

Reduced Cytokine Secretions by LAK Cells of Pulmonary Tuberculosis Patients in Response to Tumor Targets *In Vitro*

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ABSTRACT

Activation of macrophages and other immune components to release a series of proinflammatory cytokines is one of the first events in innate resistance to intracellular infections. Severe manifestations of tuberculosis (TB) could be caused by alterations in the balance of these cytokines. In this study, lymphokine-activated killer (LAK) cells of TB patients and normal individuals were generated by stimulation with cytokines *in vitro*. The LAK cells of both groups were further triggered with allogeneic tumor targets. Cytokines interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were estimated in the supernatants generated in the two groups. The aim was to see if infection with TB influenced the secretory capacity of the immune cells *in vitro*. Reduced cytokine profiles were observed in TB patients, indicating defective interactions between patient effector cells with allogeneic transformed cells compared with normal individuals. Partial restoration of IFN- γ production was seen with a combination of cytokines interleukin-2 (IL-2) and IL-12 in TB patients. Based on the *in vitro* observations, we hypothesize that *in vivo* also there is diminished immune cell activation of effector cells in response to the presence of infected macrophages. This probably leads to a diminished secretory function that can be corrected by the use of such cytokines as IL-2 and IL-12. The effector populations of TB patients are probably in a state of target-induced anergy, allowing the bacteria to thrive, and immunomodulatory cytokines that improve the host immune response toward countering mycobacterial infection.

TUBERCULOSIS (TB) REMAINS AN IMPORTANT CAUSE of morbidity and mortality worldwide, with one third of the population infected with the tubercle bacillus.⁽¹⁾ By far the most important factor that determines whether infection will ultimately lead to disease is the adequacy of the host immune response in general and the cell-mediated immune response in particular. Proof of this lies in the fact that the risk of TB is linked to cellular immunodeficiency conditions associated with age, immunosuppressive drugs, and other diseases, such as diabetes, malignancies, silicosis, renal failure, and most notably AIDS.⁽²⁾ For a successful protective immune response, the network involving infected macrophages, activated T lymphocytes, activated natural killer (NK) cells, and their respective cytokines needs to be well coordinated.⁽³⁾ The recent outbreak of multidrug-resistant TB underscores the need for novel approaches to treat and prevent TB, including the use of immunotherapeutic modalities that enhance the antimycobacterial

defense. Development of such strategies requires a detailed study of the cytokines that mediate resistance toward TB.^(4,5)

Macrophage interleukin-12 (IL-12) production was shown to be prominent in tuberculous lymph nodes, and it is known that IL-12 is a central initiator of the local Th1 response,⁽⁶⁾ as it is responsible for induction of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-8, and IL-2 cytokines from T and NK cells.⁽⁷⁻⁹⁾ It is known that *in vitro* culture of human peripheral blood lymphocytes (PBL) in IL-2 results in the generation of cytotoxic cells that lyse autologous and allogeneic tumor targets through release of a number of well-characterized slow-acting cytokines, such as natural killer cytotoxic factor (NKCF), IFN, TNF, and lymphotoxin, that are cytostatic or cytotoxic for tumors. This has been referred to as the lymphokine-activated killer (LAK) phenomenon, with NK cells being the major contributors to LAK activity.⁽¹⁰⁻¹⁵⁾ Similarly, it is pos-

sible that infected monocytes likewise initiate a cascade of cytokine release from the LAK cells generated *in vivo* under infectious conditions through release of such cytokines as IL-2. On the basis of this model, experiments were designed to see if the potential problem in the host immune response of the TB patient was a defect in the trigger of important cytokines by the patient's LAK cells. The allogeneic cell line U937 served as a tumor target model to trigger the LAK cells generated *in vitro* in the patient group and was compared with LAK cells of normal individuals. As human infection with *Mycobacterium tuberculosis* displays a spectrum of manifestations that reflect the efficacy of the immune response,⁽¹⁶⁾ understanding the mechanisms of LAK cell-mediated cytokine secretions *in vitro* will provide an insight into how the production of cytokines may be regulated *in vivo*.

