Natural Killer Cells and its Subsets in Pulmonary Tuberculosis Patients Under Treatment

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Abstract

Tuberculosis remains a major health problem in different region of the world. Egypt is one of these areas. Although the great efforts to control this infection the cases detection rate and multi-drug resistant cases are still not controlled enough to the satisfactory limit for both WHO and the Egyptian national program. One of the important steps along the way to fight T.B. is to know more about how the immune system deals with this infection. Natural killer is a key player in the innate immunity response against the infection. Each of the NK subsets has a specific function and is identified by the expression both CD56 and or CD16. This study included 20 T.B. patients and their matching control group of healthy blood donors. NK and its subsets were assessed in peripheral blood by flowcytomtery. The resuly showed significant increase in T cells, NK cells CD56+/CD16- and CD56-/CD16+ while, there was decrease in the CD56+/CD16+ subset. Further studies regarding the effect of treatment on these changes are required to understand better the role Of the Natural killer in the immunity against T.B infection.

Key Words: Natural killer cells – Pulmonary tuberculosis patients.

Introduction

TUBERCULOSIS remains a public health problem in Egypt. Although Egypt is in the era of epidemiologic transition from communicable to non-communicable diseases like many other countries, TB must still be addressed and handled as a health problem affecting large sectors in the society [1]. Tuberculosis (TB) has afflicted mankind from the time immemorial. Evidence of spinal disease has been found in Egyptian mummies of several thousand years BC and references to TB are found in ancient Babylonian and Chinese writings [2]. Throughout history Tuberculosis (TB) remains the most hazardous bacterial infection worldwide.

Globally there are nearly 9 million new TB cases and nearly 2 million TB-related deaths each year. TB occurs throughout the world, but the incidence varies greatly. In the United States, the annual incidence is less than 5 per 100,000 persons, but in some countries in sub-Saharan Africa and Asia the annual incidence is several hundred per 100,000. The highest number of TB cases occurred in Asia (55%) followed by Africa (31 %) The World Health Organization 2009 (WHO) [3] has estimated that one-third of the total world population is latently infected with M. tuberculosis and 5%-10% of the infected individuals will develop active TB disease during their life time. However, the risk of developing active disease is 5%-15% every year and lifetime risk is 50% in HIV coinfected individuals [4]. Most of the active disease cases in low TB incidence countries arise from this pool of latently infected individuals. Primary infection with M. tuberculosis leads to clinical disease in only 10% of individuals. In the remaining cases, the ensuing immune response arrests further growth of M. tuberculosis.

Mycobacteria survive within macrophages, which can kill the bacteria if sufficiently activated, so induction of a Th1-type response, and in particular IFN-γ production, is key to protection against infection [2]. The importance of the innate response in disease is still unclear [5]. The role of NK cells during infections with intracellular bacteria has recently attracted considerable interest as there is strong evidence that the complete elimination of the bacterial pathogen from the host requires that the innate cellular system and the specific T-cell response interact efficiently [6]. Although NK cells were initially characterized for their marked lytic functions against a variety of cell targets, the contribution of NK-derived cytokines to host im-
Natural Killers constitute a distinct subpopulation of lymphocytes that is defined as CD3-, CD16+ and or CD56+ and most of NK in peripheral blood are CD56+/CD16+ and being able to break certain target cells without previous sensitization and without restriction to major histocompatibility complex. NK activation is not related to T cell so it can play a major role in immunity against certain intercellular pathogens. In the acute phase of these infections the predominance of the NK with the markers CD3-/CD56-/CD16+. As the infection progress the trend to increase number of the phe- type CD56+/CD16+ most of them are CD56 dim and about 10% are CD56 bright or CD56- [7]. Peripheral blood NK cells are divided in two subsets based on the different cell surface densities of CD56: CD56 dim and CD56 bright populations and other markers [8]. Each of these populations has a specific function, according to the surface markers (CD56 and CD16). There are indications that heterogeneous surface markers expression correlates to differentiation or function. The expression of CD16 is correlated to the antibody dependent cellular Cytotoxicity (ADCC) function of NK cells. CD56 bright is the cytokine secretor subset, its expression of CD56 is high with a small percentage expressing CD16 but mainly it is considered CD16- and it comprises less than 10%. CD56 dim is thought to be cytotoxic subset, expressing lower CD56 than the bright subset and is CD16+, and comprises around 80-90%. Finally, the CD56-16+ which is known as “the immature subset” [9].

