# Cellular Immunity in Current Active Pulmonary Tuberculosis[1-3]

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SUMMARY A group of 10 patients with recently diagnosed pulmonary TB were studied and compared to 10 bacillus Calmette-Guérin (BCG) immunized healthy individuals. Cellular immune mechanisms were explored *in vitro* utilizing fresh and precultured peripheral blood mononuclear cells exposed to PHA, PPD, and recall antigens (SK/SD and CA). Proliferative assays were also carried out in the presence of either each patient's serum (autologous serum) or cocultured with $CD3^+$-depleted adherent cells. Serum measurements of soluble interleukin-2 (IL-2) receptor and synthesis of IL-2 generated by mononuclear cells stimulated with PPD and SK/SD were also performed. Patient sera were able to inhibit autologous as well as allogeneic cell responses, and a significant adherent cell suppressive effect was observed. As a whole the group of patients showed decreased blast transformation to PPD, preserved proliferative responses to other recall antigens, and a low PPD-induced generation of IL-2. Furthermore, as possible evidence of preactivated T cells, these patients demonstrated high soluble IL-2 receptor serum levels. Early compromise of specific cell-mediated immunity, including IL-2 abnormalities, may be of significance in newly diagnosed pulmonary TB.

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## Introduction

A defective cell-mediated immune response (CMI) in the initial contact with *Mycobacterium tuberculosis* has been implicated as a cardinal predisposing factor in the progression of active tuberculosis (TB) (1). Thus in advanced tuberculosis the compromised cellular immunity is frequently associated with cutaneous anergy (1, 2). The presence of serum inhibitory factors, suppressive monocytes, and the redistribution of peripheral circulating T cell subpopulations have been suggested as modifiers of patient clinical stages (3–5). Handling of TB antigen load resides on monocyte-T cell interactions, and the evidence accumulated entails the possible existence of a purified protein derivative (PPD)-specific suppression by means of cell-mediated mechanisms that may allow the persistence of the disease-related antigen (6). We report here on findings of CMI in non-high-risk individuals with recently diagnosed current pulmonary TB, suggesting that, even at this stage, a clinically important compromise of CMI may exist.

## Methods

A group of 10 untreated patients with current pulmonary TB were studied. In table 1 the categorized clinical and pathologic findings using the American Thoracic Society criteria for TB are depicted (7). All the patients were well nourished, and none were considered high-risk individuals for other infectious diseases, being negative for the presence of human immunodeficiency virus and hepatitis B serologic markers. A group of 10 healthy individuals matched for age and sex were selected as controls. Controls had received bacillus Calmette-Guérin (BCG) vaccination in the past, and a positive tuberculin reaction (mean ± standard deviation, 9 ± 2 mm) was confirmed in the whole group.

### *Peripheral Blood Mononuclear Cells*

Peripheral blood mononuclear cells were isolated on Ficoll-Hypaque gradient (8). Cell preparations were resuspended in RPMI-1640 (Microbiological Associates, Walkersville, MD) supplemented with 10% heat-inactivated normal human serum (NHS), glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (50 mg/ml; GIBCO Laboratories, Grand Island, NY).

### *Blast Transformation Assays*

Cell proliferative assays were carried out as described elsewhere (9). Predetermined optimal concentrations of phytohemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, England), purified protein derivate (Batch No. NT 23–62; Statens Serum Institute, Copenhagen, Denmark), streptokinase streptodornase (SK/SD; Lederle Laboratories, Caracas), and *Candida albicans* (CA; Instituto de Biomedicina, Dep. Micopatologia, Caracas) were utilized. The final optimal concentrations were PHA, 2.5 µg/ml; PPD, 100 µg/ml; SK/SD, 250 IU/ml; and CA, 20 µg/ml. Cultures were set up using round-bottomed microculture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) with a final 200-µl volume containing $5 \times 10^5$ cells/well. Cultures were run using fresh cell (FC) preparations as well as precultured cells (PC), with the latter precultured for 18 h in RPMI 1640 medium supplemented with penicillin-streptomycin, glutamine, and 10% heat-inactivated human serum in an atmosphere of 95% air and 5% $CO_2$ (10, 11). Culture plates were incubated for 72 h (PHA) and 168 h (antigens) at 37° C in a humidified 5% $CO_2$ atmosphere. Cultures were pulsed with 1 µCi of tritiated thymidine (New England Nuclear, Boston, MA) 18 h before harvesting in a Mash II apparatus (Microbiological Associates, Bioproducts, Walkersville, MD). The incorporated radioactivity was determined in a β-scintillation counter (Tri-carb 3255: Packard, Chicago, IL). In addition, cultures were also set up in the presence of 10% heat-inactivated autologous serum (AS) corresponding to each patient's serum rather than 10% heat-inactivated NHS.

