IFN-γ IN PERIPHERAL BLOOD MONONUCLEAR CELLS AND BRONCHOALVEOLAR LAVAGE PATIENTS WITH PULMONARY TUBERCULOSIS BEFORE AND AFTER TREATMENT WITH ORAL ANTI-TUBERCULOSIS DRUGS

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ABSTRACT

Results of cytokine production by human T cells in response to Mycobacterum tuberculosis vary widely between studies. The present study was to determine the profile of the Th1 immune response in pulmonary tuberculosis during treatment. Nine male patients with M. tuberculosis culture positive pulmonary tuberculosis were studied. IFN-γ cytokine was measured by ELISA from culture of peripheral blood mononuclear cells and bronchoalveolar lavage fluid cells with purified protein derivative (PPD). Only nine patients completed the study. The result of IFN-γ in PBMC before treatment after challenged with PPD was 5705.33 pg/ml and 10682.71 pg/ml after treatment with oral anti-tuberculosis (OAT). The result of IFN-γ in BAL fluid after challenged with PPD was 10855.33 pg/ml and 27.17 pg/ml after completion the chemotherapy. Changes in the IFN-γ cytokine profile to Th1 in PBMC patients after treatment conflicted with the result from the BALF cells. This study showed that in these patients the Th2 immune response still present at the site of the disease.

Keywords: *pulmonary tuberculosis, Th1/Th2, IFN-γ, PBMC, BALF*

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INTRODUCTION

Tuberculosis (TB) remains a common infectious disease, with nearly one-third of the world's population being infected by *Mycobacterium tuberculosis* (*M. tuberculosis*) and 8 million new TB disease cases occurring each year despite the development of anti-tuberculosis drugs, which have been available for more than 50 years (Raju 2008). In 2003, there were 8.8 million new cases of TB, of which 3.9 million were smear positive; 674,000 of the patients were coinfected with HIV. There were a total of 15.4 million cases, of which 6.9 million were smear positive. An estimated 1.7 million people died from TB, including 229,000 people coinfected with HIV (WHO 2006).

Understanding the host cellular immune response would be valuable for developing new treatments to improve clinical outcome, reduce the long duration of chemotherapy, and inhibit reactivation of latent infection. Tuberculosis usually affects the lungs (pulmonary TB) although may involve any organ. The most common route of infection is by inhalation of droplets carrying *M. tuberculosis*. *M. tuberculosis* activates the cellular immune system and results in a local lung immune response that generally contains the infection. The mechanisms of protective immunity against *M. tuberculosis* in humans have not been fully clarified. Cell-mediated immunity (CMI), especially CD4+ T lymphocyte function, is crucial in eliminating the mycobacteria in tuberculosis. Other T lymphocytes, such as γδ, CD8+ and CD1-restricted T lymphocytes have roles in the immune response to *M. tuberculosis* infection (Kaufmann 2004).

T lymphocytes can be divided into at least two phenotypic classes: T-helper (Th) 1 and Th2. Th1 CD4+ lymphocytes secrete interferon-gamma (IFN-γ) and contribute to growth control of *M. tuberculosis* infection in the lung. Interleukin (IL)-12 promotes IFN-γ production and Th1 development via signaling pathways that lead to signal transducer and activator of transcription-4 (STAT-4) activation. Th2 lymphocytes typically produce IL-4, IL-5 and IL-10, which enhance antibody synthesis of B cells and play a role in allergic diseases. Mycobacteria predominantly induce CD4+ Th1 cells and CD8+ cytotoxic T cells with a Th1-like cytokine profile of elevated IL-2 and IFN-γ levels. The Th1/Th2 hypothesis postulates that two different and reciprocally inhibitory lymphocyte responses operate after stimulation with environmental or bacterial

antigens and that these phenotypes are defined by specific cytokine production. The progression of disease by *M. tuberculosis* in infected individuals is based on the outcome of the immune responses. There are no well-defined immunological correlates of protective immunity in human tuberculosis (TB) and despite robust site-specific Th1 responses the disease progresses. (Rook 2001). In this study, IFN-γ was measured from the bronchoalveolar lavage and peripheral blood cells in TB patients before and after treatment.

