Elevation of $CD56^{bright}CD16^{-}$ Lymphocytes in MDR Pulmonary Tuberculosis

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ABSTRACT

Background: Protective immune responses induced in the majority of people infected with *Mycobacterium tuberculosis* enable them to control TB infection. **Objective:** The aim of this study was to investigate CD56 and CD16 positive peripheral blood mononuclear cells (PBMCs) and leukocyte subsets from multi-drug resistant pulmonary tuberculosis (MDR-TB), and compare them with nonresistant (NR) TB patients and healthy controls. **Methods:** 13 MDR-tuberculosis patients, 20 NR-TB individuals and 40 healthy subjects were included. Peripheral blood mononuclear cells were double stained with fluorochrome conjugated antibodies against CD56 and CD16 cell surface markers. The phenotype of positive cells was then analyzed by flow cytometry and the percentages of $CD56^{+}$ $CD16^{+}$, $CD56^{-}$ $CD16^{+}$, $CD56^{dim}CD16^{+/-}$, and $CD56^{bright}CD16^{+/-}$ subsets were calculated. **Results:** There was a significant decline in the percentage of $CD56^{+}CD16^{+}$ lymphocytes in both MDR and NR-TB patients compared with healthy controls. We also observed lower proportions of $CD56^{dim/bright}CD16^{+}$ in addition to higher percentages of $CD56^{dim/bright}CD16^{-}$ subsets in all TB patients ($p \leq 0.05$). In MDR-TB, our findings demonstrated a distinct phenotypic feature with increased levels of $CD56^{bright}CD16^{-}$ in comparison with both NR-TB and healthy subjects. **Conclusion:** Considering the function of CD56/CD16 expressing cells in TB, we suggest that phenotypic characteristics of PBMCs in MDR-TB may correlate with their status of drug resistance and probably with their high mortality rates.

Keywords: CD16, CD56, MDR Tuberculosis

INTRODUCTION

M. tuberculosis is responsible for about 2-3 million deaths worldwide (1). Multidrug resistant (MDR) strains of *M. tuberculosis* especially in HIV infected patients are a growing problem in many countries (2). It is estimated that one third of the world population is infected by *M. tuberculosis*, but about 90% of the infected subjects never de velop the active disease indicating the ability of human immune responses to control the

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infection (3). Active tuberculosis is associated with prolonged suppression of *M. tuberculosis* specific immune responses and expansion of regulatory T cells leading to decreased T cell IFN-γ production (4). The causative agent survives and proliferates within macrophages. These cells can be activated by cytokines such as IFN-γ secreted by NK and T cells which kill the bacteria. The inflammatory environment induced in TB infection recruits other innate effector cells such as NK, macrophages and neutrophils leading to the establishment of acquired immunity (5). Thus, initial activation of innate mechanisms is essential for TB protection. NK cells mediate early protection against *M. tuberculosis* and a variety of intracellular pathogens (6). NK cells are capable of rapidly producing IFN-γ as well as lysing target cells in the absence of prior activation. The early production of IFN-γ by NK cells at inflammatory sites activates phagocytic cells and primes antigen-presenting cells for IL-12 production (7). IL-12 regulates human macrophages and shifts the adaptive immune responses towards T helper type 1 (Th1) mechanisms necessary for elimination of intracellular *M. tuberculosis* (8,9). It has been indicated that NK cell derived IFN-γ differentially regulates T-independent resistance and granulocyte function in AIDS patients or other CD4+ T cell compromised individuals with *M. tuberculosis* infection (10).

Although, the most widely used biomarker correlated with TB protection is the IFN-γ secretion, additional biomarkers including those from innate responses may also be considered.

CD16 molecules are involved in cytotoxic activities and are expressed on different cells such as NK, NKT, monocytes, macrophages and neutrophils. CD56 or neural cell adhesion molecules are expressed on the surface of neurons, glia, skeletal muscle and natural killer cells. They have a role in cell-cell adhesion and in homing into inflammation areas. Indeed, activated cells are able to migrate to sites of infection via intercellular adhesion molecules such as CD56. On the other hand, CD56 expressing cells have been described as a subset of regulatory T cells in response to intracellular pathogens. CD56 positive lymphocytes are also the major cells in immuno-surveillance and anti tumor responses (11,12). An unusual $CD56^-$ NK subset that is greatly expanded in HIV-viremic individuals is phenotypically and functionally examined. These NK cells are reported to have association with extremely poor cytotoxic functions and reduced secretion of certain cytokines (13).

