IL-10 and IL-12 Production in Response to *Mycobacterium Tuberculosis* Total Lipid Antigens in Multidrug-Resistant Tuberculosis

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ABSTRACT

Background: *Mycobacterium tuberculosis* lipid antigens take part in pathogenicity of the bacterium but the response of monocytes/macrophages to these antigens in tuberculosis is not well known. **Objective:** The aim of current investigation was to study the *M. tuberculosis* lipid antigens in tuberculosis pathogenesis. **Methods:** In the present study *M. tuberculosis* lipid antigens were extracted. Monocytes and macrophages from multidrug-resistant tuberculosis (MDR-TB), TB patients, asymptomatic healthy individuals with positive tuberculin skin test positive and healthy individuals with negative tuberculin skin test were collected using magnetic cell sorting. The cells were stimulated by *M. tuberculosis* total lipid antigens and IL-12 and IL-10 in their supernatants were measured by enzyme-linked immunosorbent assay. **Results:** The IL-12 production by monocytes in response to *M. tuberculosis* total sonicate antigens in the MDR-TB patients did not show a considerable difference with the PPD positive healthy subjects, whereas in the active TB patients, IL-12 levels significantly decreased ($p<0.05$). IL-10 production by monocytes in TB patients in response to total lipid antigens showed a significant increase in comparison to MDR-TB patients and healthy individuals. **Conclusion:** In the MDR-TB patients, IL-10 and IL-12 production by monocytes in response to *M. tuberculosis* lipid antigens are similar to the healthy subjects.

Keywords: IL-10, IL-12, Lipid, Macrophage, Monocyte, Multidrug Resistant Tuberculosis

INTRODUCTION

According to World Health Organization (WHO) reports, prevalence of multidrug resistant

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tuberculosis (MDR-TB) is increasing in many countries. About 22% of MDR-TB patients ultimately will die of TB. On the other hand, TB Patients who are co-infected with human immunodeficiency virus (HIV) have a 50% response rate to anti-tuberculosis chemotherapy with 50% mortality due to TB (1-3).

The majority (90%) of infected individuals with *Mycobacterium tuberculosis* develop a cellular immune response to the bacterium and successfully control its growth (4). The interaction of T cells and mononuclear phagocytes is critical in this acquired protective immune response, which is mainly associated with a monocyte/macrophage proinflammatory cytokine environment (5).

Upon exposure to *M. tuberculosis* bacilli or its constituents, monocytes/macrophages secrete both proinflammatory cytokines, such as IL-1, IL-6, IL-12, TNF-α, and inhibitory ones like IL-10 and transforming growth factor-β (TGF-β) (6,7). IL-12 is an important cytokine in the host defense against mycobacterial infections. In addition, IL-12 favors the development of T helper 1 responses by enhancing IFN-γ (8-10). In murine models, administration of exogenous IL-12 enhances immunity against *M. tuberculosis* (10-15). On the other hand, IL-10 inhibits IFN-γ production and antigen specific T helper 1 proliferation responses, up-regulates T helper 2 responses and prevents macrophage activation (16,17). According to the previous studies, *M. tuberculosis* lipid antigens promote immediate effective immune responses (8,9). In spite of the importance of mycobacterial lipid antigens, little is known about the ability of the antigens to induce IL-10 and IL-12 production by monocytes and macrophages, which are the principal host targets of *M. tuberculosis* in MDR-TB patients. Therefore, the present study was undertaken to determine the ability of *M. tuberculosis* total sonicate and total lipid antigens in producing IL-10 and IL-12 by monocytes and macrophages in MDR-TB patients.

MATERIALS AND METHODS

Mycobacterial Antigen Preparation and Evaluation of Lipid Antigens. *M. tuberculosis* strain *H37Rv* total sonicate and total lipid antigens were prepared as previously described (18). Evaluating the possible contamination of the lipid extract with proteins was done by using Lamelli SDS-PAGE and checking for endotoxin contamination in the total sonicate and the total lipid antigen extract was done by using LAL assay (Cambrex Bio Science, Walkersville, MD, USA) according to the manufacturer's instructions as described elsewhere (18).

