Role of TNF-α in host immune response in tuberculous pleuritis

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Tumour Necrosis Factor (TNF)-α, a pro-inflammatory cytokine has a dual role in host immunity and immunopathology of tuberculosis and is considered to be pivotal for determining the clinical course of the disease, either beneficial or detrimental. The assessment of TNF-α in pleural tuberculosis will help us to understand its role in host defence mechanism against Mycobacterium tuberculosis (MTB) infection. In this study, TNF-α and IFN-γ levels were measured in plasma and pleural fluid of both tuberculosis (TB) and non-TB patients and in the supernatants of blood and pleural fluid mononuclear cells (PBMCs and PFMCs) stimulated in vitro with PPD, culture filtrate and heat-killed (MTB). In addition, apoptosis induced by PPD and MTB was also studied. TNF-α and IFN-γ were significantly elevated in pleural fluid than in plasma of pleural tuberculosis patients, suggesting the compartmentalization of Th1 cytokine-secreting cells at the site of disease. In vitro stimulation of PFMCs with PPD and MTB showed a significant increase in these cytokine levels and also enhanced apoptosis of these cells. This increase in TNF-α levels may contribute to the containment of infection by synergizing with IFN-γ to activate infected macrophages or by the regulation of T-cell apoptosis.

TUBERCULOUS pleuritis occurs in about 30% of patients with tuberculosis (TB). This percentage increases with HIV-positive conditions. HIV-positive individuals tend to develop tuberculous pleuritis during their early stage of immunosuppression. The emergence of multi drug-resistant tuberculosis (MDR-TB) has added burden to TB therapy. Currently, immunotherapeutic studies are being carried out to reduce the duration of therapy and to find an alternate treatment for MDR-TB. Tuberculous pleuritis serves as the best model to understand the immunity at the site of infection. The local cell-mediated immune (CMI) response generated by macrophages in cooperation with T-lymphocytes in tuberculous pleuritis resolves the disease without chemotherapy. The response is mediated by the secretion of complex cytokine pattern by the antigen-specific T-cells present in the pleural space. The cytokine pattern in tuberculous pleuritis is suggestive of Th1 type2,3. Cytokines like gamma interferon (IFN-γ), Tumour Necrosis Factor (TNF)-α, IL-2, IL-12 and IL-18 are the ones that play a major role in bringing about the reactions like granuloma formation, intramacrophage elimination of the bacillary antigens and fibrosis that lead to the resolving of the disease1.

TNF-α is produced by macrophages, monocytes and dendritic cells in response to Mycobacteria or its products, or to other microorganisms like Listeria monocytogenes. It has immuno-regulatory properties and brings about the activation of macrophages, granuloma formation, T-cell stimulation and regulation of chemokine induction, thus controlling infection2. High levels of TNF-α were reported in chronic TB accompanied by the elevated release of TNF-α receptors resulting in fever, necrosis and weight loss. This implies that TNF-α has both protective and immunopathologic effects6. IFN-γ, a TH1-type cytokine is essential in TB immunity, which is the single most important factor for macrophage activation and TNF-α induction3. Individuals who lack either IFN-γ or IFN-γR are highly susceptible to severe systemic infections which are important in the control of TB5,6.

Apoptosis is a crucial phenomenon occurring at the site of infection in TB. Previous studies indicate that the microenvironment of a Mycobacterium tuberculosis (MTB)-infected focus is favourable for apoptosis of mononuclear cells10,11. Infected macrophages secrete TNF-α, which acts as an important apoptotic factor. Assessment of TNF-α, together with its inducer IFN-γ, will help us formulate its functional role in apoptosis and to understand the local CMI response that results in the clearance of the bacilli. Moreover, most reports on TNF-α and IFN-γ are from either in vivo levels or about in vitro studies, but not from both12,14. Hence we assessed these cytokine levels simultaneously, both in vivo and in vitro, with an idea to confirm previous reports from in vivo studies and extend them to in vitro results.

We included 75 patients from the Government General hospital, Chennai with pleural effusion. These patients were broadly divided into two groups according to the diagnosis.

(i) A TB group consisting of 46 patients with tuberculous pleural effusion. The effusion was unilateral. Diagnosis was confirmed by the clinical picture, AFB positivity for the smear, culture positivity or positive by PCR.
(ii) A non-TB (NTB) group of 29 patients with transudative effusion due to causes other than tuberculous pleuritis. This included patients with congestive cardiac failure and liver diseases.

The pleural fluid and blood collected for therapeutic and diagnostic purposes respectively, were utilized for this study according to the ethical guidelines of the Government General Hospital, Chennai. A written, informed consent for participation in the study was obtained from each patient. Blood and pleural fluid were collected before the start of the treatment.

