Differential serum cytokine levels are associated with cytokine gene polymorphisms in north Indians with active pulmonary tuberculosis

Abhimanyu a, Irengbam Rocky Mangangcha a, Pankaj Jha b, Komal Arora a, Mitali Mukerji b, Jayant Nagesh Banavaliker c, Indian Genome Variation Consortium b, 1, Vani Brahmachari d, Mridula Bose a, *

a Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110007, India
b Genomics and Molecular Medicine, Institute of Genomics and Integrative Biology, Mall Road, New Delhi 110007, India
c Rajan Babu Institute of Pulmonary Medicine and Tuberculosis, Kingway Camp, New Delhi 110007, India
d Dr. B. R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi 110007, India

ARTICLE INFO

Article history:
Received 6 November 2010
Received in revised form 1 March 2011
Accepted 24 March 2011
Available online 1 April 2011

Keywords:
Serum cytokine
Cytokine gene polymorphisms
Pulmonary tuberculosis
Population stratification

ABSTRACT

Globally only 5–10% of people encountering Mycobacterium tuberculosis have a lifetime risk of active disease indicating a strong host genetic bias towards development of tuberculosis. In the current study we investigated genotype variants pertaining to five cytokine genes namely IFNG, TNFA, IL4, IL10 and IL12 in the north Indian population with active pulmonary tuberculosis (APTB) and correlated the serum cytokine levels with the corresponding genotypes. Twenty five single nucleotide polymorphisms (SNPs) including six loci examined for the first time in tuberculosis were selected for genotyping in 108 patients with APTB from north India and 48 healthy regional controls (HC). Applying exclusion criteria 12 SNPs passed all the filters and were analysed further. The serum cytokine concentrations were measured by ELISA. Compared to HC mean serum IFN-γ, IL-12, IL-4, and IL-10 levels were higher in APTB (p = 0.3661, p = 0.0186, p = 0.003, p = 0.7, respectively). In contrast the mean serum TNF-α level was higher in HC (p = 0.007). Comparison of genotypes and serum levels of the corresponding cytokine genes reveal that though IFN-γ and IL-4 levels were higher in APTB the genotype variants showed no difference between HC and APTB. In contrast the genotypes of the selected rsIDs in the TNFA, IL12 and IL10 genes showed significant association with the varying serum levels of corresponding cytokines. The variant of the TNFA gene at rs3093662, the IL12 gene at rs3213094 and rs3212220 and the IL10 gene at rs3024498 did show a strong indication to be of relevance to the immunity to tuberculosis. To our knowledge this is the first report from this region relating genotypes and serum cytokine levels in north Indian population.

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1. Introduction

Tuberculosis is a highly infectious disease and a major public health problem globally. India has almost 30% of the global burden of tuberculosis (TB). One person dies of the disease every minute. The average prevalence of all forms of tuberculosis in India is estimated to be five per thousand. The prevalence of smear positive cases is 2.27 per thousand and the average annual incidence of smear positive cases is 84 per 1,00,000 annually (Chakraborty, 2004). The severity of infection with Mycobacterium tuberculosis (Mtb) reflects the balance between the bacillus and host defense mechanism in which the quality of host defense determines the outcome. Immune response to TB is regulated by a complex set of interactions between the antigen-presenting cells, lymphocyte (T CD4+ and TCD8+), macrophages, monocytes and a vast array of immune mediators, the cytokines (Ladel et al., 1995). Effective production of cytokines in response to the bacilli would result in the immune clearance of the Mycobacterium from the host cells.

During Mtb infection, Th1-type cytokines have been shown to be essential for protective immunity which is secreted after 3 weeks of infection activating the macrophages to exert an anti-microbial action leading to formation of granuloma (Henderson et al., 1997; Young et al., 2002; Pieters and Garfield, 2002). IFN–γ is one of the most important cytokines involved in macrophage activation (Schultz and Kleinschmidt, 1983), stimulating antimur and anti-microbial activities as well as expression of MHC-II (Pace et al., 1983; Basham and Merigan, 1983; Nathan et al., 1983; Torrico et al., 1991).
TNF-α is a potent pro-inflammatory and immunoregulatory cytokine that plays a key role in the initiation, regulation, and perpetuation of the inflammatory response (Orme and Cooper, 1999). TNF-α is also required for induction of apoptosis in response to mycobacterial infection (Keane et al., 1997).

IL-12, a heterodimeric pro-inflammatory cytokine produced by activated macrophages, monocytes, B-lymphocytes and dendritic cells is the principal Th1 response inducing cytokine (Abbas et al., 1996). This cytokine has been found to be important for sustaining a sufficient number of memory/effector Th1 cells to mediate long-term protection to intracellular pathogens.

IL-10 is an anti-inflammatory cytokine produced by T lymphocytes (Barnes et al., 1993) and macrophages after phagocytosis of M. tuberculosis (Shaw et al., 2000). IL-10 antagonizes the proinflammatory cytokine response by down-regulation of production of IFN-γ, TNF-α and IL-12 (Fulton et al., 1998; Gong et al., 1996; Hirsch et al., 1999).

IL-4, an anti-inflammatory cytokine has been implicated to down-regulate IFN-γ, and thus has a detrimental effect on TB patients (Lucey et al., 1996; Powrie and Coffman, 1993). It also promotes the induction of Th2 cells (Abbas et al., 1996). Owing to such vital role these cytokines was selected for the present study.

Although there are reports on ex-vivo cytokine production in response to mycobacterial antigens and their correlation with variant genotypes (Ansari et al., 2009; Wilkinson et al., 1999; Danis et al., 1995; Akahoshi et al., 2002; Lopez-Maderuelo et al., 2003; Yilmaz et al., 2005) few have studied the serum cytokine levels and its implications in the context of genotypes of the patients (Vallinoto et al., 2010; Hurme and Santilla, 1998). Though there are some recent reports from Southern Indian population (Vidyarani et al., 2006; Selvaraj et al., 2008) such data on north Indian population is lacking. Moreover, there exists a significant difference in gene pools of both the populations as indicated by IGVC (Indian Genome Variation Consortium, 2008). The present study was therefore undertaken to examine the association between SNP genotype and serum cytokine levels in patients and healthy controls in North Indian population. Here we investigated the hypothesis that in a given (north Indian) population varying genotypes of the related cytokine genes namely IFNG, TNFA, IL4, IL10 and IL12 in patients with active pulmonary tuberculosis (APTB) may account for variable levels of serum cytokines and contribute to the disease pathogenesis. Understanding of the genetic make up of the patients in this context may have a futuristic impact on better disease countering strategies. Our results, albeit based on a limited data, reveal significant bearing of the genotype variant of the cytokine genes on the corresponding serum cytokine levels in the tuberculosis patients from north India. The cytokine genes considered here play an important role in the pathogenesis and mounting of protective immunity against Mt. Among the cytokine genes studied the variant of the TNFA gene at rs3093662, the IL12 gene at rs3213094 and rs3212220 and the IL10 gene at rs3024498 did show a strong indication to be of relevance to immunity to tuberculosis. To our knowledge this is the first report from this region relating genotypes and serum cytokine levels and contributing to the picture of genotype bias in the context of tuberculosis in north-Indians.

2. Materials and methods

2.1. Ethical aspects

All individuals were briefed about the study and a signed informed consent was obtained from the patient or his or her guardians before sample collection. The study was approved by the ethical committee of Vallabh bhai Patel chest Institute, University of Delhi, India.

2.2. Study population

Venous blood was taken from 108 Active pulmonary tuberculosis (APTB) patients above 15 years of age from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis (RBIPMT), Kingsway camp, New Delhi (India) after obtaining written consent and before start of any anti-tubercular therapy. Included patients were clinically and radiologically (chest X-ray) diagnosed for pulmonary tuberculosis and confirmed by sputum microscopy and culture for Mycobacteria following the guidelines of Revised National TB Control Program (RNCTP) Ministry of Health and family Welfare, Government of India. Clinical data was obtained from patients' medical records. All patients had access to free anti-tubercular drugs under DOTS (Directly Observed Treatment, short course) regimen of the Govt. of India. Patients with any immunosuppressive presentation such as diabetes mellitus or HIV co-infection which are considered to be risk factors for tuberculosis development and patients suspected of extra-pulmonary tuberculosis were removed from the study. Out of 108 APTB cases selected for study only 44 cases (40%) could match the exclusion criteria of being devoid of any immunosuppressive presentation or HIV co-infection. 13 cases (12%) had HIV co-infection, 30 of them (27%) had both pulmonary and extra-pulmonary TB (which was diagnosed after blood collection) 10 cases (9.2%) had diabetes mellitus and HIV status of 10 (9.2%) could not be determined as they were not willing to disclose their HIV infection status and hence not considered for the study. The Healthy control (HC) group consisted of 48 randomly chosen sex and age matched students and laboratory personnel from the various departments of the University of Delhi who were enrolled in the study after their informed consent with no signs, symptoms or history of previous Mycobacterial infection.

2.3. Analysis of population stratification

In an effort to avoid any spurious results arising from population stratification the self reported ethnicity of each subject and his/her parents was carefully considered. A recent attempt was made to classify the Indian population according to region and ethnicity by a consortium called Indian Genome Variation Database (IGVDB; Indian Genome Variation Consortium, 2008). According to the IGVDB the present study group corresponds to the Indo-European group (IE) according to region and ethnicity, therefore we matched our APTB cases and HC to the IGVDB data (530 cases which of 138 were from IE group) using EIGENSTRAT principal component analysis (Price et al., 2006). The method applies principal components analysis to genotype data to infer continuous axes of genetic variation. The axes of variation reduce the data to a small number of dimensions, describing as much variability as possible; they are defined as the top eigenvectors of a covariance matrix between samples (Fig. 1). Secondly, it continuously adjusts genotypes and phenotypes by amounts attributable to ancestry along each axis, via computing residuals of linear regressions; intuitively, this creates a virtual set of matched cases and controls. Thirdly, it computes association statistics using ancestry-adjusted genotypes and phenotypes (adapted from Price et al., 2006).

2.4. SNP Selection

Twenty five SNPs covering five important cytokine genes indispensable to the process of countering tuberculosis infection were chosen. We focused on less explored intronic and 3' UTR
variants to detect some possible new associated loci for tuberculosis. SNPs were selected from the Intronic and UTR regions as some studies from north India covering promoter variants in these cytokine genes reported little or no success in finding any fruitful associations (Sharma et al., 2010). We reasoned that apart from the promoter region, variations in other parts namely coding introns and UTRs might also be key regulators of the cytokine levels by regulating their transcript level. The criteria for SNPs selection was the same as previously described (Indian Genome Variation Consortium, 2008). In addition observed heterozygosity at the loci was considered from hapmap populations in an effort to minimize selection of monomorphic loci (data not shown).

2.5. DNA and Serum Extraction

Six ml of venous blood was drawn out of which 3 ml was dispensed in BD vacutainers (BD Franklin lakes, NJ, USA) containing ethylenediaaminetetraacetic acid as anticoagulant and kept frozen until use; rest of blood was dispensed in BD vacutainers for serum. Genomic DNA was extracted from frozen whole blood using phenol–chloroform method. Extracted DNA was quantified spectrophotometrically, checked for purity and stored at −20°C until further analyses. Serum was obtained by centrifuging blood at 1600 × g for 30 min; sera were collected and stored at −80°C until they were assayed.

2.6. Assay for serum cytokine level

Cytokines (IFN-γ, TNF-α, IL-4, IL-10 and IL-12) in collected serum were assessed using human cytokine ELISA kit purchased from U-Cytech Biosciences (Yalelaan, The Netherlands). Serum samples were serially diluted and assay was carried out as per manufacturer’s instruction. The optical density readings in the linear range of the dose response curve were used for calculating the concentrations. The final concentrations (pg/ml) were obtained after multiplying the values by dilutions at which the OD was read. The sensitivity and range of cytokine detection was 6.25–400 pg/ml as reported by the manufacturer.

2.7. SNP Genotyping

Genotyping of 25 SNPs from 5 cytokine genes was performed. SNPs were genotyped using the Sequenom primer extension-based assay (San Diego, CA, United States) following the manufacturer’s instructions. Briefly, multiplex primer extension assays were designed with the SpectroDesigner software. As template, 5 ng of genomic DNA was used in a multiplex PCR reaction. The PCR product was further purified before the primer extension reaction to generate allele-specific base extension products. The base-extension products were detected in the MassARRAY time-of-flight

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mass spectrometry (MALDI-TOF) system (Sequenom) to determine genotypes.

2.8. Genetic and statistical analysis

Hardy–Weinberg equilibrium was calculated in both APTB cases and HC separately by using Haploview v4.2 (http://www.broad.mit.edu/mpg/haploview) using default conditions (HW p-value cut off = 0.0010; Minimum genotype = 75% and minimum minor allele frequency (MAF) = 0.010) (Barrett et al., 2005). The samples and variations failing this test were not selected for further analysis. We found that 4 SNPs (16% of the genotyped polymorphisms) were monomorphic in the north-Indian population, 4 SNPs were monomorphic for HC group. 1 SNP was found to be monomorphic for APTB, 2 had genotype less than 75% and 1 SNP was out of Hardy–Weinberg equilibrium (information available on request). All 13 of the above mentioned polymorphisms were excluded from analysis. Remaining 12 obeyed all the filtering criteria and their description is given in Table 1. The serum cytokine levels were expressed as mean ± standard error (SE). One way analysis of variance (ANOVA) with Bonferroni comparison was used to compare the serum cytokine levels between the different genotypes within APTB cases and HC of the corresponding cytokine gene. All computations including Bonferroni comparison were done using GraphPad Prism (version 5.00 for Windows, Graph Pad Software, San Diego, CA USA, www.graphpad.com). A two-tailed p value < 0.05 was considered statistically significant. Multiple logistic regression was done using SPSS 16.0 (SPSS for windows release 16.0, Chicago SPSS, Inc). All the reported p values are after Bonferroni comparison.

