

DEFECTIVE INTERLEUKIN 2 PRODUCTION AND RESPONSIVENESS IN HUMAN PULMONARY TUBERCULOSIS

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Some patients with active tuberculosis lack a delayed skin test reaction to PPD (1–4). The basis for this immunologic hyporesponsiveness has been investigated using in vitro systems. Tuberculin-induced blastogenic responses of lymphocytes are decreased in such patients compared with healthy tuberculin reactors (5, 6). This is not simply due to a dearth of responsive cells in the circulation. Rather, monocytes act as antigen-specific suppressor cells in patients with pulmonary tuberculosis (5) and tuberculous pleurisy (8). Fc γ R⁺ lymphocytes, i.e., cells bearing receptors for the Fc portion of IgG, also suppress PPD-induced blastogenesis in tuberculous patients (9).

T cell proliferation is critically dependent on the lymphokine, IL-2 (10, 11). This hormone is a prerequisite for clonal expansion of antigen-specific T cells (12) and activation of the effector limb of cell-mediated immune responses (13, 14). IL-2-dependent cytotoxicity may be important for containment of facultative intracellular parasites such as *Mycobacterium leprae* (15) and *Mycobacterium tuberculosis*.

We investigated IL-2 homeostasis in patients with active, newly diagnosed pulmonary tuberculosis. Depressed PPD-induced blastogenesis in these patients was associated with abnormalities in IL-2 production and IL-2-R generation restricted to the response to tuberculin PPD. In 10 of 22 patients, IL-2 generation was depressed and unaffected by adherent cell depletion; these patients showed more extensive disease by radiographic criteria. Dysregulation of production and response to the pivotal cytokine IL-2 thus may be critical factors in the defective cellular immune response in tuberculosis.

Materials and Methods

Study Population. 22 patients with newly diagnosed active pulmonary tuberculosis hospitalized at University Hospitals at Cleveland, Cleveland Veterans Administration Hospital, and Cleveland Metropolitan General Hospital, Cleveland, OH were studied. Patients with factors potentially affecting the immune response were excluded: (a) age over 65 yr, (b) antituberculosis therapy for over 14 d, (c) concomitant debilitating diseases.

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Diagnosis was based on the demonstration of acid fast bacilli in sputum or biopsy specimens and was eventually confirmed by positive culture for *M. tuberculosis*. The chest roentgenogram obtained from each patient at the time of study was graded for extent of tuberculosis according to National Tuberculosis and Respiratory Association criteria (16). A group of 14 healthy tuberculin reactors, age- and sex-matched to the patients, was studied concurrently with the patients.

Preparation of Cells. 50 ml of heparinized blood (20 U/ml) was obtained from each subject. Peripheral blood mononuclear cells (PBMC)¹ were separated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) sedimentation (17). The fraction of monocytes in PBMC was determined by peroxidase cytochemistry (18). Adherent cells were prepared by suspending PBMC (5×10^6 /ml) in RPMI-1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 10% vol/vol heat inactivated FCS (Hyclone Laboratories, Logan, UT) and incubating 5–10 ml of the cell suspension in plastic petri dishes (100 × 20 mm; Falcon Labware, Oxnard, CA) at 37°C for 1 h. After nonadherent cells were removed the adherent monolayers were dislodged by gentle scraping with a rubber policeman. The resultant adherent cell population consisted of 85–90% monocytes (peroxidase staining) and was 99% viable by trypan blue exclusion. Nonadherent cells were washed onto 600 mg acid-treated nylon wool columns and incubated at 37°C for 30 min. The cells then eluted were T cell-enriched (>85% SRBC rosetting) and monocyte-depleted (<1% peroxidase positive in both patients and healthy donors).

Antigens and Reagents. PPD was a gift of Lederle Laboratories (Div. American Cyanamid Co., Wayne, NJ) and used at a final concentration of 100 µg/ml. Streptolysin 0 was purchased from Fisher Scientific Co., Pittsburgh, PA and used at a 1:50 dilution. We found these concentrations to be optimal for stimulating both lymphocyte blastogenesis and IL-2 generation in a group of healthy donors. Indomethacin (Sigma Chemical Co., St. Louis, MO) was dissolved in 95% ethyl alcohol at 10 mg/ml, diluted at RPMI-1640, and used at a final concentration of 1 µg/ml.