### *Depletion of Adherent Cells*

PBMC ($5 \times 10^6$ cells/ml) in RPMI supplemented with 10% NHS were placed on a plastic petri dish (Falcon Labware, Oxnard, CA) at 37° C for 1 h. The nonadherent cells were recovered by means of three consecutive washes with RPMI media and were further purified through a nylon column following the Julius group's technique (12). Final cell suspension showed 95% $CD3^+$ cells as assessed by anti-CD3 monoclonal antibody (Ortho Diagnostic, Raritan, NJ). The adherent cell fraction was gently removed by a rubber policeman and washed three times with RPMI, finally showing 90% of monocytes identified

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TABLE 1

CLINICAL AND PATHOLOGIC DATA OF PATIENTS WITH CURRENT ACTIVE PULMONARY TUBERCULOSIS*

| Patient No. | Age (Yr) | Sex | Roentgenogram Findings | Bacteriologic Stain | Tuberculin Reaction (mm) | CH50 (U/ml) | IgG (mg%) | IgA (mg%) | IgM (mg%) | CIC† |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 23 | M | Micronodular infiltrate | + + | 13 | 100 | 1973 | 524 | 191 | 40 |
| 2 | 32 | F | Nodular infiltrate, bilateral cavities | + + + | 5 | 125 | 2120 | 586 | 79 | 56 |
| 3 | 32 | M | Bilateral nodules, cavities | + + | 0 | 208 | 2651 | 252 | 251 | 40 |
| 4 | 35 | M | Bilateral micronodules, cavities | + + | 0 | 227 | 1973 | 577 | 162 | 144 |
| 5 | 20 | F | Nodular infiltrate, bilateral cavities | + + | 8 | 217 | 2256 | 193 | 168 | 51 |
| 6 | 42 | M | Left apex infiltrate | + | 12 | 263 | 2271 | 448 | 221 | 124 |
| 7 | 56 | M | Right micronodular infiltrate | + + | 16 | 208 | 2994 | 477 | 379 | 130 |
| 8 | 26 | M | Nodular infiltrate, bilateral cavities | + + | 12 | 295 | 2020 | 770 | 200 | 68 |
| 9 | 26 | M | Right apex infiltrate | + | 14 | 290 | 3164 | 604 | 314 | 190 |
| 10 | 32 | M | Nodular infiltrate | + + | 13 | 263 | 1897 | 460 | 371 | 56 |

* Normal Values: CH50 = 150 to 250 U/ml; IgG = 800 to 1800 mg%; IgA = 150 to 250 mg%; IgM = 40 to 160 mg%.
† Circulating immune complexes expressed as µg aggregated human gammaglobulin/ml. Normal values < 30 µg/ml (14).

by peroxidase stain and 99% viability as assessed by trypan blue exclusion. The purified $CD3^+$ T cell suspension was stimulated with optimal concentrations of PHA and PPD in the presence of 5% autologous adherent cells.

### *T Cell Subsets*

T cell subsets were investigated through indirect immunofluorescence using monoclonal antibodies of the OKT series (Ortho Pharmaceutical, Raritan, NJ). T cell subpopulations were identified as CD3, CD4, or CD8 following the Krensky method (13).

### *Measurement of Soluble Interleukin-2 Receptor*

Soluble Il-2R levels were measured in serum by means of an enzyme-linked immunosorbent assay (ELISA; T-cell Sciences, MA).

