

## A Study of T-Cell Response and Lymphokine Profile of Children Suffering from Pulmonary Tuberculosis

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The present work was undertaken to study the immune profile of pre-school going children with Pulmonary Tuberculosis. 40 children visiting the OPD aged between 2-5 years, who were diagnosed as Pulmonary Tuberculosis or Pulmonary plus other site (Disseminated) Tuberculosis. Peripheral venous blood samples were taken and mononuclear cells were harvested. Lymphocyte suspensions were prepared and Lymphocyte proliferation was assessed *in vitro* after BCG stimulation. The cytokines IFN- $\gamma$ , IL-2, and IL-4 in these suspensions were assayed as per Genzyme protocol provided with the kit. The tests were repeated after 3 months and at 6 months at the end of treatment using a standard protocol.

Children with only pulmonary tuberculosis showed a higher lymphocyte proliferation index as compared to children with disseminated disease. The cytokines secreted by cells from patients with a limited and only pulmonary disease showed a significant pattern of a Th1 pattern of cytokine secretion with large amounts of IFN- $\gamma$  and IL-2 as compared to children with disseminated disease which responded with a Th2 pattern of cytokine release and mainly IL-4 secretion. Th1 cytokines in only pulmonary tuberculosis were associated with a better outcome. The presence of Th1 pattern of cytokines can be used to predict a better prognosis in pulmonary tuberculosis and a mixed pattern of cytokine secretion that is Th0 in disseminated tuberculosis is also associated with a favorable outcome in pre-school going children.

**Key words:** Pulmonary Tuberculosis, Disseminated, Lymphocyte Proliferation index, Cytokines, Interferon gamma (IFN- $\gamma$ ), Interleukins (IL-2, IL-4).

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Pulmonary Tuberculosis is a serious problem in children. Globally about two billion people are infected with Mycobacterium tuberculosis, eight to ten million of them develop active disease and two million die from TB every year.<sup>1</sup>

An overwhelming majority of tuberculous patients reside in developing countries which suffer from marked poverty, lack of healthy living conditions, and inadequate medical facilities.<sup>1</sup> To control tuberculosis we require more effective vaccines and BCG in spite of being an effective vaccine in tuberculous meningitis and miliary tuberculosis<sup>2,3</sup> has not reduced the global burden of the disease.<sup>4</sup> To this end it is important to study the antigen response of T-cells in patients suffering from tuberculosis specially in response to different antigenic stimulation to develop new protective vaccines<sup>1</sup>.

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## METHODS

This study was conducted at Army Hospital Research and Referral New Delhi. 40 children aged 2-5 years diagnosed as pulmonary tuberculosis/ pulmonary plus other site tuberculosis (disseminated tuberculosis) visiting the pediatric outpatient department over one year comprised the material for this study. These children were followed up for six months.

In the absence of a gold standard for diagnosis of tuberculosis in children, the study children were diagnosed as per criteria for diagnosis of tuberculosis based on criteria as per Seth V<sup>5</sup> and in accordance with another study by Rigouts<sup>6</sup> and subsequently they also satisfy the latest IAP guidelines as per the consensus statement on childhood tuberculosis<sup>7</sup>.

### Inclusion criteria

#### 1) Essential criteria

- i) Children aged 2-5 years visiting the Pediatric OPD of AHRR New Delhi.
- ii) Essential criteria of symptoms, mainly low grade fever, weight loss, and persistent cough >2 weeks.

### Important criteria

- i) Positive chest skiagrams /other site scanning
- ii) AFB seen in gastric aspirate/ sputum / any other tissue fluid .
- iii) Contact with an adult TB index case
- iv) Presence of some other supportive criteria. These were positive mantoux test, absence of BCG scar and protein energy malnutrition up to grade II.

### Exclusion criteria

Any condition likely to affect the immune response to tuberculosis was excluded .These were

- i) Severe malnutrition (grade III and IV PEM)
- ii) Any other associated disease

A thorough clinical exam was done to find the extent of disease and any associated pathology.

Of the 40 children, 31 had only pulmonary tuberculosis that is restricted disease, where as nine had pulmonary + other site tuberculosis (disseminated tuberculosis). In this second group besides pulmonary TB four of these had TB meningitis and five had involvement of the liver and spleen. These children were treated with standard ATT (EHRZ for two months followed by

HR for four months). Their clinical profile was monitored throughout the duration of treatment and compared to their laboratory profile. Cure was declared when the children after six months of treatment were totally symptom free and had gained their weight, regained their appetite, the skiagrams / CT Scans had resolved and there was resolution of all symptoms pertaining to the disease.

To study the immune profile, the cell mediated immune response in the form of lymphocyte proliferation using BCG antigen stimulation in vitro and cytokines released by them were studied. The tests were conducted before starting treatment and repeated after three months and after completion of treatment at the end of six months.

