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CXCL10 is overexpressed in active tuberculosis patients compared to *M. tuberculosis*-exposed household contacts

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ABSTRACT

Introduction: Variability in clinical outcome of tuberculosis infection is dependent, among other factors, on variation in host immunological response to the infection, which is modulated, in part by genetic variations present in the host. We undertook a study to identify host factors associated with such clinical variability.

Study design and methods: A comparative study between groups of active TB patients vs. clinically normal household contacts, family members living under the same roof with the patients for a long period of time, was carried out. We screened 22 candidate cytokines and chemokines in the plasma of 119 pairs ("discovery set") of TB patients and their asymptomatic household contacts. Identified associations were validated in an independent cohort of 78 patient-household contact pairs ("validation set"). Validated associations were further cross-validated by gene expression assays using RT-PCR and *in-vitro* whole blood stimulation by mycobacterial antigens ESAT6 and Rv2031c, two well-characterized antigens that are expressed in active and latent phases of disease, respectively. In a concomitant SNP association study, we have sequenced the validated gene in these patients and household contacts.

Result: CXCL10 was found to be the most significantly ($p = 0.0002$) elevated chemokine – discovered and validated – in patients' plasma compared to their household contacts. We found that *CXCL10* was overexpressed by 5-fold at the RNA level in patients compared to asymptomatic household contacts ($p = 0.004$). On stimulation of whole blood collected from normal healthy volunteers with mycobacterial antigens ESAT6 and Rv2031c, we found that production of CXCL10 by ESAT6 was significantly higher ($p = 2.8 \times 10^{-12}$) than Rv2031c. The production of CXCL10 was 20-fold more than IFN-γ, the most widely validated cytokine, by ESAT6 stimulation ($p = 4.6 \times 10^{-8}$). One of the polymorphisms in promoter of *CXCL10*, rs4508917 (−1447 A > G), was identified as a proteinQTL (pQTL). Reduced expression of CXCL10 was observed among individuals with GG genotype, but the reduction was statistically significant only among controls, but not among patients. Among patients, the expression level was very high compared to the controls irrespective of the genotypes at this locus.

Conclusion: Plasma level of CXCL10 is predictive of the active phase of TB infection.

1. Introduction

Tuberculosis (TB), a disease of antiquity, continues to persist in spite of mounting of massive eradication efforts. It still remains as a global threat with estimates of 1.8 million deaths and 10.4 million new cases in 2015 [1]. South-East Asia accounts for nearly half of the world's TB burden. In most regions of high prevalence, non-detection and under-reporting are major problems [1]. Improved and early diagnosis of infection may lead to reduction in spread of TB disease.

Clinical disease manifests in only a subset of individuals infected with *Mycobacterium tuberculosis* (*M.tb.*) [2]. Three possible outcomes of infection are a) development of clinical symptoms, b) containment of the pathogen in an asymptomatic, latent condition and c) spontaneous clearance of infection [2]. These outcomes are orchestrated by a number of factors that include immunological characteristics and genetic environment of the host [3]. Surveillance studies based on positive delayed-type hypersensitivity responses to *M.tb.* purified protein derivative (PPD) indicate that one third of the world population is latently infected with *M.tb.* [1]. These latent individuals have 10% lifetime risk of developing clinical disease and 10% annual risk if they become immune-suppressed [1]. Currently, Tuberculin skin test (TST) and Interferon Gamma Release Assay (IGRA) are the two indirect diagnostic assays based on host immune response that are available for detection of latent TB infection. But, in a high endemic setting, the efficiency of these two diagnostic tests is not satisfactory [4]. A recent report suggested that the diagnostic efficiency of IGRA can be enhanced

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by the addition of more cytokines [5].

Upon infection, the elimination of *M. tb.* mainly depends on the nature of interaction between infected macrophages and T lymphocytes. Phagocytic cells initiate and direct the adaptive T-cell immunity by presentation of mycobacterial antigens and expression of co-stimulatory signals and cytokines and chemokines. Following this initial interaction, bacteria migrate to the draining lymph node where they initiate antigen-specific T cells. These cells then differentiate into cytokine-producing cells [6]. These antigen-specific T cells release cytokines that activate the infected cells to kill the *M. tb.* [6].

Thus, chemokines and cytokines are critical to ensure the control of bacterial infection without the development of detrimental inflammation in the lung tissue [7]. The balance and interplay of cytokine-chemokine network is of paramount importance in understanding of TB pathogenesis [8,9]. Although some past studies have demonstrated that some cytokines have higher levels in the serum of patients than uninfected normal [9], most of the studies considered only a small number of cytokines. Our study design rests on the hypothesis that an individual, living in close proximity (in a small dwelling unit) with a TB patient for an extended period of time, but not exhibiting clinical symptoms, must have some protective factors. Such factors for protection might manifest in the altered signature of one or more cytokine. We therefore selected as comparison groups: a set of TB patients (cases) and a set of individuals living under continuous exposure of TB infection but did not develop frank disease. Thus, the overarching aim of our study is to compare the circulatory level of cytokines between TB patients and their household contacts, free of clinical symptoms of TB. Here, we have systematically evaluated a large number (22) of cytokines using a study design that is potentially powerful to robustly detect associations of cytokine levels with outcomes of infection.