NK cells are phenotypically characterized by $CD56^+$, $CD3^-$ and $CD16^+$ molecules. Based on the density of CD56 and CD16 (FCγR111) surface markers, two major functional subpopulations of NK cells are identified in humans. Approximately 90% of peripheral NK cells are low density CD56 ($CD56^{dim}$) and high density CD16 ($CD16^{bright}$), which are involved in Antibody Dependent Cell Mediated Cytotoxicity (ADCC) responses. A minor percentage of NK cells expressing high levels of CD56 ($CD56^{bright}$) produce IFN-γ and other cytokines with a low cytotoxic activity (11). $CD56^{bright}$ NK cells are abundant in human secondary lymphoid organs and inflammatory sites where they may interact with T cells and thereby contribute to the control of the disease activity (14).

Since NK cells are the most abundant CD56/CD16 positive cells in peripheral blood mononuclear cells, in this study these two markers were employed to determine the phenotypic status of NK cells. Due to the involvement of CD56 and CD16 molecules in protection against intracellular pathogens, the aim of this study was to investigate the phenotypic differences of lymphocytes from MDR-TB and NR-TB patients regarding

these two markers. Moreover, based on the reports indicating the implication of other leukocytes in TB infection (3,10,11), we compared the percentages of monocytes, neutrophils and total lymphocytes from MDR and NR patients and healthy controls. Determination of lymphocytes and NK subpopulations may be helpful to understand the condition of host immunity. These findings may also be useful to predict drug resistance in TB patients.

MATERIALS AND METHODS

Participants. Two groups of patients i.e. MDR-TB and NR-TB were selected from the Masih Daneshvari hospital according to their clinical and paraclinical findings. Patients consisted of 33 tuberculosis patients (aged 18-50 years) with positive bacilloscopy and radiological thorax alterations. They had at least one period of TB treatment, two positive sputum smear tests and a positive sputum culture. MDR-TB subjects (n=13) were resistant to isoniazid and rifampin. Twenty TB patients considered in this study were drug nonresistant. Forty blood donors aged 20 to 48 years were considered as healthy controls**.** Because of the BCG vaccination in Iran, all healthy blood donors were tuberculin reactive (PPD positive skin test). Study was approved by the ethical review board of the National Research Institute of Tuberculosis and Lung Disease (NRITLD). All subjects in this study showed negative serology test for HIV and gave informed consent to enter the study.

Sample Preparation. 5 ml of peripheral blood was collected in EDTA as anticoagulant. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll-hypaque density gradient centrifugation. PBMCs were washed once in RPMI 1640 supplemented with 5% FCS and once in phosphate buffered saline (PBS) plus 1% FCS at 4ºC. Cells were then resuspended in ice-cold staining buffer (PBS, 3% FCS) at 5×10^6 cells/ml. Cells were stained in aliquots of 100 μL with fluorochorome-conjugated monoclonal antibodies for 20 minutes. Anti-human CD16 Ab (VEP13) conjugated with phycoerythrin (Miltenyi Biotec, Germany) and monoclonal Ab to human CD56 antigen conjugated with fluorescein isothiocyanate (FITC) from Invitrogen (Canada) were applied for cell staining according to manufacturers' instructions. Cells were then washed once by centrifugation at 540 ×g and resuspended in 0.2 M PBS prior to flow cytometry.