3. Results

3.1. Demographic characteristics of the study groups

One hundred and eight patients suffering from active pulmonary tuberculosis (out of which only 44 could pass the exclusion criteria for this study) from north India and 48 healthy age-matched regional controls were included in this study. The age of APTB cases ranged from 18 to 75 years with a median of 35 years while that of HC ranged from 21 to 36 years with a median age of 27 years. Age and sex had no significant effect on serum cytokine levels among the genotypes but a trend was observed with AA genotype being the higher producer compared to TA genotype and AA accounting for the low serum IFN-γ producer. Among HC also AA genotype was found to be the lowest serum IFN-γ producer and GA genotype being highest serum IFN-γ producer (Table 2).

3.4. TNFA polymorphisms and serum TNF-α levels

The mean serum TNF-α level was significantly higher in HC than APTB cases (p = 0.007) (Fig. 2). Within the HC group when compared among the genotypes for the rs3093662 the AA genotypes were high TNF-α producers and GG genotypes were significantly low (p < 0.05) producers. APTB cases did not show any significant variation in the serum TNF-α levels among the genotypes but a trend was observed with AA genotype being the higher producer followed by GA genotype and GG being the low producer of serum TNF-α (Table 2).

3.5. IL4 gene polymorphism and serum IL-4 levels

APTB cases compared to HC showed significantly higher (p = 0.003) mean serum IL-4 level (Fig. 2). The variant rs2243266 studied for the first time in TB did not show any significant association of different genotypes with serum IL-4 levels in either APTB cases or HC. Nonetheless, GA genotype showed high serum IL-4 levels closely followed by GG and AA genotypes respectively in APTB cases and HC (Table 2).

3.6. IL10 gene polymorphisms and serum IL-10 levels

APTB cases showed higher (p = 0.7) mean serum levels for IL-10 which was not significantly different from HC (Fig. 2). Analysis of correlation between serum cytokine level and genotypes showed that GA genotype of variant rs3024498 demonstrate significantly high serum levels (p < 0.05) as compared to GG among APTB cases. No such association was observed for HC but GA genotype was higher producer of serum IL-10 followed by AA while GG genotype shows low IL-10 production similar to APTB cases. (Table 2) The IL10 gene variants at rs3024496 and rs3024490 did not show any association with the levels of serum IL-10; however a trend was clearly evident from the data obtained. Among APTB cases CC genotype accounted for high serum IL-10 levels followed by TT genotype with CT genotype showing lower serum IL-10 level for rs3024496. Interestingly CT genotype was the higher producer of serum IL-10 among HC showing a reverse trend as compared to
AFTP cases (Table 2). For variant rs3024490 among AFTP cases, the GT genotype accounted for higher serum levels followed by TT genotype and GG being lowest producer. In HC TT genotype accounted for least serum IL-10 levels preceded by GG and GT genotype, respectively (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Active pulmonary TB (AFTP) cases</th>
<th>Healthy controls (HC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene name</strong></td>
<td><strong>db SNP</strong></td>
</tr>
<tr>
<td><strong>TNF-α rs3093662</strong></td>
<td>AA (35)</td>
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<tr>
<td>GA (3)</td>
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</tr>
<tr>
<td>GG (3)</td>
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</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td><strong>rs2430561</strong></td>
</tr>
<tr>
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</tr>
<tr>
<td>AA (4)</td>
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<tr>
<td><strong>rs2069718</strong></td>
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<tr>
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<tr>
<td>CC (5)</td>
<td>9.2</td>
</tr>
<tr>
<td><strong>IL-10 rs3024496</strong></td>
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<tr>
<td>CT (11)</td>
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<tr>
<td><strong>rs3024490</strong></td>
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<tr>
<td>TT (17)</td>
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</tr>
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<td>TT (18)</td>
<td>290.2</td>
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<tr>
<td><strong>IL-4 rs2243266</strong></td>
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<tr>
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<tr>
<td>AA (3)</td>
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<tr>
<td><strong>IL-12 rs3213094</strong></td>
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<tr>
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<tr>
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<tr>
<td>AA (4)</td>
<td>219</td>
</tr>
</tbody>
</table>

Numbers in parantheses indicate individuals studied for that particular genotype. All reported p-values are after Bonferroni multiple comparison test. Significant p-values are in bold.

*dbSNP, the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP).*

The mean IL-12 level in the serum was significantly higher (p = 0.0186) in AFTP cases as compared to HC (Fig. 2). Varying genotypes among the subjects accounted for differential serum IL-12 levels for variants rs3213094 and rs3212220 of the IL-12 gene. Among the AFTP cases at rs3213094 significantly decreased serum IL-12 level (p < 0.05) was seen for GG genotype as compared to AA genotype. For the same variant the serum IL-12 levels did not vary significantly among genotypes of HC (Table 2). For rs3212220 among AFTP cases TT genotype showed significantly higher serum IL-12 level when compared to either GT (p < 0.05) or GG (p < 0.01) genotype. However the serum IL-12 levels did not vary significantly according to genotypes among HC (Table 2). For rs2853694 among AFTP cases AA genotype showed trend towards higher serum IL-12 level in contrast to a reverse trend observed in HC where AA accounted for lowest serum IL-12. Other genotypes were not found to be significantly differing in cytokine levels in either AFTP cases or HC for this variant (Table 2). For variant rs3181216, among AFTP cases the TT genotype recorded the highest serum IL-12 level followed by TA and AA genotype, respectively. Among HC, a reverse trend was observed. The AA genotypes were higher producers as compared to TA and TT, in that order (Table 2). The variant at rs730690 failed to give any significant association between varying serum IL-12 level with varying genotypes among both AFTP cases and HC, but a trend was observed among AFTP cases with AA genotype accounting for high serum IL-12 levels followed by GA and GG genotype, respectively (Table 2).

### 4. Discussion

Cytokines as key mediators of immune response play a central role in homeostasis and any variation may account for a less effective immune response affecting the outcome of tuberculosis infection in humans (Flynn and Chan, 2001). In the present study we investigated the serum cytokine levels in active pulmonary tuberculosis patients and the possible effect of gene variants of the corresponding cytokine genes on serum cytokine levels. On the
basis of our results we were able to define a certain higher or lower producer phenotypes.

When analysing a genetic dataset a surmounting challenge is to determine whether the samples are from a random homogeneous population or there is any evidence of a structured population which is genetically heterogeneous. Admixed or structured population check should be included in all genetic analysis (Devlin and Roeder, 1999) as structured population can induce high false positive results (Heiman et al., 2004) and give confounding genetic associations (Choudhry et al., 2006). In our study we used a principal component analysis as illustrated by Price et al. (2006) and found that the study population formed a homogenous group when related genetically thereby reducing chances of false positive results.

In our patient group (APTB) there was significant elevation of mean serum cytokine levels for IL-12, and IL-4. Though IFN-γ and IL-10 levels were higher in APTB cases the difference as compared to HC was not statistically significant. Surprisingly TNF-α was found to be significantly higher in HC. The cytokine profile observed in our patients is generally consistent with the earlier reports except for serum IL-4 levels which is reported to be low in other studies but was significantly (p = 0.003) elevated in APTB cases in our study. Elevated serum levels of IFN-γ, IL-6 and IL-10 and a decreased serum level for IL-12 has been reported in tuberculosis patients by Verbon et al. (1999). They also found that IL-4 serum levels were low in APTB cases. Another study by Deveci et al. (2005) on changes in serum cytokine level in active tuberculosis patients undergoing treatment has reported elevated serum levels for IFN-γ, IL-10, IL-12 and TNF-α in APTB cases but not IL-4. The serum cytokine level of IL-4, important down-regulator of Th1 response, was found to be significantly higher in APTB cases here probably indicating suppression of Th1 response leading to disease progression in these cases.

India being an endemic country for tuberculosis large sections of population harbor an asymptomatic sub-clinical infection with the tubercle bacilli resulting in an elevated basal level of the cytokines even in HC and might very well account for the lack of significant difference observed for IFN-γ and IL-10 between patients and healthy north Indians. These results support the view that altered cytokine balance should be considered in tuberculosis rather than elevation or decrease of just any particular cytokine as pointed out by Verbon et al. (1999).

A recent study relating plasma IFN-γ levels and the IFNG gene promoter variant rs2430561 in Brazilian subjects (Vallinoto et al., 2006) showed that TT genotype accounted for higher plasma IFN-γ as compared to AT or AA genotype with AA accounting for the lowest plasma IFN-γ level. Although not statistically significant we also found a similar trend in our study with TT genotype showing higher serum IFN-γ levels as compared to AT and AA; with AA genotype showing the lowest serum IFN-γ level. Studies from south India (Vidyarani et al., 2006; Selvaraj et al., 2008) for the IFNG +874 polymorphisms also could not find any significant correlation between the IFNG variant genotypes and mycobacterial antigen induced IFN-γ levels. The other IFNG variant rs206971B studied here has been implicated in a case–control association study in the west-African population for tuberculosis (Cooke et al., 2006). The polymorphism is adjacent to an octamer transcription factor 1 (OCT-1) binding site and alters a TAAA transcription motif that might be clinically relevant. However, that study did not explore any association of the cytokine level and variants. For the same variant in our study we observe that the genotypes had no bearing on the serum IFN-γ level.

The TNFA variant rs3093662 included in the present study, although studied in relation to the outcome of sepsis (Wurfel et al., 2008) and type 1 diabetes in south Croatian population (Boraska et al., 2009) has not been reported so far in pulmonary tuberculosis. Our results indicate that this locus may be important in protection against tuberculosis as serum TNF-α level for AA genotype was significantly high as compared to GG genotype among HC (p < 0.05). AA genotype being higher producer of TNF-α might enjoy certain degree of tolerance to tubercle bacilli. APTB cases did not show any significant variation in the serum TNF-α levels although GG genotype was certainly lower producer of TNF-α.

IL-10 is an anti-inflammatory cytokine which suppresses Th1 response and has macrophage deactivation property. Out of the three variants studied here, namely rs3024498, rs3024496 and rs3024490 only rs3024496 showed significant correlation between genotype and the serum IL-10 level. The GA genotype showed higher serum IL-10 levels (p < 0.05) than GG among APTB cases. This observation suggests a role for GA genotype in determining outcome of tuberculosis as APTB cases as this genotype might have a suppressed Th1 response against Mtb infection. Although both AA and GA genotype had high serum IL-10 levels, the difference between GA and GG was statistically significant (p < 0.05). Notably this variant has not been studied in tuberculosis so far; only implicated in obesity and colorectal cancer (Tsididis et al., 2009). The variant rs3024490 has also not been studied in relation to tuberculosis till date. Present in 3’ UTR of the IL10 gene the locus has been subject to study in asthma (Bosse et al., 2009). Variant rs3024496 also present in 3’ UTR of the IL10 gene has been studied in relation to tuberculosis in a case–control association study in the Korean population (Shin et al., 2005) and very recently also in South Africans (Möller et al., 2010). In our study we did not find any association of disease with these variants.

IL-12 is an important cytokine in mediating protective immunity to tuberculosis and its variants have been reported in many studies including from south India (Selvaraj et al., 2008), north India (Morahan et al., 2007) and Honkong Chinese population (Tso et al., 2004). However, these studies did not explore any association with serum IL-12 level. Among five variants of the IL12 gene studied here only two were observed to show correlation with serum IL-12 level. These two are novel variants studied for the first time in tuberculosis patients. Among the APTB patients studied here the GG genotype of variant rs3213094 showed significantly low IL-12 level in comparison to AA genotype (p < 0.05). Although the patients in each genotype group was less, the patients for GG genotype were more (n = 15) as compared to AA genotype (n = 5). Interestingly even among healthy controls the GG genotype individuals were more (n = 19) than AA genotype (n = 3). It is possible that AA genotype of north Indians would mount a better immune response than GG genotype and the GG genotype individuals being more in number add to TB susceptible population. This point needs to be probed further in a larger study group. As for rs3212220, only five APTB patients showing high serum IL-12 level were of TT genotype whereas seventeen patients were of GG genotype that showed lowest serum IL-12 level. Among the HC also the GG genotype individuals showed low serum IL-12 level. This observation further suggests that in north Indian population these two variants may play a role in immune response to TB. Our observation is further strengthened by a recent report from South Africa which appeared while this manuscript was being prepared (Möller et al., 2010) in which the variant rs3212220 contributed to a haplotype and has been shown to be relevant to tuberculosis.

In addition when an attempt was made to find out genotype profile of individual patients considering rs3213094 and rs3212220 we found three common combinations of probable predictor genotypes for low serum IL-12 level which are GA–GT (n = 12), GA–GG (n = 10) and GG–GG (n = 9) of both the variants, respectively. Incidentally both the variants are in strong linkage
disequilibrium (LD) $r^2 = 1$ and contribute to common multiallelic haplotypes (data not shown) further accentuating the importance of these loci in influencing serum IL-12 in patients. The genotypes of HC did not show any significant association hence not analysed for predictors.

Previous studies from India have sought to investigate the ex vivo cytokine production after stimulation by mycobacterial antigen (Vidyarani et al., 2006; Selvaraj et al., 2008) but none have to our knowledge looked into the serum cytokine levels and associated gene variants. The serum cytokine level correlation with corresponding gene variants has been considered in only a handful of studies and analysed mostly at single polymorphic locus (Vallinoto et al., 2010; Hurme and Santilla, 1998). Thus to our knowledge this is the first study to have implicated the serum cytokine levels variation with their gene variants in a large number of cytokine genes.