LAK cells were generated by the method of Grimes and Hersh's group.⁽¹⁷⁾ Eight patients smear and culture positive for TB and eight normal individuals (25–50 years of age) were included in the study. Peripheral blood mononuclear cells (PBMCs) from both groups were obtained by the standard Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation method and adjusted to 10×10^6 cells/ml in complete RPMI 1640 culture medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 5 mM HEPES, 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 20 μ g/ml gentamicin at pH 7.2–7.4 (Sigma Chemical Co.). PBMC (2.5×10^6) were cultured in 12-well tissue culture plates (Costar, Cambridge, MA) in complete RPMI medium in the presence of IL-2 (100 U/ 10^6 cells), IL-12 (50 U/ 10^6 cells), and IL-2 + IL-12 (50 U + 10 U/ 10^6 cells) for 3 days at 37°C in a humidified 5% CO₂ atmosphere. rIL-2 was purchased from Genzyme (Cambridge, MA), and rIL-12 was a kind gift from G. Trinchieri (Philadelphia, PA). At the end of the incubation period, nonadherent cells and the supernatants were collected and centrifuged at 1800 rpm. The supernatants were gently aspirated and stored at –70°C in small aliquots, taking care to avoid repeated freeze-thaw cycles.

After removal of the supernatants of the LAK cells, the effector cells (E) were thoroughly washed and resuspended in complete medium. They were then mixed with 5×10^6 U937 cells (tumor target cell line [T] obtained from the National Facility for Animal Tissue and Cell Culture, DBT, Pune, India). The E:T ratio was maintained at 1:2, and the mixture was incubated for a further period of 18 h. The mixed population was harvested, and the supernatants were gently collected and stored at –70°C in small aliquots. Appropriate controls were maintained.

The LAK supernatants collected before and after coculturing with tumor targets were analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of the cytokines IFN- γ , TNF- α , and GM-CSF according to the standard protocol using ELISA reagents (Endogen, Boston, MA). The results were analyzed using Student's *t*-test.

In Figure 1, cytokine production by LAK cells in normal individuals and TB patients is illustrated. IL-2 and IL-12 were potent inducers of IFN- γ , producing 5000 ± 2000 pg/ml and 8000 ± 2000 pg/ml IFN- γ , respectively, in normal individuals. IL-12 appears to be more potent than IL-2, and the two cytokines together further enhance the production of IFN- γ , up to 13000 ± 6500 pg/ml in normal individuals, compared with either of them alone ($p < 0.01$). In the case of TB patients, IFN-

γ levels in response to IL-2 and IL-12 were around 4000 ± 2000 and 7000 ± 1000 pg/ml, respectively. The IFN- γ level was 8000 ± 1000 pg/ml in response to the two cytokines together. These results indicate that TB patients also produce IFN- γ in response to IL-2/IL-12 stimuli, but the levels of IFN- γ were significantly reduced ($p < 0.05$) compared with normal individuals. It was found that in normal individuals, an average of 8000 pg/ml IFN- γ was produced in response to prestimulation with IL-2 alone, and 16,000 pg/ml was produced in response to IL-2 + IL-12, but when IL-12 alone was used, the response was not so dramatic. The levels dropped to 2500 pg/ml, indicating that by itself IL-12 was unable to prestimulate the cells to produce IFN- γ in response to the target cells. Similarly, TB patients also showed reduction in IFN- γ levels when triggered with tumor targets when prestimulated with IL-12 alone. IL-2 also failed to prestimulate the cells to produce IFN- γ in response to tumor targets, in contrast to normal individuals. However, the response to a combination of IL-2 and IL-12 was found to be 7000–9500 pg/ml. The results indicated that there was a significant reduction in cytokine secretion by TB patients in response to tumor targets, compared with normal individuals ($p < 0.001$). It follows that IL-2 and IL-12 are potent inducers of IFN- γ , acting in a synergistic manner. Patients respond well to a combination of IL-2 and IL-12 prestimulation, although the response was significantly less than that in normal individuals. IFN- γ production is probably one of the most physiologically relevant functions of IL-12, and many of IL-2 *in vivo* effects are mediated, at least in part, through IFN- γ .⁽¹⁸⁾ It is well known that IL-2 stimulates the secretory activities of cytotoxic lymphocytes. IL-12 also strongly synergizes with other stimuli in inducing maximum levels of IFN- γ production and is a known Th1 inducer. These observations have been corroborated in the present study.

