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Original Article

Elevated expression of Tim-3 in $CD56^{dim}CD16^{+}$ 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 $CD4^{+}$ and $CD8^{+}$ 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 $CD56^{dim}CD16^{+}$ NK cells during TB infection. Our results revealed that pulmonary TB patients had significantly elevated Tim-3 expression on $CD56^{dim}CD16^{+}$ NK cells compared with healthy controls. The expression of Tim-3 on $CD56^{dim}CD16^{+}$ NK cells was significantly higher in TB patients with advanced disease than patients with mild-to-moderate disease. Tim-3-expressing $CD56^{dim}CD16^{+}$ NK cells had a functional defect. Spontaneous apoptosis in $Tim\text{-}3^{+}CD56^{dim}CD16^{+}$ NK cells was higher than in $Tim\text{-}3^{-}$ cells. Blocking of Tim-3 signaling resulted in increased expression of CD107a on $CD56^{dim}CD16^{+}$ NK cells. In conclusion, the elevated expression of Tim-3 on $CD56^{dim}CD16^{+}$ cells correlates with functional defects of $CD56^{dim}CD16^{+}$ 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 subpopulations can be defined on the basis of the relative expression of the markers CD16 and CD56: $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{+/-}$ [5]. The $CD56^{dim}CD16^{+}$ 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 $CD56^{bright}CD16^{+/-}$ subset and cytolytic activity is the functional properties of $CD56^{dim}CD16^{+}$ subset [6]. However, recent studies demonstrate that $CD56^{dim}CD16^{+}$ NK cells not only have cytolytic activity but also early IFN-γ 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