Five ml of heparinised blood was collected and mononuclear cells were separated using ficoll-hypaque gradient centrifugation technique. Lymphocyte cell suspensions were prepared. BCG was used to stimulate the suspension. All the samples were cultured for three days at 37°C. At the end Thymidine uptake which is an index of lymphocyte proliferation was measured using liquid scintillation spectroscopy.

Data was expressed as Stimulation Index.

$S.I = \frac{\text{Mean CPM of antigen containing cultures}}{\text{Mean CPM of controlled cultures}}$

CPM = Counts per minute, scintillation count.

Cytokine IFN -  $\gamma$ , IL-2 and IL-4 were assayed as per genzime protocol provided with the kit .

The difference between the groups with continuous variables was statistically tested using student's t test. Phenotypic distributions between the study groups were compared using the Chi-square test. Two tailed P values <0.05 were considered statistically significant.

### Informed consent of parents were taken

The study was approved by the Institute Ethics Committee.

## RESULTS

The Stimulation Index in test cases showed that proliferation of lymphocytes was different in subjects with pulmonary tuberculosis only and in those with disseminated tuberculosis<sup>8</sup>. As seen in Table no I. 28/31 children with restricted disease and only pulmonary tuberculosis had a S.I. > 2. The range was 2.1 to 9.3 with a mean of 5.6

except in three cases out of 31 where it was <2. On follow up the lymphocyte proliferation continued to be the same in those which initially had a S.I.>2. However in the three cases of only pulmonary tuberculosis which initially showed a poor proliferation two improved later and the Stimulation Index became >2 after six months of therapy. (Table 2) One patient in this group was lost to follow up.

In eight out of nine children with disseminated tuberculosis proliferation response was poor with a (S.I. < 2). After three months five out of these had a stimulation index >2 and at the end of six months six out of nine had a stimulation index >2 One child died and he was a case of TB meningitis. Two patients continued to show a S.I.<2.

A significant pattern of cytokine secretion from these lymphocytes was seen. As seen in (Table

**Table 1.** Table showing clinical status of test subjects as correlated with lymphocyte proliferation response (stimulation index to BCG)

Site	S.I.<2	S.I.>2	Total
Pulmonary	03	28	31
Pulmonary + other site	08	01	09
total	11	29	40
			P<0.05

Disseminated disease correlates significantly with poor lymphocyte proliferation response (S.I. <2)

**Table 2.** Showing lymphocyte proliferation response to BCG stimulation initially and on follow up

Disease site	Initial S.I.<2	Initial S.I.>2
Pulmonary only	0 month	3
	3 month	1
	6 month	0
Disseminated	0 month	8
	3 month	3
	6 month	2

The response in most cases moves to a higher stimulation index. One patient in disseminated TB with S.I.<2 died within 1 month of starting therapy. He had TB Meningitis. One patient each from pulmonary and disseminated Tb was lost to follow up. Table 2.

**Table 3.** Showing cytokine response by T cells of test cases stimulated by BCG initially and on follow up

No of cases initially	T cells response initially	Follow up at 3 months	Follow up at 6 months	Remarks
26	Th 1	25	25	Remains Th1. One patient lost to follow up
5	Th 2	4	2	Two patients convert to Th1 and One died
9	Th0(mixed)	5	8	One patient lost to follow up. Rest all convert to Th1.

**Table 4.** Showing Clinical Response Vis a Vis T-Cell Response

No of Cases	T-Cell Response initially	Clinical Features	Response to Therapy	End Result
26	Th1	PulmonaryTuberculosis	Satisfactory	25 Followed up. All Cured. One lost to follow up
5	Th2	Disseminated TB	Un-satisfactory	1 Death 2 cured2 Not Yet Cured.
9	Th0	5 Pulmonary/4 Disseminated	Satisfactory	8 Followed up All Cured. One lost to follow up

3), a majority 26/40 showed a large amount of IFN- $\gamma$  (mean level 34 ng/ml) and IL-2 (26 ng/ml). This is a Th1 response in children<sup>9</sup>. Five cases showed a Th2 response<sup>10,11,12</sup> with predominantly IL4 secretion (mean level 20 ng/ml). A third group of 9/40 patients responded to BCG stimulation by producing all three cytokines-IFN- $\gamma$ , IL-2 and IL-4. Such a response is described as Th0 or a mixed response and has been seen in other studies<sup>9,12</sup>.

A follow up cytokine response was done after three months of therapy. This study reveals (Table III) that of 26/40 cases that showed a Th1 response initially, 25 continued to do the same after three months of therapy and one was lost to follow up. In 5/40, cases which showed an initial Th2 response one died. Remaining four continued to show a Th2 type of response. In the third group out of 9/40 cases showing an initial mixed response eight could be followed up. Five of them showed a Th1 response at the end of three months.