### *Interleukin-2 Generation*

IL-2 was generated by stimulation of PBMC ($1 \times 10^6$ cells/ml) from patients and controls with PPD (100 µg/ml) or SK/SD (250 IU/ml) for 48 h at 37° C using microculture plates. Cell-free supernatants were obtained by centrifugation of the plates at 400 × g for 10 min and kept at −20° C until tested for IL-2 activity. IL-2 activity was evaluated using IL-2-dependent T cell blasts generated by stimulation of T cells with PHA (1 µg/ml) for 72 h and maintained with human recombinant IL-2 (Biogen, MA). Before IL-2 testing, T cell blasts were washed twice to eliminate residual IL-2. Subsequently, $1 \times 10^4$ cells were incubated with 100 µl experimental or control supernatant for 2 h at 37° C in microtiter plates. Cells were labeled with 1 µCi of [$^3$H]thymidine 6 h before the end of the incubation period; finally, cells were lysed with distilled water, and DNA was harvested in a Mash II cells harvester (Microbiological Associates). The incorporated radioactivity was measured in a β-scintillation counter (Packard Tri-Carb 3255).

IL-2 was quantified through extrapolation on a dose-response curve prepared with known amounts of human recombinant IL-2.

### *Statistics*

Differences between groups were analyzed by means of Student's *t* tests and by analysis of variance, when required.

## Results

### *T Cell Subsets*

Phenotypic characterization of T cell subsets showed absolute values comparable to matched healthy individuals (CD3, 1.536 ± 280; CD4, 814 ± 122; CD8, 460 ± 80 × mm³). Healthy control values were CD3, 1.434 ± 433; CD4, 860 ± 257; CD8, 407 ± 136 × mm³.

### *Blast Transformation Assays*

Fresh PBMC from the patient group showed PHA responses similar to those in the control group but responses to PPD were lower (figure 1). This observation was restricted to PPD, since blasto-

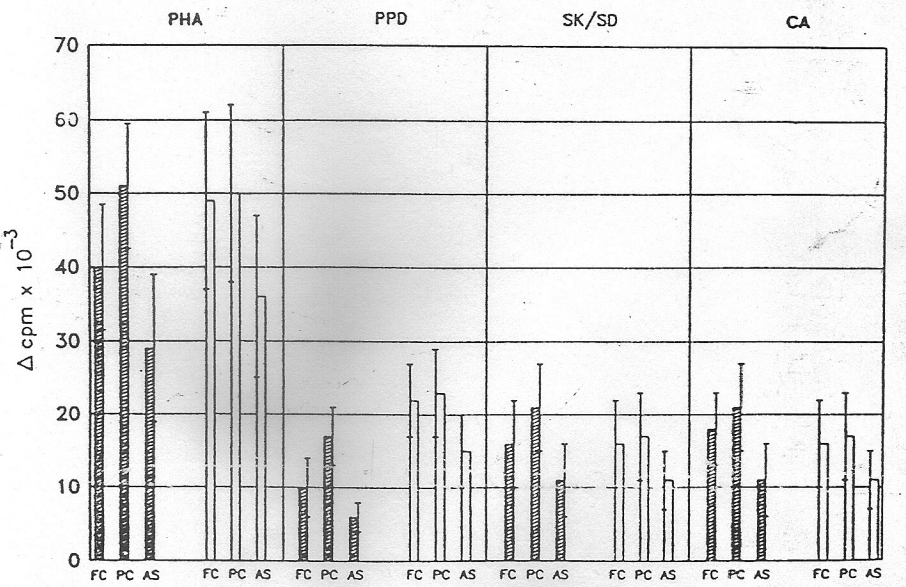


Fig. 1. TB patient blastogenic response of PBMC to different stimuli. Comparison with control cells under same culture conditions. FC = fresh cells; PC = precultured cells; AS = autologous serum (patient serum). Fresh PBMC = patients versus controls to PPD, $p < 0.005$. Patient FC versus PC to PHA, PPD, SK/SD, CA, $p < 0.005$. Effect of AS on patient (hatched bars) or control (open bars) FC, $p < 0.005$.