MATERIALS AND METHODS

This study was an observational prospective with pretest-posttest design. The study was done in the outpatient of the Pulmonary Department of Dr Soetomo General Hospital and was approved by the Ethics Committee of the Dr Soetomo General Hospital. Each case was examined twice during 6 months treatment with oral antituberculosis (OAT) drugs using standard directly observed treatment short-course according to the guidelines for treatment of tuberculosis by WHO (WHO 1997). The first examination was taken directly after the patient diagnosed as moderately-advanced pulmonary tuberculosis (Anonymous 1969) and the second examination done after the patient finished the 6 months treatment. Twelve male new culture-positive for *M. tuberculosis* pulmonary TB patients, age between 18-40 years old were tested for the inclusion criteria and bacteriology. The blood and bronchoalveolar lavage fluid (BALF) patients were collected before and after 6 months treatment.

Ten milliliters of intravenous blood was collected from ante-cubital vein into 10-ml syringe and diluted with phosphate buffer saline (PBS). Separation of the mononuclear cells was done by centrifugation with 2:1 dilution using Histopaque 1077. After washing with PBS, resuspension of the peripheral blood mononuclear cells (PBMC) with 1 ml or more of complete RPMI-1640 culture medium and concentrated to 1 x 10^6 for further examination.

Bronchoalveolar lavage was performed using a fiber-optic bronchoscope (Olympus Co., Tokyo Japan) with the administration of local anesthetic (2% lidocaine) by the Standard Operation Procedure of the Pulmonary Department Dr Soetomo Teaching Hospital. Four times of 50 ml aliquots of warm sterile normal saline were introduced through the bronchoscope, aspirated and collected into a plastic bottle. Cell isolation from the BALF was performed under hypothermic conditions in a sterile environment. Following volume measurement, the BALF was filtered through a sterile gauze and centrifuged at 600 g for 5 min in 4^0C temperature, after which the supernatant was separated. The cells were washed twice in complete RPMI-1640 culture medium and after centrifugation the pellet was resuspended in 1 ml of the culture medium. The mononuclear cells were counted with Trypan Blue in a Neubauer chamber. For IFN-γ cytokine analysis, the concentration of cells suspension was adjusted to 1x10^6 in 1 ml of BALF.

The antigen used for challenge was Purified protein derivative (PPD) batch RT44 and purchased from Statens Seruminstitute and was used at a final concentration of 10 µg/mL. The antigens were all tested to determine the optimal concentration for use. Cytokine production and assays were done using the suspension of 1 x 10^6 cells/ml from PBMC and BALF in complete RPMI culture medium in 96 well-plated and were incubated with 100 µl of 10 µg/ml of PPD in 37^0C and 5% of CO_2 for 4 days. Supernatants were stored at 80^0C deep-freezer. The total amount of IFN-γ present in culture supernatant was analyzed by ELISA according to the instructions of the manufacturer with sensitivity 1-2 pg/ml (CLB Pelikine Kit IFN-γ, Amsterdam). Statistical analysis was done using the Pearson correlation test for analyzing normal distribution data sets. The Spearman test was used for data sets that were not normally distributed. $P < 0{\cdot}05$ was considered significant.

RESULTS

There were 12 male patients from the Outpatient of the Pulmonary Department Dr Soetomo General Hospital on the first examination, but only 9 patients finished the second examination (75%). The patients were from 18 to 40 years old and the mean was 27.56 years old with deviation 3.97. Mean bodyweight was 51.22 kg with deviation 5.24. The result of IFN-γ in PBMC after challenged with PPD before treatment was 5705.33 pg/ml and after treatment with OAT was 10682.71 pg/ml (Fig 1). The result of IFN-γ in BAL fluid after challenged with PPD before treatment was 10855.33 pg/ml and after treatment with OAT was 27.17 pg/ml (Fig 2). IFN-γ production increased from the PBMC but decreased from BALF cells after treatment and the result was not correlated (p>0.05) (Fig 3).