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

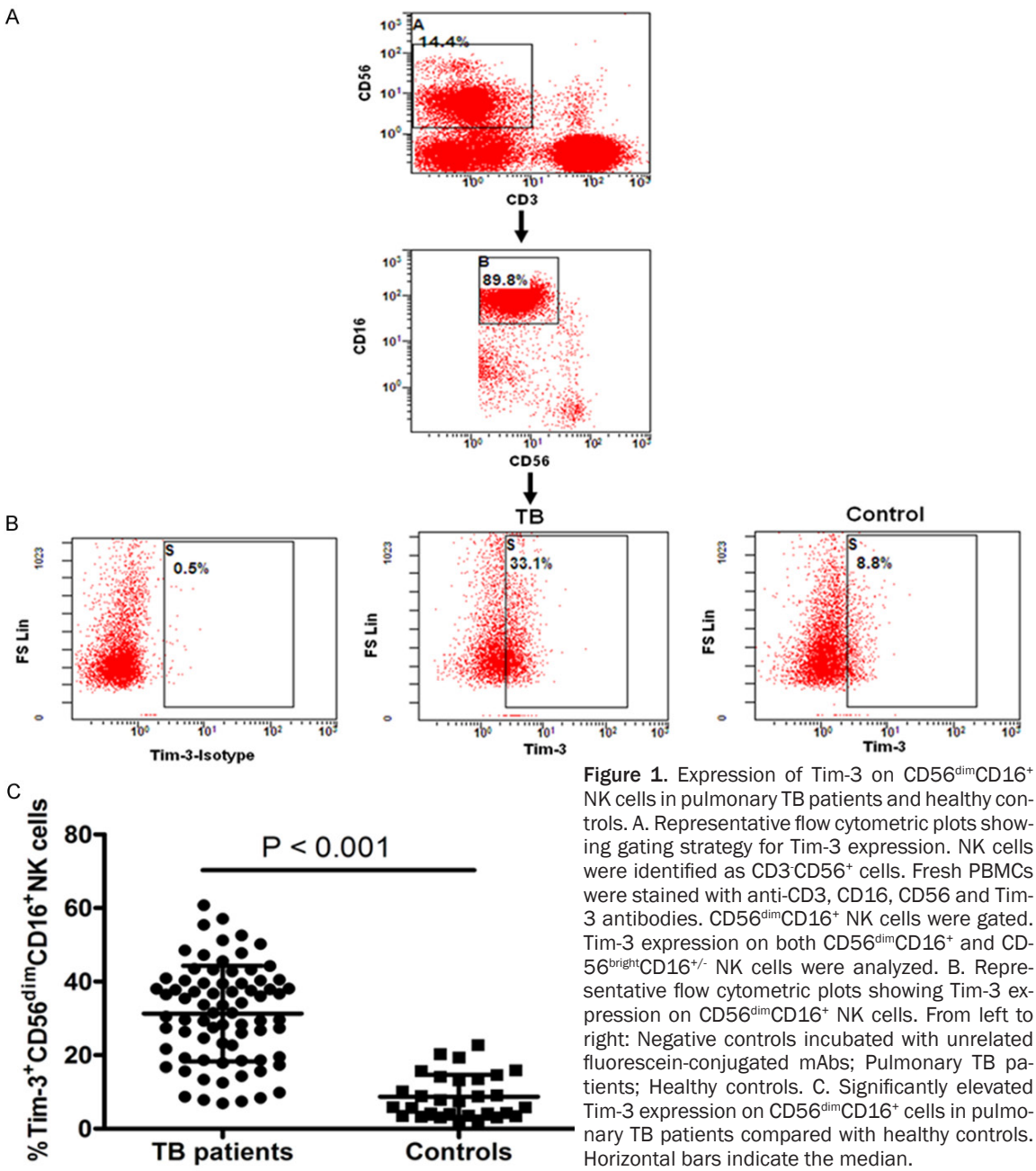

Figure 1. Expression of Tim-3 on $CD56^{dim}CD16^{+}$ NK cells in pulmonary TB patients and healthy controls. A. Representative flow cytometric plots showing gating strategy for Tim-3 expression. NK cells were identified as $CD3^{-}CD56^{+}$ cells. Fresh PBMCs were stained with anti-CD3, CD16, CD56 and Tim-3 antibodies. $CD56^{dim}CD16^{+}$ NK cells were gated. Tim-3 expression on both $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{+/-}$ NK cells were analyzed. B. Representative flow cytometric plots showing Tim-3 expression on $CD56^{dim}CD16^{+}$ NK cells. From left to right: Negative controls incubated with unrelated fluorescein-conjugated mAbs; Pulmonary TB patients; Healthy controls. C. Significantly elevated Tim-3 expression on $CD56^{dim}CD16^{+}$ cells in pulmonary TB patients compared with healthy controls. Horizontal bars indicate the median.

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

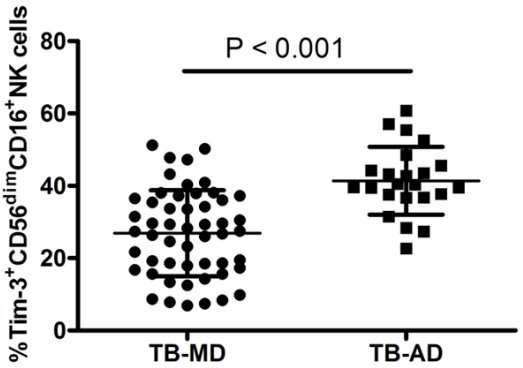

Figure 2. Expression of Tim-3 on $CD56^{dim}CD16^{+}$ NK cells in pulmonary TB patients correlated with disease severity. PBMCs from PTB patients with mild-to-moderate disease (TB-MD) and advanced disease (TB-AD) were isolated by Ficoll gradient centrifugation and the Tim-3 expression on $CD56^{dim}CD16^{+}$ NK cells were analyzed by flow cytometry. Each symbol denotes a single subject, and the mean ± SD for each study population is shown.

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 $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{+/-}$ subsets. The frequencies of Tim-3-expressing $CD56^{dim}CD16^{+}$ 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 $CD56^{bright}CD16^{+/-}$ NK cells were similar among active TB patients and healthy controls ($P > 0.05$).

Association of Tim-3 expression on $CD56^{dim}CD16^{+}$ NK cells with disease severity of pulmonary TB

The frequencies of Tim-3 expressing $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ NK cells in TB patients recovered to the levels of those in healthy volunteers. These results suggested that Tim-3 expression on $CD56^{dim}CD16^{+}$ NK cells correlated with disease severity of pulmonary TB.

