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Multiplex analysis of cytokines/chemokines as biomarkers that differentiate healthy contacts from tuberculosis patients in high endemic settings

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ABSTRACT

Differentiation of latent tuberculosis infection (LTBI) from active disease is one of the crucial elements in the control of tuberculosis. Earlier in Indian population which is tuberculosis endemic, we identified that 10 *Mycobacterium tuberculosis* secreted protein fractions, induced IFN-γ response only in healthy contacts of TB patients (HCs) and not in tuberculosis patients (TB). These fractions were termed as "Contact Specific Fractions" ("CS" fractions) and found useful for differentiating HC from TB. Proteomic analysis revealed that "CS" fractions have 16 different proteins, of which three were novel T cell antigens. Using these "CS" fractions as stimulants, earlier IFN-γ, TNF-α and IL-4 cytokine responses were studied. In the present study, in order to identify the other useful cytokine biomarkers that were differentially expressed between HC and TB, Cytokine/chemokine response to "CS" fractions were analyzed using multiplex cytokine assay system. This preliminary investigation in our tuberculosis endemic population showed six cytokine (G-CSF, IL-6, IL-7, IL-8, IL-9, and PDGF) and one receptor antagonist (IL-1Ra) that were differentially expressed between HC and TB, for the first time. Especially IL-6 and PDGF were more promising biomarkers. IL-6 measurement identified seven as HC out of 10 HC analyzed. The measurement of PDGF identified eight as TB out of 10 TB tested. Studies are underway to further validate these biomarkers for the differentiation of LTBI from active tuberculosis.

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1. Introduction

Tuberculosis is still one of the most important infectious diseases in the world and it is the only infectious disease which was declared as global emergency by World Health Organization (WHO) in 1999 [1]. Incidence of tuberculosis is increasing at a rate of 1% per year. Worldwide 9.4 million new cases and 1.8 million deaths occurred due to tuberculosis in 2008 [2]. Although effective treatment for tuberculosis is available; it is expensive and with long duration. Due to the above drawbacks, noncompliance of treatment occurs in some cases, which will cause the spread of drug resistant strains, which would further complicates control of tuberculosis [3].

Tuberculosis is a communicable disease. Infection is initiated by inhalation of droplet nuclei, which are particles of 1–5 μm in diameter containing *Mycobacterium tuberculosis*, expectorated by patients with active pulmonary tuberculosis (PTB). Approximately 30% of the population exposed to *M. tuberculosis* will have evidence of infection by tuberculin skin test (TST) [4]. Among those infected, approximately 10% will develop clinical manifestations of active tuberculosis disease within 2 years post-exposure. In the remaining cases, the ensuing immune response arrests further growth of *M. tuberculosis* [5]. However, the pathogen is completely eradicated in only ~10% of infected, while the immune response in the remaining ~90% of individuals succeed in only containment of infection. In those people, *M. tuberculosis* remains in nonreplicating (dormant or latent) state at the site of infection. This process is termed as latent tuberculosis infection (LTBI). The WHO estimated that one-third of the total world population is latently infected with *M. tuberculosis* and 5–10% of the infected individuals will develop active tuberculosis disease during their life time [2,6].

LTBI is defined by the evidence of immunological response to mycobacterial proteins in the absence of clinical symptoms of active disease. Earlier, *in vivo* skin test response against purified protein derivative (PPD) was used to identify the LTBI. The presence of cross reactive antigens in PPD, prior BCG vaccination, as well as exposure to nonpathogenic mycobacterial exposure cause false positives in TST [7]. The Quantiferon TB Gold assay (QFT-IT) which measures the *in vitro* release of IFN-γ in response to *M. tuberculosis* specific antigens is now used widely for the diagnosis

Abbreviations: IFN-γ, interferon gamma; IEF, iso electric focusing; AFB, acid fast bacilli; CFP, culture filtrate protein; IL-8, interleukin-8; LTBI, latent tuberculosis infection; "CS" fractions, "contact specific" fractions; HC, healthy contact (subjects having latent tuberculosis infection); TB, tuberculosis patients; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; ESI, electrospray ionization; BCG, bacille calmette-guérin; BAL, bronchoalveolar lavage.

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of LTBI. In endemic setting, both TST and QFT-IT does not distinguish between LTBI and TB [8].

Earlier, we aimed at the identification of antigens specific for these two infection states (LTBI and TB). For that purpose, we separated culture filtrate proteins of *M. tuberculosis* using two dimensional liquid phase electrophoresis (2D-LPE). These protein fractions were subjected to *in vitro* immunological characterization in LTBI (Healthy contacts, HC) and tuberculosis patients (Active disease, TB). It was found that 10 fractions specifically induced IFN-γ response only in HC ("CS" fractions). Using the "CS" fractions as stimulants, earlier we had studied three key cytokines (IFN-γ, TNF-α and IL-4) response in HC and TB. Many researchers observed that, other cytokines were also differently expressed between two infection states [9]. To identify the other cytokines/chemokine which were differentially stimulated by "CS" fractions in HC and TB, Cytokine/chemokine response to "CS" fractions were analyzed using multiplex cytokine assay system. Twenty four cytokines/chemokines were tested in supernatants obtained from diluted whole blood cultures, which were stimulated with "CS" fractions. We found that seven biomarkers were differentially expressed between the groups, which have the potential to be used for the differentiation of LTBI and TB.