M. tuberculosis-activated NK cells produce IFN-γ, activated NK cells stimulated infected monocytes to produce IL-15 and IL-18, and production of IL-15 and IL-18 were inhibited by anti-IFN-γ. These findings suggest that NK cells maintain the frequency of M. tuberculosis-responsive CD8+IFN-γ T cells by producing IFN-γ, which elicits secretion of IL-15 and IL-18 by monocytes. These monokines in turn favor expansion of Tc1 CD8+ T cells. The capacity of NK cells to prime CD8+ T cells to lyse M. tuberculosis-infected target cells required cell-cell contact between NK cells and infected monocytes and depended on interactions between the CD40 ligand on NK cells and CD40 on infected monocytes. NK cells link the innate and the adaptive immune responses by optimizing the capacity of CD8+ T cells to produce IFN-γ and to lyse infected cells, functions that are critical for protective immunity against M. tuberculosis and other intracellular pathogens [8].

NK cells in the pleural effusion of patients with tuberculosis are enriched for CD56hi cells with reduced expression of CD16 and perforin, which may be attributable to selective apoptosis of CD56dim cells induced by as yet unidentified soluble factors in pleural fluid [10]. In accordance with the CD56hi subset of NK cells being associated with high cytokine production, NK cells from pleural effusions spontaneously produced IFN-c and responded strongly to re-exposure to MTb by producing IFN-c, and this IFN-c production correlated with disease severity [10]. Thus, in active disease, NK cells exhibit reduced cytotoxicity but increased IFN-c production, perhaps because of selective activation of NK cell subsets. The question is if these changes are reflected on the peripheral blood NK and whether NK subsets can help to follow-up T.B. patients. Accordingly this study aims to study NK subsets in T.B. patients under treatment.

Aim of work:

This study aimed to study NK subsets (CD56 bright, CD56+/CD16+, CD56+/CD16- and CD56/16+) in T.B. patients under treatment.

Subjects and Methods

The study population consisted of 20 patients with diagnosed TB disease, who were undergoing TB treatment at the chest hospital-Ismailia during 2010. The diagnostic criterion for the presence of TB disease was defined as the presence of at least one of the following: 1) Clinical and radiological findings consistent with TB disease and positive sputum smears for acid-fast bacilli on at least two separate occasions; and 2) Culture positivity of sputum, bronchial lavage and/or pleural for M. Tuberculosis. The control group consisted of 20 unrelated subjects from blood bank donors who had been investigated and been found to be healthy. The inclusion criteria for the control group were the absence of acute or chronic pulmonary disease and a negative history for TB disease. Demographic data, including sex and age, and clinical data, including TB localization, method of diagnosis of TB disease, results of sputum examination and, if available, relapsed TB diagnosis were recorded. Written informed consent was obtained from all patients and control subjects. The bacteriological work was done at the Microbiology department while the Flow cytometry studies were done at the Zahran WA Clinical Pathology Lab in Faculty of Medicine Suez Canal University.
Patients were diagnosed and followed-up by positive sputum smears for acid fast bacilli by Zeal Nelson stain. Sputum samples were collected in the early morning in clean containers for three successive days. Those who had positive smears were confirmed by culture by using BBL MGIT. Samples were incubated and read daily from the day of incubation using a long wave UV light. Growth is detected by the observation of fluorescence and also by the presence of non homogenous turbidity or small grains or flakes in the culture medium. There are approximately $10^4-10^7$ CFU/ml of mycobacterium present at time of positivity [11,12].