TABLE 2

TB PATIENT BLASTOGENIC RESPONSES: INTRAGROUP COMPARISON*

| | PHA | PPD | SK/SD | CA | Tuberculin Reaction ($mm^2$) |
|---|---|---|---|---|---|
| | 37.823 | 8.816 | 13.645 | 13.161 | 5 |
| | 29.976 | 6.837 | 14.861 | 11.027 | 0 |
| | 48.837 | 3.986 | 18.033 | 14.451 | 0 |
| | 34.468 | 9.957 | 17.694 | 14.304 | 8 |
| $\bar{X} \pm SD$ | 38.879 ± 9.475 | 7.399 ± 2.265 | 16.058 ± 1.860 | 13.236 ± 1.370 | 3 ± 3 |
| $\bar{X} \pm SD$ (6) | 41.299 ± 8.464 NS | 12.571 ± 2.759† $p < 0.02$ | 18.328 ± 6.021 NS | 20.740 ± 3.863 $p < 0.01$ | 13 ± 1.3 |

* Results expressed in cpm.
† Compared to controls (21.515 ± 5.262), $p < 0.001$.

genic responses to SK/SD and CA from TB patients exhibited values comparable to those of the control group (figure 1). Patients 2, 3, 4, and 5, who showed little or no tuberculin skin reaction, were compared with the six patients with large positive tuberculin reaction: as can be seen in table 2, the former TB patients showed the lowest responses to PPD. Comparison of the latter TB group with the PBMC control responses to PPD still were significantly lower (table 2). The four hyporesponsive TB patients maintained comparable intragroup PHA and SK/SD responses, but cell proliferation to CA was lower. This observation has already been described in TB patients (4). By using precultured cells, we tried to establish possible differences in PBMC reactivity obtained with fresh cell preparations. Consequently, preculturing significantly enhanced PBMC proliferative responses to PPD, PHA, SK/SD, and CA (figure 1); increased ranges varied from 17 to 81% for TB patients but in controls the ranges were zero to 12%. It should be noted that precultured TB cells were still unable to reach the level of response showed by the healthy controls. To investigate the influence of the patients' autologous serum on the PBMC blastogenic responses, fresh PBMC from both groups (patients and controls) were set up in the presence of each patient's serum. Serum inhibitory influence was noted both in autologous (patients) or allogeneic (control) cell cultures, and the suppressive serum effect was observed to either stimuli (PHA, PPD, SK/SD, and CA; figure 1).

### *Depletion of Adherent Cells*

To investigate possible suppressive effects mediated by monocytes, fresh purified $CD3^+$ cells were cultured adding only 5% of adherent cells plus the optimal concentrations of PHA and PPD. As shown in figure 2, the depletion of adherent cells generated a significant increase in the response of TB patients to either stimuli (17 to 67% and 32 to 204% to PHA and PPD, respectively), whereas the control group responses did not show a significant enhancement (zero to 13% and zero to 10% to PHA and PPD, respectively).

### *Soluble IL-2R*

Serum soluble IL-2R was measured as a marker of lymphocyte activation. TB patient IL-2R serum levels were significantly higher than those values obtained in the control group (table 3).

### *IL-2 Generation by Activated PBMC*

PBMC from patients stimulated with PPD showed a lower IL-2 production compared to PBMC from healthy individuals (table 4). However, the same PBMC cells stimulated with SK/SD produced IL-2 levels similar to those in the control group. As explained in Methods, both assays were carried out using predetermined PPD and SK/SD optimal concentrations.

## Discussion

The pathogenesis of tuberculosis seems to be linked to functional abnormalities of specific T lymphocytes (1–2, 15). In particular, patients with advanced tuberculosis commonly may show antigen-specific anergy associated with antigen stimulation (1, 15–17). Several host factors may simultaneously operate, modifying the sequence of a CMI reaction to the *M. tuberculosis* challenge. Some factors, such as autologous plasma inhibitors (3), suppressive cells (4), and redistribution of the peripheral T cell pool (5), have been stressed.