The results presented here indicate that single nucleotide polymorphisms in the cytokine genes play a role in alteration of the levels of the corresponding cytokines in the serum in patients with active pulmonary tuberculosis. Many variants included in the study are novel loci which have been included in a tuberculosis polymorphism study for the first time and includes variants of the TNFA gene at rs3094662, the IL10 gene at rs3024498, rs3024490, the IL12 gene at rs3213094, rs2853694, rs3181216 rs730690 and the IL4 at rs2243266. These loci need to be further probed for relative risk for developing tuberculosis. The variants in the TNFA gene, the IL12 gene and the IL10 gene may have a role in varying serum cytokine levels in the north-Indian population that may have a bearing on the genetic proneness or resistance to tuberculosis in this population group.

**Author’s contributions**

Conceived the study: MB. Experimental design: MB, VB and MM; Carried out the experiments: A, IRM, KA; analysed the data: A, PJ, MM; Wrote the paper: A and MB; Sample Collection supervision: JNB; Sample collection: IRM and A

**Acknowledgement**

The work is financially supported by Council for scientific and industrial research (CSIR), New Delhi, Project No: 60/0081/06/ EM-II. The Authors thank CSIR for providing Junior Research Fellowship to Mr. Abhimanyu. We are indebted to the patients and industrial research (CSIR), New Delhi, Project No: 60(0081)/06/ for predictors.

**References**


Basham, T., Merigan, T.C., 1983. Recombinant interferon-g increases HLA-DR expression on human monocytic cells. Cytokine 12, 483–486.


Intracellular survival of Mycobacterium tuberculosis in macrophages is modulated by phenotype of the pathogen and immune status of the host

Monika Sharma a,1, Mridula Bose a,*, Abhimanyu a, Latika Sharma a, Amit Diwakar b, Sujeet Kumar a, Shailendra Nath Gaur b, Jayant Nagesh Banavalikar c

a Department of Microbiology, Vallabhbhai Patel Chest Institute, New Delhi, India
b Department of Respiratory Medicine, Vallabhbhai Patel Chest Institute, New Delhi, India
c Rajan Babu Institute of Pulmonary Medicine and Tuberculosis, Kingsway Camp, New Delhi, India

ABSTRACT

Emerging evidence indicates that the causative agent of tuberculosis is more genetically and functionally diverse than appreciated previously. The impact of this variation on the clinical manifestation of the disease remains largely unknown. In addition, there exists significant variability in the immune status of the host governing susceptibility to tuberculosis. The effect of these variations on the host pathogen interaction was investigated by taking varying pathogen phenotypes (virulent H37Rv, a-virulent H37Ra and a multidrug resistant strain #591) and varying host (18 MDR-TB and 16 fresh TB patients and 10 healthy, BCG-vaccinated individuals). The key question was whether the intracellular survival of Mycobacterium tuberculosis (MTB) in human monocyte-derived macrophages (MDM), an attribute of pathogenic potential, can be modulated by the immune status of the hosts or phenotype of MTB.

The findings of this study indicate that induction of TNF-α may not be a global indicator of virulence of a strain. TNF-α release may be differentially regulated in response to the same strain depending upon the immune status of the host. Moreover, the phenotype of the infecting MTB and the host's immune status played a comparable role in the intracellular survival of MTB. This picture supports the hypothesis that in addition to the phenotype variation of the mycobacteria, the immune status of an individual will greatly influence the outcome of the host–pathogen interaction. These results may have a bearing on the future endeavors in vaccine development and TB control strategy.

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* Corresponding author. Address: Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110007, India. Tel.: +91 11 27667120; fax: +91 11 27666549.
E-mail addresses: Monika_sharma18@rediffmail.com (M. Sharma), mridulabose@hotmail.com (M. Bose), mannu.abhimanyu1986@gmail.com (Abhimanyu), drlatikasharma@gmail.com (L. Sharma), dramitdiwakar@gmail.com (A. Diwakar), sujeet13@hotmail.com (S. Kumar), SNGaur@yahoo.com (S.N. Gaur), jnbanavaliker@yahoo.com (J.N. Banavalikar).

1 Present address: Department of Zoology, Miranda House, University of Delhi, India.

Abbreviations: CFU, colony-forming unit; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; IFN-γ, interferon-gamma; IL-10, interleukin 10; IL-12, interleukin-12; iNOS, inducible nitric oxide synthase; MDM, monocyte-derived macrophages; MIRU, mycobacterial interspersed repetitive units; MDR-TB, multidrug-resistant tuberculosis; MTB, Mycobacterium tuberculosis; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; PGL, phenolic glycolipids; PI, propidium iodide; RFLP, restriction fragment length polymorphism; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; RPMI-1640, Roswell Park Memorial Institute medium-1640; TNF-α, tumor necrosis factor-alpha

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http://dx.doi.org/10.1016/j.ijmyco.2012.02.001
Introduction

The rational design of a more effective vaccine and better therapeutic agents against tuberculosis requires an understanding of the pathogenesis and immune response [1]. The relative inefficiency of BCG vaccine and the recent spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) necessitates an in-depth analysis of the host and the pathogen factors that would finally be relevant as considered parameters towards the ultimate goal of TB control.

Central to understanding the pathogenesis of TB is the dynamics of the interaction between Mycobacterium tuberculosis (MTB) and macrophages where both the pathogen and the host factors contribute to the final outcome.

Strain variation of MTB has drawn attention recently as a probable causal factor to elicit variable degrees of severity of the disease in the human hosts. Moreover, MTB isolates from patients with similar characteristics had remarkable variation in transmission rates when studied in animal models, again consistent with the idea of intrinsic biological differences between strains [2]. The W/ Beijing, HN878 and CDC1551 are some of the MTB strains that have been studied in detail to understand the effect of genetic and phenotypic variations of MTB on the virulence of the organism and severity of the disease [3]. Initial mouse infection studies demonstrated that CDC1551 replicates more rapidly in the lung than another reference strain known as Erdman [3]. However, subsequently it was shown that CDC1551 replicate at a comparable level as H37Rv, Mycobacterium bovis and other clinical isolates [4,5]. Experiments with human monocytes revealed that different clinical isolates show variability in rate of replication and survival in this model, too [6].

Macrophages are the first line of defense in the lungs that counter MTB. Ingestion of mycobacteria induces the release of proinflammatory cytokines and chemokines, which eventually leads to the induction of an immune response. A key player in the generation of Th1 response is interleukin-12 (IL-12), a cytokine produced mainly by the macrophages. IL-12 has been shown to be crucial in the development and sustenance of protective immunity against TB in mouse and human studies [7–9].

Reactive nitrogen intermediates (RNI) are important effector molecules released by the macrophages in response to mycobacterial infection. RNI are more important than reactive oxygen intermediates (ROI) to control the mycobacterial growth at least in case of Murine TB [10]. Tumor necrosis factor-alpha (TNF-α) controls intracellular mycobacteria in Murine macrophages through TNF-α inducible nitric oxide synthase (iNOS)-dependent as well as TNF-α/iNOS-independent pathways [11]. It was previously shown that MTB induces a high production of nitric oxide (NO) along with the production of TNF in patients with fresh active TB, but not in MDR TB [12]. TNF-α is a pleiotropic cytokine produced primarily by monocytes and macrophages and plays an important role in the host immune response to tuberculosis [13].

In 1949, Haldane [14] proposed that the maintenance of multiple genes that confer relative susceptibility of the host to infectious diseases would be favored by evolution. Several case–control studies have identified the association between TB and candidate genes potentially involved in the immune response to TB [15,16]. A strong hypothesis is in favor of a role played by host factors in TB susceptibility because only 10% of the infected individuals develop active clinical disease [17]. Therefore, an integrated approach is important and necessary to understand the role played by the diversity of the host in the context of the immune response and the variable pathogenic potential of MTB strains.

In the present study, the differential effect of host and pathogenic variability was investigated in an ex-vivo system of host–parasite interaction. The pathogen variability represented by the virulent H37Rv, non-virulent H37Ra and a clinical MDR strain of MTB, #591 (a TBd-1 positive and EAI clade spoligotype). The host category included MDR-TB patients, fresh active pulmonary TB patients and healthy volunteers. The intracellular survival of all the above-mentioned strains in MDM (monocyte derived macrophages) from different categories of hosts and the immunologic response parameters were assayed in terms of IL-12, TNF-α and NO release. Interferon-gamma (IFN-γ) receptor expression was analyzed on the CD14 positive MDM as an indicator of activation.

Materials and methods

Subjects

Informed consents were obtained from patients and volunteers who took part in this study. Twenty-one HIV negative patients, clinically and bacteriologically positive for MDR-TB and 17 newly diagnosed patients suffering from fresh active TB from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis (RBIPMT), Kingsway Camp, Delhi, India, were included in the study. All the MDR-TB patients were infected with tubercle bacilli resistant to at least INH and RIF. The age of the patients ranged from 20 to 60 years, the average age being 40.5 ± 12.27 years.

Ten healthy subjects of comparable age group without any previous history of tuberculosis or any other disease were also included in the study. All healthy volunteers had received the BCG vaccination at birth. The study was approved by the ethical committee of the Vallabhbhai Patel Chest Institute, where this study was conducted.

Isolation of PBMC and monocytes

Venous blood from TB patients and healthy volunteers was drawn into heparinized vials, diluted 1:2 in Roswell Park Memorial Institute (RPMI)-1640 medium, layered onto ficoll-hypaque and centrifuged for 30 min at 2000 rpm. Peripheral blood mononuclear cells (PBMC) were isolated from the interface and washed in RPMI-1640. Approximately 2 × 10⁶ PBMC were placed in 6-well culture plates and incubated overnight at 37 °C. The next day non-adherent cells were removed by washing in RPMI-1640.
The MDM isolated as mentioned earlier in Section 2.2 (Isolation of PBMC and monocytes) were recharged with complete RPMI medium supplemented with 10% autologous serum without any antibiotic and were infected with single-cell suspension cultures of #591 strain or H37Ra at MOI of 1:10 and incubated overnight. The supernatants were spun down at 10,000 rpm to remove any cell debris. The cytokines TNF-α and IL-12 were assayed in cell culture supernatants at 48 h by a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs according to the manufacturer’s instructions (Prolab, Germany).

CFU assay

The number of ingested and internalized mycobacteria within the MDM was calculated by lysing the infected cells from one of the wells in 1% Triton X 100. Serial dilutions of the lysate were inoculated on LJ medium slants in duplicates. The slants were incubated at 37 °C for 3 weeks. At the end of 3 weeks, slants were taken out and colony forming units (CFU) were calculated from the number of colonies of MTB on the LJ slants, taking into consideration the dilution factors.

Nitric oxide release

The concentrations of NO in the culture supernatant of the mycobacteria-infected macrophages were measured at 48 h. The concentration of nitrite produced by MDM in response to mycobacterial infection as a measure of the production of NO was determined using Griess reagent at 540 nm by a spectrophotometer. Briefly, 100 µL supernatant was removed from culture wells, centrifuged at 400g for 10 min to make it cell free and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, 2.5% H3PO4) at room temperature for 10 min. The concentration of nitrite was determined by using sodium nitrite as the standard.

Cytokines assay

To determine the release of IL-12 and TNF-α, the supernatants were harvested from the MDM culture of different study groups infected with MTB phenotypes at 48 h of infection. The supernatants were spun down at 10,000 rpm to remove any cell debris. The cytokines TNF-α and IL-12 were assayed in cell culture supernatants at 48 h by a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs according to the manufacturer’s instructions (Prolab, Germany).

IFN-γ receptor expression

Expression of IFN-γ receptor was observed on MDM at 48 h of infection by using phycoerythrin (PE)-labeled antibody to IFN-γ receptor-1 (α chain, CD119) (BD Biosciences). MDM were identified using fluorescein isothiocyanate (FITC)-labeled CD14 antibody (BD Biosciences). The cells were labeled and fixed. The data was acquired by flow cytometry using fluorescence-activated cell sorter (FACS) Calibur and analyzed by CELLQuest Software.

Statistical analysis

The in vitro NO release, cytokine release and CFU were analyzed and the results were expressed as mean with standard error of mean (mean ± SEM). Student’s t-test and Pearson coefficient of correlation were used to assess significance using Graph Pad Prism software (http://www.graphpad.com).
Results

Survival of MTB phenotypes (MDR-TB) strain, H37Rv and H37Ra in MDM of different study groups

To examine the survival of phenotype variants of the MTB intracellularly within the host cells, MDM from MDR-TB patients, fresh active TB patients and healthy individuals were infected with #591, H37Rv and H37Ra separately at MOI of 1:10. After 48 h of infection, the intracellular mycobacteria were released from the cells, serially diluted, plated on LJ slants and CFU was counted after 21 days of incubation at 37°C. The isolate #591 grew faster in the MDM of MDR-TB patients, and the growth rate was least in MDM of healthy volunteers (Fig. 1). This difference was statistically significant (*p* < 0.005). H37Rv grew at the same rate as the #591 in MDM of MDR-TB patients. In comparison, the growth of the attenuated strain H37Ra was greatly restricted by the MDM of MDR-TB patients (Fig. 1). This difference in the rate of survival of #591 strain and H37Rv as compared with H37Ra was significant (**p** < 0.05) in the case of MDR-TB patients. However, when the growth rate (CFU) of all three phenotypes were compared between MDR-TB patients and the healthy individuals, the CFU of H37Ra was found to be significantly higher in MDR-TB MDM (*p* < 0.05) indicating thereby that MDR-TB patients’ ability to restrict even the attenuated mycobacterium were compromised.

Notably, in the MDM of fresh active TB patients, the rate of growth in terms of CFU was comparable for all three phenotypes. However, the CFU of the #591 strain was much lower than that in the MDM of MDR-TB patients (*p* = 0.05) (Fig. 1), thus indicating that fresh active TB patients could control the growth and survival of the #591 strain more efficiently than the MDR-TB patients.

