

## Original Articles

### T-LYMPHOCYTE SUBPOPULATIONS IN TUBERCULOSIS

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**Background:** Tuberculosis is associated with both qualitative and quantitative defects in the cell mediated immune response. The changes that occur in the lymphocyte profile in blood in children with tuberculosis are not well understood. **Design:** Prospective study. **Setting:** Referral hospitals. **Methods:** Lymphocyte subpopulations were determined by flow cytometry in 17 healthy tuberculin positive children, in 22 children with newly diagnosed pulmonary tuberculosis and in 8 of these children after antituberculosis therapy, **Results:** Absolute numbers and percentages of CD3+ and CD4+ T cells were reduced in children with tuberculosis, compared to controls. CD4+ counts increased significantly following antituberculosis treatment, compared to baseline values. In contrast, the proportion of T cells expressing the  $\alpha\beta$  T cell receptor was similar in tuberculosis patients and controls. **Conclusion:** Children with tuberculosis have a systemic decrease in the proportion and number of CD3+ and CD4+ T cells which reverses during therapy.

**Key words:** Immune response, Lymphocyte subsets, T-cells, Tuberculosis.

**B**ECAUSE cell mediated immunity plays a central role in human defenses against mycobacteria, the manifestations and clinical outcome of human infection with *Mycobacterium tuberculosis* are believed to depend on the efficacy of the cell mediated immune response. T-lymphocytes contribute to immunity against mycobacteria by recognizing foreign protein via antigen receptors and subsequently releasing immuno-regulatory cytokines. Protective immunity against tuberculosis is believed to be mediated primarily by CD4+ T-cells, particularly Th1-cells that produce interferon- $\gamma$  (1,2). Children who lack the interferon-gamma receptor succumb to

disseminated mycobacterial disease(3,4) and mice that do not express interferon-gamma because of gene disruption are exquisitely susceptible to tuberculosis(5). CD8+ cytotoxic T-cells and T-cells expressing the  $\gamma\delta$  T cell receptor probably also contribute to protective immunity against tuberculosis(6,7).

Prior studies of the distribution of T-cell populations in tuberculosis patients have evaluated adults(8-12). Because most adults were infected in the distant past, the distribution of T-cell subpopulations during the initial response to infection cannot be assessed. In addition, many adult tuberculosis patients have coexistent illnesses, e.g., diabetes or alcoholism

which may affect T-cell subpopulations. The study of tuberculosis in children provides a unique opportunity to evaluate T-cell subpopulations during the initial immune response to infection with *Mycobacterium tuberculosis*. To investigate this question, we measured T-lymphocyte subpopulations in children with pulmonary tuberculosis and in age matched healthy tuberculin positive controls.

### Subjects and Methods

**Patients:** Children attending the Outpatient Department of the Institute of Social Pediatrics, Government Stanley Hospital, Chennai and the Institute of Child Health, Chennai were considered for this study which was conducted between July 1996 and February 1997. For children aged 1-12 years old who had signs and symptoms suggestive of tuberculosis (persistent fever, cough, failure to thrive or loss of weight), a Chest X-ray was performed and a Mantoux test with PPD-RT23 1 TU was placed and read after 48-72 hours. Gastric lavage was done on an empty stomach on 2 consecutive mornings and examined for AFB smear and culture for *M. tuberculosis*. All patients who were diagnosed to have tuberculosis during this period were eligible for the study. Tuberculosis was diagnosed in 39 children during this period of whom 22 were willing for participation (blood collection, follow up, etc.) and were enrolled in the study. The diagnosis was culture proven in 17 cases and was based on clinical and radiographic features in 5 cases (X-ray abnormalities persisting after 2 weeks of broad spectrum antibiotics, household exposure to tuberculosis and positive tuberculin skin test results). All patients were negative for HIV by ELISA.

Patients were treated with standard short course anti-tuberculosis regimens (2 months of Isoniazid, Rifampicin, and Pyrazinamide daily followed by Isoniazid and Rifampicin for 4

months). After completion of therapy, blood samples were obtained from the eight children who were available for follow-up, all of whom had an excellent response to therapy.

We selected 17 healthy tuberculin-positive children as controls including children attending the hospital for minor complaints and siblings of the study patients who were found not to have tuberculosis. The controls selected were matched for socioeconomic strata and age group to the study patients. Venous blood was obtained from tuberculosis patients and controls in heparinized tubes and sent to the laboratory within 1 hour for analysis. Blood was obtained from patients after they had received less than one week of antituberculosis therapy. The study was approved by the Institutional Review Board of the Tuberculosis Research Center and informed consent was obtained from the parents of the study participants.

