Original Article

Elevated expression of Tim-3 in CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells correlates with disease severity of pulmonary tuberculosis

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Abstract: Tim-3 is a member of the T cell immunoglobulin and mucin domain (Tim) family of immune cell receptors and it plays an important role in modulating CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell immunity. However, little is known about Tim-3 pathway regulates Natural killer (NK) cell function in tuberculosis (TB) patients. In this study, we investigated expression and functions of Tim-3 on CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells during TB infection. Our results revealed that pulmonary TB patients had significantly elevated Tim-3 expression on CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells compared with healthy controls. The expression of Tim-3 on CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells was significantly higher in TB patients with advanced disease than patients with mild-to-moderate disease. Tim-3-expressing CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells had a functional defect. Spontaneous apoptosis in Tim-3\textsuperscript{+}CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells was higher than in Tim-3\textsuperscript{-} cells. Blocking of Tim-3 signaling resulted in increased expression of CD107a on CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells. In conclusion, the elevated expression of Tim-3 on CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells correlates with functional defects of CD56\textsuperscript{dim}CD16\textsuperscript{+} cells and disease severity of pulmonary TB.

Keywords: Tuberculosis, immunity, Tim-3, NK cells

Introduction

Tuberculosis (TB), an infectious disease caused by Mycobacterium tuberculosis infection, remains a leading cause of morbidity and mortality worldwide [1]. It is estimated that 10.3 million cases of TB occurred in 2015 and that 1.4 million died of TB [2]. Despite the high rate of M. tuberculosis infection in humans, especially in developing countries, only 5-10\% of infected people develop active TB in their life time [3]. A delicate interplay between M. tuberculosis and the host’s innate and acquired immune system can influence the outcome of the infection, which ranges from pathogen elimination to the establishment of a latent infection or a progressive disease.

Natural killer (NK) cells are innate lymphocytes that are crucial for the early control of some pathogen infections and malignancies [4]. In human peripheral blood, two major NK cell sub-populations can be defined on the basis of the relative expression of the markers CD16 and CD56: CD56\textsuperscript{dim}CD16\textsuperscript{+} and CD56\textsuperscript{bright}CD16\textsuperscript{-}/ [5]. The CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells represent at least 90\% of all peripheral blood NK cells and are therefore the major circulating subset. It is generally accepted that cytokine production is mainly associated with CD56\textsuperscript{bright}CD16\textsuperscript{-}/ subset and cytolytic activity is the functional properties of CD56\textsuperscript{dim}CD16\textsuperscript{+} subset [6]. However, recent studies demonstrate that CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells not only have cytolytic activity but also early IFN-\gamma producing capability [7]. Despite several studies that have been conducted on M. tuberculosis-NK cells interaction [8-10], one should acknowledge that our understanding of the mechanisms deployed by M. tuberculosis to modulate the host cell’s response to infection is still incomplete.

It has been proven that NK cell functions are regulated by different activating and inhibitory...
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Surface receptors, in order to maintain a balanced immune response toward infection [11]. T cell immunoglobulin domain and mucin domain (Tim)-3, initially identified on terminally differentiated Th1 cells, negatively regulates the T cell response by inducing T cell apoptosis [12]. Recently, Tim-3 was also shown to play important roles on innate immune cells, including macrophages/monocytes, dendritic cells (DCs), and NK cells [13]. Wang et al. [14] reported that pulmonary TB patients had significantly elevated Tim-3 expression on total CD8+ T cells and on antigen-specific CD8+ T cells compared with tuberculin-positive healthy controls. The elevated expression of Tim-3 on CD8+ T cells correlates with functional defects of CD8+ T cells and disease severity of pulmonary TB. However, little is known about Tim-3 pathway regulates NK cell function in TB patients. Therefore, in this study, we investigated expression and functions of Tim-3 on NK cells during TB infection.

Materials and methods

Subjects

Patients with pulmonary TB (43 male and 32 female) were recruited from the Jiangxi Chest Hospital and the First Affiliated Hospital of Nanchang University from January 2015 to December 2015. The subjects ranged in age from 15 to 60 years (mean age [± SD], 27.5 ± 8.2 years). Patients with TB were classified as mild-to-moderate disease (TB-MD) and advanced disease (TB-AD) according to the extent of disease evident on chest radiography. Fifty-two of these patients were TB-MD and 23 patients were TB-AD. All of these patients were diagnosed with TB on the basis of the typical TB clinical symptoms, bacterial culture, and imaging examinations, and in accordance with the Health criteria in People’s Republic of China: The legal diagnostic criteria of infectious diseases (WS288-2008) and the revised international definitions in TB control of the World Health Organization (WHO) [15]. Individuals with malignant tumor, HIV infections were excluded.

