Diminished Absolute Counts of CD56^{dim} and CD56^{bright} Natural Killer Cells in Peripheral Blood from Egyptian Patients with Hepatocellular Carcinoma

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Natural killer cells (NK) as components of the innate immunity substantially contribute to anti-tumor immune responses. NK cell subpopulations can be defined on the basis of the relative expression of CD16 and CD56 markers. Earlier research demonstrated a dramatic reduction in the frequency of peripheral blood CD56^{dim}CD16^{+} NK subsets in hepatocellular carcinoma (HCC) patients compared with healthy subjects. We aim to assess the relative and absolute counts of natural killer cells subsets in hepatitis C-related HCC among Egyptian patients. Flowcytometric analysis of peripheral blood NK subsets was performed for HCV with HCC patients (n=20) and HCV without HCC patients (n=14) as compared to healthy control subjects (n=15). We found that HCC patients displayed a marked reduction in the relative frequency of peripheral CD56^{dim} subsets compared with healthy subjects. Moreover, there was a significant reduction in the absolute counts of CD56^{dim}^{+}, CD56^{dim}^{-} and CD56^{bright}. In conclusion, this study demonstrated that the absolute counts of dim and bright NK cell subsets were decreased in different proportions in patients with HCV-related HCC that refers to a possible role for these cells, particularly CD 56^{bright} cells, in the immune response to HCC. This might aid in developing new therapeutic strategies targeting both NK subsets for HCC.

Natural killer (NK) cells are a major component of innate lymphocytes that play a critical role in innate resistance against tumors (Smyth et al., 2002). Upon activation, NK cells exocytose cytotoxic granules containing perforin and various granzymes leading to perforation of the target cells and subsequent apoptotic death (Lieberman, 2003). Besides their killing ability, it has become clear in recent years that NK cells can assist in T-cell polarization, dendritic cell (DC) maturation, and innate immune control by their ability to secrete immunomodulatory cytokines such as interferon-γ (IFN-γ) (Strowig et al., 2008).

About 5 to 20% of peripheral blood lymphocytes comprise NK cells, whereas intrahepatic lymphocytes comprise about 30 to 50% NK cells (Gao et al., 2008). NK cells are usually defined as lymphocytes that are negative for CD3 and positive for CD56, the 140-kDa isoform of neural cell adhesion molecule (Kita et al., 2001). In human peripheral blood, five NK cell subpopulations can be defined on the basis of the relative expression of CD16 and CD56 markers (Caligiuri, 2008). These include; CD56^{bright} CD16^{−} cells, CD56^{bright} CD16^{+} cells, CD56^{dim} CD16^{−} cells, CD56^{dim} CD16^{+} cells and CD56^{bright} CD16^{−} cells. The CD56^{dim} NK cell subset kills target cells upon proper recognition and secretes only low levels of IFN-γ. In contrast, CD56^{bright} NK cell subset upon stimulation with pro-inflammatory cytokines produces large amounts of cytokines, including IFN-γ, necrosis factor (TNF) and granulocyte macrophage-colony stimulating factor (GM-CSF), however, such cells acquire...
cytotoxicity only after prolonged activation (Cooper et al., 2001a & b). CD56\textsuperscript{bright} NK subset is more abundant in the human liver than in peripheral blood. The CD56\textsuperscript{bright} NK subset was also shown to be markedly enriched in secondary lymphoid organs, accounting for up to 75% of NK cells in lymph nodes (LN) and 50% in the spleen (Caligiuri, 2008). This abundance may result from the specific expression of secondary lymphoid organ homing markers such as CC-chemokine receptor 7 (CCR7), CD62 ligand (CD62L) and CX-chemokine receptor 3 (CXCR3) on the CD56\textsuperscript{bright} NK cell subset (Campbell et al., 2001).