We complemented these studies with *in-vitro* whole blood stimulation assays using two classes of mycobacterial antigens; ESAT-6, representing the active phase of infection and Rv2031c, expressed during dormancy or latent condition [10–13]. Finally, we genotyped individuals at polymorphic loci in relevant cytokine genes to assess their role in determining cytokine expression.

2. Methods

2.1. Ethical statement

The study was approved by Institutional Ethics Committees of National Institute of Biomedical Genomics, Kalyani, India. Biological specimens were collected from the study participants with written informed consent.

2.2. Study design

We have adopted a case vs. control (household contact) study design. All individuals recruited into this study were older than 18 years. The criteria for inclusion of an individual as a "case" in this study were: (a) the individual should have reported with clinical symptoms of TB to a clinic established by the Government of India for TB control, (b) should have been ascertained by the clinic to be *M. tb*. infected, (c) should have self-declared to be treatment naive, with no past history of infection, and (d) should be tested to be HIV negative. The spouse of the patient, if biologically unrelated to the patient, and sharing the dwelling unit with the patient, was enrolled as a household contact. The inclusion criteria for accepting such an enrollee as a *control* were (a) no past history of tuberculosis, (b) currently free of clinical symptoms, and (c) has remained free of clinical manifestations of TB for the three months after enrolment into this study. If either a case or a control did not remain in her/his normal residence for five consecutive days during the three months after enrolment, the pair of individuals was excluded from the study. Because of the stringent criteria adopted in accepting an individual as a "control", such an individual is actually a "super-control" since she/he has remained free of disease in spite of regular exposure by having lived with a clinically active TB patient.

Sputum Acid Fast Bacilli (AFB) smear test was done to diagnose TB. Patients were classified in four classes following World Health Organization (WHO) guidelines according to the number of smear positive bacteria in microscopic fields: AFB (+++), AFB (++), AFB (+) and Scanty. Among the household contacts, Interferon Gamma Release Assay (IGRA) was performed by measuring Interferon-γ produced by T cells in response to antigens specific to *M. tb*. IGRA positivity is an indirect indicative of latent infection, i.e., infection without any clinical manifestation.

2.3. Collection of samples

Pairs of TB patients and their household contacts (Discovery set, n = 119 pairs; Validation set, n = 78 pairs) were enrolled into this study with voluntary, written informed consent, following the inclusion and exclusion criteria described in the previous section. A 10 ml sample of blood was collected in EDTA Vacutainer tubes by trained phlebotomists from each individual (patient or control) recruited. Plasma was isolated from venous blood by centrifugation of vials at 1900 g in 4 °C for 10 min. The supernatant was carefully aspirated, aliquoted into fresh vials, labeled and stored with protease inhibitor (cOmplete™ mini, Roche) at −80 °C immediately. All plasma samples were preserved frozen until cytokine assays were done and were thawed only once.

Genomic DNA was isolated from the study participants using QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's protocol. DNA concentration was determined using Qubit™ software (Invitrogen, USA) with Qubit™ DNA Assay Kits according to the manufacturer's instructions.

2.4. Cytokine assay

Levels of 22 cytokines, in the plasma were assayed using Bio-Plex® Multiplex Immunoassays as per the manufacturer's instructions (BioRad). These cytokines, along with chemokines, were chosen from Th1, Th2, Th17 and Treg pathways and were assayed using three panels; an eighteen-plex panel to measure levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IFN-γ, CXCL10/IP-10, MCP-1, MIP-1α, RANTES and TNFα; a three-plex panel for IL-12p40, IL-18 and CXCL9/MIG, and a single-plex assay for TGF-β1. All samples were assayed in duplicate. Mean values of the two wells estimated for each were used for further data analysis. Samples from both case and control groups were assayed together in the same batch to minimize technical bias. A 4-parameter logistic standard curve was generated in each assay.

2.5. Real time PCR

Peripheral Blood Mononuclear Cells (PBMC) were separated from whole blood by Ficoll (Histopaque® −1077, Sigma) density gradient centrifugation and stored at −80 °C until RNA extraction. RNA was isolated by RiboPure™ RNA Purification Kit (Ambion) according to manufacturer's instruction and treated with DNase I (Ambion). Subsequently, 300 ng of total RNA was transcribed to cDNA using SuperScript® III Reverse Transcriptase (Invitrogen) with random hexamer primers, in accordance with the manufacturer's protocol and stored at −80 °C until use. PCR was conducted in 10 µl of volume with 1 µl of cDNA using Power SYBR® Green PCR Master Mix (Applied Biosystems), as per the manufacturer's protocol. A 'no template' control was always included in each assay. The expression of a gene of interest (e.g. *CXCL10*) and that of a reference gene *GAPDH* were measured in duplicates by real-time qPCR using SYBR Green incorporation (Applied BioSystem). The following primers were used: *CXCL10* sense, 5′-CTG-CAA-GCC-AAT-TTT-GTC-CA-3′; antisense, 5′-ATC-CTT-GGA-AGC-ACT-GCA-TCG-3′; *GAPDH* sense, 5′-GAC-CAC-TTT-GTC-AAG-CTC-ATT-TC-