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood (10 U/ml) by Ficoll-
Hypaque (Pharmacia) density gradient centrifugation, as described elsewhere. To obtain pleural fluid mononuclear cells (PFMCs), the heparinized pleural fluid (1 U/ml) was layered over Ficoll-Hypaque and centrifuged at 1800 rpm for 30 min. The interphase was collected and washed twice with HBSS (Whittaker) at 1800 rpm for 10 min. PBMCs and PFMCs were suspended at the concentration of $1 \times 10^8$ cells/ml in RPMI 1640 (Sigma Chemical Co.) supplemented with 10% autologous serum. Viability of the cells was assessed by trypan blue exclusion method. The cell-free plasma and pleural fluid were stored at -70°C for assessment of cytokines.

Only cells from those patients who were positive by all the diagnostic criteria (clinical, smear, culture and PCR) and for whom the cells were adequate were taken for in vitro studies. Accordingly, 18 out of 46 TB samples of tuberculous pleuritis were included in this study. Next, $1 \times 10^8$ PBMCs and PFMCs were distributed in 48 well round-bottom plates (Nunclon) and incubated with different mycobacterial antigens like PPD (Weybridge, UK), culture filtrate (CF) and heat-killed MTB at 10 μg/ml concentration. These cultures were incubated at 37°C in 5% CO₂. The CF antigen and heat-killed MTB were prepared in our laboratory. Mononuclear cells incubated with medium alone served as control. Cell-free supernatants were collected after 48 h at which the cytokine concentration were found to be maximum, and stored at -70°C for cytokine estimation. TNF-α and IFN-γ levels were measured in plasma and pleural fluid and in supernatants of in vitro experiments using a commercial sandwich ELISA kit (R&D Systems, Minneapolis, USA) and following the manufacturer’s instruction. Diagnostic PCR using IS6110 primers (Bangalore Genei Private Limited, India) was performed to find the positivity for the presence of MTB bacilli.

For the detection of apoptosis $1 \times 10^8$ cells (PBMCs and PFMCs) of 10 out of the 18 TB patients chosen for in vitro studies, were treated with known apoptosis-inducing agent like Dexamethasone (100 μg/ml) and mycobacterial antigens (PPD and MTB – 50 μg/ml) for three days. The cells were harvested, permeabilized and stained with 500 μl of fluorochrome solution (200 μg RNAase A (Amersham Corporation) and 100 μg propidium iodide (Sigma Chemical Co.) in Ca²⁺, Mg²⁺-free PBS and were incubated at 37°C for 1 h. Acquisition was done with FACSORT (Becton Dickinson, CA) and analysis was done using CellQuest software. The PI staining intensity of cells was determined by measuring the red fluorescence. The percentage of cells in the less than 2N region (hypoploidy) was taken as the quantitative measure of the apoptotic cells, as described previously.

Comparison between groups was done using paired or unpaired Student’s t test as appropriate for normally distributed data. Wilcoxon’s rank sum test was performed for the data that were not normally distributed. The values were expressed as mean ± standard error of the mean, both in the text and in the figures.

With a view to understand the local in vivo role of TNF-α in relation with IFN-γ, the mean TNF-α and IFN-γ concentrations in the plasma and pleural fluid of TB and NTB groups were assessed, the results are shown in Figure 1. The mean levels of TNF-α and IFN-γ in pleural fluid were significantly higher ($P < 0.05$) than the plasma levels in patients with tuberculous pleuritis. There was no significant difference between the plasma and pleural fluid levels in transudative effusions from the NTB patients. A number of studies have been reported on the protective role of IFN-γ and TNF-α in TB, and higher levels of these cytokines in pleural fluid than in plasma of tuberculous pleuritis patients. The selective increase observed in these cytokine levels in pleural fluid supports these studies. The high concentration of these cytokines in pleural fluid reflects the stimulation of local, immuno-reactive cells by mycobacterial components that facilitate bacillary elimination and granuloma formation. Moreover, it also suggests that there is a compartmentalization of TH1 cytokine-secreting cells, as reported earlier. This compartmentalization occurs because of the migration of TNF-α and IFN-γ secreting cells to the site of the disease and hence not accessible through the sampling of plasma of peripheral blood, thus attributing to the lesser plasma levels of these cytokines in tuberculous pleuritis patients than in NTB patients. The comparable in vivo levels of TNF-α in pleural fluid of NTB patients with that of TB patients may be due to the generalized TNF-α secretory response to any inflammatory conditions, but not due to compartmentalization. This is evident from the

![Figure 1](image_url)
similar levels of TNF-α noticed in the plasma and pleural fluid of NTB patients.