Flowcytomtery studies:

Peripheral blood mononuclear cells (PBMCs) were prepared from heparanized blood samples by centrifugation over Ficoll-Hypaque density gradients (Pharmacia, Uppsala, Sweden). Cells were washed well with RPMI 1640 medium (Life Technologies, Gaithersburg, MD), and were resuspended in phosphate buffered saline (PBS) with 1% heat-inactivated fetal calf serum (FCS; Life Technologies). Cells were stained with the following mAbs anti-CD56 PE, anti-CD16 FITC, anti-CD3 APC (Becton Dickinson Biosciences, San Jose, CA), and incubated for 20min, on ice. After incubation the cells were washed with PBS. Cells were analyzed on the 4 colours FACSC alibur flowcytometry (Becton Dickinson, immunosystems) and cells were acquired per tube. Lymphocytes were determined according to their light scatter properties, followed by gating on CD56+CD3- population and this gate was used to detect the CD56+16-, CD56+16+ and CD56-16+ population. The percentage of positive cells was calculated using CellQuest software.

Statistical analysis:

The collected data were analyzed for statistical purposes through specialized computer software (SPSS) utilizing the *t*-test to evaluate the differences in means between the studied groups.

**Results**

There was no significant difference between the studied groups and the control regarding the age, sex and demographic data. The mean age of males was 38.1±12.8 years and of females 34.0±11.8 years matching with those of the control group. Of the 22 study patients, 10 (45.5%) were female and 12 (54.5%) male. Their Mean±SD age was 35.4±13.5 yrs.

-CD3+ cell, CD3- cell, and NK:

Lymphocytes were identified through their light scatter properties and were gated accordingly. After gating on Lymphocytes, the CD3+, CD3- cells were identified. CD3- cells were further gated and total NK and it subsets percent were identified. The percentages of CD56+bright and CD56+/CD16- and CD56+/CD16+ were obtained by gating on desired populations after gating on all of CD3- populations. There was statistically significant increase in NK cells percent in the T.B. group and control respectively. Yet, the studied groups showed significant statistically increase in NK cells percent in the T.B. group with mean of 21.6±6.15 while, the control group had a mean of 15.63±7.45.

**Table (1): Lymphocytes subset and NK percent in both studied groups.**

<table>
<thead>
<tr>
<th>T Cell type</th>
<th>T.B. patients (n=20) Mean±(SD)</th>
<th>Controls (n=20) Mean±(SD)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ lymphocytes</td>
<td>38.5±12</td>
<td>58.2±7.8</td>
<td>&lt;0.05 0.011*</td>
</tr>
<tr>
<td>CD3- lymphocytes</td>
<td>48.35±11.51</td>
<td>44.23±10.44</td>
<td>&gt;0.05 0.237</td>
</tr>
<tr>
<td>CD3+CD56+ NK</td>
<td>21.6±6.15</td>
<td>15.63±7.45</td>
<td>&lt;0.05 0.011*</td>
</tr>
</tbody>
</table>

*Asterisk denotes significant difference.

**Table (2): NK subsets in both studied groups.**

<table>
<thead>
<tr>
<th>NK Cell subsets %</th>
<th>Infected (n=20) Mean±(SD)</th>
<th>Controls (n=20) Mean±(SD)</th>
<th><em>p</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56+/CD16- cell</td>
<td>17.90±10.69</td>
<td>2.85±1.82</td>
<td>&lt;0.001 0.000*</td>
</tr>
<tr>
<td>CD56+/CD16+ cell</td>
<td>56.58±15.00</td>
<td>87.78±6.86</td>
<td>&lt;0.001 0.000*</td>
</tr>
<tr>
<td>CD56 bright cell</td>
<td>7.50±5.96</td>
<td>3.76±3.45</td>
<td>&lt;0.05 0.018*</td>
</tr>
</tbody>
</table>

*Asterisk denotes significant difference.
**NK subsets and duration of treatment:**