Although MDM of healthy volunteers could restrict the growth of all three phenotypes of MTB, namely, #591, H37Rv and H37Ra, the reduction of CFU was more in the case of H37Rv and H37Ra. In contrast, #591 strain could grow more efficiently in MDM of volunteers, and this difference was statistically significant (**p** < 0.05).

The results from the three study groups point toward a host-dependent differential ability to control the growth and survival of different phenotypic variants of MTB (Fig. 1). This observation may be an indication that the status of the immune response of the host is one of the deciding factors for the survival of mycobacteria in the host cells.

Expression profile of IFN-γ receptor on the MDM of patients and healthy volunteers in response to phenotypes of M. tuberculosis

In order to understand whether the variation observed above is owing to a difference in the state of activation of the MDM in different subject groups, MDM of MDR-TB patients, fresh active TB patients and healthy individuals were infected with #591, H37Rv and H37Ra in separate experiments. The percentage of CD14 positive MDM expressing the IFN-γ receptor I (CD119) at 48 h post infection were determined by flow cytometry.

The level of IFN-γ receptor-I expression on the CD14 positive MDM was significantly high in MDR-TB patients (40.75 ± 5.57) as compared with healthy subjects (20.00 ± 6.84). In the case of fresh active TB patients, the percentage of IFN-γ receptor-I positive MDM was also higher (36.18 ± 5.984) as compared with healthy subjects (20.00 ± 6.84), although the difference was not statistically significant. When the MDM derived from MDR-TB patients, fresh active TB patients or healthy individuals were infected with the three phenotypic variants of MTB, the percentage of IFN-γ receptor positive MDM did not show any further significant change. These results indicate that once internalized, the intracellular growth and survival of the phenotypic variants of MTB may not regulate the expression of IFN-γ receptor on the CD14 positive MDM (Fig. 2).

![Fig. 1 – The intracellular survival of the MTB strains in the MDM. MDM (monocyte-derived macrophages) from different subject categories: MDR-TB (n = 10), fresh active TB (n = 10) and healthy subjects (n = 8) were infected with M. tuberculosis (MTB) strains, clinical drug resistant (#591), H37Rv and H37Ra at MOI of 1:10 in independent experiments. Results expressed as ‘mean ± SEM (standard error mean). Significant values are indicated as symbols. *p* < 0.05; **p** < 0.05; ***p** < 0.005; ****p** < 0.0005; ***p** < 0.005. Results show higher survival of #591 and H37Rv in MDM of MDR-TB patients as compared to MDM of healthy subjects.](image-url)
Nitric oxide (NO) released in response to drug-resistant and drug-sensitive phenotypes of MTB (#591, H37Rv and H37Ra) was estimated at 48 h post-infection. The spontaneous release of NO by MDM of healthy individuals was higher (15.70 ± 2.19 μM/L), though not statistically significant, as compared with the spontaneous release of NO by MDM from MDR-TB and fresh active TB patients (Fig. 3).

In response to infection with #591, the NO released by MDM of MDR-TB, fresh active TB patients and healthy individuals was at basal level (Fig. 3). In response to the virulent laboratory strain, the NO release was at the basal level. Interestingly, however, in response to infection with attenuated laboratory strain H37Ra, the release of NO from MDM from both MDR-TB and fresh active TB patients was still at basal level, whereas healthy volunteers’ MDM produced significantly higher NO (34.70 ± 7.76 μM/L, p < 0.05).

IL-12 release by MDM of patients and healthy subjects in response to phenotypes of MTB

MDM of MDR-TB, fresh active TB patients and healthy individuals were either infected with #591, H37Rv and H37Ra or left uninfected. At 48 h post-infection, supernatant from the wells of infected/uninfected cells were collected and assayed for the release of IL-12.

The basal level of IL-12 release was comparable in the MDR-TB patients and healthy individuals (114.0 ± 37.38 and 164.8 ± 66.71 pg/ml, respectively). In contrast, basal level of IL-12 in the MDM supernatant of fresh active TB patients (26.88 ± 4.99 pg/ml) was significantly depressed as compared with healthy subjects (p < 0.05) (Fig. 4).

MDM of MDR-TB patients, when infected with the #591 strain, released a higher concentration (221.5 ± 47.89 pg/ml) of IL-12 as compared with the basal level (114.2 ± 37.38 pg/ml). Following infection with H37Rv, the release of IL-12 was also significantly up regulated (279.7 ± 53.54 pg/ml) (p < 0.05), whereas in response to H37Ra, the release remained at the basal level (133.1 ± 37.61 pg/ml).

MDM of fresh active TB patients, in response to infection with #591 strain, released significantly higher concentration of IL-12 (276.7 ± 64.48 pg/ml) as compared with depressed spontaneous release (26.88 ± 4.99 pg/ml) (p < 0.0005). H37Rv and H37Ra also induced significant up regulation of IL-12 secretion from MDM of fresh active TB patients (156.0 ± 46.35 and 129.5 ± 34.32 pg/ml, respectively). However, although relatively up regulated, since the initial concentration was very low, the absolute IL-12 levels were much lower when compared to the concentration of IL-12 from the MDM of the healthy individuals and MDR-TB patients.

In the case of healthy volunteers, the scenario presented with a clear contrast; #591 drastically down regulated the release of IL-12 (39.05 ± 8.95 pg/ml) from MDM of healthy volunteers as compared with spontaneous release (164.8 ± 53.14). Interestingly, however, in response to H37Rv and H37Ra, the release of IL-12 was not much affected and remained comparable with spontaneous release of IL-12 (Fig. 4). Thus, in healthy subjects, the #591 showed a definite down regulation of the IL-12 release, whereas in the TB patients (MDR-TB and fresh active TB), the same strain demonstrated an up regulatory response.

TNF-α released by MDM of patients and healthy subjects in response to phenotypes of M. tuberculosis

MDM of MDR-TB, fresh active TB patients and healthy individuals were either infected with #591, H37Rv and H37Ra or left uninfected to assay for spontaneous release of TNF-α. At 48 h post-infection, supernatant from the wells of infected/uninfected cells were collected and assayed for the release of TNF-α.

Fig. 2 – Expression of CD14 and IFNγR-I as markers of activation on the MDM (monocyte-derived macrophages). The study included 21 MDR-TB patients, 17 fresh active TB patients and 10 healthy subjects. Results are expressed as percentage. * p < 0.05.
**Fig. 4 – Trends of IL-12 release by MDM of patients and healthy individuals.** MDM (monocyte-derived macrophages) of MDR-TB patients (n = 21), fresh active TB patients (n = 17) and healthy subjects (n = 10) were assayed for the release of IL-12, in response to infection with #591, H37Rv and H37Ra. Significant values are indicated with symbols. **p < 0.05; hhh p < 0.0005; hh p < 0.005; h p < 0.05. (a) Shows the variable IL-12 release by MDM of MDR-TB patients in response to MTB phenotypes. (b and c) Represents the trend of IL-12 released by MDM of fresh active TB patients and healthy volunteers respectively, in response to MTB phenotypes. It may be noted that in response to infection with #591, IL-12 release shows sharp down regulation in the MDM of healthy individuals, whereas, in contrast, #591 infection significantly up regulates IL-12 release by MDM of fresh active TB patients. However, H37Rv, a virulent laboratory maintained strain, elicited high IL-12 release in all three groups.

**Fig. 3 – Nitric oxide estimation.** MDM (monocyte-derived macrophages) culture from different subject categories; MDR-TB patients (n = 21), fresh active TB patients (n = 17) and healthy subjects (n = 10). The results are expressed as mean ± SEM. *p < 0.05; hp < 0.05.
The spontaneous release of TNF-α from uninfected MDM of MDR-TB patients and fresh active TB patients was depressed significantly (53.44 ± 16.84 and 27.56 ± 11.85 pg/ml, respectively) as compared with the same from healthy volunteers (150.8 ± 48.83 pg/ml).

The #591 induced an enhanced release of TNF-α from MDM of MDR-TB patients (113.6 ± 30.74 pg/ml) as compared with the spontaneous release (53.44 ± 16.84 pg/ml). The virulent strain H37Rv also induced a significantly higher release of TNF-α as compared with spontaneous release (p < 0.005). The avirulent strain H37Ra induced a high but not-significant release of TNF-α (Fig. 5).

The MDM of fresh active TB patients in response to #591 released a significantly higher concentration of TNF-α (223.8 ± 59.49 pg/ml) as compared with the spontaneous release (27.56 ± 11.85). In response to H37Rv, the release was significant, and in response to H37Ra, the release was high but not significant (Fig. 5).

On the other hand, in the case of healthy volunteers, the #591 down regulated the TNF-α release from the MDM so drastically that the concentration (38.20 ± 21.27 pg/ml) reached below the level of spontaneous release (150.8 ± 48.83 pg/ml). In response to H37Rv and H37Ra, however, the TNF-α release remained comparable with the concentration of spontaneous release (Fig. 5).

Discussion

Variability in the severity and extent of the disease course in TB has fueled speculation regarding the respective contributions of host resistance and bacterial virulence [19]. Also, it has remained an intriguing question to understand the precise role played by the immune status of the host in such an interaction. There is no clear explanation to date as to why only a fraction of the population exposed to MTB develops clinical disease.

Comprehensive knowledge of the genetic and molecular bases of virulence is central to understanding the pathogenesis of diseases owing to intracellular pathogens. The virulent MTB strain H37Rv and its avirulent counterpart H37Ra have been studied extensively in animal models, and the genetic and phenotypic differences between these two strains are under intensive investigation [20]. In addition to the standard reference strains, studies have also been conducted using phenotypic variants of clinical isolates of MTB, such as W/Beijing strain, CDC1551, etc. [5]. All of these studies focused on

![Fig. 5](image-url)
the genetic and phenotypic variations of these strains in the context of virulence and their effect on the immune parameters either in animal models or ex-vivo cultured human monocytes and MDM. Virulence of MTB strains has traditionally been assessed in terms of the ability of bacilli to replicate within specific organs of mice and guinea pigs following aerosol infection. Such studies are time-consuming and expensive. Furthermore, although mouse and guinea pig models can effectively assess the virulence of MTB strains, animal studies may be less helpful in understanding virulence in the context of human TB and clarifying the mechanism by which the bacilli interact with or subvert host immune response [5].

Li et al. [6] described a method for low level of infection of human blood monocytes in which patterns of intracellular growth of virulent MTB (H37Rv), attenuated vaccine strain M. bovis BCG, and avirulent MTB (H37Ra) correlated with the observation in the animal model [21].

In the present study, this ex vivo low-level infection model was applied to infect MDM derived from healthy volunteers and TB patients. The virulence of a clinical MDR-TB strain #591 isolated from a patient suffering from pulmonary TB was assessed in the host parasite interaction using this model. This strain is a multidrug-resistant “old world” strain [22] that showed a three-banded pattern (low banding pattern) by IS6110 RFLP and which belonged to the East African-Indian (EAI) clade by spoligotyping (data not shown), which is a common spoligotype found in the Delhi region [18]. The standard virulent laboratory strain H37Rv and the attenuated strain H37Ra were included as phenotype variants of MTB. The healthy individuals and pulmonary TB patients, both fresh active TB and MDR-TB, were the host subjects included in the study. The question asked was whether the intracellular survival of MTB in human MDM, an attribute of pathogenic potential of the MTB strains, can be modulated by the variability in the immune status of the hosts or the phenotype of the pathogen?

It was found that the intracellular growth rate of the #591, in terms of CFU, differed greatly in the MDM of the three study groups. Strain #591 grew faster in the MDM of MDR-TB patients, as shown by a very high CFU (4.14 × 10³) compared with fresh active TB patients (2.7 × 10³) and healthy individuals (2.05 × 10³) (Fig. 1). Zhang et al. [20] in a study with human macrophages observed that all the four patients isolated in their study proliferated more rapidly inside the macrophages than H37Rv. Their study, however, did not include any patient groups and used macrophages from healthy subjects only. Moreover, in that study no immune parameters were investigated.

It was surprising to note that while the growth rate of #591 was comparable in fresh active TB patients and healthy volunteers, the immune response parameters showed a strikingly reverse trend. TNF-α and IL-12 were assessed as the parameters of immune response because both in vivo and in vitro studies have suggested a role for TNF-α in containment of intracellular MTB [23]. In the present study, it was observed that following the challenge with #591 the TNF-α level took a significant sudden dip (p < 0.05) in the case of healthy volunteers. Such a significant down regulation of TNF-α suggests that MDM from immune-competent healthy subjects is able to contain the growth of the clinical strain #591 and regulate the pro-inflammatory cytokine TNF-α, supporting the hypothesis that the ability of MTB to stimulate production of TNF-α may serve primarily to promote pathogenesis rather than protection [28]. In contrast, the same strain, #591, shot TNF-α release from the MDM of fresh active TB patients many fold higher from the basal value (p < 0.005) (Fig. 5). These patients were suffering from active infection, and the MDM were already in a state of activation, as evidenced by the IFN-γ R1 level (Fig. 2). The challenge with the clinical strain #591 led to a significant up regulation of TNF-α release. In the case of MDR-TB, the TNF-α level was up regulated, although this increase was not statistically significant, possibly because the basal level of TNF-α in MDR-TB patients was already high.