Flow Cytometry. Data acquisition was carried out by a Partec flow cytometer. Lymphocytes were gated into the window named R1 on size versus granularity dot plots. Information on the 20000 events for each sample was stored. The phenotype of different cell subpopulations was determined based on CD56 and CD16 intensity and results were expressed as the percentage of cell subsets within the lymphocyte population. A flow cytometric pattern from one of the cases and the method for drawing the quadrants is illustrated in Figure 1. For each antigenic determinant, the percentage of positive cells stained above the negative control values was determined. The proportions of $CD56^{+}$ $CD16^{+,}$ $CD56^{dim}$ $CD16^{+/-}$ and $CD56^{bright}$ $CD16^{+/-}$ subsets were calculated using dot plots. As shown in figure 1, different gating was used for calculating the percentages of cell subsets according to the density of CD56 and CD16 as follows:

$CD56^{+}$ /$CD16^{+}$ = Q2, $CD56^{-}$ /$CD16^{-}$ = Q3, $CD56^{bright}$ /$CD16^{bright}$ = QC2, $CD56^{-}$ /$CD16^{bright}$ = QB4, $CD56^{bright}$ /$CD16^{-}$ = QA4, $CD56^{bright}$ /$CD16^{dim}$ = QA2-QC2, $CD56^{dim}$ /$CD16^{bright}$ = QC4-QB4, $CD56^{dim}$ /$CD16^{-}$ = QA3-Q3, $CD56^{dim}$ /$CD16^{dim}$ = QA4- Q4 – ($CD56^{dim}$ /$CD16^{bright}$), $CD56^{-}$ /$CD16^{dim}$ = QC3-QA3-($CD56^{dim}$ /$CD16^{dim}$), $CD56^{dim}$

/CD16$^{+/-}$ = (CD56dim /CD16^{bright}) + (CD56^{dim} /CD16^{dim}) + (CD56^{dim} /CD16^{-}), CD56 ^{bright} /CD16$^{+/-}$ = (CD56^{bright} /CD16^{bright}) + (CD56^{bright} /CD16^{dim}) + (CD56 ^{bright} /CD16^{-}).

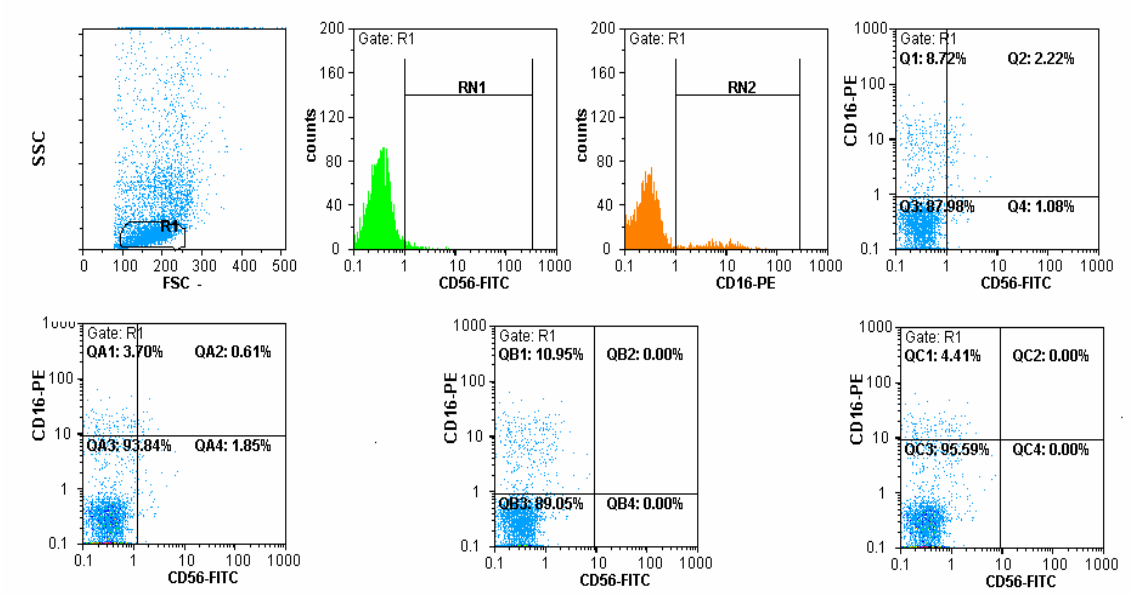

Figure 1. A sample of flow cytometric profile of PBMCs. Peripheral blood mononuclear cells were stained with CD56-FITC and CD16-PE mAbs. Cells were gated on lymphocytes for each experiment. Distinct cell subpopulations for CD56 and CD16 markers are calculated from percentages given in different quadrants.

