Intracellular granzyme A expression of peripheral blood lymphocyte subsets in pulmonary tuberculosis

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Cell-mediated immunity is a key weapon of host defence against tuberculosis (TB). Granzyme A (GzmA), a serine protease, present in the granules of cytotoxic cells induces caspase-independent cell death. We estimated the proportion of GzmA producing lymphocyte subsets in peripheral blood from 59 normal healthy volunteers and 48 pulmonary TB (PTB) patients using flow cytometry. When compared with normal healthy subjects, we observed a significantly higher percentage of GzmA-positive CD56$^+$ cells ($P = 0.01$) in PTB patients. However, when the absolute number was compared between the two groups, a significantly decreased number of GzmA-expressing CD16$^+$ ($P = 0.01$) and CD56$^+$ ($P = 0.0001$) cells was observed in patients and this could be explained by the significantly reduced number of total lymphocytes ($P = 0.0009$) seen in the patients. There was no significant difference in the number of CD4$^+$ and CD8$^+$ GzmA double-positive cells between the two study groups. CD56 is a natural killer cell marker and these cells represent innate immune response to TB. We report an increased percentage of CD56$^+$ cells expressing GzmA in TB patients, which shows the relevance of the GzmA-mediated pathway of apoptosis in immunity against *Mycobacterium tuberculosis*.

Keywords: Cytotoxic cells, granzyme A, peripheral blood, pulmonary tuberculosis.

TUBERCULOSIS (TB) remains a serious global health threat despite effective treatment strategies. *Mycobacterium tuberculosis* (MTB), an intracellular pathogen has the ability to persist in the host by successfully evading the defence mechanisms. A coordinated immune response involving innate and cell-mediated immunity is required to control the infection. Eradication of the pathogen requires lysis of the infected cells by cytotoxic T lymphocytes (CTL) and natural killer cells (NK cells). Recent studies with MTB-reactive CD8 T-cells have shown that cytolytic function was associated with inhibition of intracellular MTB growth[1]. The granule exocytosis pathway is an important mechanism by which the cytotoxic cells cause target cell lysis and death. The CTL granules contain a pore-forming protein called perforin, and a group of serine proteases called granzymes in a proteoglycan matrix[2]. Granzymes enter the target cell with the help of perforin and accumulate in the nucleus, where they can activate pathways of nuclear damage[3]. Granzyme B causes caspase-dependent cell death, whereas granzyme A (GzmA) mediates caspase-independent cell death[4].

GzmA causes DNA damage by introducing single-stranded nicks in the DNA[5]. It destroys the lamins and opens up the DNA for degradation by targeting the histones[6]. GzmA causes irreversible damage to the target cell by cleaving the SET complex, which is involved in the repair response to oxidative stress[7]. Cells that are resistant to caspase-mediated cell death are sensitive to the apoptotic effects of GzmA[5]. Studies have also shown that GzmA signals monocytes to have enhanced phagocytic activity and to produce elevated levels of IL-6, IL-8 and TNF-α, which suggests that granzymes may have important regulatory roles in cell mediated immunity[8].

The aim of our study was to examine the proportions of peripheral blood lymphocyte subsets expressing GzmA in normal healthy subjects (NHS) and pulmonary tuberculosis (PTB) patients, which could reflect the *in vivo* situation in TB.

The study group included 48 PTB patients (mean age $\pm$ SD is 35.5 $\pm$ 10.6) and 59 NHS (mean age $\pm$ SD is 30 $\pm$ 8). Patients attending Tuberculosis Research Centre (TRC), Chennai with respiratory symptoms and radiographic abnormalities suggestive of PTB and sputum-positive for MTB by both smear and culture were included for the study. All the patients were HIV-negative. Among the 48 patients, 32 were males (mean age $\pm$ SD is 35.8 $\pm$ 11) and 16 females (mean age $\pm$ SD is 34.8 $\pm$ 10). NHS were volunteers who were clinically normal at the time of blood collection and the NHS group comprised 41 males (mean age $\pm$ SD is 31.3 $\pm$ 8.7) and 18 females (mean age $\pm$ SD is 25.8 $\pm$ 5). Blood samples were collected after obtaining informed consent and before starting chemotherapy. The study has been approved by the ethical committee of TRC.