Interestingly, the CFU for the other two strains of MTB, namely H37Rv and H37Ra, in the MDM of fresh active TB patients were similar and comparable with that of #591 (2.5 × 10³ and 2.04 × 10³, respectively), although the TNF-α release showed a variable response. The TNF-α release was least in response to H37Ra and higher in response to H37Rv (Fig. 5). This differential expression of TNF-α correlating with the increase in the virulence of the challenge strains has been noted by others also [21]. Rook et al. [24] observed a greater induction of TNF-α by human monocytes in response to H37Rv than to BCG. Cho et al. [25] reported higher levels of TNF-α mRNA in guinea pig macrophages infected with H37Rv compared with H37Ra. In the past, there was some controversy surrounding the question as to whether attenuated or virulent mycobacteria induce higher levels of TNF-α [26]. In that context, the results of this study indicate that the host’s immune status plays a very important regulatory role in the release of TNF-α, because attenuated MTB strain H37Ra up regulates TNF-α release in healthy subjects in comparison with minimal up regulation in fresh active TB and MDR-TB patients (Fig. 5). Thus, the emerging picture suggests that the up or down regulation of TNF-α in response to mycobacteria will depend on the immune status of the individuals as well as the infecting MTB strain phenotype. While standard laboratory strains may give comparable results, the wild type of clinical strains circulating in the population possibly may elicit a more variable response.

The results of the present study using #591, a drug-resistant clinical old world strain, representing a common spoligotype circulating in the local population, is of sharp down regulation of TNF-α in healthy subjects and up regulation in TB patients. The IL-12 release, as an indicator of MDM activation, was also included in the present study. IL-12 enhances IFN-γ production and cytotoxicity of NK and T cells, including γδ (CD4+ and CD8+) and γδ T-cell receptor bearing cells [27,28]. IL-12 also favors the development of Th-1 like T-cell responses by antagonizing IL-4 and IL-10 and down regulating Th-2 responses [29,30]. In Murine models, administration of exogenous IL-12 enhances immunity against MTB. Not much is known, however, about the ability of mycobacteria to induce IL-12 production by human macrophages, the principal host target for the mycobacteria. The results of the IL-12 assay in this study showed a trend that is similar to the profile observed for TNF-α release; #591 drastically down regulated the IL-12 level in healthy subjects whereas in fresh active TB
patients, IL-12 was significantly up regulated. In MDR-TB patients, the IL-12 level was also raised above the basal value in response to infection with #591 that ran in parallel to the profile of TNF-α release. The present observation that IL-12 release is modulated by the different phenotypes of MTB infecting MDM from different types of hosts draws support at least from two recent studies [31,32]. The study by Fulton et al. [31] demonstrated that the IL-12 production by human monocytes is dependent on phagocytosis of MTB by the monocytes. The second study, by Li et al. [32], used MDM from healthy volunteers to study the uptake of MTB under different environmental conditions. They observed significantly lower IL-12 and TNF-α release by MDM infected by MTB grown under different environmental conditions in comparison with H37Rv cultured under standard laboratory conditions. The results of this study reporting highly significant down regulation of TNF-α and IL-12 by the clinical strain #591 in healthy subjects corroborates with their findings.

The overall emerging scenario reveals that in response to the challenge by the three different strains of MTB, the cytokine profile of the MDM of healthy volunteers was the reverse of that in fresh active TB patients; the picture somewhat blurred in MDR-TB patients. Here, all three phenotypes of MTB lead to up regulation of IL-12 and TNF-α. But this rise had no effect on the control of intracellular growth of MTB. In spite of high IL-12 and TNF-α, the #591 and H37Rv (and also H37Ra) grew very efficiently inside the MDM of the MDR-TB patients. This could mean that the MDM from these patients are on a state of sustained activation, but the intracellular machinery to control mycobacterial growth is impaired. High IFN-γRI expression but generally unaltered NO levels (Fig. 2) as observed in the MDR-TB patients may be an indication of this possibility. The findings of this study clearly indicate that the release of TNF-α may not be a global indicator of virulence of a strain. TNF-α release may be differentially regulated in response to the same strain, depending upon the immune status of the responding host. Thus, this picture further supports the hypothesis that in addition to the phenotype variation of the mycobacteria, the immune status of an individual will greatly influence the outcome of the host-pathogen interaction. These results may have a bearing on the future endeavors in vaccine development and TB control strategies.

Authors’ contributions


Acknowledgements

One of the authors (M. Sharma) is a recipient of the Senior Research Fellowship from the Council of Scientific and Industrial Research, India. We are thankful to Dr. Urvashi Balbir Singh and, the All India Institute of Medical Sciences, New Delhi, India, and Dr. Nalin Rastogi, Tuberculosis and Mycobacteria Unit, the Institut Pasteur de Guadeloupe, Guadeloupe, France, for spoligotyping of the clinical isolate #591.

REFERENCES

Footprints of genetic susceptibility to pulmonary tuberculosis: Cytokine gene variants in north Indians

Abhimanyu, Mridula Bose, Pankaj Jha* & Indian Genome Variation Consortium

Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi & *Genomics & Molecular Medicine, CSIR-Institute of Genomics & Integrative Biology, Delhi, India

Received March 1, 2011

Background & objectives: Tuberculosis is (TB) responsible for high morbidity and mortality worldwide. Cytokines play a major role in defense against *Mycobacterium tuberculosis* infection. Polymorphisms in the genes encoding the various pro- and anti-inflammatory cytokines have been associated with tuberculosis susceptibility. In this study we examined association of 25 sequence polymorphisms in six candidate cytokine genes namely *IFNG*, *TNFB*, *IL4*, *IL1RA*, *IL1B* and *IL12* and their related haplotypes with risk of developing pulmonary tuberculosis (PTB) among north Indians.

Methods: Pulmonary TB (n=110) patients and 215 healthy controls (HC) from north India were genotyped. Purified multiplex PCR products were subjected to mass spectrometry using Sequenom MassARRAY platform to generate the genotypes in a population-based case-control study.

Results: Using multiple corrections, significant overall risk against PTB was observed at seven loci which included variants in *IFNG* at rs1861493 and rs1861494; *IL4RA* at rs4252019, *IL4* variant rs2070874, *IL12* variants rs3212220, rs2853694 and *TNFB* variant rs1041981. Analysis of gene structure revealed two haplotype blocks formed by *IFNG* variants rs1861493 and rs1861494. The TA haplotype was significantly over-represented (*P*=0.011) in the cases showing a two-fold risk in the current population (Odds ratio=1.59 CI=1.101 to 2.297) and *TNFB* variants at rs2229094 and rs1041981 contributed to two haplotypes which were in strong linkage disequilibrium (LD) with AT haplotype showing a three-fold risk (*P*=0.0011, Odds ratio=3, CI=0.1939 to 0.7445) of developing PTB in north Indians.

Interpretation & conclusions: Our study showed six novel associations of cytokine gene variants with susceptibility to PTB in north Indians. Variants of *IFNG* and *TNFB* emerged as factors imposing a significant risk of developing PTB in north Indians apart from risk indicated by *IL1RA*, *IL4* and *IL12*.

Key words Cytokine gene variant - haplotype - *Mycobacterium tuberculosis* - pulmonary tuberculosis - single nucleotide polymorphisms

Tuberculosis (TB) causes significant morbidity and mortality throughout the world1. The vast majority of individuals infected with *Mycobacterium tuberculosis* (up to 95%) remain healthy, probably because of mounting an effective immune response against *M. tuberculosis*. In 1949, Haldane proposed that the maintenance of multiple genes that confer relative susceptibilities on the host to infectious diseases would be favoured by evolution. In support of this hypothesis, certain populations appear to be
at risk for both increased susceptibility to infection\textsuperscript{2} and progressive clinical disease due to mycobacteria\textsuperscript{3}. Several case-control studies have identified association between TB and candidate genes potentially involved in immune response to TB\textsuperscript{4,5}. A growing body of evidence supports a role of host genetic components in the development of tuberculosis. The observation of familial clustering of disease with higher concordance of tuberculosis disease in monozygotic versus dizygotic twins\textsuperscript{4}, the ethnic clustering of tuberculosis disease with a higher prevalence of tuberculosis in individuals of recent African descent\textsuperscript{2}, as well as the demonstration of both common polymorphisms and rare mutations which confer susceptibility to mycobacterial species in humans\textsuperscript{5} point significantly in this direction. These studies suggest that unique environment and natural selective factors may be responsible for the development of ethnic-specific host genetic factors associated with TB.

The first step in innate host defense is cellular uptake of \textit{M. tuberculosis}, which involves different cellular receptors and humoral factors. The subsequent inflammatory response is regulated by the production of pro- and anti-inflammatory cytokines and chemokines. Interferon-gamma (IFN-γ) one of the most important cytokines involved in macrophage activation, stimulating anti-tumour and anti-microbial activities as well as expression of MHC-II\textsuperscript{8,9}. Interleukin-4 (IL-4), an anti-inflammatory cytokine has been implicated to downregulate IFN-γ, and thus has a deleterious effect on TB patients\textsuperscript{10}. It also promotes the induction of Th2 cells\textsuperscript{11}. IL-12, a heterodimeric pro-inflammatory cytokine produced by activated macrophages, monocytes, β-lymphocytes and dendritic cells is the principal Th1 response inducing cytokine\textsuperscript{11}. This cytokine is important for sustaining a sufficient number of memory/effector Th1 cells to mediate long-term protection to intracellular pathogen. Like tumour necrosis factor-alpha (TNF-α), IL-1β is mainly produced by monocytes, macrophages, and dendritic cells\textsuperscript{12}. In tuberculosis patients, IL-1β is expressed in excess\textsuperscript{13} and at the site of disease\textsuperscript{14}. Implicated mainly in tuberculosis pleurisy, a usually self-resolving type of primary tuberculosis, one may hypothesize that an increased IL-1β /IL-1Ra ratio protects against a more severe form of tuberculosis.

TNF-β or lymhotoxin-alpha (LTα) is considered to be a proinflammatory cytokine and it is shown that secreted LTα is essential for the control of an intracellular bacterial infection\textsuperscript{15}. Recently Allie et al\textsuperscript{16} suggested that LTα might not have a critical role in host defense to acute mycobacterial infection, independent of TNF, but certainly a contribution of LTα in the control of chronic \textit{M. tuberculosis} infection is observed\textsuperscript{17}.

Association studies from north India probing multiple loci across the spectrum of candidate cytokine genes are scanty. The present study, therefore, was aimed to bring in focus certain unexplored polymorphisms in the context of tuberculosis susceptibility in north Indian population. The role and importance of genetic background in tuberculosis has now become univocal with ethnicity playing a crucial role. Probing new loci relating to tuberculosis susceptibility could suggest novel approach in pharmacogenomics and therapy to combat this pathogen. Also it could provide an insight into predicting individual’s genetic proneness to tuberculosis and of being future diagnostic tool for preventive therapy against tuberculosis.

\textbf{Material & Methods}

\textit{Study population}: PTB patients above 18 yr of age (n=110) were enrolled randomly in the study between 2010-11 from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis (RBIPMT), Kingsway Camp, New Delhi (India). The study was carried out in Department of Microbiology, V.P. Chest Institute, University of Delhi, Delhi. Enrolled patients were category I cases, clinically and radiologically (chest X-ray) diagnosed for pulmonary tuberculosis and confirmed by sputum microscopy and culture for \textit{Mycobacterium} following the guidelines of Revised National TB Control Programme (RNCTP), Ministry of Health and Family Welfare, Government of India (http://www.tbcindia.nic.in). All patients were given free anti-tuberculosis drugs under DOTS (Directly Observed Treatment, short course) regimen of the Government of India. The mean age of PTB cases was 31.89 ± 2.6 yr while the ratio of male : female was 47:53.

Patients having any immunosuppressive presentation such as diabetes mellitus or HIV co-infection which are considered to be risk factors for tuberculosis development, and patients suspected to have extra-pulmonary tuberculosis along with pulmonary tuberculosis were excluded from the study. Structured questionnaires were used to document all other relevant information such as age, sex, ethnicity, socio-economic status, BCG vaccinations, and previous family history of tuberculosis. The healthy control (HC) group consisted of 215 randomly chosen
nonconsanguineous BCG vaccinated students and laboratory personnel from the various departments of the University of Delhi who were willing to participate in the study with no signs, symptoms or history of previous mycobacterial infection. For HC mean age was 29.31 ± .82 yr and the ratio of male : female was 43:57.

Analysis of population stratification: Serious effort was made to avoid any false-positives arising as a result of population stratification. The self reported ethnicity of each subject and his/her parents was carefully considered. In addition, the genotype data were subjected to EIGENSTRAT principal component analysis for population stratification correction as illustrated by Price et al.18.

All individuals were briefed about the study and a signed informed consent was obtained from the patient or his or her guardians before sample collection. The study was approved by the ethics committee of Vallabhbhai Patel Chest Institute, University of Delhi, India.

DNA extraction: Three ml of venous blood was collected in BD vacutainers containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant and kept frozen until use. Genomic DNA was extracted from frozen whole blood using QiaAMP DNA kit (Qiagen, Germany). Extracted DNA was quantified by spectrophotometry, checked for purity and stored at -20°C until further analyses.

SNP selection and genotyping: Six candidate cytokine genes namely IFNG, TNFB, IL4, IL1RA, IL1B and IL12B, were selected owing to their suggested role in tuberculosis pathogenesis. All single nucleotide polymorphisms (SNPs) selected for genotyping were accessed from the public dbSNP (http://www.ncbi.nlm.nih.gov) and the HapMap (http://www.hapmap.org/). Most of the selected SNPs are from the intronic regions of the corresponding genes. We reasoned that not only the changes in the promoter but also of other unexplored regions of the gene may hamper its normal functioning leading to disease. The parameters taken into account while SNP selection were the frequency of <0.01 in dbSNP, reported allele frequency of at least 20 per cent in two world populations (from Hapmap), average spacing 1 kb but in closely spaced minor allele frequency was carefully considered. In addition, reported heterozygosity was considered in an effort to minimize selection of homozygous loci.