Thirty healthy individuals (17 male and 13 female) who attended the hospital for a physical examination during the same time period, were recruited as controls. They ranged in age from 17 to 58 years (mean age [± SD], 28.6 ± 7.5 years). Nineteen healthy controls had positive results of a purified protein derivative (PPD) test; findings of peripheral blood examination and chest radiography were normal.

This study was approved by the Ethical Review Committees of the First Affiliated Hospital of Nanchang University. Written informed consent was obtained from all adult subjects or guardians on behalf of the minors enrolled in this study. All of the procedures were performed in compliance with the Declaration of Helsinki.

Cell preparations

Fresh peripheral whole blood obtained from TB patients and control individuals was treated with heparin to prevent coagulation. After sample collection, peripheral blood mononuclear cells (PBMCs) were freshly isolated by density gradient centrifugation on Ficoll-Paque (Sigma, USA). 1 × 10^6 PBMCs were washed twice with RPMI 1640 (GIBCO, USA) respectively, and then resuspended in RPMI 1640 supplemented with 10% human serum (Sigma, USA).

Surface antibody staining and flow cytometric analysis

The PBMCs were stained with phycoerythrin-Texas Red (ECD)-conjugated anti-CD3, phycoerythrin-Cyanin 5 (PE-Cy5)-conjugated anti-CD56, fluorescein isothiocyanate (FITC)-conjugated anti-CD16, and phycoerythrin (PE)-conjugated anti-Tim-3 (ebioscience, San Diego, CA, USA) at dark for 30 min. Cells incubated with PE-conjugated mouse IgG were used as isotype controls. All flow samples were analyzed with a CYTOMICS FC 500 flow cytometer (Beckman Coulter Inc., Brea, CA, USA) and associated software programs (CXP).

Analysis of spontaneous apoptosis of NK cells

The PBMCs were incubated in complete RPMI 1640 medium overnight and then stained with ECD-, PE-Cy5-, PE-Cy7- and PE-conjugated anti-human CD3, CD56, CD16 and Tim-3 monoclonal antibodies. FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) was used to determine apoptosis of cells by following manufacturer’s instruction.

Lytic degranulation

CD107a (Lysosome-Associated Membrane Protein 1, LAMP-1) expression was performed to measure NK cell degranulation, as described
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elsewhere [16]. Briefly, PBMCs were overnight in complete RPMI 1640 medium containing IL-2 (PeproTech, Rocky Hill, USA) and IL-12 complex (each at 100 U/ml) or Tim-3 blocking antibody alone (ebioscience, San Diego, CA, USA) or together. Cells were incubated with the target cell line K562 at 5:1 ratio in the presence of PE-Cy7-labeled anti-human CD107a monoclonal antibody (BD Biosciences) for a total of 6 h. After 1 h of incubation, monensin (BD Biosciences) was added. PBMCs incubated under the same conditions but without target cells were used to measure spontaneous degranulation. At the end of incubation, cells were stained with ECD-, PE-Cy5-, FITC and PE-conjugated anti-human CD3, CD56, CD16 and Tim-3 antibodies. CD107a expression on NK cells was analyzed by flow cytometry.

Intracellular staining of IFN-γ

For intracellular staining of IFN-γ, PBMCs were incubated together with K562 cells at 5:1 ratio for 6 h and monensin was added after 1 h of incubation.
incubation. Cells were first stained with ECD-, PE-Cy5-, FITC and PE-conjugated anti-human CD3, CD56, CD16 and Tim-3 monoclonal antibodies, and then were fixed and permeabilized with fixation/permeabilization solution kit (BD Biosciences) by following manufacturer’s manual and stained with PE-Cy7-conjugated anti-IFN-γ monoclonal antibodies (ebioscience, San Diego, CA, USA). Negative control samples were incubated with corresponding PE-Cy7-conjugated isotype mAbs in parallel with experimental samples. Cells were analyzed by FC-500 flow cytometer.

**Statistical analysis**

Mann-Whitney and paired t-test analysis were used for statistical analysis between groups by using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Data were shown as mean ± standard derivation (SD). All tests were two-tailed and P < 0.05 was considered significant.

