Interferon-\(\gamma\) and Interleukin-17 production from PPD-stimulated PBMCs of patients with pulmonary tuberculosis

Abstract

**Purpose:** The purpose of this study was to evaluate Interferon (IFN)-\(\gamma\) and Interleukin (IL)-17 profiles in patients with different clinical presentations of pulmonary tuberculosis (TB) and to compare them with those of tuberculin-negative and tuberculin-reactive healthy controls.

**Methods:** Peripheral blood mononuclear cells (PBMCs), isolated from patients (n=52) and controls (n=30), were stimulated *ex vivo* with purified protein derivative (PPD) and IFN-\(\gamma\) and IL-17 levels in the supernatant were measured.

**Results:** At baseline, PBMCs from patients with TB released a significantly lower amount of IL-17 \((p=0.043)\) than PBMCs from healthy controls, whereas IFN-\(\gamma\) levels were similar in the two groups. After PPD stimulation, a significant rise in IL-17 levels was found only among healthy controls \((p=0.02)\). This rise in IL-17 levels was similar between tuberculin-reactive and tuberculin-negative subjects. After PPD stimulation, patients with infiltrative TB secreted higher levels of IL-17 and IFN-\(\gamma\) than those affected with chronic, miliary and cavitary TB \((p<0.01)\). IFN-\(\gamma\) production from patients with infiltrative TB was even higher than for healthy controls \((p<0.01)\). PBMCs from tuberculin-reactive patients released higher levels of IFN-\(\gamma\) than tuberculin-negative subjects after PPD stimulation \((p<0.01)\).

**Conclusion:** *Ex vivo* PPD stimulation of PBMCs from patients with pulmonary TB does not significantly stimulate IL-17 release; however, higher IL-17 and IFN-\(\gamma\) levels are found in patients with infiltrative disease, in comparison with those affected with miliary, cavitary and chronic TB.
Despite the high infectivity of *Mycobacterium tuberculosis* (MtB), only 5-10% of infected individuals develop clinical symptoms and radiological evidence of active disease [1]. Also, clinical presentation in patients with active tuberculosis (TB) varies substantially from mild symptoms to severe or fatal TB, the former being characterized by an infiltrative-exudative pulmonary lesion and the latter by large, cavitory lung lesions or miliary TB.

Cell-mediated immunity represents the main component of host defense against MtB [2]; the T-helper 1 (Th1) immune response, which is characterized by the production of IL-12, TNF-α and IFN-γ [3-6], implies a close interplay between CD4+ T-cells and macrophages and is usually able to control bacterial proliferation and to prevent TB clinical progression. In particular, IFN-γ, produced by CD4+ T-cells, plays a key role in the host response to MtB, given its activating effect on macrophages which is responsible for the intracellular killing of mycobacteria [7,8]. Recent studies have focused on a new CD4+ T-cell subset, Th17 cells, as an important mediator of immune responses against MtB [9]. Th17 cells produce IL-17, a potent proinflammatory cytokine that has been implicated in many autoimmune and inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, asthma and systemic lupus erythematosus [10,11]. Generation of human Th17 cells depends on IL-23, IL-1β, TGF-β and IL-6 stimulation [12-14]. In the lungs, IL-17 triggers neutrophil chemotaxis and induces the production of chemokines, growth factors and adhesion molecules [15]. The IL-23-IL17 axis has recently been shown to participate in the immune response to MtB [16-19]. Both in vitro and in vivo experiments have shown defective TH1 and TH17 response in patients with TB in comparison with healthy tuberculin reactors, and a negative correlation between IFN-γ and IL-17 levels and disease severity [14,16,21-24]. A clear-cut shift towards TH2 immune response has been demonstrated [25]; in fact, increased levels of IL-4, the prototypical TH2 cytokine, as well as IL-5 and IL-10, have been found in patients with TB and have been related to a more severe clinical state [26-28]. Other studies have reported opposing results, describing higher levels of IL-17-producing CD4+ T cells among subjects with active TB and a direct correlation between the frequency of IL-17 secreting cells and disease severity [29-31].