All SNPs were genotyped using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom Inc., USA). Assays for all SNPs were designed using SpectroDESIGNER software (Sequenom Inc., USA). All SNPs were genotyped using the iPLEX assays (www.sequenom.com/iplex). Briefly, as template, 5 ng of genomic DNA was used in a multiplex PCR reaction. The PCR product was further purified before the primer extension reaction to generate allele-specific base extension products. The base-extension products were detected in the MALDI-TOF mass spectrometer to determine genotypes.

Genetic and statistical analyses: Hardy-Weinberg equilibrium was calculated in both PTB cases and HC separately to ensure that the samples were within allelic population equilibrium by using Haploviev v 4.2 (http://www.broad.mit.edu/mpg/haploviev/). A stringent cut-off offered by the Haploviev v 4.2 was used for further analysis (minimum genotype =75% and minimum minor allele frequency 0.0010). The samples and variations failing this test were not selected for further analysis. PLINK v 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) was used to test for multiple comparison and P value after Bonferroni corrections was considered significant. Haplotype block generation was performed using the algorithm by Gabriel et al implemented in the Haploviev software which was also used for initial association testing. The statistical significance of P value of haplotypes was assessed by permutation analysis (N=10,000) with Haploviev v 4.2.

Genetic association testing was done using a 2 x 2 contingency table. Odds ratio, two tailed P value was calculated for alleles. 2 x 2 Computations were done using GraphPad Prism (version 5.00 for Windows, Graph Pad Software, San Diego California, USA; www.graphpad.com). Two-tailed P<0.05 was considered statistically significant.

Results

Table I shows the location and characteristics of the SNPs included in the study and Table II shows the associations after multiple corrections carried out using PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) which were found to be associated with susceptibility to PTB in north Indians in this study.

Population stratification correction: To access any underlying structure in the study population that could
confound the apparent genetic association population stratification correction was carried out using Eigenstrat Principal Component analysis method as illustrated by Price et al. The method models ancestry difference between cases and controls and any other compared group based on the supplied genotype data. Our cases and controls formed a homogenous group devoid of any stratification. According to Indian Genome Variation Consortium (IGVC) north Indians fall into Indo-European lineage. Our cases and controls matched with supplied marker data of Indo-European ancestry thereby ruling out completely any underlying structure in the population.

**Allelic association of cytokine SNPs and the risk of pulmonary tuberculosis:** Among the 25 studied SNPs, from six candidate cytokine genes the variants of IFNG, IL1RA, IL4, IL12 and TNFB were found to be associated with susceptibility to PTB in north Indians. All studied variants passing the exclusion criteria were in Hardy-Weinberg equilibrium in both cases and controls. Allelic association when probed in variants passing the exclusion criteria yielded six loci showing high risk for PTB susceptibility.

**IFNG polymorphism and PTB susceptibility:** After adjusting for multiple testing corrections the IFNG intronic variants at rs1861493 \( (\chi^2 = 12.089, P_{\text{bonferroni}} = 0.006593, \text{odds ratio (95%CI)} = 3.8 (1.7 - 8.6)) \) and rs1861494 \( (\chi^2 = 10.466, P_{\text{bonferroni}} = 0.01581, \text{odds ratio (95%CI)} = 3.0 (1.5 - 5.6)) \) showed a significant risk of developing pulmonary tuberculosis in north Indians with over-representation of the associated A and T alleles among PTB patients, respectively. Investigation of the gene structure and linkage disequilibrium pattern showed haplotypes formed by IFNG variants rs1861493 and rs1861494 which were in high linkage disequilibrium (LD) (Fig.). Three combinations of haplotype were seen namely TC, CC and TA, of which TA haplotype was over-represented in the cases and imposed a two-fold risk of developing pulmonary tuberculosis in north Indians (Table III).

### Table I. Location and base-pair positions of single nucleotide polymorphisms (SNPs) of various cytokine genes passing the exclusion criteria and minor allele frequency (MAF) in controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>db SNP(^a) rsID</th>
<th>Base change</th>
<th>Chromosome position</th>
<th>Location</th>
<th>MAF controls</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNG</td>
<td>rs1861493</td>
<td>A/G</td>
<td>Intron 4</td>
<td>0.13</td>
<td>New; this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1861494</td>
<td>C/T</td>
<td>Intron 4</td>
<td>0.14</td>
<td>New; this study</td>
<td></td>
</tr>
<tr>
<td>IL4</td>
<td>rs2070874</td>
<td>C/T</td>
<td>5'- UTR</td>
<td>0.25</td>
<td>Moller et al, 2010(^b)</td>
<td></td>
</tr>
<tr>
<td>TNFB</td>
<td>rs1041981</td>
<td>A/C</td>
<td>Exon 4</td>
<td>0.26</td>
<td>New; this study</td>
<td></td>
</tr>
<tr>
<td>IL12</td>
<td>rs2853694</td>
<td>A/C</td>
<td>Intron 4</td>
<td>0.5</td>
<td>New; this study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs3212220</td>
<td>G/T</td>
<td>Intron 4</td>
<td>0.28</td>
<td>Moller et al, 2010(^b)</td>
<td></td>
</tr>
<tr>
<td>IL1RA</td>
<td>rs4252019</td>
<td>C/T</td>
<td>Intron 5</td>
<td>0.05</td>
<td>New; this study</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)db SNP, the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP)

### Table II. Allelic associations in after adjustment for multiple testing

<table>
<thead>
<tr>
<th>Gene</th>
<th>db SNP(^a) rsID</th>
<th>Case ( (n=110) ), control ( (n=215) ) frequencies</th>
<th>Odds ratio (95%CI)</th>
<th>Chi square</th>
<th>( P ) value(^*)</th>
<th>( P_{\text{bonferroni}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNG</td>
<td>rs1861493</td>
<td>0.962, 0.869</td>
<td>3.8 (1.7 - 8.6)</td>
<td>12.089</td>
<td>5.00E-04</td>
<td>0.00659</td>
</tr>
<tr>
<td></td>
<td>rs1861494</td>
<td>0.946, 0.859</td>
<td>3.0 (1.5 - 5.6)</td>
<td>10.466</td>
<td>0.0012</td>
<td>0.01581</td>
</tr>
<tr>
<td>IL4</td>
<td>rs2070874</td>
<td>0.387, 0.255</td>
<td>1.8 (1.3 - 2.6)</td>
<td>10.708</td>
<td>0.0011</td>
<td>0.01387</td>
</tr>
<tr>
<td>TNFB</td>
<td>rs1041981</td>
<td>0.356, 0.238</td>
<td>1.7 (1.2 - 2.6)</td>
<td>8.649</td>
<td>0.0033</td>
<td>0.03618</td>
</tr>
<tr>
<td>IL12</td>
<td>rs2853694</td>
<td>0.607, 0.478</td>
<td>1.6 (1.2 - 2.4)</td>
<td>8.854</td>
<td>0.0029</td>
<td>0.0399</td>
</tr>
<tr>
<td></td>
<td>rs3212220</td>
<td>0.419, 0.263</td>
<td>2.0 (1.4 - 2.9)</td>
<td>14.572</td>
<td>1.00E-04</td>
<td>0.00175</td>
</tr>
<tr>
<td>IL1RA</td>
<td>rs4252019</td>
<td>1.000, 0.935</td>
<td>14.0 (1.8 - 103.5)</td>
<td>13.643</td>
<td>2.00E-04</td>
<td>0.00287</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism; \(^*\)unadjusted \( P \)-value; \(^\circ\)\( P \)-value after bonferroni multiple testing correction; \(^a\)db SNP, the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP); \( P<0.05 \) was considered significant
**Table III.** Haplotype blocks and frequencies

<table>
<thead>
<tr>
<th>Blocks</th>
<th>Haplotype frequency</th>
<th>Case (n=110), control (n=215) frequencies</th>
<th>Chi square</th>
<th>Permutations P value *</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>0.41</td>
<td>0.36, 0.43</td>
<td>2.82</td>
<td>0.093</td>
<td>0.75 (0.53 - 1.1)</td>
</tr>
<tr>
<td>CC</td>
<td>0.32</td>
<td>0.29, 0.33</td>
<td>0.86</td>
<td>0.353</td>
<td>0.85 (0.59 - 1.2)</td>
</tr>
<tr>
<td>TA</td>
<td>0.27</td>
<td>0.34, 0.24</td>
<td>6.46</td>
<td>0.04</td>
<td>1.59 (1.1 - 2.3)</td>
</tr>
<tr>
<td>Block 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>0.88</td>
<td>0.95, 0.86</td>
<td>10.66</td>
<td>0.005</td>
<td>2.9 (1.5 - 5.6)</td>
</tr>
<tr>
<td>GC</td>
<td>0.10</td>
<td>0.05, 0.13</td>
<td>8.85</td>
<td>0.017</td>
<td>0.38 (0.2 - 0.7)</td>
</tr>
</tbody>
</table>

*P value after performing permutation (n=10,000); P<0.05 was considered significant

**IL4 polymorphism and PTB susceptibility:** IL4 variant rs2070874 \(\chi^2=10.708, \ P_{\text{bonferroni}} = 0.01387, \) odds ratio (95%CI) = 1.8 (1.3 - 2.6) showed a two-fold risk by T allele in north Indians. The other studied IL-4 variant rs2243270 passing the exclusion criteria did not show any association towards susceptibility to pulmonary tuberculosis in this population.

**IL1RA polymorphism and PTB susceptibility:** The significantly associated locus ofIL1RA included intronic variant at rs4252019 \(\chi^2=13.643, \ P_{\text{bonferroni}} = 0.00287, \) Odds ratio (95%CI) = 14.0 (1.8 - 103.5) showing a 14-fold risk. Other variant such as rs315919 and rs380092 did not show any association towards susceptibility to pulmonary tuberculosis in this population.

**IL12 polymorphism and PTB susceptibility:** IL12 variants rs3212220 \(\chi^2=14.572, \ P_{\text{bonferroni}} = 0.00175, \) Odds ratio (95%CI) = 2.0 (1.4 - 2.9) and rs2853694 \(\chi^2=8.854, \ P_{\text{bonferroni}} = 0.0399, \) odds ratio (95%CI) = 1.6 (1.2 - 2.4) showed a two-fold risk associated with T and A alleles, respectively.

**IL1B polymorphism and PTB susceptibility:** The selected IL1B variants did not show any direct influence on PTB susceptibility in north Indians.

**TNFB polymorphism and PTB susceptibility:** TNFB variants at rs1041981 \(\chi^2=8.649, \ P_{\text{bonferroni}} = 0.03618, \) Odds ratio (95%CI) = 1.7 (1.2 - 2.6) a synonymous change showed a two-fold risk of association for PTB in north Indians. Interestingly rs1041981 contributed to a haplotype block with rs2229094 confirming the importance of this locus in risk of developing PTB in north Indians. The two haplotypes observed were AT and GC of which AT was over-represented in PTB cases and imposed a three-fold risk of developing PTB in north Indians.

**Discussion**

The host genetic bias contributing to susceptibility and progression of pulmonary tuberculosis might
involve interactions between multiple alleles located on different genes and chromosomes. In order to overcome this drawback we planned selection of different cytokine gene and multiple loci to cover a wide spectrum of immune response associated cytokines.

Case-control studies involving carefully chosen locus across ethnicities are valiant means of identifying novel associations pertaining to disease susceptibility. Association that arises may be a result of the polymorphism in question being functional or it being in linkage disequilibrium with another functional allele or a result of confounding association due to population stratification. To overcome such false positives, we carefully considered the self reported ethnicity of the study groups and further checked for any genetic heterogeneity in our data by Eigenstrat principal component analysis illustrated by Price et al. and found that the present data were free from any underlying population structure. Thus, this uniform data represent north Indian population for association analysis.

The IFN- being a crucial cytokine in immunopathogenesis of TB has been subject to several polymorphisms studies for pulmonary tuberculosis susceptibility. The locus probed here namely rs1861494 has not been studied in susceptibility to PTB but extensively studied in many other diseases such as leprosy and asthma. Kumar et al. found an association of this locus with susceptibility to asthma in Indians and could identify a haplotype. They also showed that alleles of rs1861494 A/G have differential affinity to bind putative nuclear factor. In the present study, we found significant risk for the locus in susceptibility to PTB. The other probed locus rs1861493 has been studied in idiopathic inflammatory myopathy and asthma but not in pulmonary tuberculosis. We also identified a risk haplotype contributed by rs1861493 and rs1861494 emphasizing the importance of the above mentioned loci as risk factors for developing pulmonary tuberculosis in north Indians.

**IL1A** locus rs2070874 has been an important locus of investigation in various diseases including asthma and rheumatoid arthritis. Its role in TB was reported not to be significant in Iranian pulmonary TB patients and recently in South Africans TB patients also the locus did not show any association. In the present study this locus showed a two-fold risk in the north Indian population.

**IL1RA** locus rs4252019 has shown significant risk of development of pulmonary TB in north Indians. The variant rs4252019 has been shown to be associated with prostate cancer risk but not pulmonary tuberculosis. Interestingly, the variant showed a 14-fold risk of developing PTB in the population studied here and emerged as a major locus to look out for in further studies.