Statistical Analysis. For all variables, mean and standard deviations were calculated. The statistical significance of the immunophenotypic differences observed between cell subsets was established using non-parametric Mann Whitney test. P values less than 0.05 were considered statistically significant.

RESULTS

Leukocytes analysis is shown in Table 1. As seen, the percentages of monocytes were elevated in both MDR and NR-TB patients but, neutrophils increased only in NR-TB group. Moreover, we found that, lymphocyte percentages from all TB patients depressed significantly in comparison with normal controls ($p \leq 0.05$).

Table 1. Differential leukocyte percentages from patients with multidrug-resistant and drug non-resistant tuberculosis, and from healthy controls.

	MDR-TB %	NR-TB %	Healthy control %
Neutrophils	59 ± `8	72 ± 10*	51 ± 7
Lymphocytes	19 ± 4*	16 ± 2*	34 ± 4
Monocytes	10 ± 1.9*	7.4 ± 1*	4 ± 1

*Statistically significant with respect to normal subjects. Data are expressed as mean ± SD of the percentages.

Data on CD56 and CD16 molecules obtained from flow cytometer were used to calculate the percentages of whole $CD56^+$ $CD16^+$ and also the proportion of each cell subset in gated lymphocyte population. We found that the percentages of $CD56^+CD16^+$ cells in MDR- and NR-TB patients were significantly lower than those in normal controls ($p \leq 0.05$). Furthermore, the proportion of $CD56^{bright}CD16^+$ and $CD56^{dim}CD16^+$ cells showed significant decreases in both MDR and NR patients in comparison with healthy controls. Conversely, $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^-$ cells showed higher percentages in patient groups compared to those in the healthy control ($p \leq 0.01$). Additionally, a significant rise was detected in $CD56^{bright}CD16^-$ cells of MDR-TB patients in comparison with NR-TB (3.0% vs. 0.4%). To highlight the significant differences among the results numerically listed in Table 1, the changes are represented in the form of bar graphs in Figure 2. Collectively, as demonstrated in Figure 2, we found that in both types of TB patients, all the $CD56^+CD16^+$ ($CD56^{dim/bright}CD16^+$) subsets were significantly lower, and all the $CD56^+CD16^-$ ($CD56^{dim/bright}CD16^-$) subsets were higher than those in normal controls ($p \leq 0.05$). Furthermore, the $CD56^-CD16^+$ subset showed higher percentages in TB patients compared with normal individuals ($p \leq 0.005$).

DISCUSSION

The mechanism of protective immunity, especially those related to innate responses against *M. tuberculosis* in humans, is not fully understood (3). During TB infection dendritic cells and macrophages secret pro-inflammatory cytokines which in turn recruit NK, neutrophil and other innate effector cells. Due to the involvement of CD56 and CD16 bearing cells in the production of cytokines and cytotoxic activities against TB, we have investigated the phenotypic differences of these cells in peripheral blood from MDR-TB, DR-TB and healthy controls (15). First, we found that all subsets of $CD56^+$ $CD16^+$ cells from TB patients had lower percentages compared to normal controls (Figure 2a). As the majority of NK cells in peripheral blood express CD56 and CD16 molecules, the suppression shown in $CD56^+CD16^+$ subsets could be attributed to NK cells. However, since we did not use the triple staining of PBMCs, CD3 positive cells were not excluded from $CD56^+CD16^+$ populations. Therefore, activated CD16 NKT, the other effector cells in TB may be included in $CD56^+CD16^+$ subsets. Thus, a decrease in CD16 positive cells is likely to indicate the suppression of cytotoxic activities of NK and NKT cells against TB infection. According to Yildiz (16), MDR-TB may result from either insufficiency of host cellular immune response or Mycobacterial mechanisms. Confirming the role of CD16 expressing cells, we found a significant decline in $CD56^{dim/bright}CD16^+$ in addition to the expansion of $CD56^{dim/bright}CD16^-$ lymphocytes in peripheral blood of TB patients in both MDR and NR-TB (Figures 2c, 2e, 2d and 2f). Interestingly, in the case of $CD56^{bright}CD16^-$ lymphocytes, MDR-TB patients showed higher percentages than NR patients (Figure 2f). Indeed, increase of all $CD56^+CD16^-$ subsets in peripheral blood of TB patients could confirm the lower cytotoxic activity, especially in MDR tuberculosis. Similar findings have been previously reported in TB and other diseases by other investigators (17-19).