In the present study, IFN-γ and IL-17 levels were determined in the supernatant of peripheral blood mononuclear cells (PBMCs) isolated from patients with different clinical and radiological features of pulmonary TB and stimulated in vitro with purified protein derivative (PPD). IFN-γ and IL-17 expression profiles for patients with pulmonary TB were compared with those of a group of tuberculin-negative and tuberculin-reactive healthy controls.

### Materials and Methods

#### Study subjects

Fifty-two consecutive patients with pulmonary TB, diagnosed at the Unit of Infectious Diseases of Catania, Eastern Sicily, Italy, were enrolled for the study between January 2010 and January 2012; diagnosis was based on clinical history, chest X-ray, intradermal tuberculin PPD test and positive detection of acid-fast bacilli in sputum smear or isolation of mycobacteria in sputum cultures. All patients with a positive sputum culture for mycobacteria also had molecular characterization of MtB as determined by polymerase chain reaction (PCR). On the basis of specific clinical and radiological features, patients were divided into four subgroups as follows: 1), patients with infiltrative TB (n=17); 2), patients with cavitory TB (n=15); 3), patients with miliary TB (n=10); and 4), patients with chronic fibrous TB (n=10).

- **Subgroup 1:** Cases of infiltrative TB were characterized by radiological evidence of apical exudative infiltration without micro- or macronodules or cavitations.
- **Subgroup 2:** Patients with cavitory TB had single unilateral (n=8) or multiple bilateral (n=7) lung cavities.
- **Subgroup 3:** Patients with miliary TB were defined on the basis of the presence of widespread micronodules (<2 mm) on chest CT scan, with or without extrapulmonary involvement (2 out of 10 miliary cases had a concurrent meningeval disease).
- **Subgroup 4:** Radiological evidence of lung fibrotic bands and/or pleural calcifications associated with a history of previously untreated or incorrectly treated TB, with repeated positive sputum smears throughout a period of at least 72 months, was consistent with the diagnosis of chronic fibrous TB.

Blood samples were taken from all TB patients at diagnosis, within two days of hospital admission and before starting antitubercular treatment.

The control group consisted of 30 healthy subjects, 15 of whom were PPD-positive and 15 were PPD-negative. Participants had not been vaccinated with BCG, were serologically negative for HIV infection and had no known condition associated with overt immune depression, such as steroid or other immunosuppressive drug intake, cancer or onco-haematological disease, primitive immune deficiency, chronic or acute renal failure, or poorly controlled diabetes.

Written informed consent was obtained from patients and control subjects for blood sampling and PPD skin test.
The main clinical and epidemiological characteristics of the study population are shown in Table 1.

**Cell preparation and in vitro cultures**

Heparinized peripheral blood, drawn from patients and healthy controls, was diluted 1:4 with PBS, overlayed on Ficoll-Hypaque (Sigma, Milan, Italy) and centrifuged at 1600 rpm for 20 minutes. The mononuclear cell layer was then removed from the plasma/Ficoll interface, washed twice with PBS and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with fetal calf serum (10%), penicillin/streptomycin (1%) and L-glutamine (1%). Cells were plated in 24-well tissue culture plates (Falcon) and incubated with or without PPD (1 μg/mL, Biocine PPD lioflo, Sclavo, Siena, Italy) for 72 hours, at 37°C in a 5% CO₂ humidified atmosphere. After 72-hour incubation, supernatants were collected, centrifuged and stored frozen (-20°C) until use.

