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Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy

Imran Hussain Chowdhury[a,1], Albin Mostaque Ahmed[b], Subhadip Choudhuri[a], Aditi Sen[a], Avijit Hazra[c], Nishith Kumar Pal[d], Basudev Bhattacharya[a], Bojlul Bahar[b,e,*]

[a] Department of Biochemistry, Institute of Postgraduate Medical Education and Research (IPGME&R), 244B, AJC Bose Road, Kolkata 700020, India
[b] School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Dublin, Ireland
[c] Department of Pharmacology, Institute of Postgraduate Medical Education and Research (IPGME&R), 244B, AJC Bose Road, Kolkata 700020, India
[d] Department of Microbiology, Institute of Postgraduate Medical Education and Research (IPGME&R), 244B, AJC Bose Road, Kolkata - 700020, India
[e] School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Dublin, Ireland

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ABSTRACT

Active pulmonary tuberculosis (APTB) is associated with a failure of the host immune system to control the invading *Mycobacterium tuberculosis (Mtb)*. The objective of this study was to quantify and assess the role of serum inflammatory cytokines in active pulmonary tuberculosis patients following anti-tuberculosis drug (ATD) therapy.

Blood samples were collected from APTB patients and normal healthy subjects (NHS) (total $n = 204$) at baseline and 2, 4 and 6 months post-therapy and the abundance of serum inflammatory cytokines were measured by cytokine specific ELISA.

Compared to NHS, APTB patients at baseline had higher levels of serum pro-inflammatory cytokines IL-12p40 ($P < 0.001$), IFN-γ ($P < 0.001$), TNF-α ($P < 0.01$), IL-1β ($P < 0.001$) and IL-6 ($P < 0.001$) and anti-inflammatory cytokines IL-10 ($P < 0.001$) and TGF-β1 ($P < 0.001$) while there was no change in the level of IL-4. In APTB patients, the serum levels of IFN-γ, TNF-α, IL-6 and TGF-β1 directly relate to the bacterial load while the TNF-α, IL-1β, IL-6 and TGF-β1 relate to radiological severity. At baseline, the IL-6 level in NHS and APTB patients differed most and following ATD therapy, this level rapidly decreased and stabilized by 4-month in APTB patients.

It is concluded that a subtle reduction in the serum level of IL-6 of the APTB patients following ATD therapy might play a vital role in immune-protection of the host against *Mtb* infection and hence the serum IL-6 level can be a useful marker to diagnose the effectiveness of therapy in the patients.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) causes life-threatening pulmonary and extra-pulmonary tuberculosis (TB) in humans (Ellis, 2004). Mechanisms underlying host defense to *Mtb* infection is poorly understood (Grange, 1992). In response to infection, the host immune cells secret a number of cytokine and chemokines signals (Cooper and Khader, 2008), which play active roles in initiation and regulation of the immune response at the various stages of the disease development (Ameglio et al., 2005). Following an anti-tuberculosis drug (ATD) therapy, it is likely that the cytokine mediated cell signaling alters which ultimately lead to the recovery of the TB infection. Therefore, delineating the relationship of the major cytokines at various stages of disease progression is necessary to understand the mechanism of host response to *Mtb*.

In the *Mtb* infected hosts, macrophages and primary immune effector cells [neutrophils, natural killer (NK) cells and dendritic cells (DC)] serve as the main reservoir of the organism (Johnson and McMurray, 1994). The interaction of infected macrophages with T lymphocytes is central to protective immunity against *Mtb* and this interaction is mediated by a number of inflammatory cytokines produced by both cell types (Munk and Emoto, 1995). The major cytokines produced by immune cells during the effector phases of the immune response includes interleukin (IL)-12, interferon

Abbreviations: APTB, active pulmonary tuberculosis; ATD, anti-tuberculosis drug; *Mtb*, *Mycobacterium tuberculosis*; TB, tuberculosis; IL, interleukin; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alfa; Th, T helper; NK, natural killer; TGF-β, transforming growth factor beta; NHS, normal healthy subjects; AFB, acid-fast bacilli; ZN, Ziehl–Neelsen; RNTCP, Revised National Tuberculosis Control Programme; DC, dendritic cell; TLRs, toll like receptors.

* Corresponding author at: School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Dublin, Ireland. Fax: +353 17166258.
E-mail address: bojlul.bahar@gmail.com (B. Bahar).
[1] Present address: Department of Microbiology and Immunology, School of Medicine, University of Texas Medical Branch, Galveston, Texas, USA.

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gamma (IFN-γ), and tumor necrosis factor alfa (TNF-α) (Boom et al., 2003). The secretion of TNF-α by macrophages, DCs and T lymphocytes mainly contribute to a pro-inflammatory response (Raja, 2004). Macrophages also secrete IL-12p40, which has been implicated in the pathogenesis of T-cell-mediated response. IL-12p40 causes maturation of antigen-naive T helper (Th) cells into Th type 1 (Th1) cells (Trinchieri, 1994). The IFN-γ is secreted by Th1 cells as well as NK cells (Cooper et al., 2011). The IFN-γ activates alveolar macrophages to produce a variety of molecules involved in inhibition of growth and killing of *Mtb* (Denis, 1991; Conesa-Botella et al., 2012).