Our research protocol was designed to

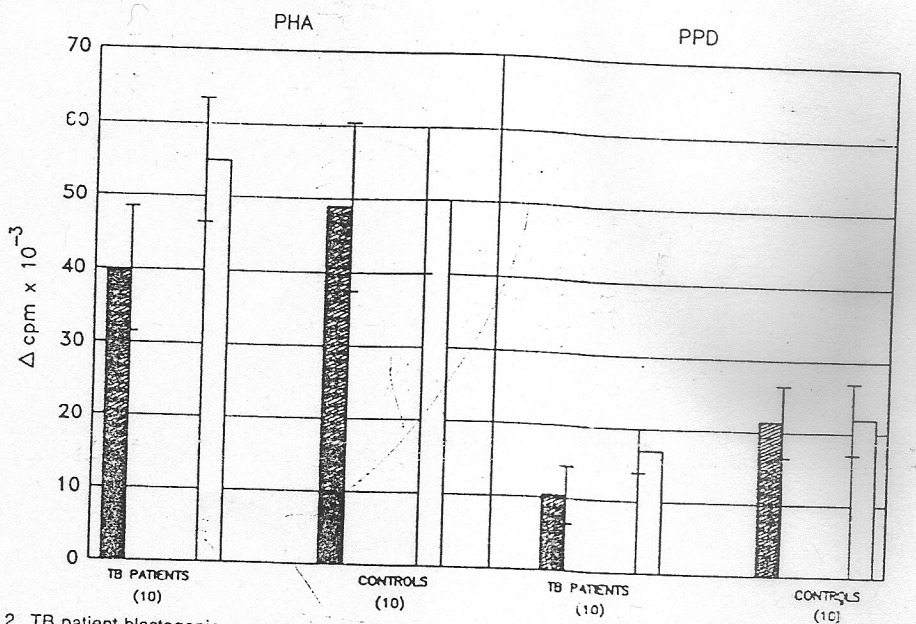


Fig. 2. TB patient blastogenic responses of PBMC to PHA and PPD. Effect of adherent cell depletion. Patient PBMC versus $CD3^+$ + 5% M to PHA, $p < 0.005$; to PPD, $p < 0.025$.

TABLE 3

MEASUREMENT OF SOLUBLE IL-2R LEVELS IN SERUM FROM TB PATIENTS VERSUS CONTROLS*

| Patients | Controls |
|---|---|
| 450 | < 50 |
| 900 | < 30 |
| 1,420 | 180 |
| 1,720 | ND |
| 800 | 180 |
| 770 | 260 |
| 450 | 160 |
| 1,370 | 170 |
| 720 | 270 |
| 913 ± 442† | 162 ± 82† |

* Results expressed as U/ml. ND = not determined.
† $p < 0.005$, $\bar{X} \pm SD$.

TABLE 4

*IN VITRO* SYNTHESIS OF IL-2 BY PBMC STIMULATED BY PPD AND SK/SD: COMPARISON BETWEEN TB PATIENTS AND CONTROLS*

| PPD | | SK/SD | |
|---|---|---|---|
| Patients | Controls | Patients | Controls |
| 7 | 24 | 73 | 48 |
| 2 | 16 | 36 | 50 |
| 2 | 18 | 73 | 18 |
| 2 | 73 | 24 | 21 |
| 3 | 140 | 16 | 48 |
| 6 | 14 | 71 | 48 |
| 2 | 21 | 18 | 24 |
| 2 | 147 | 21 | 13 |
| 21 | 36 | ND | ND |
| 3 | 48 | ND | ND |
| 5 ± 6† | 54 ± 50† | 42 ± 26 | 34 ± 16 |

* Results expressed as U/ml. PPD concentration, 100 U/ml; SK/SD concentration, 250 U/ml.
† $p < 0.005$, $\bar{X} \pm SD$.
ND = not determined.