The duration of treatment ranged from 2 month to 10 month. The NK subsets were compared to the duration of treatment. All patient included in the study were under treatment. 13% of them received treatment for less than 2 months (group 1), 63% of them received treatment for a period ranging from 2 to 6 months (group 2), while 24% were under treatment for more than 6 months (group 3). There was a positive correlation between the duration of treatment and the (r=0.6), among group 2, with an average mean fluorescence intensity of 280.9±55. In this group cultures were negative in all 14 cases, while 10 cases of them showed negative ZN smears, and the other 4 cases were positive, at time of study. The group who received treatment for less than 2 months showed both positive cultures and smears at time of the study, while those who had treatment for more than 6 months and were considered non responder to treatment showed TLR2 MFI of 183±20 and both culture and smears positive at time of study (Table 4). There was a significant difference between group 2 and the other two groups concerning the MFI of TLR2.

**Table (3): NK subsets CD56-/CD 16+ in both studied groups.**

<table>
<thead>
<tr>
<th>Controls (n=20)</th>
<th>infected (n=20) (Mean±SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56-/CD16+ cell</td>
<td>9.94 ± 6.58</td>
<td>5.61 ± 4.63</td>
</tr>
</tbody>
</table>

* Asterisk denotes significant difference.

**Discussion**

The interplay between M. tuberculosis and the human host determines the outcome after infection. With respect to the human host, both innate and adaptive defense mechanisms are involved. After uptake of M. Tuberculosis in alveolar macrophages, several possible scenarios may be envisaged. M. Tuberculosis may be destroyed immediately, in which case no adaptive T-cell response is developed. When infection is established, however, a focal nonspecific inflammatory response follows. This response is regulated by a network of pro- and anti-inflammatory cytokines and chemokines. Most of the mediators at this point are derived from macrophages or dendritic cells, but IFN-gamma has several cellular sources, including NK cells. This initial response determines the local outgrowth of M. Tuberculosis (sometimes dissemination) or containment of infection.

Activated NK cells play a critical role in the recruitment of T cells to the infected sites and kill infected cells via perforin/granzyme, and FasL pathways and produce proinflammatory cytokines, which can induce the T cell response and activate the macrophage. Thus, NK cells could potentially contribute toward infection control. It would not be surprising that in the face of an adequate NK cell response, pathogens such Mycobacterium Tuberculosis may be controlled even in the absence of specific immune responses. This study aimed to study the effect of T.B. infection on the NK subsets (CD5 6+/CD 16+, CD56+/CD16- and CD56-/CD 16+).

In this study, the T cell showed significant statistically decrease in the TB patients when compared to the control groups. There was significant statistically difference between the two studied groups. CD3- cells (B cells+NK cells) percent in the control group had a mean of 44.23±10.44 while in the T.B. group it had a mean of 48.35±11.51. There was no significant statistically difference between the two studied groups regarding their CD3- cells percent. Jadoon et al. (2004) showed...
that the activated T cells were decreased in the TB patients included in their study when compared with normal controls. Barcelo et al., [7] agreed as their study revealed that the Lymphocytes subsets in pulmonary tuberculosis that there was significant decrease in CD3 and significant increase in the CD19+ cells. and Zahran et al., [12,13] disagreed when they showed that T cell population didn’t statistically differ in TB patients than the control group.

Peripheral blood NK cells are divided in two subsets based on the different cell surface densities of CD56: CD56 dim and CD56 bright populations and other markers [8]. About 10% of the peripheral blood NK cells express high levels of CD56 (CD56+ bright) and low levels of CD16 on their surface, and the reverse is true for the remaining 90% of the NK cells (CD56+ dim) and expresses CD16. The CD56+dim subset acts as the main cytolytic subset and expresses high levels of perforin and natural cytotoxicity receptors [11]. The expression of CD16 is correlated to the antibody dependent cellular Cytotoxicity (ADCC) function of NK cells. CD56 bright is the cytokine secretor subset while CD56 dim 16+; is thought to be cytotoxic subset, expressing lower CD56 than the bright subset and is CD16+, and comprises around 80-90%. Finally, there is a subset CD56-16+ which is known as “the immature subset” [11].