2. Materials and methods

2.1. Antigens

M. tuberculosis culture filtrate proteins (CFPs) were prepared and separated by 2D-LPE as described previously [10]. Briefly, 1 g CFPs was separated using liquid phase IEF system in the first dimension (Rotofor, Bio-Rad Laboratories, Hercules, CA, USA), based on the isoelectric point [11]. The separated protein fractions were again resolved using Preparatory sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) based on the molecular weight and were eluted using whole gel elutor (Bio-Rad Laboratories, Hercules, CA, USA). The 2D-LPE procedure separated CFPs into 600 fractions. A minimum of 200 μg of protein was required for immunological as well as proteomic characterization. Of the 600 2D LPE fractions, 350 possessed 200 μg or more of protein, and these were selected for further immunological analyses. Immunological analysis showed that 10 of these 350 fractions (7_28, 8_2, 8_29, 8_30, 9_24, 9_26, 10_11, 11_24, 12_21 and 12_24) induced IFN-γ response only in HC, not in TB [10]. Only these 10 "CS" fractions were chosen for the immunological analysis in the present study.

2.2. Mass spectrometric analysis

Five microgram of each "CS" fractions was subjected to overnight digestion with 1 μg of trypsin (Hoffmann-La Roche, Basel, Switzerland). Digestion was terminated by adding 3 μL of 10% trifluroacetic acid (TFA) and dried in vacuum concentrator. The sample was dissolved in 10 μL of 10% acetonitrile and mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/mL in 50% acetonitrile containing 0.1% TFA). The samples were hand painted onto 96-well plates (Applied Biosytems, MA, USA) and were allowed to air-dry for MALDI-TOF-TOF analysis.

Single Mass Spectrum (MS) and Tandem Mass Spectrum (MS/MS) analyses were performed utilizing an Applied Biosystems 4800 Proteomics Analyzer (Framingham, MA, USA). Mass accuracy of the instrument was internally calibrated with the 4800 Proteomics Analyzer Calibration Mixture of peptides (mass accuracy 35 ppm). Using the 4000 series Explorer Software (Applied Biosystems, MA, USA), MS spectral data was acquired from the samples and a MS/MS list was automatically generated for further analysis based on the top most five intense ions present (trypsin and major keratin ions were excluded).

The MS and MS/MS spectral data obtained were exported from the 4800 Proteomics Analyzer using the Global Proteome Server (GPS) Explorer Software (Applied Biosystems, MA, USA). The data was then submitted to a local MASCOT search engine for protein database searching against NCBInr and the *M. tuberculosis* complex databases for identification. Methionine oxidation was allowed as a variable modification in MS/MS analysis. Precursor tolerance was set to 150 ppm and fragment tolerance was set at 0.25 Da.

For the positive identification of proteins, two criteria have been considered. First, total protein score of reported proteins should be above 51. Secondly, the protein should have at least one peptide with an ion score $\geqslant$20. All mass spectrometry data were manually analyzed for this.

2.3. Study population

The study was approved by the Institutional Ethics Committee of Tuberculosis Research Centre (TRC) and an informed written consent was obtained from all the subjects who were enrolled in this study.

Ten patients with PTB were enrolled at the TRC clinic with age ranging from 26 to 52 years and the male to female sex ratio of 7:3. Two spot and one overnight sputum specimens were collected from each patient. Sputum specimens were examined for AFB by fluorescent microscopy (Auramine O phenol staining). For *M. tuberculosis* culture identification, the sputum specimens were processed by modified Petroff's method and inoculated onto Lowenstein Jensen (L-J) medium and incubated for up to 8 weeks at 37 °C [12]. The blood samples were collected from these subjects before the start of treatment. All the TB patients showed positive results by sputum smear microscopy and culture.

TST using 2 Tuberculin units of PPD (Statens Serum Institut, Copenhagen, Denmark) was performed on all subjects, and an induration of $\geqslant$15 mm after 48 h was considered positive. TST Induration $\geqslant$15 is specifically due to *M. tuberculosis* infection and not by environmental mycobacteria exposure [13]. Out of the 10 PTB patients, seven were TST negative and three were positive.

Seven healthy house hold contacts (HCs) whose age ranged from 28 to 55 years were recruited from families where there was at least one sputum positive PTB patient (index case) living in the same household, for at least 3 months before the start of their treatment [14]. Because of their proximity and prolonged exposure they have high probability of infection. The male to female ratio was 5:2. Three heavily exposed health care workers, working closely with PTB patients for at least 2 years at TRC, were also included in the study. Their age ranged from 28 to 35 years, and all were males. These individuals had no history of tuberculosis on the basis of personal history; physical examination, chest X-ray, and negative acid fast bacilli sputum smear microscopy. All contacts were TST positive.

After recruitment and drawing blood, they were followed up for a period of 6 months and none broke down with tuberculosis, which confirmed their disease free status at the time of participation in the study. The above explained parameters show that these people were infected, but not developed clinical tuberculosis. Therefore the HC were considered as LTBI group in the present study.

All subjects were HIV negative as determined by Tridot (J. Mitra & Co., India) and Retroquic (Qualprodiagnostics, India) assays with serum. Heparinized blood (21 mL) was collected from all the subjects for the immunological assays presented in this study.

2.4. Collection of culture supernatants for immunological analysis

Whole blood was cultured by diluting 1/10 in RPMI-1640 medium (Sigma Chemical Company, St. Louis, MO, USA), supplemented

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