**Results**

**Elevated Tim-3 expression on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells in patients with pulmonary TB**

To determine the expression of Tim-3 on NK cells, PBMCs were stained with fluorochrome-labeled anti-human Tim-3, CD3, CD16 and CD56 antibodies and analyzed by flow cytometry. Totally, 75 patients with confirmed diagnosis of pulmonary TB and 30 healthy controls were studied. Tim-3 was expressed on both CD56<sup>dim</sup>CD16<sup>+</sup> and CD56<sup>bright</sup>CD16<sup>-/–</sup> subsets. The frequencies of Tim-3-expressing CD56<sup>dim</sup>CD16<sup>+</sup> NK were significantly higher in active TB patients than in healthy controls (P < 0.001) (Figure 1), while the expression levels of Tim-3 on CD56<sup>bright</sup>CD16<sup>-/–</sup> NK cells were similar among active TB patients and healthy controls (P > 0.05).

**Association of Tim-3 expression on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells with disease severity of pulmonary TB**

The frequencies of Tim-3 expressing CD56<sup>dim</sup>CD16<sup>+</sup> NK cells showed much wider range in TB patients (from 7.4% to 60.8%) than in healthy controls. We asked whether Tim-3 expression was associated with disease severity of pulmonary TB. Patients with active TB were recruited and classified as mild-to-moderate disease (TB-MD) and advanced disease (TB-AD) according to the extent of disease evident on chest radiography. Analysis of Tim-3 expression on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells showed that patients with TB-AD had significantly elevated expression of Tim-3 than patients with TB-MD (26.9% ± 11.9 vs. 41.4% ± 9.4) (P < 0.001) (Figure 2). Furthermore, we compared the frequency of Tim-3 expressing CD56<sup>dim</sup>CD16<sup>+</sup> NK cells in new active pulmonary TB patients who had not received anti-TB therapy and those who had different durations of anti-tuberculosis therapy. As shown in Figure 3, the percentages of Tim-3 expressing CD56<sup>dim</sup>CD16<sup>+</sup> NK cells in TB patients following 3 months- anti-TB therapy were significantly lower than those in patients who had not received anti-TB therapy (P < 0.001). After 6-months of anti-TB therapy, the percentage of Tim-3 expressing CD56<sup>dim</sup>CD16<sup>+</sup> NK cells in TB patients recovered to the levels of those in healthy volunteers. These results suggested that Tim-3 expression on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells correlated with disease severity of pulmonary TB.

**Functional analysis of Tim-3-expressing CD56<sup>dim</sup>CD16<sup>+</sup> NK cells**

Cytolytic activity and early IFN-γ production are functional properties of CD56<sup>dim</sup>CD16<sup>+</sup> NK cells. To determine whether Tim-3 has any influence on NK cell function, cytolytic activity and IFN-γ production was compared between Tim-
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3+ and Tim-3 CD56dimCD16+ NK cells. CD107a is expressed on surface of NK cells during degranulation and can be used as a marker for cytotoxic activity. To study relationship of cytotoxic activity of CD56dimCD16+ NK cells and Tim-3 expression, PBMCs from TB patients were incubated with target cell line K562, and Tim-3 and CD107a expression were analyzed by flow cytometry. Tim-3+ subset had much less CD107a-expressing CD56dimCD16+ NK cells than Tim-3 subset (P = 0.029) (Figure 4). Flow cytometric analysis showed that Tim-3+CD56dimCD16+ NK cell subset had significant-

Spontaneous apoptosis rate of Tim-3+CD56dimCD16+ NK cells was higher than Tim-3 cells

Spontaneous apoptosis of NK cells were determined by staining with Annexin V antibody and propidium iodide (PI), and analyzed by flow cytometry. We found that the percentage of apoptotic cells was significantly higher in Tim-3+CD56dimCD16+ NK cells than in Tim-3 cells (P = 0.005) (Figure 6).

Blocking Tim-3 signaling resulted in increased expression of CD107a on CD56dimCD16+ NK cells

To determine whether blocking of Tim-3 signaling pathway could rescue functional defects of CD56dimCD16+ NK cells, PBMCs were incubated with target cell line K562 in the presence of anti-Tim-3 blocking antibody or isotype control antibody. Blocking of Tim-3 signaling resulted in increased expression of CD107a on CD56dimCD16+ NK cells (P = 0.007) (Figure 7).

Discussion

NK cells are involved in innate immunity and play an important protective role against pathogen infection and cancer. This effect is achieved through a complex mosaic of inhibitory and

Figure 3. The frequency of Tim-3+CD56dimCD16+ NK cells decline after successful anti-tuberculosis therapy. Scatter plot showing longitudinal analysis of Tim-3+CD56dimCD16+ NK cells during course of anti-tuberculare treatment. Each symbol represents an individual case. A significant level of decline in frequency of Tim-3+CD56dimCD16+ NK cells was observed at 3 months and subsequent follow-up.

