Original Article

INCREASED ACTIVATION AND EXPANSION OF A CD57+ SUBSET WITHIN PERIPHERAL CD8+ T LYMPHOCYTES IN *MYCOBACTERIUM TUBERCULOSIS*-INFECTED PATIENTS

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Background: ***Mycobacterium tuberculosis*-specific CD8+ and CD4+ T lymphocyte responses restrict the spread of extracellular pathogens by limiting *M.tuberculosis* replication. Alterations in cytolytic function, inappropriate maturation/differentiation, and limited proliferation could reduce their ability to control *M.tuberculosis* replication.**

Methods: In an attempt to further characterize the immune responses during *M.tuberculosis* infection, we enumerated γδ and αβ receptor-bearing T cells expressing CD8 or CD4 phenotype and analyzed the differentiation phenotypes of CD8+ and CD4+ T lymphocyte subpopulations in 47 cases (23 new cases and 24 multidrug resistant patients) and 20 control subjects, using flowcytometry.

Results: We found that the CD4/CD8 ratio was significantly lower in newly-diagnosed *M.tuberculosis* patients compared to multidrug resistant and control subjects ($P < 0.003$). Also, we found that a large proportion of CD8+ T lymphocytes in newly-diagnosed patients was defined by increased surface expression of CD57 as compared to the two other settings ($P < 0.002$). This increase was more profound in patients with an inverted CD4/CD8 ratio. Analysis of the late activation antigen revealed that this was predominantly HLA-DR+ ($P < 0.003$). No significant changes were observed in the percentages of CD8+CD57+ T cells between the different settings. Moreover, the co-stimulatory molecule CD28+ tended to be underexpressed by CD8+ T cells in multidrug resistant patients when compared to newly-diagnosed subjects ($P < 0.002$), but not to the control subjects. In contrast, the frequency of CD28+ marker on CD4+ T cells was higher in the setting of multidrug resistant compared with those of new cases ($P < 0.0001$). No significant changes were observed in percentages of γδ receptor-bearing T cells between different groups.

Conclusion: We suggest that the increase in the proportion of CD57+ within CD8+ T cells in newly-diagnosed patients results from *M.tuberculosis* antigenic stimulation, which is a hallmark of many infections and that the protracted accumulation of CD57+ T lymphocytes might reflect an end-stage differentiation phenotype.

Archives of Iranian Medicine, Volume 9, Number 1, 2006: 53 – 57.

Keywords: CD4+CD28+ T cell • CD8+CD57+ T cell • *Mycobacterium tuberculosis*

Introduction

Phagocytosed *Mycobacterium tuberculosis* either replicate within the endocytic compartment of mononuclear phagocytes or they are destroyed by the host immune cells.[1] Protection against *Mycobacterium* depends on a complex interaction between the host cells and the pathogen. Clinical outcomes of pulmonary tuberculosis are variable, ranging from asymptomatic lifelong infection to parenchymal lung destruction, resulting in cavitary lesions. A role for gamma/delta receptor-bearing T cells and αβ receptor-bearing T lymphocytes expressing CD4 or CD8 phenotype in an adaptive immune response to infection with *M. tuberculosis* has been suggested by several lines of evidence.[2, 3] Furthermore, several studies of various infections

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Accepted for publication: 18 April 2005

and diseases have indicated that chronic antigenic stimulation has been associated with peripheral expansion of CD8+ T lymphocytes characterized by CD57 expression and down-regulation of CD27 expression.[4, 5] In addition, reports have shown that CD8+CD57+ T cells are efficient producers of a variety of cytokine.[6, 7] In a recent study, it was shown that the increased expression of CD57 on antigen-specific CD8+ T cells defines replicative senescence and antigen-induced apoptotic death of CD8+ T lymphocytes.[8] Additionally, in the presence of decreased levels of CD4 cells, elevation of the CD8+CD57+ subpopulation has been associated with a combination of preacquired immune deficiency syndrome known as AIDS-related complex (ARC).[9, 10]

The CD28 antigen is present on approximately 60% – 80% of CD3+ T lymphocytes. Both CD4+ and CD8+ T lymphocyte subsets can be separated into two functionally distinct subsets based on CD28 antigen expression density. These subsets differ in their cytotoxic and lymphokine-production capabilities. For instance, CD4+ lymphocytes that express high levels of the CD28 antigen lack CD3-mediated cytotoxic ability and produce only minimal amounts of cytokines. On the other hand, CD4+ lymphocytes that express low levels of the CD28 antigen possess strong CD3-mediated cytotoxicity and produce IL-2, IFN-γ, and TNF-α/β.[11, 12] Expression of the CD28 antigen also divides the CD8+ lymphocyte subset into two functional groups. CD8+CD28+ T lymphocytes tend to have long-term proliferative capacity *in vitro* and *in vivo*, while CD8+CD28- lymphocytes are thought to have effector function with limited proliferative capacity and shortened telomeres.[13, 14] Because, CD8+ and CD4+ T cell subsets function, differentiation, and maturation are critical to immune-mediated control of many infections, they are often impaired in many infectious diseases.[15, 16] Taken together, we, therefore, examined the phenotype of the T cell subpopulation subsets in the peripheral blood of *M. tuberculosis* patients, with a focus on CD57, co-stimulatory molecule (CD28), and the late activation marker expression within CD8+ T lymphocytes.