Human Subjects. The study consisted of 80 individuals; out of which 20 were healthy tuberculin reactor PPD-positive donors (12 females, 8 males, mean age, 33.23 years), 20 were healthy tuberculin skin test negative (PPD-negative) subjects (11 males, 9 females, mean age, 35.7 years), 20 were new smear positive cases of TB (10 males, 10 females, mean age, 46.7 years) and 20 were MDR-TB patients (12 males, 8 females, mean age, 42.2 years). Positive skin test was confirmed if the diameter of induration at the site of injection was >10 mm. New smear positive TB patients had positive results for the smear and the culture examinations. MDR-TB cases had the following inclusion criteria: they had a history of at least one previous period of TB treatment under the centers direct observation (6 months documentation), two positive sputum smear tests and a positive sputum culture. Their susceptibility testing showed resistance to isoniazid and

rifampin, and their chest X-ray and clinical symptoms were compatible with pulmonary tuberculosis. Exclusion criteria for all the subjects were: HIV and HCV antibody positive, HBsAg positive, any known concurrent infection, allergy and asthma, organ transplanted individuals and subjects younger than 14 or older than 70 years. All patients were selected from the Masih Daneshvari Hospital, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Beheshti University of Medical Sciences (Tehran, Iran). This study was approved by the Institutional Review Board (IRB) and the Ethical Review Board (ERB) of NRITLD. Informed consents of patients and healthy volunteers were obtained to collect 15-20 ml of their heparinized peripheral blood samples.

Preparation of the Cells. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphodex (Inno-Train, Germany) density centrifugation. The PBMCs were incubated with anti-CD14 FITC monoclonal antibody (DakoCytomation, Denmark) for 20 min. The cells were magnetically labeled with anti-FITC microbeads (Miltenyi-Biotec) for 15 min at 8ºC and then washed at 300 ×g for 10 min. The cells were loaded onto Mini Magnetic Cell Sorting (MACS) columns (Miltenyi-Biotec) and were placed in the magnetic field of a MACS separator (Miltenyi-Biotec). Purity of the monocytes was analyzed using flow cytometry. The cells were resuspended in complete RPMI-1640 medium (10 mM HEPES buffer, 200 mM L-glutamine, 50 U/ml of streptomycin-penicillin, all from Gibco-BRL) and were cultured in flat-bottomed micro titer plates at 50000 cells per well in 0.20 ml complete RPMI-1640 containing 10% human AB serum (Sigma, USA). For immunophenotyping of the monocytes, anti-CD14 and anti-CD45 antibodies (Dakocytomation, Denmark) were used. Flow cytometric analysis was performed on a fluorescent activator cell sorter (FACS Caliber) flow cytometer (Becton-Dickinson, San Jose, CA, USA) and the CELLQUEST software.

Cytokine Assay. Monocytes and macrophages were cultured in flat microtiter plates at 50,000 per each well in 0.2 ml of complete medium containing 10% human AB serum (Sigma, USA). *M. tuberculosis* total sonicate and total lipid extracted antigens were added at a concentration of 20 μg/ml and incubated at 37ºC for 48 h. PMA (Sigma, USA) was used as a positive control. The supernatants were collected after 48 h and frozen at -70ºC until cytokine assay. Concentrations of IL-12 and IL-10 were measured in pg/ml using Quantikine human immunoassay kits (R&D, USA) according to the manufacturer's instructions. The enzyme-linked immunosorbent assay (ELISA) was performed in triplicates for each sample and the cytokine concentrations were calculated using standard curves.

Statistical Methods. Statistical analysis was performed by nonparametric analysis and Mann-Whitney U test. A value of $p \leq 0.05$ was considered as significant.

RESULTS

Monocytes Enrichment. As stated under materials and methods section, the $CD45^{+}CD14^{+}$ cells were separated from PBMCs by magnetic cell sorting. More than 95% of the cells from magnetic cell sorting expressed CD14 according to the flow cytometric analysis (Figure 1, A2).

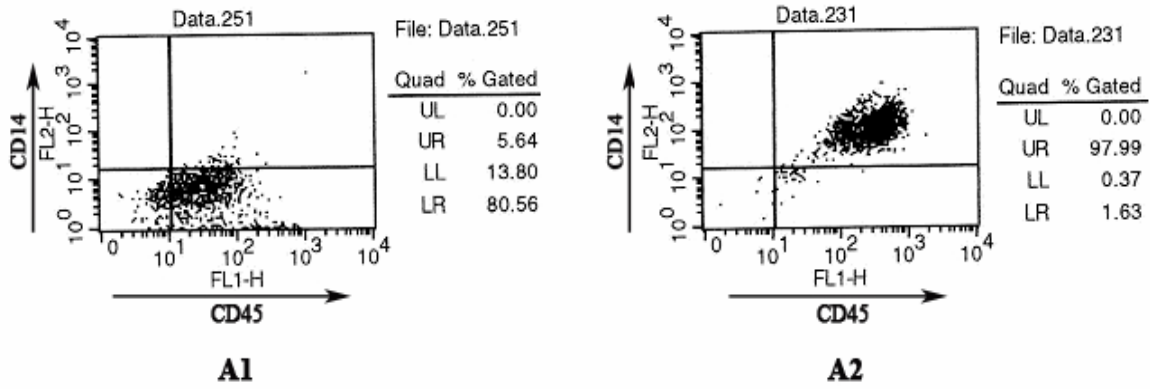

Figure 1: Monocyte enrichment. Monocytes were enriched from peripheral blood mononuclear cells using magnetic cell sorting and purity was evaluated by flow cytometric analysis. Monocyte purity before isolation was shown in A1 dot plot and after enrichment in A2 dot plot. According to these results purity of monocytes was more than 97%. (FL1-H = anti-CD45 FITC and FL2-H= anti-CD14 PE).