Generation of IL-2-containing Supernatants. PBMC or T cells were incubated in 24-well Linbro plates (Flow Laboratories, Inc., McLean, VA) at 3×10^6 /ml/well in RPMI-1640 supplemented with L-glutamine (2 mM), penicillin (50 U/ml), gentamicin (50 µg/ml), and 2% vol/vol heat-inactivated pooled human serum. Cell cultures were stimulated by antigens (PPD or streptolysin 0). After 48 h, supernatants were separated from cells by centrifugation at 500 g for 10 min and stored at –20°C until assay.

IL-2 Assay. The IL-2-dependent cytotoxic murine T cell line CTLL-20 was used for assay purposes. This cell line has been kept in culture with IL-2 containing supernatants of con A-activated rat splenocytes for over 2 yr, and was a gift of Dr. J. Finke (Cleveland Clinic Foundation, Cleveland, OH) (19). CTLL-20 cells were washed extensively, and suspended in DME (4.5 gm/liter glucose) (M. A. Bioproducts) at 10^5 /ml. 100 µl of the cell suspension was placed in flat-bottomed microtiter wells (3072; Falcon Labware) along with 100 µl of supernatants. IL-2 determinations were performed in triplicate. The total duration of culture was 24 h; 1 µCi of [³H]thymidine sp act 6.7 Ci/mmol (ICN Laboratories Inc., Irving, CA) was added per well for the last 5 h of culture. Cells were then harvested with a Mash II harvester (M. A. Bioproducts) and ³H activity was assayed by liquid scintillation spectrometry.

IL-2 activity was expressed as [³H]thymidine incorporation of CTLL-20 in culture with 1:2 dilutions of supernatants of antigen-stimulated cell cultures. Study of serial dilutions of supernatants of antigen-stimulated cells revealed that maximal [³H]thymidine incorporation of the indicator cells occurred at the 1:2 dilution, which was consistently in the linear portion of the dose-response curve; unlike supernatants of mitogen stimulated cells, prozone inhibition was not apparent (20).

Assessment of IL-2-R Generation. The mAb, anti-Tac (21, 22), a gift from Dr. T. Waldmann (Bethesda, MD), was used for assessment of IL-2-R. PBMC in RPMI-1640 (10^6 /ml) were stimulated with PPD for 5 d, then incubated with 50 µl of a 1:100 dilution of anti-Tac antibody for 30 min at 4°C. Washed cells were further incubated with FITC-

¹ Abbreviations used in this paper: LP, low producer of IL-2; NP, normal producer of IL-2.

TABLE I
Antigen-induced IL-2 Production and Blastogenesis in PBMC from Tuberculosis Patients and Healthy Donors

Antigen	IL-2 activity (cpm \pm SEM)*		Blastogenesis (cpm \pm SEM; [3 H]TdR)	
	TB patients (n = 22)	Healthy donors (n = 14)	TB patients (n = 22)	Healthy donors (n = 14)
PPD	3,571 \pm 790	17,947 \pm 4,406	6,655 \pm 1,410	18,187 \pm 2,132
Streptolysin 0	53,493 \pm 16,636	55,781 \pm 17,409	36,561 \pm 15,813	37,592 \pm 15,344

* [3 H]Thymidine incorporation of CTLL-20 after incubation with a 1:2 dilution of supernatants of antigen-stimulated PBMC.

conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) at a 1:20 dilution. Fluorescence of washed cells was then analyzed with a FACS EPICS V.

Assay of [3 H]Thymidine Incorporation. Cells were cultured at 7.5×10^5 /ml in RPMI-1640 supplemented with 20% vol/vol heat-inactivated pooled human serum. 100 μ l of cell suspension was incubated in round bottomed microtiter plate wells (Falcon Labware). PPD and streptolysin 0 were added to quadruplicate wells. In some experiments, purified IL-2 (Electro-Nucleonics Inc., Silver Springs, MD) was added to wells. This preparation of IL-2 has comparable activity (640 half-maximal U/ml) to the National Institutes of Health IL-2 standard and was used at final concentrations of 1–10%. When assayed with CTLL-20, a 10% vol/vol concentration of this preparation consistently produced maximal responses, whereas a 1% vol/vol concentration showed IL-2 activity uniformly exceeding 50% of maximum. Cultures were incubated at 37 °C in 5% CO₂ for 5 d. 18 h before the termination of culture, 1.0 μ Ci of [3 H]thymidine, sp act 6.7 Ci/mmol (ICN Laboratories, Inc.), was added per well. Cultures were then harvested and [3 H]TdR assessed by liquid scintillation spectrometry.