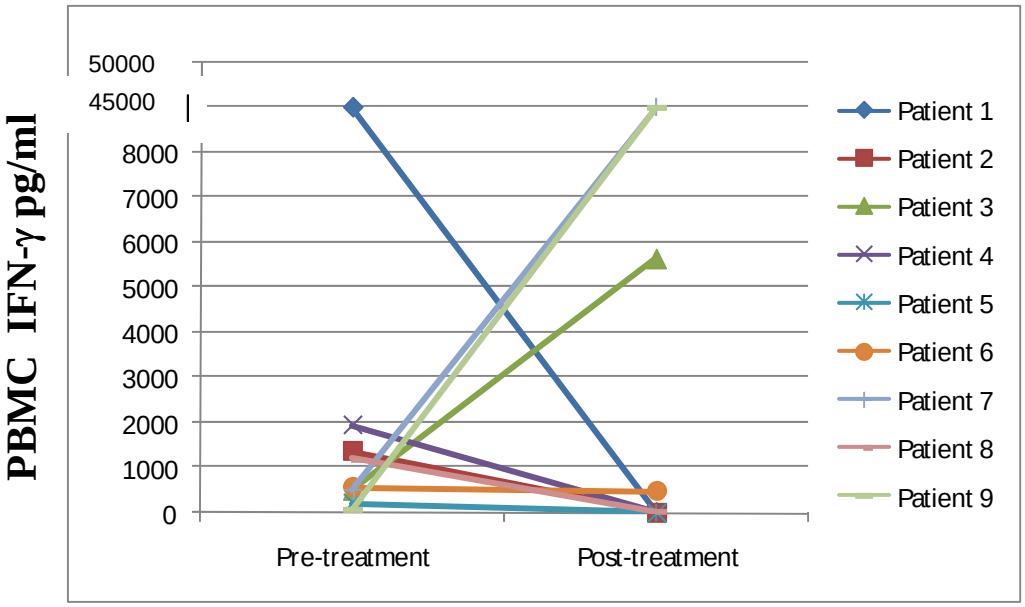

Figure 1. IFN-γ from PBMC challenged with PPD
before and after treatment with Oral Anti-tuberculosis

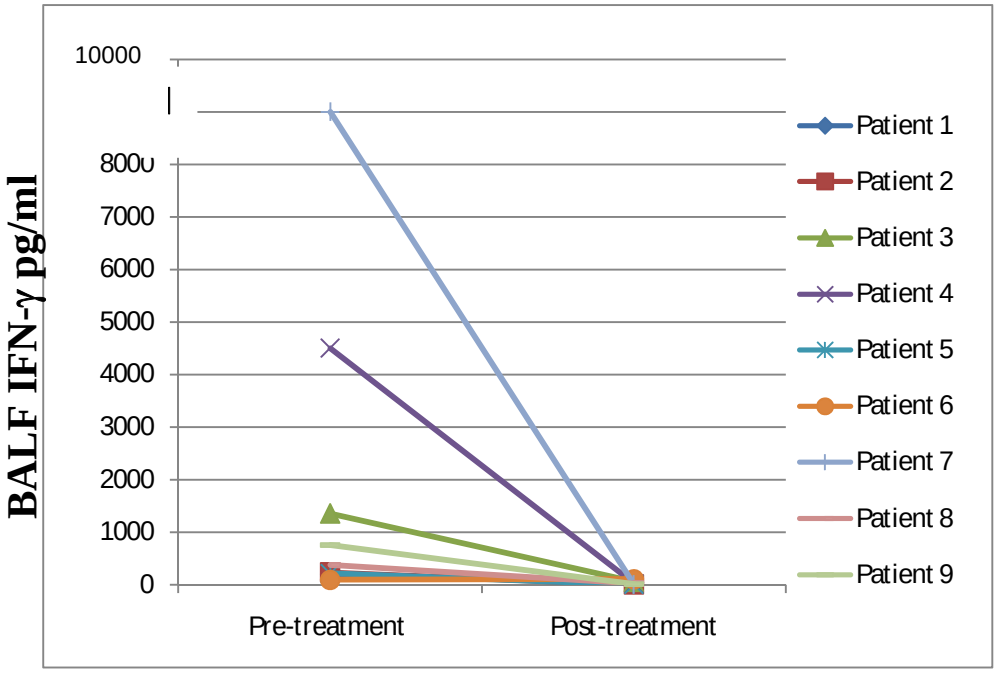

Figure 2. IFN-γ from BALF cells challenged with PPD
before and after treatment with Oral Anti-tuberculosis