**Enzyme linked immunosorbent assay (ELISA) for human IFN-γ and IL-17**

IL-17 concentration in cell supernatant was measured using a solid-phase enzyme amplified sensitivity immunoassay performed on microtiter plate, purchased from Biosource Europe S.A. (Biosource IL-17 Cytoscreen kit, Nivelles, Belgium), following the manufacturer’s instructions. The lower limit of detection of the assay was 2 pg/mL. IFN-γ concentration in cell supernatant was measured by ELISA (Predicta, Genzyme Diagnostics, Cambridge, USA). The lower limit of detection for the assay was 3 pg/mL.

**Statistics**

Data were expressed as means ± standard deviation (SD). Categorical variables were analyzed by using the Fisher’s exact test. Intergroup comparisons were performed using the paired and unpaired Student’s t test. Statistical analysis was performed using Prism software (GraphPad, La Jolla, USA).

**Results**

IFN-γ and IL-17 levels in the supernatant of PPD-stimulated PBMCs from patients with different patterns of pulmonary TB and healthy controls were analyzed.

**Baseline and post-PPD levels of IL-17**

Baseline levels of IL-17 were significantly lower among patients with TB in comparison with healthy controls (205 ± 57 vs 75 ± 11 pg/mL, p=0.05). Mean IL-17 concentration after PPD-stimulation was 153 ± 88 pg/mL in TB patients, which was again significantly less (p=0.043) than in healthy controls. After PPD stimulation, a significant rise from basal IL-17 values was found among healthy controls (p=0.02), whereas in patients with TB no significant increase was seen in comparison with baseline values (Figure 1a). PPD-induced IL-17 produc-

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**TABLE 1. Clinical and epidemiological characteristics of the study population**

<table>
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<td>30</td>
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<td>Age (years±SD)</td>
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<td>39±14</td>
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<td>Detection of Mycobacterium tuberculosis by PCR*</td>
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<td>Clinical features of TB</td>
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<td>Infiltrative disease*</td>
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<td>Cavitary disease*</td>
<td>15(28.8)</td>
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<td>Miliary disease*</td>
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<tr>
<td>Chronic fibrous disease*</td>
<td>10(19.2)</td>
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*N(%)
FIGURE 1.

a. IL-17 levels in the supernatant of peripheral blood mononuclear cells (PBMCs) from patients with TB (n=52) and healthy controls (n=30), at baseline and after 72-hour stimulation with PPD (purified protein derivative). Baseline levels were significantly higher in healthy controls in comparison with patients with TB (**p=0.05). A significant rise in IL-17 levels was found only in healthy controls (**p=0.02). After PPD stimulation, IL-17 levels were significantly higher in healthy controls in comparison with patients with TB (**p=0.043).

b. IL-17 levels in the supernatant of peripheral blood mononuclear cells (PBMCs) from healthy controls whose tuberculin skin test was positive (n=15) and negative (n=15) at enrollment. A significant rise in IL-17 levels was found in tuberculin-negative (p=0.05), as well as tuberculin-reactive subjects (**p<0.01), when comparing baseline and post-PPD values.

c. IL-17 levels in the supernatant of peripheral blood mononuclear cells (PBMCs) from healthy controls (n=30) and patients with pulmonary TB (n=52), divided into four groups on the basis of their clinical state, at baseline and after 72-hour stimulation with PPD (purified protein derivative). Baseline (p<0.01) and post-PPD levels (p<0.01) were significantly higher in healthy controls in comparison with the other groups; in addition, post-PPD levels were significantly higher in patients with infiltrative TB (n=17), compared with those with cavitary (n=15), miliary (n=10) and chronic fibrous (n=10) TB (**p<0.01). After PPD stimulation, a significant rise in IL-17 levels was found in patients with infiltrative (α p<0.01), cavitary (β p=0.048) and chronic fibrous (γ p=0.038) TB, but not in patients with miliary TB.
FIGURE 2.

a. IFN-γ levels in the supernatant of peripheral blood mononuclear cells (PBMCs) from patients with TB (n=52) and healthy controls (n=30), at baseline and after 72-hour stimulation with PPD (purified protein derivative). A significant rise in IFN-γ levels was observed in TB patients (**p<0.01), as well as in controls (‘p<0.01). PPD-induced IFN-γ production was significantly higher in controls, in comparison with TB patients (**p<0.01).