Statistical Analysis. Group means were compared using the Student's *t* test unless otherwise indicated.

Results

IL-2 Activity in Supernatants of Antigen-stimulated PBMC. Supernatants were prepared from antigen-activated PBMC of patients with active tuberculosis and a group of healthy tuberculin positive individuals. The IL-2 activity of such supernatants is shown in Table I. Mean PPD-induced IL-2 activity was 81.2% lower in patients with active disease as compared with healthy donors ($p < 0.001$). In contrast, streptolysin 0-induced IL-2 activity was similar in supernatants of PBMC from both groups. Table I also shows antigen-induced [3 H]-thymidine incorporation in PBMC for patients and controls. Again, mean PPD-induced blastogenesis was significantly lower in patients ($p < 0.001$), while streptolysin 0-induced blastogenesis was comparable to controls. Thus, patients with active tuberculosis showed a concomitant defect in both IL-2 production and blastogenesis by PBMC, which was restricted to the disease-related antigen, PPD.

The observation of low IL-2 activity in supernatants of PPD-activated PBMC in tuberculosis could reflect a factor in these supernatants interfering with IL-2-mediated proliferation of the assay cells. To exclude this possibility, CTLL-20 cells were cocultured with PBMC supernatants plus serial dilutions (1:2–1:32) of the rat IL-2 (supernatants of Con A-stimulated rat splenocytes) used to maintain

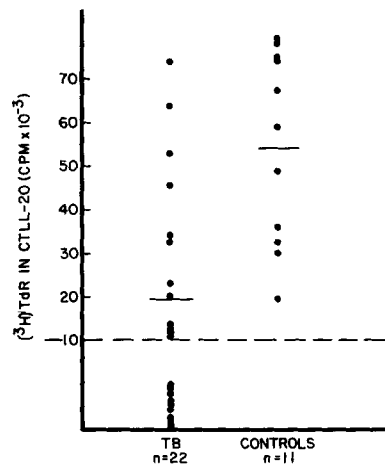


FIGURE 1. IL-2 activity in supernatants of PPD-stimulated T cell cultures for tuberculosis patients (left) and controls (right). Dashed line indicates mean IL-2 activity of controls $- 2$ SD. This line can be used to segregate tuberculosis patients into NP (above line) and LP (below line).

this cell line. Supernatants of PPD-stimulated PBMC from four patients with tuberculosis had no effect on IL-2-mediated proliferation of CTLL-20 (data not shown).

Role of Adherent Cells in Regulation of IL-2 Production in Tuberculosis. To examine the role of adherent cells in PPD-induced IL-2 production in tuberculosis, PBMC were depleted of such cells by two sequential procedures: plastic adherence and nylon-wool column incubation as outlined in Materials and Methods. T-enriched cells thus obtained were comparable in monocyte content ($<1\%$ peroxidase positive) in both the patient group and the controls. Supernatants of PPD-stimulated T cells showed significantly more IL-2 activity as compared with PBMC supernatants for both the patients ($19,186 \pm 4,736$ vs. $3,571 \pm 790$) ($p < 0.01$) and the healthy subjects ($54,604 \pm 6,714$ vs. $17,946 \pm 4,406$) ($p < 0.001$). IL-2 activity in supernatants from PPD-stimulated T cells of patients, however, remained significantly lower than that of healthy donors ($p < 0.001$). Fig. 1 is a scattergram of IL-2 activities in supernatants of PPD-stimulated T cells for both patients (left) and controls (right). The dashed line indicates mean PPD-induced IL-2 activity of T cell supernatants from controls minus two standard deviations. This line was used to separate the tuberculosis patients into two categories. In 12 patients, PPD-stimulated T cell production of IL-2 could not be distinguished from healthy donors; this group showed a significant increase in IL-2 activity on adherent cell removal, from $4,572 \pm 1,314$, to $32,973 \pm 6,350$, ($p < 0.001$) mimicking the pattern observed in PPD-reactive individuals. They were designated normal producers of IL-2 (NP). The effect of adherent cell depletion on blastogenic responses was also examined. In these experiments T cells were reconstituted with 5% adherent cells to assure adequate accessory activity. Adherent cell depletion resulted in increased PPD-induced blastogenesis in NP patients from $9,789 \pm 2,681$ in PBMC to $15,563 \pm 3,370$ in cultures of T cell plus 5% adherent cells ($p < 0.01$).