investigate the immunopathologic status of a group of patients with recently diagnosed untreated tuberculosis. Their immunoclinical data showed elevated levels of both IgG, IgA, and IgM and circulating immune complexes associated with a significant specific hyporesponsiveness to PPD and normal CD3, CD4, and CD8 lymphocyte absolute numbers. Three aspects were particularly searched: the *in vitro* response pattern of fresh and precultured PBMC to mitogen and recall antigens, the influence of autologous monocytes and serum inhibitors, and the *in vitro* synthesis of IL-2 along with serum-soluble IL-2 receptor levels. The preculture period showed a statistically significant difference in the *in vitro* cell reactivity only of the patients for all stimuli, probably allowing a memory response to be fully expressed. Previously we observed recovered blastogenic responses of precultured cells in paracoccidioidomycosis (18), onchocerciasis (19), and schistosomiasis (20). Furthermore, Mohagheghpour and colleagues showed that precultured cells exhibit a strong proliferative response to *Mycobacterium leprae* antigen in patients with lepromatous leprosy (21). Shedding of membrane-bound molecules, the transition to a state of resting T cells expressing low-affinity IL-2 receptors, and the elimination of short-lived suppressor cells have been identified as probable mechanisms mediating the effect of preculturing (10, 11, 18–21).

Membrane-bound factors may contribute to the hyporesponsiveness to PPD as evidenced by the nonspecific inhibitory action of autologous serum on *in vitro* responses to PPD, SK/SD, and CA. Inhibition exerted by autologous plasma has been previously reported in TB patients (3). The nature of TB plasma or serum inhibitors remains unknown, although immunosuppressive properties of circulating immune complexes have been suggested (3). Using the C1q solid-phase method significantly elevated levels of CIC were found in 100% of our TB patients, which may account for the inhibitory serum effect observed. It is also possible that in our group of TB patients, who also fit in the intermediate form of the disease (22), specific PPD hyporeactivity could be due to the existence of a preactivated state, which may prevent blastogenic responses to new specific stimulation. In fact, the highest enhancing effect of precultured PBMC before the specific challenge was observed in response to PPD. Furthermore, high levels of soluble IL-2R in the sera of patients with *M. tuberculosis* is possibly associated with a preactivated state. Soluble IL-2R seems to emerge from the deletion of the Tac protein being released in a stable form by means of activated T cells (23). In consequence, as a marker of T cell activation, chronically TB-stimulated lymphocytes may release greater amounts of soluble IL-2R. The other relevant aspect found related to IL-2 was the significant lower *in vitro* IL-2 synthesis when stimulated with PPD. This finding is in agreement with the report by Toosi and coworkers on patients with active TB (24). The nature of the latter remains unknown; a specific PPD-related defect may be stressed since patient lymphocytes stimulated with SK/SD generated IL-2 comparable to that in control cells. In addition, both soluble and membrane IL-2 receptors have the ability to bind circulating IL-2. Thus an excess quantity of soluble IL-2R may prevent IL-2 from binding to membrane receptors, perhaps interfering with *de novo* IL-2 synthesis and response of an expanded PPD antigen-specific lymphocyte pool. Decreased production of IL-2 could also be due to the presence of activated macrophages with suppressive action (6). Within this context, depletion of adherent cells increased IL-2 synthesis in TB patients considered high responders to tuberculin (6, 24). In pulmonary TB, as in other disease models, this macrophage inhibitory effect has been attributed to the secretion of an inhibitor factor, such as prostaglandin $E_2$, by the adherent mononuclear cell fraction (3, 25). We have found that the addition of naproxen to PBMC cultures from TB patients increased the cell responses to PHA and PPD in a similar fashion compared to the depletion of adherent cells (data not shown). It should be noted, however, that both *in vitro* conditions were unable to provoke a TB cell response to PPD at the same level as control cells, confirming the existence of other immunomodulatory factors besides the monocyte-mediated influence.

In conclusion, newly diagnosed current TB is associated with an *in vitro* antigen-specific compromise of CMI. The results, including systemic anergy, may probably depend on the final balance of the host immunoregulatory circuits already impaired by the concomitant influence of both cellular and humoral factors.

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