b. IFN-γ levels in the supernatant of peripheral blood mononuclear cells (PBMCs) from healthy controls whose tuberculin skin test was positive (n=15) and negative (n=15) at enrolment. A significant rise in IFN-γ levels was detected only in tuberculin-reactive subjects (‘p<0.01). Post-PPD levels were significantly higher among tuberculin reactive subjects, in comparison with tuberculin negative patients (**p<0.01).

c. IFN-γ levels in the supernatant of peripheral blood mononuclear cells (PBMCs) from healthy controls (n=30) and patients with pulmonary TB (n=52), divided into four groups on the basis of their clinical state, at baseline and after 72-hour stimulation with PPD (purified protein derivative). Baseline levels were similar. Post-PPD levels were higher among patients with infiltrative TB in comparison with the other groups (‘p<0.01). Patients with cavitary (n=8), miliary (n=5) and chronic (n=5) TB did not show a significant increase in IFN-γ levels, whereas significant differences were observed for patients with infiltrative (n=7) TB (**p<0.01).
tion varied significantly among patients with different TB clinical features. In fact, patients with miliary TB did not exhibit a significant increase in IL-17 production after PPD stimulation (61 ± 7 vs 71 ± 38 pg/mL); in contrast, IL-17 levels rose significantly after PPD stimulation among those subjects with infiltrative TB (87 ± 13 vs 350 ± 99 pg/mL, p<0.01), cavitary TB (51 ± 10 vs 132±78 pg/mL, p=0.048) and chronic fibrous TB (81 ± 10 vs 178 ± 68 pg/mL, p=0.032) (Figure 1c).

Among healthy controls, baseline and PPD-induced IL-17 did not significantly differ between tuberculin-reactive and tuberculin-negative subjects. After PPD challenge, IL-17 levels significantly rose from baseline both in tuberculin-negative (p=0.05) and in tuberculin-reactive (p<0.01) subjects (Figure 1b).

Baseline and post-PPD levels of IFN-γ

At baseline, IFN-γ levels were similar between healthy controls and patients with TB; after PPD stimulation, a significant increase in IFN-γ levels was observed for both TB patients (from 19.6 ± 9.4 pg/ml to 121.8 ± 159.1 pg/ml, p<0.01) and healthy controls (from 17.8 ± 6.3 pg/ml to 205.5 ± 85.8 pg/ml, p<0.01) (Figure 2a). In detail, IFN-γ concentration in PBMCs supernatants was significantly higher after PPD-stimulation in tuberculin-reactive controls in comparison with those tuberculin-negative patients whose levels did not rise significantly from baseline (Figure 2b). When considering only the TB cohort, subgroup analysis showed a significant increase in IFN-γ levels among patients with infiltrative TB (p<0.01). In contrast, PPD stimulated-PBMCs from patients with chronic, miliary and cavitary TB did not produce higher levels of IFN-γ in comparison with unstimulated PBMCs (Figure 2c).

Discussion

In the present study, PBMCs from patients with TB were found to release a significantly lower amount of IL-17 than PBMCs from healthy controls; moreover, our findings support the existence of a clear-cut correlation between IL-17 levels and clinical presentation of pulmonary TB. In fact, in patients with infiltrative TB, usually considered as the clinical prototype of a strong but pathologic cell-mediated response to Mtb (mostly described among young and immunocompetent adults [4]), a consistently higher production of PPD-induced IL-17 was seen in comparison with other clinical forms of pulmonary TB, such as cavitary and miliary TB, but not in comparison to healthy controls. In addition, a significantly higher production of IFN-γ was observed in subjects with infiltrative disease, when compared with healthy controls and subjects with other clinical features of TB, in keeping with the major role of Th1 cytokines during the host response to Mtb [8].