TABLE II
Clinical Features of Patient Groups

Subject	Age (yr)	Male/female ratio	Chest x-ray				Initial PPD (5 TU)		Percent PBMC peroxidase-positive
			Far advanced	Moderately advanced	Minimal	Miliary	Positive	Negative	
NP tuberculosis patients (<i>n</i> = 12)	50.5	12:0	2*	9	0	1	7/8 [‡]	1/8	25.6 ± 6.4 [‡]
LP tuberculosis patients (<i>n</i> = 10)	48.7	8:2	8*	1	0	1	2/4	2/4 [‡]	27.6 ± 5.1 [‡]

* Differ by Fisher's exact scientific test, $p = 0.027$.

[‡] Not significantly different.

The second group of tuberculosis patients was composed of 10 individuals in whom PPD-induced IL-2 activity was significantly depressed and remained low despite adherent cell removal: $2,075 \pm 579$ PBMC vs. $2,642 \pm 502$ T cell cultures; they were designated low producers (LP). Adherent cell depletion also augmented PPD-induced blastogenesis slightly in these patients ($2,720 \pm 880$ PBMC vs. $7,570 \pm 2,534$ T cells + 5% adherent cells) but this failed to achieve statistical significance ($p > 0.05$).

Clinical features of the two categories of tuberculosis patients are shown in Table II. The major difference between the two groups was the significantly higher number of LP patients with far advanced disease on chest x-ray ($p = 0.027$). Each group contained one patient with miliary tuberculosis and neither included patients with minimal lesions. No difference was observed between the two groups in the fraction of PBMC identified as monocytes by peroxidase staining. Data on PPD skin test reactivity did not show significant differences between the two categories.

Comparison of Adherent Suppressor Cell Activity in Patients with Tuberculosis and Controls. Depletion studies indicated that adherent suppressor cells contributed to low PPD-induced IL-2 production by PBMC in our NP tuberculosis patients. In order to quantify the suppressive capacity of adherent cells, T cells were cocultured with graded numbers of adherent cells (2 and 25% of T cells) and PPD-induced IL-2 activity determined. Results are shown in Fig. 2. Adherent cells from NP patients were more suppressive of IL-2 production than was the case in healthy donors; the presence of as few as 2% adherent cells in T cell cultures caused a 50% drop in IL-2 activity, from $32,973 \pm 6,350$ to $15,267 \pm 5,216$ ($p < 0.05$). In PPD-reactive healthy controls, on the other hand, IL-2 activity of supernatants of antigen-stimulated T cells and T cells + 2% adherent cells was comparable ($59,079 \pm 7,415$ vs. $40,722 \pm 6,498$) ($p > 0.05$); a significant 5.3-fold suppression of IL-2 activity was apparent, however, when 25% adherent cells were present in T cell cultures ($p < 0.001$). Indomethacin ($1 \mu\text{g/ml}$) present in cultures of T cells with 25% adherent cells did not significantly block sup-

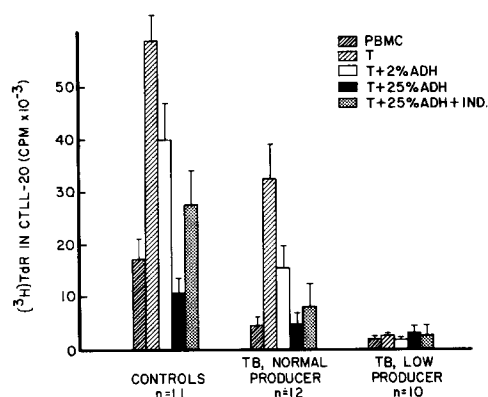


FIGURE 2. PPD-induced IL-2 activity in cultures of PBMC, T cells (T), T cells + 2% adherent cell (ADH), T cells + 25% adherent cell, and T cells + 25% adherent cells in the presence of 1 μ g/ml indomethacin. Each bar represents mean \pm SEM.