Schierloh and colleagues reported that increased susceptibility to apoptosis of $CD56^{dim}CD16^+$ NK cells induces the enrichment of IFN-γ producing $CD56^{bright}$ cells (19).

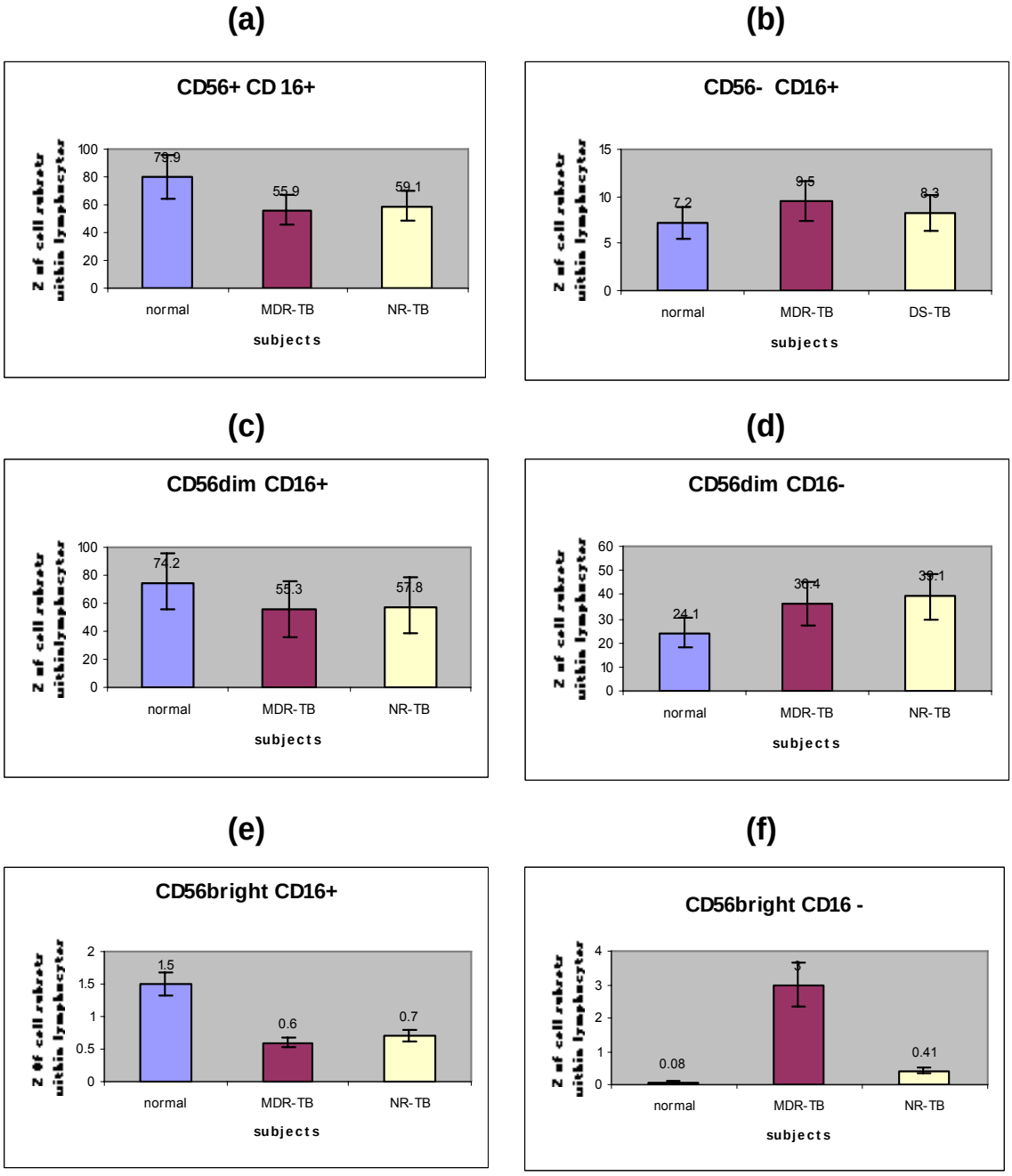

Figure 2. Peripheral blood mononuclear cells from 13 multidrug resistant tuberculosis (MDR-TB), 20 drug nonresistant (NR-TB) and 40 healthy subjects were stained with CD56-FITC and CD16-PE mAbs. Lymphocyte subsets were then determined by flow cytometry. Here, the results from those subsets with statistically significant differences ($p \leq 0.05$) are illustrated.

Accordingly, we found the suppression of $CD56^{dim}CD16^{+}$ and the expansion of $CD56^{bright}CD16^{-}$ cells especially in MDR patients. Hence, we suggest that higher percentage of $CD56^{bright}CD16^{-}$ subsets in peripheral blood of MDR-TB may be due to the less migration of these cells into the infection sites and to low local IFN-γ secretion. These findings could be considered as mechanisms involved in the onset of drug resistance in tuberculosis.

It is known that IFN-γ is a key mediator in providing protective immunity to *M. tuberculosis*, thus recombinant IFN-γ has been reported to activate human alveolar macrophages in vivo (20). Since the main route of entry of this organism is the respiratory system, alveolar macrophages are important cell types which combat this pathogen (21). Thus, we suggest that less migration of $CD56^{bright}$ cells into the lung of MDR-TB patients leads to a decrease in local IFN-γ and the suppression of anti tuberculosis immunity. Accordingly, Grahmann et al. have recently introduced a new protocol for IFN-γ therapy in MDR-TB patients (20).

It has been known that signalling via CD16 (FC-γR111) results in the production of cytokines and several chemokines, and causes degranulation of NK and NKT cells (5). Although the cytotoxic activity was not measured in our study, the reduction of CD16 subsets shown in this study is consistent with the work of others who reported decreases of NK cell activities in tuberculosis (22). Consequently, we conclude that TB patients may have a failure in whole cytotoxic activities of $CD56^+$ lymphocytes.