It is well known that a switch from Th1 to Th2 cell response takes place in clinically overt TB. Some recent studies suggested a role for impaired Th17 response in determining the severity of pulmonary TB. Chen et al. [18] reported the Th17 response to be suppressed in patients with active TB: flow cytometry analysis showed that the frequency of a nonspecific Th17 response was significantly lower in patients with active TB disease than in healthy controls and subjects with latent TB infection (LTBI) and, of importance, suppressed Th17 response correlated with the severity of disease since those patients with pulmonary TB exhibiting meningeal complications had a significantly lower frequency of Th17 cells than patients with uncomplicated pulmonary TB. Kumar et al. [22] found similar results in a population of children affected with active TB: children with TB had diminished Th1 and Th17 cytokine responses in comparison with controls, either spontaneously in vitro or following PBMCs stimulation with mycobacterial antigens. In addition, children with active TB complicated by microbial dissemination and neurological involvement showed the most profound defect in production of both IFN-γ and IL-17. Stern et al. [32] reported a 9-fold increase in the levels of IL-17 mRNA after PPD stimulation of PBMCs drawn from individuals with LTBI. Consistent with the results of the present study, Scriba et al. [16] reported a reduced Mtb-specific Th17 response in patients with active TB in comparison with healthy donors. Other authors reported opposing results: Jurado et al. [29] recently demonstrated that in patients affected with TB, stimulation of lymphocytes with sonicated Mtb antigens induced a significantly lower amount of IFN-γ and higher concentration of IL-17 than healthy controls. The authors showed that IL-17 was secreted by CD4+ IFN-γ+ IL-17+ lymphocytes, whose proportion directly correlated with the severity of disease. Similarly, Marin et al.[30] reported that patients with active TB had a higher frequency of circulating IL-17-producing CD4+ T cells after PPD stimulation than subjects with LTBI. In contrast to the results of the present study, Sargentini et al. [33] did not observe any significant difference in IL-17 release from PBMCs between healthy controls and patients with active TB after 72-hour stimulation with PPD; however, in this study, a very limited number of individuals (14 patients and 6 controls) was enrolled. In a recent paper of Cowan et al. [34], the authors found that the frequency of IL-17-producing CD4+ T cells was lower in patients with active TB in comparison with healthy controls and subjects with LTBI. After a 7-day stimulation of PBMCs with my-.
cobacterial antigens, Cowan and coworkers detected a significant higher increase in IL-17 expressing CD4+ T cells among patients with active TB, in comparison with healthy controls. In contrast with our observation of similar response to PPD stimulation among tuberculin-negative and tuberculin-reactive subjects, Babu et al. [35] found that the production of Th17 cytokines, such as IL-17 and IL-23, was significantly lower in tuberculin-reactive individuals after challenging with PPD and Mtb culture filtrate.

Our study has a number of limitations: firstly, it was conducted on a relatively small number of patients. Secondly, since PPD, which is not a Mtb specific antigen, was used to stimulate PBMCs, further research is required to establish whether our findings may be reproduced when stimulating PBMCs with specific Mtb antigens, such as ESAT-6 and CFP-10. Thirdly, cytokine production was measured by ELISA so further evaluation by using surface and intracellular cytokine flow cytometry should be worthwhile. In addition, it would be interesting to focus on the possible role of macrophages and dendritic cells, considering that their interaction with Mtb antigens and their subsequent cytokine secretion may modulate host response to Mtb [36].

In conclusion, IL-17 production is inhibited in patients with pulmonary TB, with particular reference to cavitary or miliary TB, both of which are characterized by a weaker cell-mediated immune response.

The protective and pathological mechanisms of immune response to Mtb are complex and still unclear. More research is needed to understand how Th17 cytokines may influence the severity of TB presentation. Further investigations are required in order to shed light on the complex network of cytokine responses that mediate resistance or susceptibility to Mtb infection and determine the variability of clinical presentation of pulmonary TB.

References


