoting partnerships, and mobilizing resources; ii) Evidence-based policy and data for action; iii) Prevention of transmission; and iv) Screening, care and treatment.

Further information about this document and the Global Hepatitis Programmes is available at: http://www.who.int/topics/hepatitis/en/