Materials and Methods

Study population

The National Research Institute of Tuberculosis and Lung Disease Review Board of Research Associates approved the use of human subjects for this study. We enrolled 47 subjects with pulmonary tuberculosis (23 newly diagnosed and 24 multidrug resistant [MDR]) and 20 control subjects. Screening included history and physical examination, complete blood count and chemistries, HIV testing, a chest X-ray, and three sputum acid-fast bacilli smears. Sputum smear-positive individuals with symptoms and radiographs consistent with pulmonary tuberculosis, Karnofsky score > 50%, and without prior tuberculosis treatment (in the case of newly-diagnosed patients) were enrolled.

Sample preparation and flowcytometric analysis

Whole blood samples (2 mL) were obtained by venipuncture in a sterile blood collection tube. Flowcytometric analysis was performed using a FACSCalibur flowcytometry (Becton-Dickinson, Mountain View, Ca, USA). Pairs of monoclonal antibodies (FITC/PE conjugated), CD45/CD14, IgG1/IgG2a, CD3/CD19, CD3/CD4, CD3/CD8, CD3/CD16+56, CD3/gamma-delta, CD8/HLA-DR, CD8+/CD57+, CD8/CD28, and CD4+/CD28+ (all from Becton-Dickinson) were used. Twenty μL of an appropriate monoclonal antibody were mixed with 100 μL of patient's sample and incubated for 20 minutes at room temperature. Then 2 mL of BD FACS lysing solution were added, followed by centrifugation for 5 minutes. The cells were then washed with PBS and immediately subjected to flowcytometric analysis using Simultest software.

Statistical analysis

The results were analyzed using Pearson's regression model. For each subset of lymphocytes, the data were expressed as the mean ± standard error of mean (SEM). The reproducibility of each subset was determined using Pearson's correlation coefficients. The differences of the mean were calculated by the Student's *t*-test, using SPSS analysis with a P value of < 0.05 regarded as significant.

Results

Table 1 presents the phenotypic analysis of lymphocyte subpopulations, expressed as percentages of cells in each of the subpopulations. The expression of CD57+ (subset marker), CD57–,

Table 1. Percentages of lymphocyte subpopulation subsets in *M.tuberculosis*-infected and in control subjects (results are expressed as mean ± SEM).

Lymphocyte population	Control	Newly diagnosed	MDR
CD3+	70.62 ± 7.29	74.16 ± 4.89	75.41 ± 3.96
CD3+/CD4+	42.11 ± 6.95	33.90 ± 4.5	40.33 ± 3.44
CD3+/CD8+	28.41 ± 8.58	45.9 ± 5.67	38.5 ± 3.98
CD4/CD8	1.61 ± 0.6	0.85 ± 0.05	1.37 ± 0.1
CD3+/γδ-T cell	10.1 ± 2.36	11.9 ± 1.28	13.8 ± 2.19
CD3+/HLA-DR+	8.56 ± 2.31	30.81 ± 6.87	14.83 ± 4.58
CD8+/CD28+	15.6 ± 1.21	21.8 ± 8.5	13.6 ± 2.65
CD8+/CD57+	20.1 ± 2.65	35.1 ± 7.63	14.85 ± 3.2
CD8-/CD57+	6.85 ± 2.53	7.0 ± 1.24	6.71 ± 1.39
CD4+/CD28+	54.2 ± 1.23	26.36 ± 5.34	51.2 ± 6.5

CD28+, and HLA-DR within CD8+ were studied. Most notably, newly-diagnosed *M.tuberculosis* patients had lower CD4/CD8 ratios and harbored significantly higher percentages of CD8+ and HLA-DR+ T lymphocytes than the MDR *M.tuberculosis* patients and control subjects ($P < 0.003$). Concordantly, significantly greater numbers of CD57+ expressing T cells were found within peripheral CD8+ T cells than the controls and MDR subjects ($P < 0.002$). This was more profound in newly-diagnosed patients with low CD4/CD8 ratios. Furthermore, CD28+ was expressed by higher percentages of peripheral blood CD8+ T lymphocyte subsets in newly-diagnosed patients than in MDR subjects ($P < 0.003$). On the contrary, CD4+ T lymphocytes of MDR patients expressed significantly higher levels of CD28 antigen as compared to newly-diagnosed *M.tuberculosis* patients ($P < 0.0001$). No significant shift in the relative proportions of CD3, CD19, CD16+56 (data not shown), or CD8-CD57+ was detectable amongst the different study groups. Moreover, no significant differences in the relative numbers of γδ receptor-bearing T cells was noticed amongst the different study groups.