The amounts of TNF- α were also compared in both groups in the same manner as described for IFN- γ . As seen in Figure 1, LAK cells produced by three different stimulation conditions secreted no more than 420 ± 300 pg/ml of TNF- α in normal individuals, whereas in TB patients, the average TNF- α levels in unstimulated and stimulated cells were around 2000–3500 pg/ml. These results indicate that TB patients produce significantly higher levels of TNF- α than do normal individuals ($p < 0.01$). On coculturing the prestimulated cells overnight with target U937 cells at a stimulator/effector ratio of 2:1, there was a substantial increase in cytokine production in normal individuals. It was found that 200–2000 pg/ml TNF- α was produced in response to prestimulation with IL-2 + IL-12, but when IL-12 alone was used, the response was not so dramatic. The levels dropped to 15–90 pg/ml, indicating that by itself IL-12 was unable to prestimulate the cells to induce them to produce TNF- α in response to the target cells. There was, however, no TNF production in response to tumor targets among the TB patients even after prestimulation with the cytokines. Therefore, it is clear that there was a significant reduction in the secretion of TNF- α by TB patients in response to tumor targets ($p < 0.001$) compared with normal individuals. In mycobacterial infection, TNF- α is required for control of bacillary growth and the protective granulomatous response, but it may cause an immunopathologic condition. Results from experiments using murine models suggest that the relative amount of TNF- α at the site of infection determines whether the cytokine is protective or destructive.⁽¹⁹⁾ TNF- α is even implicated in the synergism between *M. tuberculosis* and HIV infection.^(20,21) The role