About 1 ml of blood was heparinized and used for intracellular and surface-staining by flow cytometry and hematological profile using electron profile counter (ABX micros CE Haematalgie, France). Flow cytometric detection of intracellular GzmA in whole blood was performed based on a method described earlier[9]. Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, CD8 and CD16 antibodies and phycoerythrin (PE)-conjugated anti-CD56 antibody (Becton Dickinson, California, USA) were used for surface-staining followed by lysis of red blood cells (RBC) with RBC lysis solution (Becton Dickinson). Leucocytes were fixed in 1% para formaldehyde (PFA) for 10 min followed by permeabilization with 0.1% saponin in phosphate buffered saline (PBS). Intracellular staining was performed by incubating the cells with PE or FITC-conjugated anti-GzmA antibody (Becton Dickinson) at 4°C for 30 min. Stained cells were washed twice in PBS,

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fixed with 1% PFA and acquired in Becton Dickinson FACSort flow cytometer. Using scatter pattern, 10,000 events were counted in the lymphogated region and analysed using Cell Quest Pro software. Results were expressed as arithmetic mean ± standard error (SE) and analysed using Student's t test to examine the significance of results between the two study groups. P value less than 0.05 was considered statistically significant.

Dot-plot pictures showing scatter pattern of GzmA-positive cells and their immunophenotypes from a NHS and a PTB patient are shown in Figure 1. The proportions of CD4^{+}, CD8^{+}, CD16^{+} and CD56^{+} cells expressing GzmA were compared between the study groups. When compared with NHS, there was a significant increase in the percentage of CD56^{+} GzmA-positive cells in PTB patients ($P = 0.01$). The percentage of CD4^{+} and CD8^{+} GzmA-positive cells was slightly higher in PTB patients, but the difference was not significant. In contrast, the percentage of CD16^{+} GzmA-positive cells was slightly decreased in PTB patients (Figure 2 a).

The absolute number of each subset was calculated from the total lymphocyte count. When we compared the absolute numbers between the two groups, a significantly decreased number of CD16^{+} ($P = 0.01$) and CD56^{+} ($P = 0.0001$) cell population, which stained positive for GzmA, was observed in PTB patients. This may be due to a significant reduction in the number of total lymphocytes ($P = 0.0009$) in PTB. There was no significant difference in the number of CD4^{+} and CD8^{+} GzmA double-positive cells between the two study groups (Figure 2 b).

Host defence against TB involves NK cells and CTLs. Recent studies have revealed that the CTLs deliver highly microbicidal proteins into infected macrophages, thereby killing the virulent pathogen, and lyse the target macrophages by granule-mediated perforin mechanism of apoptosis which co-delivers bactericidal proteins[10]. One such granule-associated protein is GzmA, which causes cell death by introducing single-stranded DNA nicks. Most circulating CD56^{+} NK cells, almost half of CD8^{+} T-cells and a few CD4^{+} T-cells express GzmA[9]. GzmA expression is constitutive in many NK cells and continues to be expressed long after T-cell activation[11].