Observing the enhanced response of TNF-α in vivo, we further decided to investigate its in vitro response to the widely used crude mycobacterial antigens. The levels of TNF-α and IFN-γ in the supernatants of PBMCs stimulated with PPD, CF and MTB did not show any difference when compared with their control levels. This implies that the TNF-α and IFN-γ secreting cells present in the PBMCs population are not specific for MTB antigens. However, PFMCs stimulated with PPD and MTB but not with CF, showed significant increase when compared with their control levels (P < 0.05; Figure 2). This suggests that these cells are specific for mycobacterial components. The increase in the cytokine response may be due to the memory response of the cells towards the mycobacterial antigens. Overall, the in vitro levels of TNF-α in the supernatants of PBMCs were higher than those found in the supernatants of PFMCs under all stimulated conditions.

Apoptosis is a feature of tuberculous granuloma in pulmonary TB. Previous in vitro study from TB patients has reported that there occurs T-cell hyperresponsiveness linked to spontaneous or MTB-induced apoptosis of T-cells. Thus PFMCs at the site of infection constitute the mycobacterial antigen-sensitized population and hence are more prone to apoptosis. Our results showed the induction of apoptosis of PFMCs by mycobacterial antigens (Figure 3). As expected, we observed maximum spontaneous apoptosis (21%) occurring in PFMCs compared to PBMCs (5%) when there is no stimulation. Also, a known apoptotic agent like Dexamethasone induced maximum apoptosis (43%) in PFMCs. With mycobacterial antigens, marginal apoptosis (12–18%) was seen in PBMCs while PFMCs showed significantly high (34–45%) levels of apoptosis. We have also observed that it is the T-cell population of PFMCs that undergoes enhanced apoptosis (S. Das unpublished data). Such spontaneous or MTB-induced apoptosis of T-cells has been previously documented. In another study based on pleural tuberculosis, the loss of IFN-γ-producing cells was found to be limited specifically to MTB-responsive cells. These studies provide evidence that T-cell apoptosis is biologically significant in the disease process.

Most studies indicate a critical role for TNF-α and IFN-γ in the apoptosis mediated by MTB. Association between the high levels of these cytokines and enhanced apoptosis of PFMCs in our study indicates that these cytokines have a dual role at the site of infection. As known, IFN-γ induces TNF-α, which plays an immunoprotective role by containing MTB infection and facilitating granuloma formation. On the other hand, it can also bring about the apoptosis of sensitized T-cells, that has immunopathologic outcomes. The immunopathologic role of IFN-γ is also supported by the recent observation of its role in upregulating the transcription of genes involved in apoptosis (FasL, Fas, Bak).

It is reported that the TNF-α-mediated pathology occurs only in the presence of IL-4 in certain inflammatory conditions, including TB, at the systemic level. We extend this finding to the localized response in TB. Supporting this, we found an increase in in vitro levels of IL-4 (data communicated) in these patients, suggesting the tilt of immune response towards TH2/Th0 type. This increase in IL-4 production in response to MTB possibly influences the sensitzation of T-lymphocytes to apoptosis by a TNF-α-mediated pathway, as suggested by Seah and Rook. Cytokine profile-dependent action of TNF-α also suggests that the tissue damage was caused by TNF-α in the mixed TH1 + TH2/Th0 response.

In conclusion, the threshold levels of TNF-α may contribute to the containment of the infection by synergizing

![Figure 2](image1.png)

**Figure 2.** In vitro production of TNF-α (a) and IFN-γ (b) by PBMCs and PFMCs of TB patients stimulated with PPD, culture filtrate (CF) and heat-killed Mycobacterium tuberculosis (MTB); *P < 0.05.

![Figure 3](image2.png)

**Figure 3.** Apoptosis of PBMCs and PFMCs in 10 TB patients detected by flow cytometry. Cells were treated with known apoptosis-inducing agent – Dexamethasone (DEX) and with mycobacterial antigens PPD and heat-killed MTB.
with IFN-γ to activate infected macrophages, or by the regulation of T-cell apoptosis; whereas the selective depletion of mycobacterium-reactive cells that secrete TNF-α may lead to enhanced susceptibility to MTB infection and result in the dissemination of the bacilli. Thus, TNF-α plays an immunoregulatory role in TB. In addition, we speculate that the high levels of IL-4 observed in our studies may also contribute to apoptosis in a TNF-α-dependent manner. It is interesting to note from our studies that the two antagonistic cytokines, viz. IFN-γ and IL-4 bring about the same effect of apoptosis mediated through TNF-α. However, further experiments should focus on finding the conditions and mechanisms by which these cytokines bring about apoptosis to elucidate the functional role of TNF-α in apoptosis. Understanding such basic mechanisms would help us explore new therapeutic strategies for TB.

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