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Despite recent advances in new therapeutic modalities, a significant number of HCC patients show frequent recurrence and progress to an advanced stage with the development of significant resistance to conventional chemotherapy. This opens the door for immuno-modulatory approaches to be additive therapeutic modalities in the future (Llovet et al., 2003). In this regard, Cai et al. (2008) studied NK subsets in HCC patients and found a dramatic reduction in peripheral CD56\textsuperscript{dim}CD16\textsuperscript{+} NK subsets compared with healthy subjects. They suggested that decrease in NK cells might be responsible for the failure of anti-tumor immune responses. Similarly, we aim to assess the relative and absolute counts of natural killer cells subsets in hepatitis C related HCC Egyptian patients.

**Subjects and Methods**

Subjects in this study were categorized into three groups; group (1) included 20 patients with HCV-related HCC, group (2) included 14 patients with HCV without HCC and group (3) included 15 healthy volunteers not suffering any acute or chronic illness. Before starting this study a written or verbal consent was taken from each patient to participate in this study or to withdraw whenever they want. Patients were subjected to thorough medical history taking and physical examination as well as abdominal ultrasonography. Routine laboratory investigations including complete blood count and liver function tests (ALT, AST, total bilirubin, direct bilirubin, serum albumin, alkaline phosphatase and alpha fetoprotein). The diagnosis of HCC was based on ultrasound-guided biopsy and histopathology. Control subjects were age- and sex-matched to patients and had normal ultrasonography and normal liver function tests including normal alpha fetoprotein with no evidence of liver diseases.

Study patients did not receive any anti-tumor or anti-viral therapy. We excluded from this study patients with auto-immune hepatitis as indicated by specific tests (antinuclear antibodies (Abs), antismooth muscle Abs, antimitochondrial Abs).

Five ml of venous blood samples were withdrawn from each subject, and divided into tubes with or without EDTA. Serum was collected following the centrifugation of coagulated blood and was stored at -20°C until use.

Alpha-fetoprotein was assessed by enzyme linked fluorescent assay (ELFA) using VIDAS autoanalyzer, according to the manufacture’s instructions (Biomerieux, France).

Viral markers: HCV, HIV antibodies were assessed by microparticle enzyme immunoassay (MEIA, Abbott Laboratories,) and HB\textsubscript{Ag} by chemiluminescent immunoassay (Elecys1010, Roche Diagnostic GmbH) according to the manufacture’s instructions.

Flowcytometry analysis: Natural killer cell subsets were assessed by flowcytometry using FACSCalibur (BD Bio-sciences, San José, CA, USA). Allophycocyanin (APC)-labeled anti-CD3, fluorescein isothiocyanate (FITC)-labeled anti-CD16 and phycoerythrin (PE)-labeled anti-CD56 antibodies were purchased from IQ Product (Groningen, Netherlands). A sample of 20µl from each monoclonal reagent was added to 100 µl of whole EDTA blood and incubated at room temperature for 15 min in the dark. Lyses red blood cells was performed using FACS Lyse reagent (BD, USA) followed by centrifugation at 2000 rpm for 5 min. The supernatant was discarded and the cells were washed twice with phosphate-buffered saline (PBS) and then suspended in 300 µl of PBS. Data were analyzed using CELL Quest software. The typical forwards and side scatters were used for lymphocytes gating. NK cells were sub-grouped into CD56\textsuperscript{bright} or CD56\textsuperscript{dim} cells according to the level of CD56 expression and further defined by CD16. Calibration of the instrument settings were performed with
CaliBRITETM 3 Beads (BD Biosciences, San Jose, CA).

Statistical Analysis
Statistical analysis was done by using SPSS (Statistical package for Social Science) program version 11. Data were presented as mean ± standard deviation. Values of \( P < 0.05 \) were considered as significant.