**IL12** variants rs3212220 and rs2853694 showed a significant risk associated with development of PTB in north Indians. The variant rs3212220 has been shown to contribute to a haplotype by Moller et al. We have also predicted its importance in our previous study. Based on the analysis of serum IL-12 level, we demonstrated that for **IL12** variant rs3212220 TT genotype among active PTB cases showed significantly higher serum IL-12 level when compared to either GT or GG. The present study revealed T allele to be a risk allele in the present population. Similarly, rs2853694 a novel variant in the context of developing tuberculosis was predicted to be of importance and was validated in the present study. For rs2853694 among active PTB cases AA genotype showed a trend towards higher serum IL-12 level in contrast to a reverse trend observed in HC where AA accounted for low serum IL-12. The present study showed A allele at rs2853694 to be a risk allele for the north Indian population in the context of PTB susceptibility. An interesting observation was that both the higher serum cytokine producers i.e. TT genotype for rs3212220 and AA genotype for rs2853694 emerged as respective risk alleles T and A for this population, indicating that overproduction of IL-12 by these individuals might be interfering with the cytokine homeostasis and thus affecting the immune function of the cytokine in these individuals making them prone to infection. Our observation was further supported by the work of Leandro et al., who indicated that role of IL-12 as potent inducer of IFN-γ lied in its efficacy at low concentrations. In the present study it is observed that the PTB patients with IL12 risk allele genotypes are not efficient inducers of IFN-γ which in turn interferes with the protective immunity in these individuals, whereas a low profile of IL-12 in HC elicits an effective and optimal immune response rendering these individuals healthy.

**TNFB** though not usually considered for PTB association studies, was taken up in the current study because of its role in control of intracellular bacterial infection. The variant rs1041981 emerged as a
significant risk locus for PTB susceptibility in north Indians. The variant also contributed to a haplotype with rs2229094 and reinstated the role of TNFB polymorphisms in PTB.

Overall, five of the loci namely rs1861493 and rs1861494 (IFNG), rs4252019 (ILIRA) rs1041981 (TNFB) and rs2853694 (IL12) studied in patients of pulmonary tuberculosis showed a significant risk towards susceptibility to pulmonary tuberculosis in north Indians. We also report here the significant risk imposed by IL4 variant rs2070874 in the active PTB patients. Six new associations and three new associated haplotypes contributing to the spectrum of cytokine gene polymorphisms and risk of developing tuberculosis in general and north Indians in particular, were detected.

Acknowledgment
The authors thank all patients and volunteers for participating in this study. The support of the Medical Superintendent and staff at Rajan Babu Institute of Pulmonary Medicine and Tuberculosis (RBIPMT), Kingsway Camp, New Delhi (India) for the help in sample collection is acknowledged. Authors acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, for financial support. The first author was the Junior Research Fellow (JRF) in the CSIR project.

References


LETTER TO THE EDITOR

Reporting Genetic Association Studies: The Roadblocks and Guiding Rules for Robust Results

Abhimanyu · Mridula Bose

Received: 11 April 2012 / Accepted: 2 July 2012
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Congratulations to Dr. Birbian and colleagues for their article “GSTT1 and GSTM1 Gene Polymorphisms as Major Risk Factors for Asthma in a North Indian Population” [1]. Theirs was the first published study on GSTT1 and GSTM1 genetic polymorphisms in a north Indian population using a case–control design. The study was carried out on a good sample size and a number of conclusions were drawn. However, we felt that the authors should have followed the international guidelines for reporting genetic association studies (STREGA) [2] to add lasting value to their findings.

Case–control association studies are valiant way to detect associations in a resource-limited setting, but in reporting the results of such studies there are several roadblocks that must be cleared and addressed before arriving at robust and useful conclusions. The ultimate goal of all such studies is to arrive at very futuristic individualized medicine and an immunotherapeutic approach to disease.

Transparency and accuracy of the reported results of medical research must be ensured to enhance the value of such studies. The formation of the STrengthening the REporting of Genetic Association (STREGA) studies guidelines was a significant step in that direction; they were published in many journals simultaneously [2]. There are a number of considerations but three of the most important that have been shown time and again to be of great value are as follows:

1. **Population stratification check**: The mere mention of ethnicity does not suffice and the presence of any substructured underlying population must be checked.

The population substructure has been shown to confound the findings and result in spurious associations [3].

2. **Hardy–Weinberg Equilibrium (HWE)**: Departure from HWE must be checked in both cases and controls. Theoretically, a disease-free control group from an outbred population should follow HWE. The key inference in a genetic association study would be compromised if HWE is violated.

3. **Multiple correction testing**: In the interpretation of the results of association studies, the fundamental statistical problem is that single nucleotide polymorphism markers might be spuriously flagged as significant. A common method of dealing with these false positives is to raise the significance level for the individual tests for association of each marker. Any such adjustment for multiple testing is ultimately based on a more or less precise estimate for the actual overall type I error probability which should be taken into account before reporting associations.

In the report of Dr. Birbian et al. [1] there was neither any reference to STREGA guidelines nor were those applied in the study. These lacunae may push this study to nonsignificance over a period of time. The authors could have addressed these concerns in order to establish a robust nonspurious association.

**Conflict of interest** The authors have no conflicts of interest to disclose.

**References**

Response to Reporting genetic association studies: the roadblocks and guiding rules for robust results

Dear Editor,

We would like to assure Dr Bose that the STREGA guidelines were followed in the study “GSTT1 and GSTM1 Gene Polymorphisms as Major Risk Factors For Asthma In A North Indian Population” [1]. Merely not mentioning the term “STREGA” does not imply that the necessary guidelines for conducting the case-control studies have not been followed. The manuscript contains all the necessary ingredients of STREGA guidelines which are mentioned in the abstract, objectives, data collection/sampling, diagnostic criteria, exclusion criteria, baseline characteristics, genotyping methods and discussion summarizing the key inferences, along with funding/grant support.

As regards to statistical issues, the results have been clearly and transparently reported as OR with 95% confidence intervals, as quantifiable with the software available SPSS v 17.0 and Epi Info v 3.4.3. The Hardy–Weinberg law is also very well being followed when the GSTT1 and GSTM1 genes are analysed in combination yielding or \( \chi^2 = 0.0058 \) and \( \chi^2 \) test \( p \) value= 0.939 (\( p > 0.05 \)). So there is neither an error in sampling nor in processing.

As regards to ‘sub-populations in North India’, a study conducted among three Brahmin sub-populations of Uttar Pradesh using ApoB3’ HVR revealed no variations in the allele frequencies in North India [2]. Moreover, mtDNA variation based studies have reported genetic similarities amongst the various Indian sub-populations [3]. The genetic differences exist predominantly only between the ‘Indo-European-speaking North Indian population’ and ‘Dravidian-speaking South Indian population’ [4].

Care has already been taken to strictly recruit ‘North Indian population’ only. So the question of ‘confounding due to population stratification’ does not arise as the genetic ancestry of North India is the same according to data available.

Jagtar Singh and Niti Birbian
Panjab University, Chandigarh, Uttar Pradesh, India

*Corresponding author: Niti Birbian
E-mail: nitibirbian@yahoo.com

References for Rebuttal letter

Genetic association study suggests a role for SP110 variants in lymph node tuberculosis but not pulmonary tuberculosis in north Indians

Abhimanyu a, Pankaj Jha b, Ashima Jain a, Komal Arora a, Mridula Bose a,*

a Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, New Delhi 110007, India
b Genomics and Molecular Medicine, Institute of Genomics and Integrative Biology, Mall Road, New Delhi 110007, India

A R T I C L E   I N F O

Article history:
Received 23 December 2010
Accepted 31 March 2011
Available online 20 April 2011

Keywords:
Pulmonary tuberculosis
Lymph node tuberculosis
SP110
Variant
Single nucleotide polymorphisms
Mycobacterium tuberculosis

A B S T R A C T

Ethnic specificity is a key determinant in understanding the association of genetic variants with outcome of disease susceptibility. SP110, a component of the nuclear body, has been subjected to association studies with conflicting results. In this study we probed SP110 variants in pulmonary (PTB) and lymph node tuberculosis (LNTB) cases to explore their role in controlling susceptibility to Mycobacterium tuberculosis infection in north Indians. We genotyped 24 SP110 variants in over 140 north Indian tuberculosis cases and 78 ethnicity-matched controls. The SP110 gene variants were available from public databases. The cases and controls were free of any population stratification when subjected to Eigenstrat principal component analysis. Genotyping was carried out using the Sequenom MassARRAY platform. Applying exclusion criteria, 11 single nucleotide polymorphisms (SNPs) of the LNTB panel and 13 SNPs of the PTB panel passed all filters and were analyzed further. No significant association was observed between SP110 variants and PTB. Surprisingly, we discovered evidence of an association of SP110 variants with LNTB, a form of extrapulmonary tuberculosis, at 3 loci, namely, rs6436915, rs1427294, and rs1346311. When permutations analysis \( n = 10,000 \) of allelic \( p \) values was undertaken, only rs1427294 passed the test with its \( p \) value remaining statistically significant. The C allele of rs1427294 exhibited a 5-fold risk of developing LNTB. No significant haplotypes were observed. In the pilot study presented here, our results provide evidence for the first time that SP110 may be a risk determinant locus in LNTB while confirming a doubtful role of SP110 in PTB in north Indians. In general, the results might indicate a role of SP110 variants in extrapulmonary tuberculosis rather than PTB.

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1. Introduction

The role of human genetic factors in influencing susceptibility to tuberculosis has been conclusively demonstrated in a large number of studies [1–3] that have also confirmed a role of ethnicity governing the outcome of infection. A variant that may be a risk factor for a certain disease in one ethnic population might not be a risk at all in another ethnic population. This calls for ethnic validation of certain disease in one ethnic population might not be a risk at all in another ethnic population. This calls for ethnic validation of

The SP110 (Speckled 110) gene, located on chromosome at 2q37.1, which encodes the SP110 nuclear body protein (also known as transcriptional coactivator SP110 or interferon-induced protein 41/75), is a component of cellular structures called nuclear bodies. Nuclear bodies are located within the nuclei of cells, where they help to control the activity of certain genes containing retinoic acid response elements. Nuclear bodies are also involved in the regulation of cell division, the self-destruction of cells that are damaged or no longer needed (apoptosis), and the normal function of the immune system [6].

SP110 nuclear body protein is active primarily in immune system cells called leukocytes and in the spleen, as well as in many other tissues, although at a lower level [7,8]. SP110 likely helps regulate the activity of genes needed for the body's immune response to foreign invaders.

The SP110 locus was identified through a murine model and holds 41% sequence homology to the mouse lpr1 (intracellular pathogen resistance-1) gene present at the sst1 (supersusceptibility to tuberculosis 1) locus on chromosome 1. The sst1 locus has been demonstrated to essentially regulate susceptibility to Mycobacterium tuberculosis (M.tb) infection. Ipr1 might play an important role in preventing tuberculosis by mediating control of M.tb within its prime target cell, the macrophage [9].

The presence of structural motifs like the N-terminal SP110-like domain and the conserved chromatin-interacting SAND (Sp110, AIRE-1, NucP41/75, and DEAF-1 suppression) domain in SP110 points to its role in protein–protein interactions with other nuclear body proteins [10,11] and transcription regulation through DNA binding, respectively [12,13]. A nuclear hormone receptor motif LXXL and nuclear localization sequence are characteristics of the

* Corresponding author.
E-mail address: mridulabose@hotmail.com (M. Bose).
SP110 nuclear body protein. The gene has two splice variants, SP110a and SP110b, which differ from each other in exhibiting a C-terminal bromodomain [8]. SP110a is thought to have a role in the differentiation of myeloid cells and acts as a transcription co-activator. SP110b, lacking the bromodomain, has been demonstrated to act as a transcriptional repressor of retinoic acid response elements [14].

Association studies on SP110 and susceptibility to pulmonary tuberculosis have yielded conflicting results. A family-based study by Tosh et al. [15] has reported an association of the rs2114592 and rs3948464 single nucleotide polymorphisms (SNPs) in up to 365 families from the Gambia, Republic of Guinea, and Guinea-Bissau. Other studies that used a community-based study design [16–18] did not report any association between SP110 variants and susceptibility to PTB. Since most of the studies mentioned above did not produce any fruitful association, we decided to examine the possible role of SP110 in tuberculosis patients from north India. We included PTB and extrapulmonary tuberculosis (here lymph node tuberculosis [LNTB]) cases from the population under study here.

We investigated whether common sequence variants in the SP110 gene could influence the genetic susceptibility to PTB and LNTB in north Indians. This is a pilot study and the first attempt from India toward understanding the possible role of the SP110 gene variants in the Indian population. Here we report for the first time evidence of an association of SP110 variants with LNTB.

2. Subjects and methods

2.1. SNP selection

We genotyped 24 SNPs covering the SP110 gene that were chosen for genotyping on the basis of information available in public databases http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp&cmd=search&term= and a literature search based on previous studies carried out on SP110 (listed in Supplementary Table 1).

2.2. Study population and sample collection

Venous blood was obtained from 110 PTB patients more than 15 years of age from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis, Kingsway Camp, New Delhi, India, and 32 LNTB patients from the Chest Clinic, Lok Nayak Hospital, New Delhi, after obtaining informed written consent. PTB patients included in the study were clinically and radiologically (chest X ray) diagnosed for PTB and confirmed by sputum microscopy and culture for mycobacteria following the guidelines of the Revised National TB Control Program (RNTCP), Ministry of Health and Family Welfare, Government of India. Clinical data were obtained from patients’ medical records. All patients had access to free antitubercular drugs under the Directly Observed Treatment, Short course (DOTS), regimen of the Government of India. Patients with any immunosuppressive presentations, such as diabetes mellitus or human immunodeficiency virus coinfection, that are considered risk factors for tuberculosis development were excluded from the study. Ustom care was taken to select patients with LNTB or, more specifically, cervical tubercular lymphadenopathy for the study. Samples were drawn for fine-needle aspiration cytology from the enlarged lymph node for identification of tubercular granuloma as well as for culture confirmation of mycobacteria. The isolates were biochemically tested for confirmation of M. tuberculosis. The healthy control (HC) group consisted of 78 randomly chosen sex- and age-matched students and laboratory personnel from the various departments of the University of Delhi who were enrolled in the study after their informed consent was obtained. The HC had no signs, symptoms, or history of previous mycobacterial infection. All other relevant information, such as age, ethnicity, socioeconomic status, and BCG vaccination, was carefully recorded in a structured questionnaire.