Figure 4. Comparison of degranulation in Tim-3+ and Tim-3+CD56dimCD16+ NK cells. Tim-3+CD56dimCD16+ NK cells had significantly lower CD107a expression than Tim-3+CD56dimCD16+ NK cells when cells were incubated with target cell line K562. Wilcoxon matched pairs test was used for statistical analysis.

Figure 5. Comparison of IFN-γ production in Tim-3+ and Tim-3+CD56dimCD16+ NK cells. Tim-3+CD56dimCD16+ NK cells had significantly reduced IFN-γ production compared with Tim-3+CD56dimCD16+ NK cells. Wilcoxon matched pairs test was used for statistical analysis.
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activating receptors expressed by NK cells that ultimately determines the magnitude of the NK cell response. A recent study has evaluated the role of programmed death-1 (PD-1), PD-L1, and PD-L2 on NK cells from active TB patients and shown that \textit{M. tuberculosis} stimulation leads to higher expression of PD-1/ligands on NK cells leading to inhibition of their effect or functions like cytokine production [17]. Furthermore, the functional defect of NK cells against \textit{M. tuberculosis} can be rescued by blockade of the PD-1 pathway. Tim-3 and PD-1 are co-inhibitory receptors involved in the so-called T cell exhaustion [18]. In contrast to the relatively clear mechanisms of PD-1 pathway in TB infection, the role of Tim-3 in modulating TB immunity remains largely unclear. In this study, we studied functional role of Tim-3 on NK cells during human TB infection.

Tim-3 was initially identified on activated Th1, Th17, and Tc1 cells and induces T cell death or exhaustion after binding to its ligand, Gal-9 [19]. Tim-3 expression is associated with a dysfunctional T cell phenotype in many viral infections such as HIV and hepatitis C and B [20-22]. Several reports have shown that specific block-

Figure 6. Spontaneous apoptosis in Tim-3+ and Tim-3-CD56dimCD16+ NK cells. The percentage of Annexin V+PI apoptotic cells was significantly higher in Tim-3-CD56dimCD16+ NK cells than in Tim-3+CD56dimCD16+ NK cells. Wilcoxon matched pairs test was used for statistical analysis.

Figure 7. Blocking Tim-3 signaling resulted in increased expression of CD107a on CD56dimCD16+ NK cells. Cells were incubated with target cell line K562 in the presence of anti-Tim-3 blocking antibody (anti-Tim-3) or isotype control antibody (IgG). Blocking of Tim-3 signaling resulted in increased expression of CD107a on CD56dimCD16+ NK cells. Wilcoxon matched pairs test was used for statistical analysis.

It was recently reported that in TB infection, Tim-3 expression was elevated on NK cells when compared with healthy controls [28]. The number of TB patients recruited was only 15 in the study and clinical significance of Tim-3
associated with TB is unclear. In this study, we investigated 75 patients with active TB and demonstrated that Tim-3 was highly expressed on neither CD56\textsuperscript{dim}CD16\textsuperscript{+} NK nor CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells during human TB infection. In addition, we found that the frequency of Tim-3-expressing CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells in patients with advanced TB was significantly higher than those in patients with mild-to-moderate disease and that anti-TB therapy significantly decreased the percentage of Tim-3-expressing CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells in TB patients. These results revealed a correlation between Tim-3-expressing CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells levels and the severity of pulmonary TB. This preliminary study does highlight the potential of elevated levels of Tim-3-expressing CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells to serve as a biomarker of pulmonary TB severity.

We examined whether blockade of Tim-3 has any effect on IFN-γ production of CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells. No significant difference was found in levels of IFN-γ produced from CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells cultured in the presence of Tim-3 blocking antibody or isotype control antibody. It is possible that Tim-3 might have more potent role in modulating cytotoxic effect of CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells stimulated with K562 cells.

In summary, our study demonstrated that Tim-3 expression on CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells was significantly higher in active TB patients than in healthy controls. Tim-3-expressing CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells had decreased cytotoxicity and reduced production of IFN-γ, and blockade of Tim-3 signaling could rescue functional defect of CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells. The results suggested an inhibitory role of Tim-3 on CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells during active TB infection in humans.

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Disclosure of conflict of interest

None.

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References

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