Results
In group 1 (HCV with HCC) age ranged between 45 and 65 (53±5.9) years, 16 patients were males and 4 were females. Serum levels of \( \alpha \)-fetoprotein detected were >400 IU/ml in 17 patients, ranged between 200-400 in one patient and <200 in two patients. The majority of HCC patients had a high level of alpha-fetoprotein.

HCV without HCC group included 14 patients, their ages ranged between 42 and 54 years (47±3), 11 patients were males and 3 were females, their serum of alpha-fetoprotein ranged between 5 and 31 IU/ml (10±8) (data not shown). There was no statistically significant differences in the total leucocytes counts of the HCV with HCC patients and HCV without HCC patients as compared to controls (\( P > 0.05 \)), meanwhile a statistical significant difference (\( P < 0.05 \)) in the levels of ALT, AST, alkaline phosphates and platelets counts were detected (Table 1).

<table>
<thead>
<tr>
<th>Laboratory Parameters</th>
<th>HCV with HCC (n=20)</th>
<th>HCV without HCC (n=14)</th>
<th>Controls (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (u/l)</td>
<td>101±100</td>
<td>69±34</td>
<td>25±5</td>
</tr>
<tr>
<td>AST (u/l)</td>
<td>172±200</td>
<td>46±16</td>
<td>19±5</td>
</tr>
<tr>
<td>Alkaline phosphatase (u/l)</td>
<td>125±55</td>
<td>121±64</td>
<td>78±15</td>
</tr>
<tr>
<td>T-bilirubin (mg/dl)</td>
<td>4.1±6.0</td>
<td>1.2±1.8</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>D-bilirubin (mg/dl)</td>
<td>2.6±4.0</td>
<td>0.4±0.3</td>
<td>0.16±0.15</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>2.7±.4</td>
<td>4.4±0.3</td>
<td>4.3±3</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>10.3±1.9</td>
<td>14.0±0.7</td>
<td>13.5±1</td>
</tr>
<tr>
<td>Leukocytes count (cells/µl)</td>
<td>8800±7000</td>
<td>6328±1558</td>
<td>6700±1500</td>
</tr>
<tr>
<td>Platelet count x 10^3</td>
<td>139±74</td>
<td>171±53</td>
<td>240±35</td>
</tr>
</tbody>
</table>

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.
Natural Killer Subsets in HCV with HCC versus HCV without HCC and Controls

We observed that the percentages of total NK cells, CD56\textsuperscript{dim} cells and CD56\textsuperscript{dim}16\textsuperscript{+} were significantly lower in HCV with HCC patients compared with controls (Table 2). Figures 1 & 2 show FACS scan scatter plots for the frequency of total NK and NK subsets for HCV with HCC patients and a control subject, respectively. Meanwhile the percentages of CD56\textsuperscript{bright} did not show a statistical significant difference when compared with controls. The absolute numbers of total NK cells, CD56\textsuperscript{dim} cells, CD56\textsuperscript{dim}16\textsuperscript{+} cells, CD56\textsuperscript{dim}16\textsuperscript{−} cells and CD56\textsuperscript{bright} cells were significantly lower in HCV with HCC patients when compared to controls.

Relative and absolute counts of total NK cells & NK subsets in HCV with HCC patients versus HCV without HCC patients are presented in Table 2. There was no statistically significant differences in the relative counts of total NK cells and various NK subsets between the two groups, meanwhile the absolute counts of total NK cells and NK subsets were significantly lower in HCV with HCC patients compared with HCV without HCC patients.

Absolute Counts of NK and NK Subsets in HCV Patients and Controls

There was no statistical significant difference in the absolute counts of NK and both bright and dim subsets in HCV patients compared with normal controls. Figure 3 summarizes the results of absolute counts of NK & NK subsets.