TABLE III
Effect of Purified IL-2 (1% vol/vol) on PPD-induced Blastogenesis of PBMC in Tuberculosis Patients and Healthy Donors

Experiment	IL-2	PPD	Blastogenesis*
Patients (n = 12)	-	-	1.3 \pm 0.3
	-	+	5.8 \pm 1.7
	+	-	4.2 \pm 1.1
	+	+	10.5 \pm 1.9
Controls (n = 10)	-	-	1.5 \pm 0.3
	-	+	25.9 \pm 3.2
	+	-	7.9 \pm 1.8
	+	+	36.1 \pm 5.6

* [3 H]TdR in PBMC cultures. Data are shown as mean cpm \pm SEM ($\times 10^{-3}$).

pression of PPD-induced IL-2 activity in tuberculosis NP (4,873 \pm 2,523 vs. 7,945 \pm 4,662) ($p > 0.05$) while in the healthy donors, there was a partial but significant reversal of suppression by indomethacin (10,972 \pm 3,398 vs. 27,775 \pm 7,465) ($p < 0.05$).

Response of Mononuclear Cells from Tuberculous Patients to Purified IL-2. We studied the effect of purified IL-2 on PBMC responsiveness to PPD in 12 patients with tuberculosis. Purified IL-2 (Lot No. 1608-54; Electro-Nucleonics Inc.) was cocultured with PBMC at 1% vol/vol concentration in proliferation assays. This concentration of purified IL-2 contains 20 U/ml of activity as determined by modified Probit analysis in relation to an IL-2 standard that exceeds the activity in supernatants of tuberculin-stimulated adherent-depleted T cells from tuberculin reactors (mean 17.25 U/ml, $n = 5$). Results are shown in Table III. There was an apparent increase in [3 H]TdR of PPD-stimulated PBMC of patients in the presence of IL-2. This could be accounted for, however, by the increased

TABLE IV
Expression of IL-2-R (Tac Antigen) on PPD-stimulated PBMC from Tuberculosis Patients and Healthy Donors

Exp.	Percent of PBMC reactive with anti-Tac	
	TB patient	Healthy donor
1	4.36*	23.36
2	3.47	33.25
3	3.94	39.20
4	9.69	—
Mean \pm SD	5.36 \pm 2.9 [†]	31.93 \pm 8 [†]

* PBMC were cultured with PPD for 5 d. Anti-Tac-reactive cells were assessed by indirect immunofluorescence and FACS analysis.

[†] $p < 0.01$.

[³H]thymidine incorporation of PBMC cultured in the presence of IL-2 without PPD. Similar results were obtained with higher concentration of IL-2, 5 and 10% vol/vol, although the greater mitogenic activity of IL-2 alone complicated interpretation of these data. Virtually identical results were observed in healthy PPD-reactive controls. These data indicate that exogenous IL-2 was directly mitogenic for PBMC but unable to reverse hyporesponsiveness to PPD in tuberculosis.

T Cell Expression of IL-2-R in Patients with Tuberculosis. The above findings indicate not only a defect in PPD-induced IL-2 production in tuberculosis, but also an inability of exogenous IL-2 to correct tuberculin hyporesponsiveness of mononuclear cells. The latter could be secondary to lack of development of IL-2-R on PPD-activated PBMC. Accordingly, we evaluated the expression of the IL-2-R in four patients and three tuberculin reactive controls by FACS analysis using the mAb anti-Tac. Table IV shows these results. The fraction of PPD-stimulated PBMC that reacted with anti-Tac was significantly depressed in tuberculous subjects ($p < 0.01$). Three of the four patients studied were NPs of IL-2, as defined above, and one was an LP. Streptolysin 0-induced Tac expression, however, was similar in two patients as compared with their controls (data not shown). Therefore, the depression in Tac expression, as well as the defects in IL-2 production and blastogenesis, were restricted to responses to tuberculin.