Functional analysis of Tim-3-expressing $CD56^{dim}CD16^{+}$ NK cells

Cytolytic activity and early IFN-γ production are functional properties of $CD56^{dim}CD16^{+}$ NK cells. To determine whether Tim-3 has any influence on NK cell function, cytolytic activity and IFN-γ production was compared between Tim-

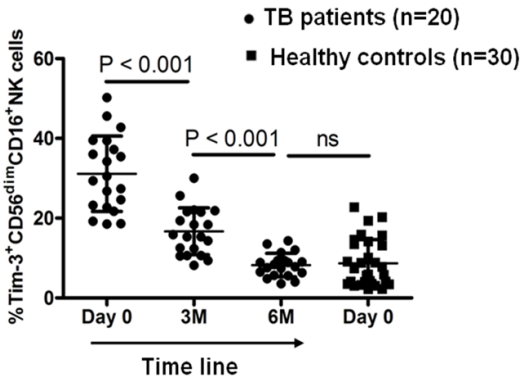

Figure 3. The frequency of Tim-3$^+$CD56^{dim}CD16$^+$ NK cells decline after successful anti-tuberculosis therapy. Scatter plot showing longitudinal analysis of Tim-3$^+$CD56^{dim}CD16$^+$ NK cells during course of anti-tubercular treatment. Each symbol represents an individual case. A significant level of decline in frequency of Tim-3$^+$CD56^{dim}CD16$^+$ NK cells was observed at 3 months and subsequent follow-up.

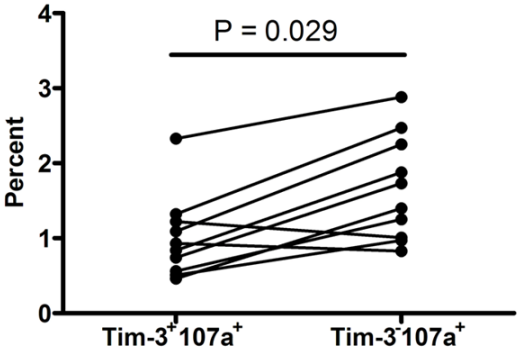

Figure 4. Comparison of degranulation in Tim-3$^+$ and Tim-3$^-$CD56^{dim}CD16$^+$ NK cells. Tim-3$^+$CD56^{dim}CD16$^+$ NK cells had significantly lower CD107a expression than Tim-3$^-$CD56^{dim}CD16$^+$ NK cells when cells were incubated with target cell line K562. Wilcoxon matched pairs test was used for statistical analysis.

3$^+$ and Tim-3$^-$CD56^{dim}CD16$^+$ 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 CD56^{dim}CD16$^+$ 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 CD56^{dim}CD16$^+$ NK cells than Tim-3$^-$ subset (P = 0.029) (**Figure 4**). Flow cytometric analysis showed that Tim-3$^+$CD56^{dim}CD16$^+$ NK cell subset had significant-

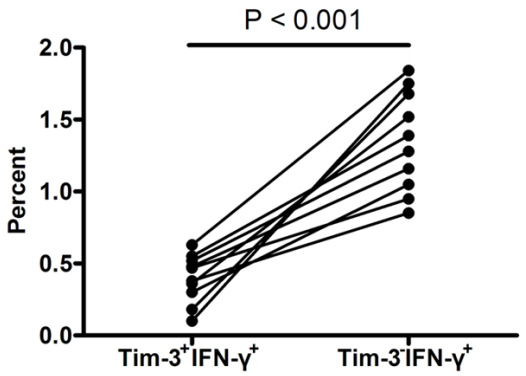

Figure 5. Comparison of IFN-γ production in Tim-3$^+$ and Tim-3$^-$CD56^{dim}CD16$^+$ NK cells. Tim-3$^+$CD56^{dim}CD16$^+$ NK cells had significantly reduced IFN-γ production compared with Tim-3$^-$CD56^{dim}CD16$^+$ NK cells. Wilcoxon matched pairs test was used for statistical analysis.

ly lower IFN-γ-producing cells than Tim-3$^-$ subset (P < 0.001) (**Figure 5**). Taken together, the results indicated that Tim-3-expressing CD56^{dim}CD16$^+$ NK cells had a functional defect.

Spontaneous apoptosis rate of Tim-3$^+$CD56^{dim}CD16$^+$ 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$^+$CD56^{dim}CD16$^+$ NK cells than in Tim-3$^-$ cells (P = 0.005) (**Figure 6**).