Table 2. Natural Killer dim and Bright Subsets in the Studied Groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>HCV with HCC</th>
<th>HCV without HCC</th>
<th>Controls</th>
<th>P*-value</th>
<th>P§-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes%</td>
<td>17.9±5.8</td>
<td>42.5±7.4</td>
<td>39±11</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Natural killer%</td>
<td>12.3±5.9</td>
<td>13.7±5.4</td>
<td>17.7±4.9</td>
<td>0.006</td>
<td>.493</td>
</tr>
<tr>
<td>CD56\textsuperscript{bright}%</td>
<td>0.66±.40</td>
<td>0.79±.39</td>
<td>0.73±.31</td>
<td>0.567</td>
<td>0.352</td>
</tr>
<tr>
<td>CD56\textsuperscript{dim}%</td>
<td>10.5±5.2</td>
<td>12.5±5.4</td>
<td>16.7±5.0</td>
<td>0.001</td>
<td>0.292</td>
</tr>
<tr>
<td>CD56\textsuperscript{dim}16\textsuperscript{−}%</td>
<td>8.9±4.8</td>
<td>10.3±5.4</td>
<td>14.4±4.8</td>
<td>0.004</td>
<td>0.430</td>
</tr>
<tr>
<td>CD56\textsuperscript{dim}16\textsuperscript{−} %</td>
<td>1.5±1.8</td>
<td>2.1±1.2</td>
<td>2.5±1.5</td>
<td>0.113</td>
<td>0.310</td>
</tr>
<tr>
<td>Absolute lymphocytes</td>
<td>1564±1287</td>
<td>2605±403</td>
<td>2486±691</td>
<td>0.011</td>
<td>0.002</td>
</tr>
<tr>
<td>Absolute NK</td>
<td>186±205</td>
<td>358±157</td>
<td>437±145</td>
<td>0.001</td>
<td>0.010</td>
</tr>
<tr>
<td>AbsoluteCD56\textsuperscript{bright}</td>
<td>10±10</td>
<td>21±13</td>
<td>18±8</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>Absolute CD56\textsuperscript{dim}</td>
<td>158±169</td>
<td>325±147</td>
<td>415±143</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Absolute CD56\textsuperscript{dim}16\textsuperscript{+}</td>
<td>141±166</td>
<td>268±145</td>
<td>352±136</td>
<td>0.001</td>
<td>0.024</td>
</tr>
<tr>
<td>Absolute CD56\textsuperscript{dim}16\textsuperscript{−}</td>
<td>17±17</td>
<td>57±40</td>
<td>59±29</td>
<td>0.001</td>
<td>0.003</td>
</tr>
</tbody>
</table>

NK & NK subsets are expressed as % of lymphocytes and absolute numbers as cells/µl.

* Student t test for HCV with HCC versus controls.
§Student t test for HCV with HCC versus HCV without HCC.
Figure 1. A FACS scan scatter plot illustrates the frequency of NK and NK subsets for a patient with HCV with HCC. a) Lymphocytes gating. b) NK cells (CD56^+CD3^-). c) CD56^{bright} & CD56^{dim}. d) CD56^{dim}CD16 & CD56^{dim}CD16.
Figure 2. A FACS scan scatter plot illustrates the frequency of NK and NK subsets for a control subject. a) Lymphocytes gating. b) NK cells (CD56$^+$CD3$^-$). c) CD56$^{bright}$ & CD56$^{dim}$. d) CD56$^{dim}$CD16$^+$ & CD56$^{dim}$CD16$^-$. 

Diminished Absolute Counts of CD56$^{dim}$ and CD56$^{bright}$ NK Cells in Peripheral Blood from Egyptian Patients with HCC
Discussion

NK cells as components of the innate immunity substantially contribute to anti-tumor immune responses (Moretta & Moretta, 2004). To eradicate tumors, NK cells perform two critical tasks, namely cytotoxicity and production of IFN-γ (Dunn et al., 2006). Adoptive transfer of NK cells has been implicated as a strategy in the treatment of HCC (Ishiyama et al., 2006).