IL-12 production by monocytes stimulated by *M. tuberculosis* total sonicate antigens. Monocytes and macrophages have important roles in *M. tuberculosis* infection by secreting versatile cytokines such as IL-12. In this study the mean of IL-12 titer in each group was measured and compared with those of the other groups using Mann-Whitney U test. According to our results, the mean of IL-12 concentration in the supernatant of the monocytes stimulated by *M. tuberculosis* total sonicate antigens in the MDR-TB patients were similar to those in the PPD-positive ($p=0.07$) and PPD-negative ($p=0.22$) healthy donors (Figure 2), while in the active TB patients, IL-12 concentration was significantly lower than that of PPD-positive and PPD-negative subjects ($p<0.05$) (Figure 2). IL-12 production in the PPD-positive and PPD-negative subjects in response to total lipid antigens did not show a significant difference with those of the MDR-TB patients, but the TB patients showed a significant decrease in IL12 production compared to the healthy subjects ($p=0.002$).

Mean IL-12 concentration in monocyte supernatants of active TB patients stimulated by total sonicate antigens was 37.22 ± 13.48 and was significantly lower than its concentration in the MDR-TB patients (55.52 ± 20.57). Mean IL-12 concentration in TB patients in response to total lipid antigens was 28.55 ± 8.55 which was significantly lower than that of the MDR-TB patients (41.11 ± 13.54) (Figure 2).

IL-10 production by monocytes stimulated by total sonicate or total lipid antigens of *M. tuberculosis.* Based on our results, IL-10 production by monocytes stimulated by *M. tuberculosis* total sonicate and total lipid antigens in MDR-TB and healthy donors were similar ($p>0.05$). However, in TB patients, mean of IL-10 concentration in the supernatants of stimulated monocytes by total sonicate antigens (198.27 ± 54.78) was significantly elevated ($p=0.008$) compared to that of the PPD-positive subjects (150.8 ± 62.22), but it did not differ significantly from that of MDR-TB patients (178.5 ± 43.23). Moreover, IL-10 production in monocytes stimulated by lipid antigens in MDR-TB patients (105.43 ± 61.55) was similar to that of PPD-positive subjects (106.23 ± 35.84), whereas in the TB patients IL-10 concentration (139.77 ± 52.98) was significantly elevated compared to the PPD-positive donors and MDR-TB patients ($p<0.05$) (Figure 3).

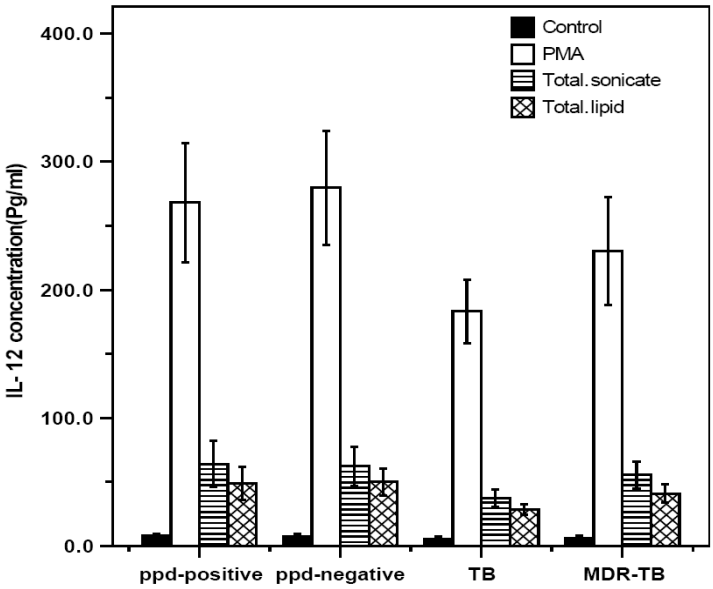

Figure 2: IL-12 production by monocytes. Enriched monocytes were cultured in complete RPMI-1640 medium according to Materials and Methods. The cells were stimulated with 20 µg/ml of M. tuberculosis total sonicate and lipid antigens. After 48 h supernatants were harvested and IL-12 was measured by ELISA in triplicate for each sample. Concentration of IL-12 is expressed on pg/ml. The concentration of IL-12 is shown as Mean ± SD.