Blocking Tim-3 signaling resulted in increased expression of CD107a on CD56^{dim}CD16$^+$ NK cells

To determine whether blocking of Tim-3 signaling pathway could rescue functional defects of CD56^{dim}CD16$^+$ 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 CD56^{dim}CD16$^+$ 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

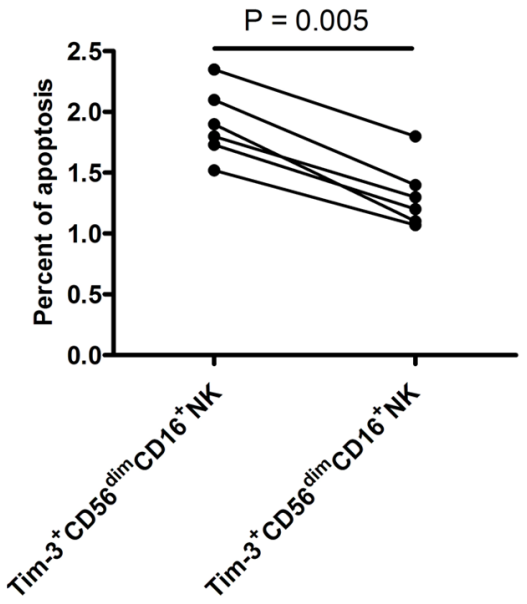

Figure 6. Spontaneous apoptosis in Tim-3$^+$ and Tim-3$^-$CD56^{dim}CD16$^+$ NK cells. The percentage of Annexin V$^+$PI$^-$ apoptotic cells was significantly higher in Tim-3$^+$CD56^{dim}CD16$^+$ NK cells than in Tim-3$^-$CD56^{dim}CD16$^+$ NK cells. Wilcoxon matched pairs test was used for statistical analysis.

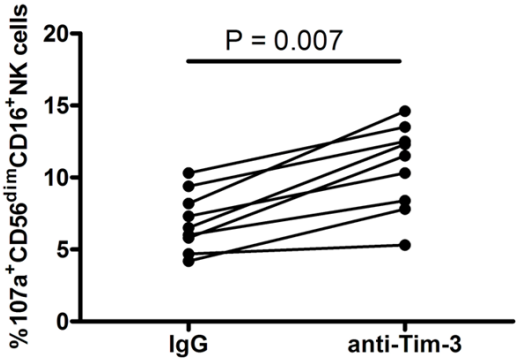

Figure 7. Blocking Tim-3 signaling resulted in increased expression of CD107a on CD56^{dim}CD16$^+$ NK cells. Cells were incubated with target cell line K562 in the presence of anti-Tim-3 blocking antibody (anti-Tim-3) or isotype control antibody (IgG). Blocking of Tim-3 signaling resulted in increased expression of CD107a on CD56^{dim}CD16$^+$ NK cells. Wilcoxon matched pairs test was used for statistical analysis.

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 *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 *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 blocking of Tim-3 signaling pathways improved T cell responses and viral control in chronically infected patients [23, 24]. Recent data have shown that the activity of NK cells can also be regulated by Tim-3. For example, Gleason et al. [25] demonstrated that Tim-3 was upregulated on human NK cells after activation and promoted IFN-γ production in response to Gal-9. However, Ndhlovu et al. [26] reported that, when Tim-3 was cross-linked on NK cells with antibodies or encountered target cells that expressed its ligand Gal-9, it suppressed NK cell-mediated cytotoxicity, showing that Tim-3 also suppresses NK cell functions. In addition, Ju et al. [27] demonstrated that chronic hepatitis B infection upregulated Tim-3 expression on NK cells and subsequently suppressed NK cell function and that this process was reversed by blockade of the Tim-3 pathway. These data suggest that Tim-3 has opposing effects on NK cells. In this study, we found that Tim-3$^+$CD56^{dim}CD16$^+$ NK cells in patients with active TB had significantly reduced degranulation and IFN-γ production, and the functional defect could be rescued by blockade of Tim-3 signaling, indicating an inhibitory role of Tim-3 on NK cells during TB infection.

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 $CD56^{dim}CD16^{+}$ NK nor $CD56^{bright}CD16^{+/-}$ NK cells during human TB infection. In addition, we found that the frequency of Tim-3-expressing $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ NK cells in TB patients. These results revealed a correlation between Tim-3-expressing $CD56^{dim}CD16^{+}$ NK cells levels and the severity of pulmonary TB. This preliminary study does highlight the potential of elevated levels of Tim-3-expressing $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ NK cells. No significant difference was found in levels of IFN-γ produced from $CD56^{dim}CD16^{+}$ 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 $CD56^{dim}CD16^{+}$ NK cells stimulated with K562 cells.

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

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Disclosure of conflict of interest

None.

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