Considering these facts, we sought to study the expression of GzmA in lymphocyte subsets of peripheral blood from NHS and PTB patients. We observed a significantly increased percentage of GzmA-expressing CD56^{+} cells in PTB patients. The difference was not significant for other cell subsets. However, due to a general decrease in the number of lymphocytes in TB, the number of CD56^{+} GzmA-positive cells was low in the peripheral blood of patients. Such a decreased total lymphocyte number in PTB patients was reported in an earlier study from our laboratory, where we have shown an increased percentage of CD8^{+} cells positive for perforin in PTB patients[12]. A similar finding has been reported in a study by Carvalho et al., where they have observed that HIV-seronegative
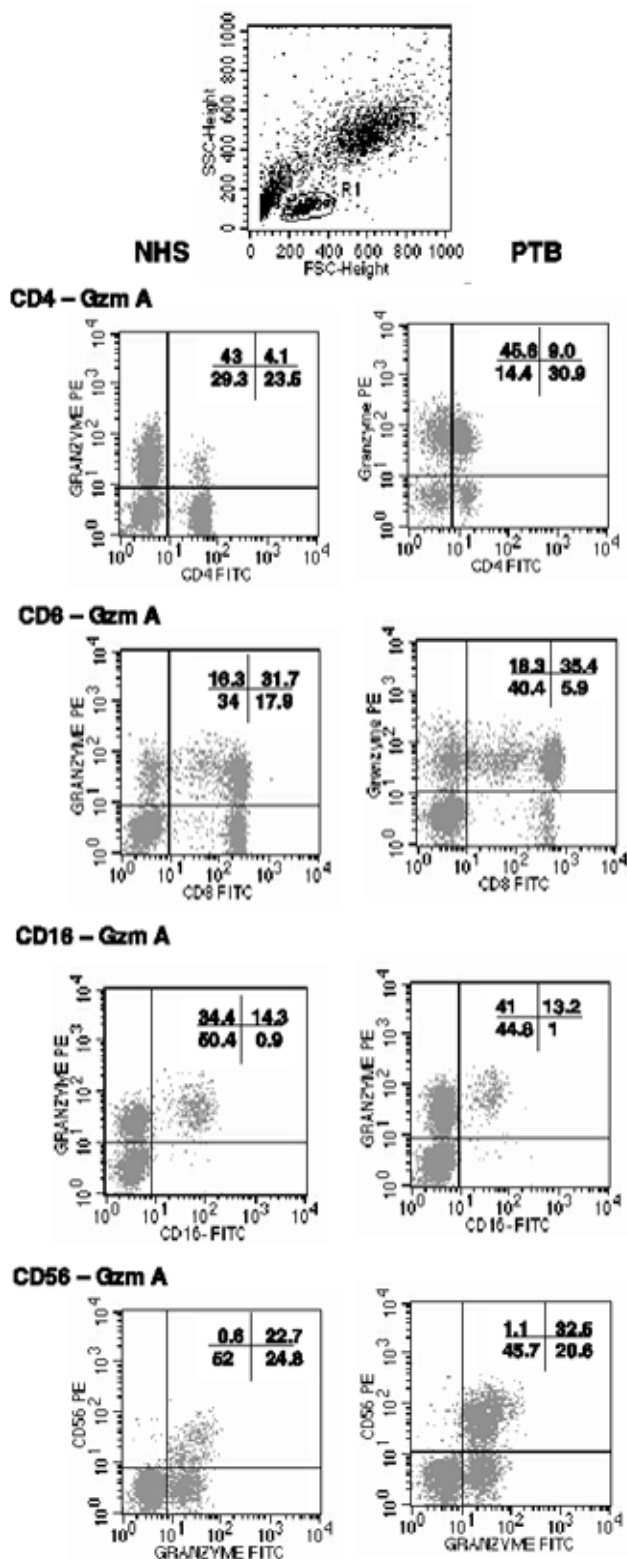