Follow up cytokine response after 6 months of therapy reveals that (Table 3) that of 26/40 cases that showed a Th1 response initially, 25 continued to do the same after six months of therapy and one was lost to follow up. In 5/40, cases which showed an initial Th2 response one died and remaining four which continued to show a Th2 type of response at three months however showed a change after six months of therapy. Two of these had increased IFN- $\gamma$  secretion after 6 months and their cytokine pattern changed to Th1 but two patients continued to have Th2 pattern of cytokines. In the third group out of 9/40 cases showing an initial mixed response eight could be followed up. All of them showed a Th1 response at the end of six months. Other studies have also suggested that initial T cell response may be mixed which later polarizes to Th1 or Th2 response pattern<sup>13</sup>.

Clinical Correlation of the cytokine response with the clinical profile shows that of 26/40 cases responding with Th1 pattern of cytokines all had only pulmonary involvement. The next group of five cases which responded by producing mainly IL-4 (Th2) were all cases of Disseminated disease. Finally the third group of nine children, which responded by producing all the three cytokines namely IFN- $\gamma$ , IL-2 and IL-4 (Th0) had four children of Disseminated Tuberculosis and five children of Pulmonary Tuberculosis.

## DISCUSSION

The study of cellular immune response of antigen stimulated T-cells in vitro in patients with Tuberculosis is important for the understanding of protective and pathological mechanisms in Tuberculosis and development of new vaccines.<sup>8</sup>

Lymphocyte proliferation showed a definite pattern. In Patients with limited pulmonary disease and who eventually responded well to therapy 90% showed a good proliferation of their lymphocytes with a stimulation index more than 2. Proliferation response at the end of 6 months also showed a significant pattern. Out of the remaining 10% two patients 6.6% converted to a S.I. >2 after 3 months and one child was lost to follow up. Apart from secreting cytokines lymphocytes and their subsets have a direct role in killing and containing mycobacteria.<sup>8</sup>

Proliferation response in those with Disseminated Tuberculosis was poor with eight out of nine children showing an initial S.I. <2. One child of TB meningitis died and two children continued to show a S.I. <2 after six months. These same children were still not declared cured and were continued on treatment and Only one child of Disseminated Tuberculosis which initially had an S.I. >2 continued to be the same. This is similar to other studies<sup>14</sup> which have shown a good lymphocyte proliferation is associated with a good immune response and better prognosis.

A significant pattern of cytokine secretion from these lymphocytes was seen. As seen in (Table 3), a majority (26/31) 85% of restricted pulmonary disease) showed a large amount of IFN- $\gamma$  (mean level 34 ng/ml) and IL-2 (26 ng/ml) to start with and the same pattern was seen after three months and six months. This is Th1 response and it continues till the end of therapy. It has been seen in several studies that protective immunity in tuberculosis is mediated by Th1 cytokines and particularly by IFN- $\gamma$ <sup>10,12,13</sup>.

5/40 cases showing a Th2 response with predominantly IL4 secretion (mean level 20 ng/ml) were all cases of disseminated tuberculosis. After three months one patient out of five that is 20% convert to Th1 and by the end of six months 40% (2/5) convert to Th1 with treatment. This is a significant finding one patient in this group died

and he was a case of TB Meningitis. A Th2 response is generally seen in cases with extensive pathology and tissue destruction but it changes to Th1 response after treatment in some cases, the time taken may be in years<sup>10,11,13</sup>.

In the third group of 9/40 patients responded to BCG stimulation by producing all three cytokines-IFN- $\gamma$ , IL-2 and IL-4. Such a response is described as Th0 or a mixed response and has been seen in other studies<sup>14, 15</sup>. This response was seen in 5/31 that is 16% of pulmonary tuberculosis and 44% (4/9) of disseminated tuberculosis. All these patients polarized to Th1 response at the end of six months of therapy. This significant finding shows that irrespective of the extent of the disease the outcome in these patients was also good. All these patients except one who was lost to follow up were declared cured. Other studies have also suggested that initial T cell response may be mixed which later polarizes to Th1 or Th2 response pattern<sup>15</sup>

These results are in agreement with various other studies which showed that protective immunity in Tuberculosis is Th1 mediated and Th2 response is associated with increased inflammatory response and tissue destruction<sup>12, 13</sup>. The early response can also be mixed Th0 and this polarizes into either aTh1 or Th2 kind of cytokine secretion<sup>13,14</sup>. Based on this study which is similar to other studies<sup>15</sup> prognosis of a case can be predicted. Children showing aTh1 response have a better prognosis.

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