In this study, we found that the relative counts of total NK cells, and CD56^{dim}16^{+} were significantly lower in HCV-related HCC patients compared with controls. These results agree with the findings of Cai et al. (2008) who reported that HCC patients displayed a dramatic reduction in peripheral CD56^{dim}CD16^{+} NK subset compared with healthy subjects. CD56^{dim} NK cells eradicate tumors by cytotoxic activity which is attributed to their high content of perforin,
granzymes, cytolytic granules and the high expression level of CD16 makes them efficient mediators of antibody-dependent cellular cytotoxicity. Also, the authors reported decreased frequency of peripheral blood CD56_{dim} NK cells with impairment of its cytotoxicity in HCC patients that was explained by increase of T-regulatory lymphocytes in this group of patients. These findings mean that NK cells lose its anti-tumor immune responses in HCC patients. On the contrary, Lucea et al. (1995) did not observe statistically significant differences in the NK cell activity or in their count among patients with hepatic cirrhosis, non treated HCC and healthy controls.

The absolute counts of CD56_{dim}CD16^{-} NK subsets were significantly decreased in HCV-related HCC patients compared with controls. The true relevance of this subsets in human health and disease is largely unknown. Up to our knowledge, this is the first study to assess this subset in HCC and their decrease in HCC may point to their role in the immune response against tumor. This subset lacks the expression of CD16, and the mechanism(s) involved in anti-tumor immune response need further investigations.

Our results concerning the relative counts of CD56_{bright} in HCC patients agreed with that reported by Cai et al. (2008), who found that the frequency of CD56_{bright} did not show significant difference among HCC patients compared with controls. However, a significant decrease of the absolute CD56_{bright} count in HCC patients compared with control was detected in this study. CD56_{bright} NK cells are the most efficient cytokine producers (Cooper et al., 2001b), where the major cytokines released are IFN-γ, TNF-, GM-CSF, IL-10 and IL-13. IFN-γ, whose major source is NK cells, participates in cancer elimination by different mechanisms that include; inhibition of cellular proliferation and angiogenesis, enhancing apoptosis, and stimulation of adaptive immune response (Dunn et al., 2006). Such role of IFN-γ produced from peripheral blood NK cells was highlighted by Cai et al. (2008) who found that NK cells of HCC patients exhibited functional deficiency in IFN-γ production.

To initiate an immune response against tumor, tumour antigens need to be presented by professional antigen presenting cells (APCs). DCs are the most potent type of APCs, Vitale et al. (2004) have shown that CD56_{bright} preferentially proliferate in coculture with immature DC, interact in LN with incoming DC from the periphery (Ferlazzo & Mu’nz, 2004), and increase significantly the percentage of TNF-α-producing monocytes upon stimulation by monokines (IL-12, IL-15, IL-18). Data obtained in this study, together point towards a possible role for CD56_{bright} in fighting against tumor cells.

In addition, CD56_{bright} express the high-affinity receptor for interleukin-2 (IL-2) and proliferate in response to picomolar concentrations of this cytokine (Cooper et al., 2001b; Carson & Caligiuri, 1996). In HCC patients, cytokine-induced killer cells transfusion after micro-invasive treatments was found to improve their immunologic function and reduce the recurrence rate (Zhou et al., 2006), that may be explained by increase NK cell counts among these patients (Rosenberg et al., 1987), including CD56_{bright} cells.

In this study, we assessed NK & NK subsets phenotype in the peripheral blood of HCC patients, further studies to assess the function(s) and intrahepatic NK cells are recommended.

In conclusion, our study demonstrated that the absolute counts of dim and bright NK cell subsets decreased in different proportions in patients with HCV-related HCC. There was a marked decrease in both CD56_{dim}CD16^{+} and CD56_{dim}CD16^{-} and in particular, the CD56_{bright}...
cells that refers to a possible role for these cells in the immune response to HCC. This might aid in developing new therapeutic strategies targeting both NK subsets for HCC.

References