### *Estimation of T-Lymphocyte Subpopulations*

The total and differential white cell counts were performed on whole blood by standard techniques. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were washed and suspended in RPMI-1640 medium with 10% Fetal Calf Serum. 100  $\mu$ l aliquots containing  $2 \times 10^5$  cells were incubated with the monoclonal antibodies for 30 minutes in the dark at 4°C washed twice with Phosphate Buffered Saline and fixed with 1% paraformaldehyde. Samples were analyzed with a FACSort flow cytometer (Becton Dickinson, CA, USA) using Cell Quest analysis software. A gate was set around the lymphocyte population based on the forward and side scatter characteristics of unstained cells. Quadrant markers were set using fluorescein isothiocyanate-conjugated (FITC) or phycoerythrin-conjugated (PE) mouse IgG (negative) isotype control antibodies. Single immunolabeling was performed with the Pan T-cell

marker FITC-CD3 and the subtype markers FITC-CD4 and PE-CD8. Double immunolabeling was performed with FITC-CD3 and PE-Pan TCR  $\gamma\delta$ , PE-V  $\delta 1$  or PE-V  $\delta 2$ . Data are expressed as per cent of total lymphocytes  $\pm$  standard error of the mean (SEM) and as absolute cell numbers/ml.

### Statistical Analysis

The absolute counts of the various lymphocyte subsets was calculated by multiplying the percentage of that subset by the total lymphocyte count. Differences between groups were compared by the Student's t-test or paired t-test as appropriate. The statistical package associated with Microsoft Excel program was used.

### Results

The mean age of the tuberculosis patients was  $5.8 \pm 3.0$  (SD) years, the mean weight was  $15.9 \pm 1.4$  kg and the mean tuberculin skin test size was  $14.4 \pm 6.6$  mm. All 22 patients had pulmonary tuberculosis and one had disseminated disease in addition. Ten patients were malnourished, defined as  $<75\%$  of their predicted body weight for age. The mean age of the controls (healthy tuberculin reactors) was  $7.1 \pm 3.2$  years ( $p = 0.2$ ), the mean weight was  $17.2 \pm 1.3$  kg ( $p = 0.5$ ) and the mean tuberculin skin test reaction was  $16.4 \pm 5.9$  mm ( $p = 0.2$ ).

Nine healthy tuberculin reactors were malnourished.

The percentage of CD3 + and CD4+ T-cells was reduced in children with tuberculosis as compared to healthy tuberculin reactors (Table I). In contrast, the percentage of CD8+ cells was increased in tuberculosis patients and the percentages of  $\gamma\delta$  T cells and the V  $\delta 1$  and V  $\delta 2$  subsets were similar in both groups. Fig. 1 shows the total lymphocyte count and absolute numbers of CD4+ and CD8+ T-cells in blood in patients and controls. Both the total lymphocyte count ( $3173 \pm 610$  vs  $5413 \pm 376$  cells/cu mm,  $p = 0.006$ ) and CD4+ T-cell count ( $971 \pm 376$  vs  $2223 \pm 333$  cells/cu mm,  $p = 0.002$ ) were significantly lower in tuberculosis patients as compared to controls. However, the absolute CD8+ counts were similar in the two groups of patients, even though the percentage of CD8+ cells was higher in tuberculosis patients.

We wished to determine if the changes in the lymphocyte subsets was related to radiographic type and extent of disease or to nutritional status. The percentage of CD3+ and CD4+ T-cells was similar in children with minimal and extensive disease on Chest X-ray(13). On comparing the T-lymphocyte subsets in children with hilar/mediastinal lymphadenopathy alone with those who had

**TABLE I-** T-Lymphocyte Subpopulations in Blood in Tuberculosis Patients and Healthy Controls

Subpopulation	Tuberculosis patients (n = 22)	Healthy tuberculin reactors (n = 17)	p value
CD3 (%)	63.2 $\pm$ 2.6	70 $\pm$ 1.7	0.03
CD4 (5%)	32.5 $\pm$ 2.0	38 $\pm$ 2.2	0.03
CD8 (%)	26.6 $\pm$ 1.8	21 $\pm$ 1.3	0.03
Pan TCR $\gamma\delta$ (%)	7.2 $\pm$ 0.8	8.2 $\pm$ 1.4	NS
V $\delta 1$ (%)	3.8 $\pm$ 0.7	3.4 $\pm$ 0.3	NS
V $\delta 2$ (%)	5.9 $\pm$ 1.0	7.5 $\pm$ 1.3	NS

NS- Not significant.