Discussion

These studies show that patients with newly diagnosed, active pulmonary tuberculosis have a defect in PPD-stimulated IL-2 production and lymphocyte blastogenesis. Responses to streptococcal antigen, however, were comparable to those of healthy donors. Deficient IL-2 production has been reported in a number of experimental settings (23–25). In humans, depressed IL-2 production has been shown in patients with the acquired immunodeficiency syndrome (26), lepromatous leprosy (27), and recurrent oral herpes simplex virus infection (28). In patients with lepromatous leprosy, both purified IL-2 and recombinant IL-2 reversed the unresponsiveness of PBMC to antigen (27, 29). Furthermore, in *Mycobacterium bovis* infected mice, IL-2 corrected defective T cell proliferation

(30). In our study, however, purified IL-2 in activities producing maximal responses in CTLL-20 and exceeding those generated by PPD-stimulated T cells from healthy tuberculin reactors failed to correct PPD-activated PBMC proliferation; in these concentrations, IL-2 was directly mitogenic for PBMC both in controls and patients. Recombinant IL-2 also has mitogenic activity (29, 31) and, in a recent study, failed to reconstitute *M. leprae* responses in lepromatous patients (31). The inability of exogenous IL-2 to correct PPD-stimulated PBMC unresponsiveness in tuberculosis was associated with deficient antigen-stimulated expression of IL-2-R identified by anti-Tac monoclonal antibody. This resembles the recent observations in lepromatous leprosy (31). Antigen-restricted defects in production of and response to IL-2 could reflect abnormal immunoregulation by adherent cells that have been shown to depress PPD-induced blastogenesis in tuberculosis (5). Adherent cell suppression, in fact, contributed to low PPD-induced IL-2 production and T cell blastogenesis in one subset of our tuberculosis patients (NP). Regulation of antigen-stimulated T cell IL-2 production by adherent cells in NP patients differed from healthy tuberculin reactors, however, in its greater magnitude and lack of indomethacin reversibility. These observations are consonant with the data on regulation of blastogenesis in tuberculosis (5, 28, 32). Prostaglandin E₂, although a known inhibitor of IL-2 production in both mitogen (33, 34) and antigen-activated cell systems (35) of healthy donors, does not appear to be a major mediator of suppression by adherent cells in tuberculosis.

In the LP patients, PPD-induced T cell IL-2 production remained low despite adherent cell depletion. LP patients were clinically distinct from NP patients in significantly higher frequency (80%) of far advanced lesions on chest x rays, indicating more extensive disease. Thus, in tuberculosis as in leprosy (36), more extensive tissue involvement was associated with more profound defects in cell-mediated immunity. The depressed T cell IL-2 production in LP patients could be due to active suppression mediated by nonadherent lymphocytes present in this population. We have previously shown the capacity of FcγR⁺ lymphocytes to function as tuberculin-specific suppressor cells in tuberculosis (10). Alternatively, the small residual monocyte content of nylon-wool-purified T cells (<1% peroxidase positive) could contribute to depressed IL-2 production. Further studies will be necessary to evaluate these possibilities.

The disturbed regulation of IL-2 homeostasis in tuberculosis must be placed in the context of other observations on the cellular interactions and cytokine cascade required for a cell-mediated immune response. In comparable clinical populations, suppression of PPD-induced blastogenesis could be ascribed to adherent monocytes (5), which also showed initially decreased surface expression of HLA-DR determinants followed by a burst in their synthesis during *in vitro* cultivation (37), and increased production of IL-1 (38). These alterations in key immunoregulatory properties of mononuclear phagocytes may contribute to the disturbances in IL-2 homeostasis in tuberculosis. The ultimate consequence of dysregulation of IL-2 metabolism on IFN-γ production and activation of the mononuclear phagocyte as an effector cell is the subject of current investigations.

Summary

Patients with newly diagnosed, pulmonary tuberculosis had a tuberculin-specific defect in IL-2 production. Mean PPD-induced IL-2 activity was 81.2% lower in patients as compared with healthy tuberculin reactors. PPD-induced expression of T cell IL-2 receptors was 5.9 times less in peripheral blood mononuclear cells of patients with tuberculosis as compared with healthy tuberculin reactors. Furthermore, purified IL-2 failed to correct PPD-induced blastogenesis in patients. Suppression by adherent cells was operative in one group of patients; adherent cell depletion increased their T cell production of IL-2 7.2-fold. A second group of patients with low IL-2 production did not have suppressor adherent cells and were clinically distinct, with more extensive disease on chest x ray. The basis for low IL-2 production in such individuals is unknown. Disordered regulation of IL-2 metabolism may be a key feature in the depressed cellular immune response of tuberculosis.

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