It has been previously described that $CD56^-CD16^+$ NK cells are functionally and phenotypically immature, but capable of maturation towards $CD56^+CD16^+$ cells (9). The elevation of this subpopulation in TB patients observed in our study (Figure 2b) is in agreement with this finding and also with a report by Veenstra et al. (18). In another study, the expansion of this NK subset from myasthenia gravis patients has been attributed to the deleterious NK cell functioning (23). However, we suggest that the differences of lymphocyte subpopulations in TB patients are likely secondary to TB infection. But, those changes which are specifically seen in MDR patients may be attributed to other reasons.

Considering previous reports on leukocytes which indicated an increase of granulocytes and granulocyte/lymphocyte ratios in TB patients (24), we compared these cells in our study groups. It is known that monocytes would play a regulatory role in the NK cell activity not only by the cytokines, but also through a signalling process mediated by costimulatory molecules (9). According to previous studies monocytes from TB patients regulate the NK function through an indirect mechanism leading to down regulation of costimulatory/adhesion molecules and/or IFN-γ production (6). In this study, we observed a significant elevation of monocytes especially in MDR-TB patients which may cause a decrease in IFN-γ production by NK cells. Moreover, it has been reported that neutrophils can mediate antimicrobial activity against *M. tuberculosis* by delivering antimicrobial peptides to macrophages. Indeed, macrophages have been reported to acquire neutrophil granules for antimicrobial activity against intracellular pathogens (25). Accordingly, we found a significant reduction in neutrophils from MDR-TB patients but not from NR-TB ones compared to healthy subjects. In addition, total blood lymphocytes were suppressed in both MDR and NR-TB patients. We conclude that these changes in leukocytes may cause the adaptive immune response insufficient especially in MDR-TB patients. Taken together, the characteristic patterns in CD56/CD16 subsets and leukocyte changes seen in MDR-TB patients may be considered as an additional cause of disease severity and high mortalities in this group of patients. However, further specific studies on CD56 lymphocyte subsets and their function using expanded sample sizes are recommended to confirm these suggestions.

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