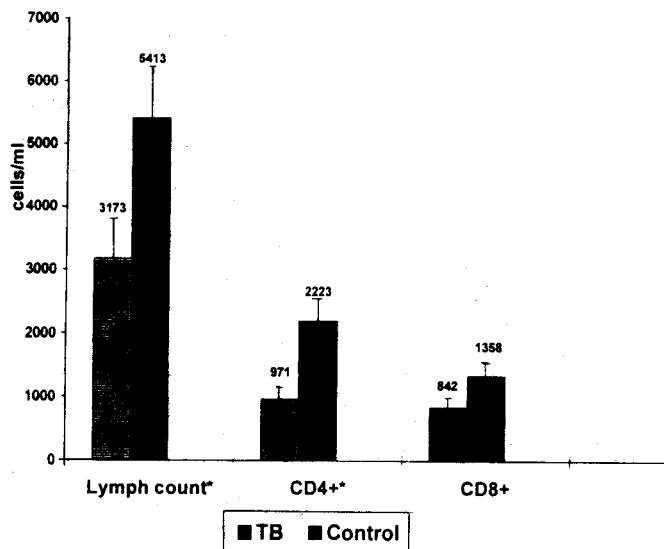


Fig. 1 Total lymphocyte count, absolute CD4+ and CD8+ counts in children with tuberculosis and healthy tuberculin reactors (controls). The mean values and standard errors are shown. \*  $p < 0.01$ .

more extensive parenchymal lesions or cavitation, we found no significant differences. However, the percentage of CD3+ cells was significantly lower in the 10 malnourished patients compared to the 12 patients who were not malnourished (Table II). The percentage of CD4+ cells was not significantly different between the two groups. When a comparison was made between malnourished tuberculin positive patients and malnourished controls, it was observed that both the CD3+ and CD4+ cell counts were significantly lower in tuberculosis patients. There was no significant correlation between lymphocyte sub-populations and nutritional status in healthy tuberculin reactors (data not shown).

We measured the lymphocyte subsets at baseline and after completion of treatment in 8 tuberculosis patients. The percentage of CD3+ cells increased after therapy to levels comparable to those of healthy tuberculin

reactors ( $60.4 \pm 4.9$  to  $67 \pm 3.3$ , NS), but the difference between values before and after treatment was not statistically significant, probably because of the small number of patients evaluated. The proportion of CD4+ T-cells also increased after chemotherapy ( $27.4 \pm 3.7$  to  $34 \pm 1.8$ ,  $p = 0.04$ ) and this difference was statistically significant. The percentages of CD8+ cells ( $25 \pm 3.7$  to  $23.4 \pm 1.8$ ) and Pan TCR  $\gamma\delta$  T cells ( $8.6 \pm 1.9$  to  $6.8 \pm 0.7$ ) showed no significant change with therapy.

## Discussion

In adult tuberculosis patients, most investigators have found that the total numbers and percentages of CD3+ and CD4+ cells are reduced as compared to healthy controls (8-13). However, because many tuberculosis patients have co-existing illnesses, it is uncertain if the changes in lymphocyte distribution are a direct

**TABLE II-** *Lymphocyte Subpopulations in Healthy and Malnourished Patients and Controls*

Subjects	CD 3%	CD 4%
TB with malnutrition (n = 10)	59 ± 4.3* <sup>^</sup>	30 ± 2.8**
TB with normal nutrition (n = 12)	67 ± 2.0*	36 ± 3.2
Malnourished tuberculin reactors (n = 9)	70 ± 2.3 <sup>^</sup>	40 ± 3.3**