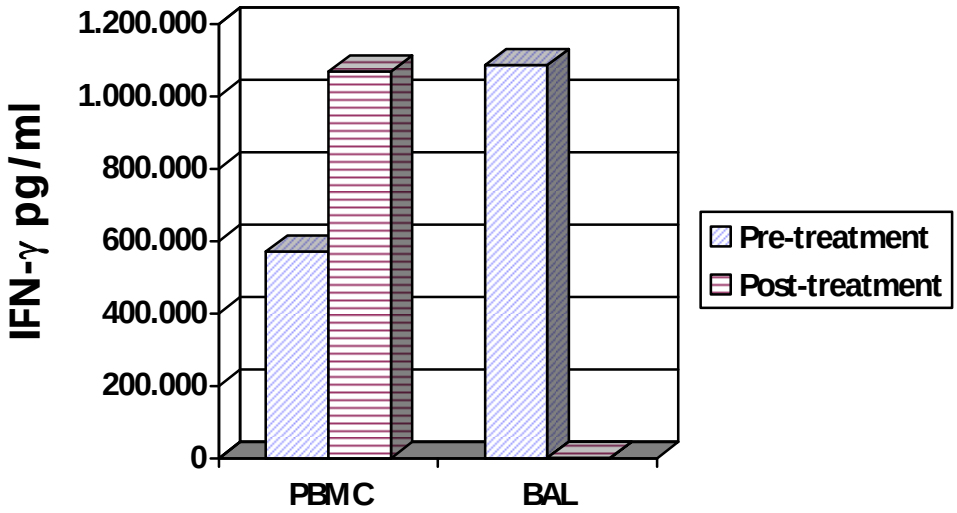

Figure 3. IFN-γ production increased from mononuclear cells in PBMC after challenged with PPD but decreased from BALF cells before and after treatment with Oral Anti-tuberculosis

DISCUSSION

In the beginning of this study there were 12 patients but only 9 patients could finished the examination. After challenged with PPD the result of IFN-γ in PBMC before treatment was 5705.33 pg/ml and after treatment with OAT was 10682.71 pg/ml. The increase of the IFN-γ in the peripheral blood may be cause by the increase of the immune response Th1 after success of chemotherapy because all of the patients were improved. After challenged with PPD the result of IFN-γ in BALF before treatment with OAT was 10855.33 pg/ml and 27.17 pg/ml after treatment. The decrease IFN-γ levels in BALF after successful treatment in this study raises the possibility that IFN-γ actually might be a marker of local inflammation in the lung.

The available reports on cytokine production by human T-cells in response to *M. tuberculosis* are conflicting. Tsao and colleagues investigate from 45 patients presenting with active pulmonary TB and 14 healthy controls the possible correlations for IFN-γ and soluble interleukin-2 receptor (sIL-2R)-α levels in epithelial lining fluid (ELF) levels from BALF, and clinical grade of pulmonary TB. Patients with higher-grade pulmonary TB (i.e., with more advanced pulmonary involvement, fever or body weight loss), revealed significantly higher ELF levels for IFN-γ and sIL-2R-α compared to those with lower grade pulmonary TB. After anti-tuberculosis chemotherapy the elevated cytokine levels for ELF and serum significantly decreased in accordance with radiographic improvement (Tsao 2002).

Boras et al. measured the production of the intracellular cytokines IFN-γ in CD4+ and CD8+ T cells obtained from peripheral blood and BALF of 20 tuberculin skin-positive patients and compared with the findings recorded in 9 tuberculin skin-negative patients with active pulmonary tuberculosis. The study demonstrated a more intensive and dominant Th1 cytokine response on PMA/ionomycin activated T lymphocytes from peripheral blood of tuberculin-negative subjects (usually a more advanced form of tuberculosis) versus a poorer response in tuberculin-positive subjects (the milder form of tuberculosis). On the other hand, a more active local immune response in the lungs, manifested by a more intensive synthesis of IFN-γ to PMA/ionomycin by BALF T lymphocytes when compared with weak responses in peripheral blood, was demonstrated in both patient groups. The results suggested a predominant and continuous Th1 cytokine profile in patients with pulmonary tuberculosis by active recruitment of tuberculosis-specific T lymphocytes to the lungs during infection (Boras 2007).

Other study by Talreja et al. in 20 new patients, 20 patients after 2 months and 20 patients after 6 months of chemotherapy with pulmonary TB, there was reduced

expression of CD54 and increased expression of CD69. CD54 is an accessory molecule expressed on antigen presenting cells, They suggests that the increased mycobacterial burden is associated with reduced expression of accessory molecules by monocytes. This may have implication relevant to pathogenesis of mycobacterial infections. The engagement of TCR in the absence of costimulatory signals may impair Th1 cell functions or enhance apoptosis. The study did not reveal any change in the Th1/Th2 cytokine profile in patients after treatment for six-months (Talreja 2003). These results are in agreement with that of Ellner who reported persistent suppression of IFN-γ production after completion of six months of anti-mycobacterial therapy (Ellner 1997). In this study the IFN-γ production was increased in PBMC but decreased in BALF after completion of chemotherapy.

Studies of immunopathogenesis of TB identified multitude of mechanisms for depressed *Mycobacterium tuberculosis* antigen-stimulated production of IFN-γ at the time of diagnosis of active TB (Gong 1996, Hirsch 1999a). However, factors responsible for the prolonged delay in recovery of *Mycobacterium tuberculosis*-specific T cell IFN-γ production by PBMC of TB patients remain unidentified.