The deleterious effect of excessive pro-inflammatory cytokines is attenuated by secretion of anti-inflammatory cytokines such as IL-10, transforming growth factor beta (TGF-β), and IL-4 (Raja, 2004). However, excessive production of these anti-inflammatory cytokines may completely suppress the pro-inflammatory response resulting in a failure of elimination of *Mtb* infection (Sharma and Bose, 2001). Currently, only limited information is available to decipher the immune signaling mediated by inflammatory cytokines, which activates inflammatory pathway in the TB patients undergoing ATD therapy. It is necessary that the levels of different cytokines are fine-tuned and the delicate balance between the pro- and anti-inflammatory cytokine signaling is established at various stages of disease progression. Hence, the objective of this study was to assess the role of inflammatory cytokines in active pulmonary tuberculosis following ATD therapy.

2. Material and methods

2.1. Study population

The study population comprised of a total of 204 individuals with 102 newly diagnosed APTB patients admitted at Bellur ESI TB hospital or Chest Medicine department of SSKM hospital (Institute of Post Graduate Medical Education and Research), both located in Kolkata, India. A total of 102 normal healthy subjects (NHS), mostly students and laboratory personnel living in the same socioeconomic environment as the APTB patients and belonging to the same ethnic group (Bengali descent of West Bengal) were included in this study. All NHS had not taken any medication four weeks prior to sampling. The ethics committee of the Institute of Post Graduate Medical Education and Research, Kolkata approved this study and written informed consent was obtained from all APTB patients and NHS prior to sample collection.

2.2. Sampling

Morning sputum samples were collected from the APTB patients on three consecutive days. TB diagnosis was based on (a) clinical sign and symptoms and radiological confirmation of pulmonary TB, (b) presence of acid-fast bacilli (AFB) in the sputum smear following Ziehl–Neelsen (ZN) staining test and (c) confirmation of the presence of *Mtb* by multiplex-PCR technique (Chowdhury et al., 2012). To minimize the possibility of any co-existing bacterial or viral infection in the APTB patients, smear from the sputum and blood samples were evaluated and viral serology and HIV testing were performed. Patients with HIV-seropositive and co-morbid illness such as diabetes and hepatitis or opportunistic infection such as Aspergillosis, Cryptococcidiosis or any other clinical manifestation of infection were excluded in this study. In addition, only those APTB patients were included who had not administered with any corticosteroids or immunosuppressive agents in the recent past. Baseline blood sample (T0) was collected from the TB patients after confirmation of the presence of active pulmonary tuberculosis and absence of any co-existing bacterial or viral infection. Subsequent blood samples were collected at the end of 2 (T2), 4 (T4), and 6 (T6) months of ATD therapy as described in the following section. Single blood sample was collected from NHS after confirmation of the absence of any infection by clinical examinations and routine blood tests.

All APTB patients were placed on ATD regimen. As first line therapy, a standard anti-tubercular regimen for all non-resistant newly diagnosed cases comprised of Isoniazid, Rifampicin, Pyrazinamide and Ethambutol (thrice weekly) for two months followed by Isoniazid and Rifampicin (thrice weekly) for four months. For relapse patients, the regimen comprised of Isoniazid, Rifampicin, Pyrazinamide, Ethambutol for three months plus Streptomycin (all thrice weekly) in initial two months followed by Isoniazid, Rifampicin and Ethambutol (thrice weekly) for five months. Suspected MDR-TB patients received Kanamycin, Ofloxacin (or Levofloxacin), Ethionamide, Cycloserine, Pyrazinamide and Ethambutol for six months.

On completion of ATD therapy for 6 months, all patients showed clinical improvement in terms of resolution of fever and cough and weight gain. Patients also showed resolution of chest X-ray lesions (described below) and three consecutive sputum AFB smears were negative before discharge. These criteria for clinical cure of the Revised National Tuberculosis Control Programme (RNTCP), India, were followed uniformly by the attending physicians.

2.3. Evaluation of chest radiograph

All APTB patients had undergone plain postero-anterior and lateral chest radiography. Chest radiographs were evaluated for the presence and distribution of signs relating to the active pulmonary tuberculosis that include miliary patterns, cavity, fibro-cavity, segmental consolidation, lober consolidation, infiltrate and patchy opacity. To minimize any biasness by the observer, the radiographs were initially assessed by two independent pulmonary physicians prior to conducting any laboratory test.

2.4. Microscopy

Prior to the start of ATD therapy, morning sputum samples were collected on three consecutive days and were investigated for bacterial load by ZN staining technique. Stratification of the APTB patients were performed based on the bacilli load as follows: 3+ for 10–99 AFB/microscopic field, 2+ for 1–9 AFB/microscopic field, 1+ for 10–99 AFB/100 microscopic fields, scanty for 1–9 AFB/100 microscopic fields or negative sputum smears where no AFB was detected. Negative sputum was further confirmed for the presence of *Mtb* infection by multiplex-PCR technique described previously (Chowdhury et al., 2012).

2.5. Cytokines assay

The abundance of serum cytokines was quantified on the blood samples collected at baseline (T0), 2 (T2), 4 (T4) and 6 (T6) months post-ATD therapy of APTB patients and at baseline of the NHS. A panel of eight inflammatory marker cytokines including five pro-inflammatory (IL-12p40, IFN-γ, TNF-α, IL-1β and IL-6) and three anti-inflammatory (IL-10, TGF-β1 and IL-4) cytokines were quantified in the serum fraction of the blood. Measurements of all cytokines were performed by sandwich ELISA method and using commercially available ELISA kits. The minimum detection limits of different cytokines are presented in Table 1. ELISA was performed following the supplier's instruction. The standards and samples for all cytokines were measured in duplicate and the absorbance values were analyzed by non-linear regression.