\*P < 0.05; \*\* P = 0.04

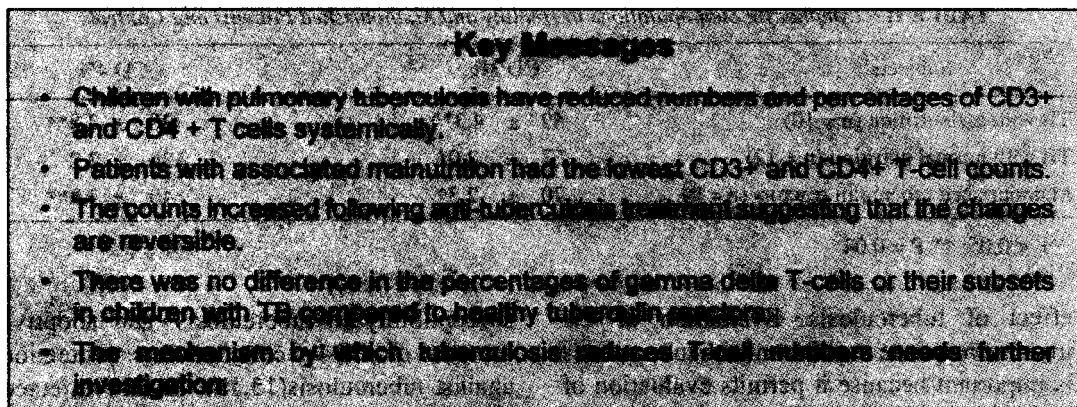
effect of tuberculosis. Evaluation of the immune response in children with tuberculosis is important because it permits evaluation of patients during the initial immune response to tuberculosis in the absence of other chronic diseases that may affect lymphocyte subpopulations.

In our study, we selected children with tuberculosis and healthy tuberculin reactors from the same socioeconomic class and with similar nutritional status. Our findings demonstrate that in children with tuberculosis, the total numbers and percentages of CD3+ and CD4+ cells are reduced suggesting that this is directly related to tuberculosis. The reduction was more marked in patients who had associated malnutrition. Patients with both malnutrition and tuberculosis had the lowest CD3+ and CD4+ T-cell percentages compared to those with tuberculosis alone or malnutrition alone. Malnutrition could have been worsened by, and therefore, reflect the severity of tuberculous disease and would have a synergistic effect on suppression of the immune response( 14). The total number of CD8+ T-cells was similar in the two groups. Even though the number of patients studied was small, our data suggest that the changes in CD3+ and CD4+ cells were reversible and resolved after anti-tuberculosis therapy.

Studies in animals and humans indicate that CD4+ T-cells play a crucial role in protective immunity against tuberculosis. In animals, depletion of CD4+ cells markedly increases

susceptibility to tuberculosis, and adoptive transfer of CD4+ cells confers protection against tuberculosis( 15,16). In HIV-infected and HIV-negative persons with tuberculosis, extent of disease is greatest in those with depressed CD4+ cell counts( 11,13). Tuberculosis enhances apoptosis of peripheral blood T-cells( 17) and provides a potential mechanism for reduction of CD3+ and CD4+ cell counts. Alternatively, the peripheral reduction in CD3+ and CD4+ T- cells may reflect recruitment of these cells to the site of disease, where these subpopulations are over represented( 18,19). The mechanism by which tuberculosis reduces CD3+ and CD4+ cell numbers needs further investigation.

In contrast to findings with CD4+ cells, we found no change in the percentages of gamma delta T-cells or their subsets in children with TB compared to healthy tuberculin reactors. The V  $\delta$ 2 subpopulation of  $\gamma\delta$  T cells is believed to contribute toward immune defenses against mycobacteria during the initial immune response. These cells recognize certain non-peptide mycobacterial antigens that are not recognized by CD4+ cells(20). Studies of peripheral blood from adult tuberculosis patients have produced conflicting results. Some authors found normal or increased percentages of  $\gamma\delta$  T cells while others found decreases in the percentage of V  $\delta$ 2 cells(21-25). These differences may reflect variations in the timing of tuberculosis infection or variability in disease extent in adults with tuberculosis. Our current results indicate that there are no



changes in the percentages of gamma delta T-cells or their subpopulations in the peripheral blood of children during the initial immune response to tuberculosis. Further studies are needed to characterize the distribution of gamma delta T-cells at the site of disease.

In summary, we found that the absolute numbers and percentages of CD3+ and CD4+ lymphocytes but not those of gamma delta T-cells were reduced in children with tuberculosis. The reduction was most marked in children with associated malnutrition. These changes appeared to be reversible and we speculate that they may be due to cell recruitment at the site of disease or to enhanced apoptosis of T-cell subpopulations.

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*Contributors:* SS was the principal investigator and was involved in both clinical and laboratory aspects and

also drafted the paper; KSN and LEH assisted with the laboratory aspects and data management, NS and PRN participated in designing the study and logistic support and PFB coordinated the study and contributed to report writing.

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