Hirsch found the low PPD-stimulated production of IFN-γ by PBMC of 24 Ugandans after 6 months and remained depressed for at least 1 year after initiation of chemotherapy. The persistence of low PPD-induced IFN-γ production beyond 6 months that dissociates from the production of immunosuppressive cytokines may indicate other mechanisms involved in the suppression of T-cell responses during TB infection/disease (Toossi 1995; Hirsch 1996; Boussiotis 2000). One explanation for this persistently low IFN-γ production is a genetic defect and environmental factors in IFN-γ production/response in susceptible individuals that may also underlie their particular risk for reactivation of a latent TB infectious focus (Hirsch 1999b; Marchant 2001). Compartmentalization of antigen-responsive cells to sites of active TB infection also may be involved in the observed peripheral blood hyporesponsiveness (Barnes 1989). However, total numbers of CD4 cells are normal in the blood from patients with TB and hyporesponsiveness persists even after completion of therapy, at a time when antigen-responsive T cells should have recirculated. Sequestration of antigen-responsive cells to sites of active TB infection is unlikely to be the sole factor responsible for T-cell hyporesponsiveness in the peripheral blood. Another possibility is that there is an active and selective depletion of circulating *Mycobacterium tuberculosis* responsive T-cells during TB, which accounts for persistently low PPD-induced IFN-γ production, even after completion of chemotherapy. During TB there is an activation of mononuclear cells and levels of TNF-RII and neopterin, which are molecules associated with monocyte and T-cell activation, are elevated at the time of diagnosis of TB. It is well established that activated mononuclear cells are prone to programmed cell death (Manfredi 1998). Therefore, it is of considerable interest that spontaneous and TB-induced programmed cell death is increased among PBMC from patients with newly diagnosed TB, compared with that of healthy control subjects (Hirsch 1999b).

The specific role of CD4+CD25+ T cells during infections may be to limit strong Th1 responses induced by microbial antigens and to prevent excessive inflammation and tissue damage (Baecher-Allan 2001; Oldenhove 2003). The immunoregulatory circuits involving Tregs may be potentially beneficial to the host in diseases with a long-term persistence of the pathogen and strong immune responses aimed at prevention of reinfection such as TB. Such Treg-mediated down-regulation of T cell responses may have been the basis for decreases of frequencies of *Mycobacterium tuberculosis* antigen-responsive IFN-γ producing T cells following successful chemotherapy of patients with active TB and of latently infected PPD-positive people (Pathan 2001). The expansion of CD4+CD25+ T cells at the time of diagnosis of TB, suggest a regulatory role for this cell type and may be an adaptive host response to counteract the intense inflammatory response at sites of active *Mycobacterium tuberculosis* infection, such as the lung. The same immunoregulatory properties may then, paradoxically, depress anti-*Mycobacterium tuberculosis* T cell responses necessary to control infection (such as IFN-γ production). This hypothesis is supported by the data, which indicate that frequencies of Tregs at the time of TB diagnosis are increased more than twofold in the lung of TB patients compared to the peripheral blood and may play a role in depressed T cell IFN-γ production even at completion of TB treatment (Mittrucker 2004; Ribeiro-Rodrigues 2006).

The T helper (Th) 1/Th2 balance in the T-lymphocyte response to PPD was evaluated in six Italian and five Gambian patients with pulmonary TB before and after antimycobacterial therapy, as well as in five Gambian and four Italian healthy subjects. After 6 mo of therapy and clinical healing, most PPD-specific clones showed a polarized Th1 profile (production of IFN- γ but not IL-4/IL-5) in both Italian and Gambian patients. The Th1 polarization was less marked in Gambian than in Italian patients and failed to occur in another group of four Italian patients who experienced treatment failure. Marchant hypothesize that a Th0/Th2 response in TB, even if not detrimental, represents an inefficient

mechanism to induce pathogen clearance and healing and suggests that immunotherapeutic approaches resulting in prompt Th1 polarization of the T-cell responses could have a positive effect on the treatment of TB (Marchant 2001).

CONCLUSION

In this study there were changes in the IFN-γ cytokine profile to Th1, showed by increased in PBMC patients with pulmonary TB, but decreased from the BALF cells after treatment for six-months with oral antituberculosis drugs. According to other studies this downregulation of the IFN-γ in BALF could be due to environmental factors, genetic sensitivity, apoptotic of the Th1 cells and regulation by subset of CD4+ T cells, as well as other differences in the, clinical form of the disease or the methods used by others. Thus, future studies may be directed at how to improve the Th1 cells by modulating the therapeutic program or by immunotherapeutic approaches during the course of active TB.

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