Discussion

The immune response against infections with *M. tuberculosis* has been studied *in vitro* and *in vivo*, but the exact mechanisms by which *M. tuberculosis* infections in humans are controlled by the cellular immune system are not clearly understood.

The data presented here indicate that, first, elevated numbers of CD8+ T lymphocytes were observed in the peripheral blood of patients with newly-diagnosed *Mycobacterium* infection when compared to MDR and control subjects. Secondly, in newly-diagnosed patients, the analysis of their late activation antigen (HLA-DR) revealed that they were predominantly HLA-DR+. The newly-diagnosed patients invariably had inverted CD4/CD8 ratios. These results are in agreement with the reports published previously.[17 – 19]

The analysis of phenotypic expansion of CD8+ and CD4+ T cells has become a widely used tool for studies of immune responses in various infectious and pathological conditions.[20, 21] In our study, flowcytometric immunopheotyping revealed significant increases in CD57+ subsets among the circulating blood CD8+ T lymphocyte subpopulation of untreated *M.tuberculosis* infected patients (newly-diagnosed) when compared to MDR and control subjects. This phenomenon showed an inverse relationship between the percentages of CD8+/CD57+ cells and CD4/CD8 ratio. While in agreement with several reports, this subset has been shown to increase in various immunopathological situations such as cytomegalovirus infection, HIV infection, alveolar echinococcosis, autoimmune diseases, IgA deficiency, multiple myeloma, chronic lymphocytic leukemia, and pulmonary sarcoidosis. [5, 8, 21 – 27] These authors concluded that CD57+ T cells delineate a subpopulation of CD8+ T cells that defines replicative senescence and, hence, antigen-induced apoptosis of CD8+ T cells. For instance, it has been reported that CD57+CD8+ lymphocytes are efficient producers of cytokines,[7, 15] and that they are capable of down-modulating cytotoxic activity.[28] Further, it has been demonstrated that CD57+CD8+ T cells can respond to CMV *in vitro* and exert virus-specific cytotoxicity.[23] Additionally, expansion of CD8+CD28+CD57+ has been demonstrated early in CMV infection[29] which, in part, supports our results with newly-diagnosed *M. tuberculosis* cases.

Lack of CD28 expression on peripheral T lymphocytes is an infrequent finding in healthy humans as CD28-expressing T cells are able to produce a variety of cytokines including IL-2.[29] In

this study, we found that CD28+ was underexpressed by MDR patients' peripheral CD8+ T lymphocytes when compared to newly-diagnosed patients. Conversely, the CD28 antigen was overexpressed by MDR patients' CD4+ T cells when compared to newly-diagnosed *M.tuberculosis* patients. Further, it has been proposed that the reduced expression of CD28 is associated with replicative senescence; a state reached by the T lymphocytes after multiple cell divisions and characterized by shortened telomeres and diminished or altered function.[30] A reduced expression of CD28, indicating T cell senescence, has been reported in the peripheral blood of patients with rheumatoid arthritis.[31] Our finding of a tendency towards reduced expression of CD28 on CD8 T cells in the peripheral blood of MDR patients compared with those of newly-diagnosed cases suggests a local *Mycobacterium* antigen stimulation of T cells, leading to generation of functionally impaired and/or functionally altered end-stage T cells. Moreover, the overexpression of the CD28 antigen on peripheral blood CD4+ T cells of MDR patients raises a question about whether CD4+CD28+ T cell subsets, which do not produce macrophage activating cytokines (e.g. TNF-α/β, IFN-γ) may not be recruited to the site of infection. Therefore, a disturbed antituberculosis immune response as a result of skewed differentiation, maturation, and/or impaired recruitment of functionally active CD8+ and CD4+ T lymphocytes may be proposed as a consequence.

Finally, we found statistically no significant differences in the relative proportion of gamma/delta receptor-bearing T lymphocytes between different study groups, although an antiinflammatory role and a high activation status for these cells have been suggested during *M.tuberculosis* infection.[2, 3, 32, 33]

In conclusion, increases in subpopulation subset within either CD4+ or CD8+ in infectious disease is a marker of an ongoing or previous immune response, but the nature of *M. tuberculosis* antigen(s) driving the evolution of CD57+CD8+ and CD4+CD28+ T lymphocytes in the two settings is obscure and needs to be investigated.

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