In the present study there was significant statistically increase in NK cells percent in the T.B. group with mean of 21.6±6.15 while, the control group had a mean of 15.6±7.45. There was statistically significant increase in CD56+/CD16- NK subset in T.B. patients when compared to the controls with a mean of 17.90±10.69 versus mean of 2.85±1.82. While, The CD56+/CD16+ showed a statistically significant decrease, in the Same group with a mean of 56.58±13.00, when compared to the control group with a mean of 87.78±6.86. There was statistically significant increase in CD56+ bright cells in T.B. infected patients than controls.

Zahran et al., [12] studied the NK in pulmonary TB patient and they found that there is no significant difference could be found in the percentages of NK cells among the studied group. Also, Veenstra et al., [13] found that NK cells were not significantly different from those of controls at diagnosis or at Week 26 of treatment, yet, NK cell counts at diagnosis showed a trend towards lower numbers and remained depressed until week 26.

While Schierloh et al., [14], found that there is enrichment of the CD56 bright subset and contributed that to the increased susceptibility to apoptosis of CD56+CD16+ NK cells Furthermore, they found that in TB patients, Mtb-induced IFN-gamma production by PF NK cells was not dependent on the presence of CD3+, CD19+, and CD14+ cells, suggesting a direct interaction of CD56 bright cells with MTB and/or the involvement of other accessory cells present at the site of MTB infection.

Barcelos et al., [7] when they performed triple labeling studies of the NK cells to identify these cells within the gated lymphocytes. It was found that after treatment that there was a decrease in the classical NK phenotype CD56+/CD16- and that the level of the pre NK (CD56-/CD16+) and mature NK (CD5 6+/CD16+) remained unchanged throughout and after the treatment. They observed larger population of the CD56 dim population with it is higher cytotoxic functions in the beginning of the treatment while the NK bright increase was observed at the end of treatment. Those data suggests the role of the cytolytic NK dim in controlling the infection.

Raja [15] suggested that during early infection, NK cells are capable of activating phagocytic cells at the site of infection. A significant reduction in NK activity was associated with multidrug resistant TB (MDR-TB). NK activity in BAL has revealed that different types of pulmonary TB are accompanied by varying degrees of depression.

As it is clear, that many studies focused on studying NK cells and its activity in different stages of the infection and treatment. Some of studies agreed with this study and other disagreed with it this disagreement could be due to the difference of the approach of the studies, or the patient selection or more detailed investigations. In this study the patients were all pulmonary T.B. and they were all under treatment but for different duration. During the study MGIT cultures and ZN smears were done as an indicator of response to treatment. Negative cultures indicated absence of viable Mycobacteria Tuberculosis, accordingly response to treatment. All of the studied group were under treatment, the range was between 2 and 10 months with average of 6.8 months. Those patients who had treatment for less 2 months were considered not responding yet to treatment and were all positive for both MGIT culture and ZN smear, also, those who were under treatment for more than 6 months were considered non responders to treatment. The percent of the not responding yet group is 13%, the non responding patients were 63% and the remaining 24% were those who receiving treatment
for a period varying from more than 2 months up to less than 6 months. The fact that the CD56+/CD16+ was decreased on all the studied group confirms that fact that most of the group was in active state of disease and still struggling with it supporting the results of both Schierloh et al., [16] and Barcelos et al., [7]. This could be due to increase CD56+/CD16+ apoptosis.

Another important NK subset is the immature CD56-/CD16+ NK. In this study this subset was decreased in the patients when compared to the healthy control. This subset needs more work to understand its function and its roles in immunity.

In conclusion, NK and its subsets (CD56+ bright and CD56+ dim CD56 are considered to play an important role and the agreement of the results of the present study and the conflict between it and other studies show the importance of the further work emphasizing on the function and cytokine pattern of each subsets and the correlation between it and the severity and the type of TB beside the treatment and multi drug resistance patients.

References
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