Figure 1. Dot-plot pictures showing scatter pattern of peripheral blood cells with lymphogate and intracellular GzmA staining in CD4^{+}, CD8^{+}, CD16^{+} and CD56^{+} cells in NHS and PTB patients. PE–anti-GzmA antibody was used with CD4, CD8 and CD16 staining and FITC–anti-GzmA antibody was used with CD56 staining. Numbers correspond to the percentage of gated cells in each quadrant and the results shown are representative data from an individual NHS and PTB patient sample.
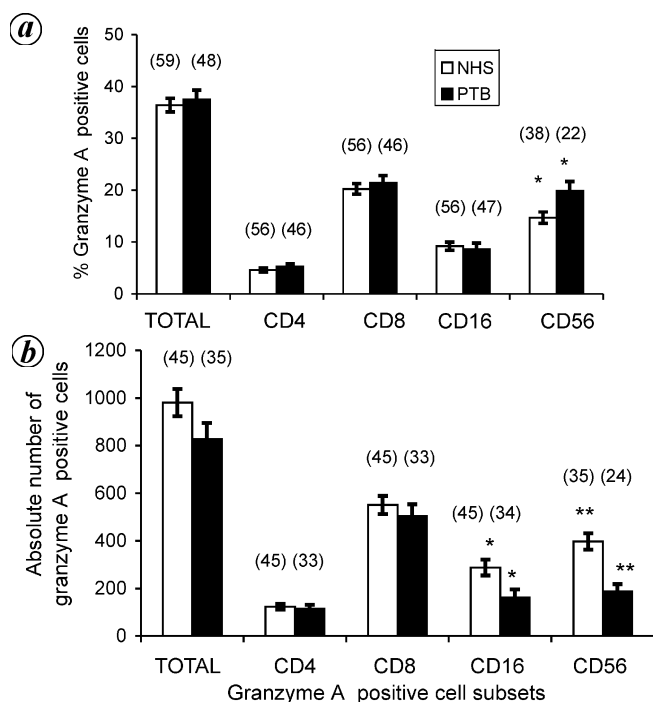
Figure 2. *a*, Immunophenotypic classification of GzmA-positive cells in peripheral blood lymphocytes of NHS and PTB patients. The percentage of total lymphocytes, CD4^{+}, CD8^{+}, CD16^{+} and CD56^{+} cells positive for GzmA in NHS and PTB patients is shown. The percentage of CD56^{+} GzmA double-positive cells was significantly high in PTB patients ($P = 0.01$) when compared with NHS. *b*, Absolute numbers of GzmA-positive total lymphocytes, CD4^{+}, CD8^{+}, CD16^{+} and CD56^{+} cells in NHS and PTB patients. In PTB patients, there was a significant decrease in the absolute number of CD16^{+} GzmA double-positive cells ($P = 0.01$) and CD56^{+} GzmA double-positive cells ($P = 0.0001$). Results are expressed as mean ± SE. Figures in parentheses represent the number of subjects studied.

TB patients had lower total lymphocyte and CD4^{+} proportions than healthy blood donors[13]. However, the percentage of CD56^{+} cells positive for GzmA is significantly higher in these patients, which is a consequence of the infection. This may be because of the increased activation and proliferation of these cells upon stimulation by the mycobacterial antigens. The surface-marker CD56 is present on NK cells, which mediate their action via cytolysis and production of Th1 cytokines. NK cells are also essential for the generation of human CD8^{+} allogenic CTL responses and this effect is mediated in part through CD56 (ref. 14). In the present study, an increased expression of GzmA in the CD56^{+} cells of PTB patients was observed. This could be due to an increased activation of these cytotoxic cells during infection or an increased production of GzmA in these cells in response to an external signal. GzmA causes apoptosis of target cells, a way to eliminate bacilli in the infected cells. In a study by Sada Ovalle *et al.*, CD8 cells with the NK cell marker CD57 obtained from PTB patients had a high expression of intracellular perforin and GzmA[15]. These cells exhibited a significant spontaneous cytotoxic activity upon autologous monocytes in the absence of antigenic stimulus. Our study reports an increased percentage of GzmA-expressing NK cells in PTB patients, suggesting the relevance of GzmA-mediated apoptotic pathway in the defence against MTB. These results indicate an important role for GzmA in the host immune response to TB, which needs to be confirmed with more functional studies.

To conclude, we report an increased percentage of GzmA-expressing CD56 cells in PTB, which supports the role played by these cytotoxic cells in the immune response mounted against the pathogen. Functional studies about the role of GzmA in infectious diseases could provide more knowledge in this aspect.

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