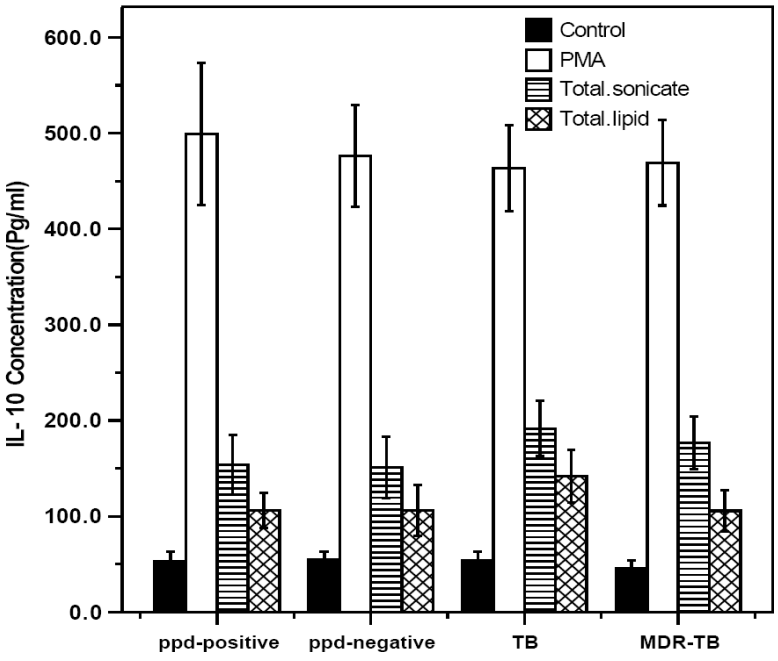

Figure 3: IL-10 production by monocytes. Enriched monocytes were cultured in complete RPMI-1640 medium according to the Material and Methods. The cells were stimulated with 20 µg/ml of *M. tuberculosis* total sonicate or lipid antigens and after 48 h, supernatants were harvested and IL-10 was measured by ELISA in triplicates for each sample. The concentration of IL-10 was calculated and expressed in pg/ml. The mean concentration of IL-10 in each group was compared with those of the other groups by Mann-Whitney U test.

DISCUSSION

According to some studies, antigen presentation and cytokine production by monocytes, macrophges and dendritic cells can determine the fate of Th1/Th2 immune responses to pathogens (19,20). In addition, the role of *M. tuberculosis* lipid antigens in effective immune response is less elucidated.

Our results showed that IL-12 production in MDR-TB patients by monocytes/macrophages stimulated by *M. tuberculosis* total sonicate and lipid antigens was similar to that of the healthy donors. In active TB patients, IL-12 production by monocytes was significantly decreased, which is consistent with other studies (20,21). Although, there was not a significant difference in IL-12 production between the MDR-TB and healthy subjects, we observed an inverse trend in the IL-10 and IL-12 titers in monocyte supernatants stimulated by total sonicate antigens in the MDR-TB patients that might be suggestive of the role of IL-10 in the suppression of IL-12. Our results are not consistent with the study performed by Lee et al. who showed that IL-12 p40 production by PBMCs stimulated by PPD antigens in advanced MDR-TB patients was significantly greater than that of the PPD-positive healthy subjects (19). The inconsistency between our results and the work by Lee et al. can be due to the fact that in our study MDR-TB patients had a history of less than one year of chemotherapy which is not considered as advanced MDR-TB; however, in their study advanced MDR-TB patients with a history of TB diagnosis of more than one year had been evaluated. In addition, the strain of *M. tuberculosis* and the cell wall lipid components can alter IL-12 production by monocytes (1).

Investigation of IL-10 production by monocytes in our study showed that the monocytes stimulated by *M. tuberculosis* total sonicate or total lipid antigens produced the same level of the cytokine in the MDR-TB patients compared with the healthy subjects, while in TB patients, IL-10 production was significantly elevated ($p<0.05$). According to some studies, although IL-10 suppresses effective Th1 immune responses to *M. tuberculosis* in TB patients, this effect may be useful in preventing immunopathologic damages due to the infection (17). Based on our results, production of IL-10 in MDR-TB is similar to that of healthy donors and this may be related to the stage of TB. It seems that in acute *M. tuberculosis* infection, production of an elevated level of IL-10 by mononuclear cells attenuates T cell responses. For elucidation of the exact role of IL-10 in mycobacterial infections more studies are necessary. In this regard, Hickman et al. (22) have shown a differential effect of dendritic cells versus macrophages in the deviation of T helper responses against *M. tuberculosis* antigens. Interestingly, this effect was influenced by the cytokine profile of these antigen presenting cells. Moreover, IL-12 production by macrophages was sensitive to the presence of IL-10.

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