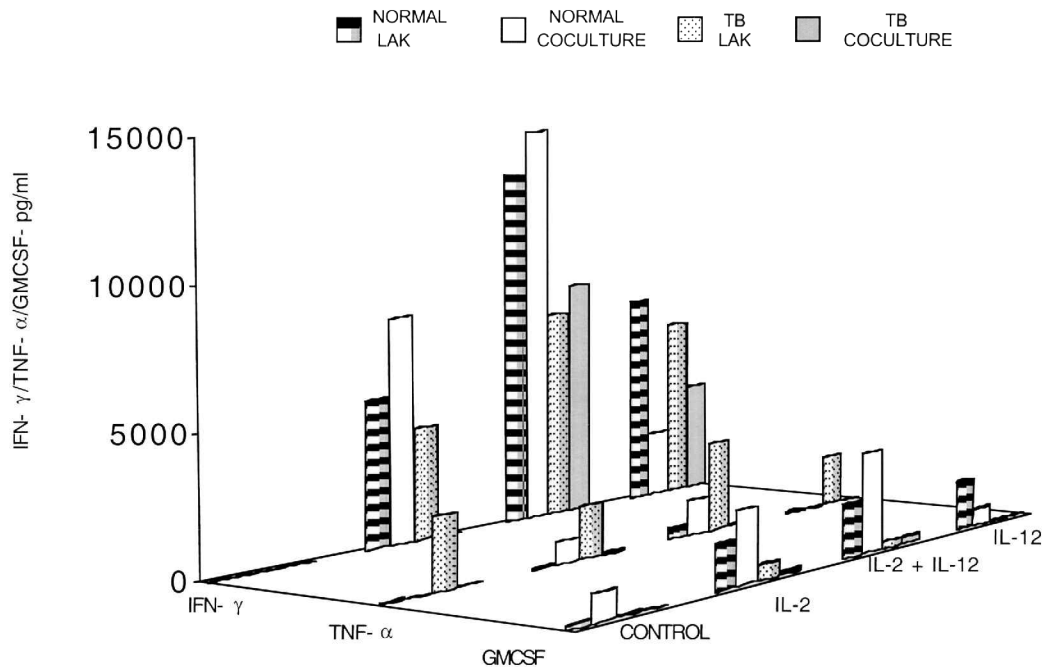


FIG. 1. Comparison of cytokine (IFN- γ /TNF- α /GM-CSF) production by LAK cells in response to stimulation by different cytokines (control/IL-2/IL-2+IL-12/IL-12) in normal individuals and TB patients, respectively. Comparison of cytokine production after LAK coculture with U937 target cells in normal individuals and TB patients, respectively ($n = 8$). Data are expressed as mean \pm SD for each category. IFN- γ production by LAK cells in response to a combination of IL-2 + IL-12 was significantly greater compared with each cytokine alone ($p < 0.01$) and significantly lower in TB patients than in normal individuals ($p < 0.05$). In response to tumor targets, the levels were significantly greater in normal individuals than in TB patients ($p < 0.001$). TNF- α production by LAK cells in TB patients was significantly higher than in normal individuals ($p < 0.01$). Coculturing with tumor targets yielded opposite results, with TB patients showing significantly lower values ($p < 0.001$). GM-CSF production by LAK cells in response to three different stimulations in normal individuals was significantly higher than in TB patients ($p < 0.05-0.01$). In normal individuals, it increased substantially after LAK coculture with U937 target cells ($p < 0.01$) and was significantly higher compared with levels in TB patients ($p < 0.001$).

of TNF- α is intriguing and needs to be further investigated before the complexities of the web of cytokine regulation of T cell function can be untangled.

GM-CSF was assessed in the same fashion as IFN- γ and TNF- α . As seen in Figure 1, LAK cells produced by three different stimulations secreted around 2500 pg/ml GM-CSF in normal individuals and no more than 50–500 pg/ml in TB patients ($p < 0.05-0.01$). On coculturing the prestimulated cells overnight with target U937 cells at a stimulator:effector ratio of 2:1, there was a substantial increase in cytokine production in normal individuals compared with the LAK values ($p < 0.01$). It was found that 200–2000 pg/ml GM-CSF was produced in response to prestimulation with IL-2 and IL-12, but when IL-12 alone was used, the response was not so dramatic. The levels dropped to 15–90 pg/ml, indicating that by itself IL-12 is unable to prestimulate the cells to a great extent to induce them to produce GM-CSF in response to the target cells. There was, however, no GM-CSF production in response to tumor targets among the TB patients even after prestimulation with the cytokines compared with normal individuals ($p < 0.001$).

Unstimulated PBMCs could not be activated by the allogeneic tumor cell line to release cytokines, as seen from the control values, and target cells did not produce these cytokines when cultured alone (data not shown).

These data indicate that the interaction between LAK cells

and tumor cells causes a large increase in the production of cytokines with known cytotoxic potential in normal individuals. However, the secretory functions of effector cells in TB patients were found to be significantly altered. It is possible that *in vivo* also the effector population of TB patients shows diminished secretory functions in response to the presence of infected macrophages. Decreased cytokine induction by PBLs in response to *M. tuberculosis* infection may lead to diminished macrophage activation and a more permissive environment for the bacteria. Patients with active TB manifest reduced activity at the T cell, monocyte, and NK cell levels compared with normal individuals. This is probably due to the decreased induction of cytokines by TB patient cells in response to mycobacterial infection.

From these results, one can speculate that the effector populations of TB patients are in a state of target-induced anergy and, hence, do not respond in an appropriate manner. This can, however, be overcome to a large extent by a combination of the two cytokines IL-2 and IL-12. Knowledge of the cytokine response to infectious diseases is important in understanding the immune basis of the host response.⁽¹⁵⁾ Major players have emerged, and immunotherapy aimed at manipulating these has already shown promise.⁽²²⁻²⁴⁾ The current challenge is to master the art of navigating the fine line between protection and pathology in TB.^(25,26)

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