2.3. Ethical aspects

All individuals were briefed about the study and a signed informed consent was obtained from the patient or respective attendant before sample collection. The study was approved by the ethical committee of Vallabhbhai Patel Chest Institute, University of Delhi, India.

2.4. DNA isolation

We collected 3 mL of venous blood in BD vacutainers (BD, Franklin Lakes, NJ) containing ethylenediaminetetraacetic acid as anticoagulant and kept frozen until use. Genomic DNA was extracted from frozen whole blood using the phenol–chloroform method and a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). Extracted DNA was quantified spectrophotometrically, checked for purity, and stored at -20°C until further analysis.

2.5. SNP genotyping

All genotyping was carried out using the Sequenom primer extension-based assay (San Diego, CA) following the manufacturer’s instructions at The Center for Genomics Applications (TCGA), New Delhi. Briefly, multiplex primer extension assays were designed with Spectro Designer software. As a template, 5 ng of genomic DNA was used in a multiplex polymerase chain reaction (PCR). The PCR product was further purified before the primer extension reaction to generate allele-specific base extension products. The base extension products were detected in the MassARRAY matrix-assisted laser desorption/ionization time-of-flight mass spectrometry system (Sequenom) to determine genotypes.

2.6. Genetic and statistical analysis

In an effort to avoid any spurious results arising from population stratification, the self-reported ethnicity of each subject and his/her parents was carefully considered. We checked for any underlying structure in our samples using Eigenstrat principal component analysis as illustrated by Price et al. [19] separately for both pulmonary and lymph node patients compared with HC. We have previously matched our cases and controls to the Indian Genome Variation Database (IGVDB) [20] with the IGVDB data (530 cases, of which 138 were from the IE group), also using Eigenstrat principal component analysis [21]. In the genotyping data, obtained, Hardy–Weinberg equilibrium was calculated in PTB, LNTB, and HC separately using Haplovie v. 4.2 [22] using default conditions (p value cutoff = 0.0010; minimum genotype = 75%, and minimum minor allele frequency = 0.0010). The samples and variations failing this test were not selected for further analysis. Eleven SNPs of the LNTB panel and 13 SNPs of the PTB panel obeyed all filtering criteria and were selected for further analysis.

Odds ratios (ORs) and allelic p values were computed using a 2 × 2 contingency table using GraphPad Prism (version 5.00 for Windows, Graph Pad Software, San Diego CA: http://www.graphpad.com). A two-tailed p value < 0.05 was considered statistically significant. Allelic association analysis was performed using Haplovie v. 4.2. The statistical significance of the p value was assessed by permutation analysis (n = 10,000) with Haplovie v. 4.2. Bonferroni correction for multiple testing was performed using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/).

Haplotypes blocks and linkage disequilibrium plots were constructed using Haplovie v. 4.2 using the default algorithm proposed by Gabriel et al. [23].
3. Results

3.1. Demographic profile of the study group

The mean age of the PTB cases was 34 years, with a range of 18 to 70 years, whereas LNTB cases were relatively younger with a mean age of 25 years and a range of 18 to 49 years. The HC group had a mean age of 28 years with a range of 21 to 65 years. There was no significant difference in age of the study groups.

3.2. Population stratification check

By careful analysis of the self-reported ethnicity and using Eigenstrat principal component analysis, we demonstrated a homogeneous case–control group in both PTB (Suppl. Fig. 1) and LNTB cases (Suppl. Fig. 2) devoid of any stratification. Previously we have reported that our cases and controls even matched the samples from IGVDB [21], further supporting the homogeneity and correct ethnic origins of our data (Suppl. Fig. 3).

3.3. SP110 variants and susceptibility to PTB

A total of 24 SNPs of the SP110 gene were examined in 110 PTB cases from north India and subjected to quality control as described under Subjects and methods. Of these, 6 SNPs were not polymorphic in this population, 3 had genotypes less than 75%, and 2 were out of Hardy–Weinberg equilibrium. In the remaining 13 SNPs passing quality control, allelic association analysis could not demonstrate any significant association between SP110 gene variants and susceptibility to PTB (Table 1). Allelic counts are indicated in Supplementary Table 2. The haplotypes, although formed (Fig. 1), were also not reported to be associated with susceptibility to PTB in north Indians.

3.4. SP110 variants and susceptibility to LNTB

When 24 SNPs of the SP110 gene were examined in 32 LNTB cases from north India and subjected to quality control as described under Subjects and methods, 3 of these SNPs were not polymorphic

---

**Table 1**

<table>
<thead>
<tr>
<th>SNP (rsID)</th>
<th>Base change</th>
<th>Allele frequencies (case, control)</th>
<th>OR (95% CI)</th>
<th>$\chi^2$</th>
<th>p value*</th>
<th>p value (Bonferroni)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6436915</td>
<td>G:T</td>
<td>0.369, 0.298</td>
<td>1.4 (0.8–2.3)</td>
<td>1.374</td>
<td>0.2411</td>
<td>1</td>
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<tr>
<td>rs13018234</td>
<td>G:A</td>
<td>0.070, 0.056</td>
<td>0.8 (0.9–3.3)</td>
<td>0.237</td>
<td>0.6262</td>
<td>1</td>
</tr>
<tr>
<td>rs2278198</td>
<td>A:G</td>
<td>1.000, 0.993</td>
<td>3 (0.12–74)</td>
<td>0.989</td>
<td>0.3199</td>
<td>1</td>
</tr>
<tr>
<td>rs1004869</td>
<td>C:A</td>
<td>0.974, 0.971</td>
<td>1.1 (0.24–5)</td>
<td>0.016</td>
<td>0.8979</td>
<td>1</td>
</tr>
<tr>
<td>rs989578</td>
<td>A:G</td>
<td>0.285, 0.258</td>
<td>1.1 (0.66–2)</td>
<td>0.234</td>
<td>0.6283</td>
<td>1</td>
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<tr>
<td>rs2114591</td>
<td>C:T</td>
<td>0.555, 0.545</td>
<td>1 (0.64–1.7)</td>
<td>0.026</td>
<td>0.8719</td>
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<tr>
<td>rs3948464</td>
<td>C:T</td>
<td>0.078, 0.063</td>
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<td>0.525</td>
<td>0.4687</td>
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<tr>
<td>rs930031</td>
<td>C:T</td>
<td>0.305, 0.273</td>
<td>1.2 (0.68–2)</td>
<td>0.304</td>
<td>0.5813</td>
<td>1</td>
</tr>
<tr>
<td>rs3769838</td>
<td>T:C</td>
<td>1.000, 0.993</td>
<td>3 (0.12–74)</td>
<td>1.004</td>
<td>0.3165</td>
<td>1</td>
</tr>
<tr>
<td>rs9061</td>
<td>G:A</td>
<td>0.216, 0.310</td>
<td>1.1 (0.61–2)</td>
<td>0.109</td>
<td>0.7408</td>
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<tr>
<td>rs11556887</td>
<td>C:T</td>
<td>0.831, 0.806</td>
<td>1.02 (0.63–2.2)</td>
<td>0.273</td>
<td>0.6015</td>
<td>1</td>
</tr>
<tr>
<td>rs7580900</td>
<td>T:C</td>
<td>0.558, 0.456</td>
<td>1.5 (0.94–2.4)</td>
<td>2.856</td>
<td>0.091</td>
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<tr>
<td>rs1346311</td>
<td>C:T</td>
<td>0.928, 0.890</td>
<td>1.6 (0.69–3.7)</td>
<td>1.182</td>
<td>0.2769</td>
<td>1</td>
</tr>
</tbody>
</table>

CI = Confidence Interval; PTB = pulmonary tuberculosis; SNP = single nucleotide polymorphisms; rsID = reference sequence ID; OR = odds ratio; 95% CI = 95% confidence interval.

*p value < 0.05 was considered significant.

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**Fig. 1.** Linkage disequilibrium plot and haplotype structure of SP110 in PTB cases. $D^*$ values are displayed within each diamond; missing values indicate $D^* = 100\%$. Color scheme gradient indicates $r^2$ values. Block length, in kilobases (kb), is indicated in parentheses. No significant association was observed between the haplotypes.
in this population with minor allele frequency <1%, and 6 had genotypes less than 75%; 4 were out of Hardy–Weinberg equilibrium. In the remaining 11 SNPs passing quality control (Table 2), allelic association analysis identified 3 SNPs in the \textit{SP110} gene with nominal \( p \) values below 0.05 (Table 2). Allelic counts are indicated in Supplementary Table 2. All associated variants were intronic, namely, rs6436915 (intron 17), rs1427294 (intron 3), and rs1346311 (intron 1). All variants conferred susceptibility to LNTB with the T allele of rs6436915 conferring a 3-fold risk (OR = 3; 95% CI = 1.3–6.3, \( p = 0.0057 \)), the C allele of rs1427294 demonstrating a 5-fold risk (OR = 5; 95% CI = 1.7–17, \( p = 0.0016 \)), and the C allele of rs1346311 demonstrating a 7-fold risk (OR = 7; 95% CI = 0.8–52, \( p = 0.0352 \)). To address the significance of our findings, a permutation analysis of allelic \( p \) values was carried out (Table 2). Only 1 of the 3 variants, rs1427294, passed the permutation test (\( n = 10,000 \)) with its \( p \) value remaining statistically significant below 0.05. Investigation of the gene structure of \textit{SP110} indicated a multiallelic haplotype block (Fig. 2) formed by rs958978, rs2114591, rs9330031, and rs9061, which was not demonstrated to be associated with LNTB susceptibility.

### Table 2

<table>
<thead>
<tr>
<th>SNP (rsID)</th>
<th>Base change</th>
<th>Allele frequencies (case, control)</th>
<th>Minor allele frequency (controls)</th>
<th>( \chi^2 )</th>
<th>OR (95% CI)</th>
<th>( p ) value</th>
<th>Permutation ( p ) value (( n = 10,000 ))</th>
<th>Bonferroni ( p ) value</th>
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<td>G:T</td>
<td>0.395, 0.183</td>
<td>0.395</td>
<td>7.634</td>
<td>3 (1.3–6.3)</td>
<td>0.0057*</td>
<td>0.071</td>
<td>0.1088</td>
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<tr>
<td>rs1004869</td>
<td>C:A</td>
<td>0.080, 0.029</td>
<td>0.08</td>
<td>2.344</td>
<td>2 (0.69–12.1)</td>
<td>0.1257</td>
<td>0.7257</td>
<td>1</td>
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<tr>
<td>rs1135791</td>
<td>T:C</td>
<td>0.625, 0.623</td>
<td>0.375</td>
<td>0.001</td>
<td>1 (0.53–1.9)</td>
<td>0.9812</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>rs958978</td>
<td>A:G</td>
<td>0.333, 0.258</td>
<td>0.333</td>
<td>1.074</td>
<td>1.3 (0.68–2.5)</td>
<td>0.3001</td>
<td>0.9681</td>
<td>1</td>
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<td>rs2114591</td>
<td>C:T</td>
<td>0.611, 0.545</td>
<td>0.389</td>
<td>0.689</td>
<td>1.4 (0.72–2.8)</td>
<td>0.4066</td>
<td>0.9936</td>
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<td>1.3 (0.66–2.6)</td>
<td>0.4165</td>
<td>0.994</td>
<td>1</td>
</tr>
<tr>
<td>rs9061</td>
<td>G:A</td>
<td>0.231, 0.210</td>
<td>0.231</td>
<td>0.095</td>
<td>1.2 (0.52–2.4)</td>
<td>0.758</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>rs11556887</td>
<td>C:T</td>
<td>0.768, 0.647</td>
<td>0.232</td>
<td>2.67</td>
<td>1.8 (0.88–3.6)</td>
<td>0.1022</td>
<td>0.6716</td>
<td>1</td>
</tr>
<tr>
<td>rs1427294</td>
<td>T:C</td>
<td>0.173, 0.037</td>
<td>0.173</td>
<td>9.92</td>
<td>5 (1.7–17)</td>
<td>0.0016*</td>
<td>0.0243*</td>
<td>0.0228</td>
</tr>
<tr>
<td>rs7580900</td>
<td>C:T</td>
<td>0.596, 0.456</td>
<td>0.404</td>
<td>2.961</td>
<td>1.7 (0.9–3.3)</td>
<td>0.0853</td>
<td>0.6247</td>
<td>0.9383</td>
</tr>
<tr>
<td>rs1346311</td>
<td>C:T</td>
<td>0.982, 0.890</td>
<td>0.018</td>
<td>4.437</td>
<td>7 (0.87–52)</td>
<td>0.0352*</td>
<td>0.3192</td>
<td>0.422</td>
</tr>
</tbody>
</table>

Significant \( p \) values are indicated in bold with an asterix.

PTB = pulmonary tuberculosis; LNTB = lymph node tuberculosis; rsID = reference sequence ID; SNP = single nucleotide polymorphisms; OR = odds ratio; 95% CI = 95% confidence interval.

4. Discussion

Some of the major loci controlling mycobacterial infection in mice have been subsequently demonstrated to also have a role in humans. Though not a natural host to mycobacteria, the findings obtained from the murine model have helped immensely in understanding tuberculosis pathogenesis and certain observations have been confirmed in humans [24]. One example of the successfully replicated loci from the mouse model is NRAMP1 [25]. It was after the identification of the \( sst1 \) locus and \( Ipr1 \) gene in immunity to TB in mice, its human homologue (with 41% sequence similarity), \textit{SP110}, was hailed as a promising candidate locus for gene–disease association.
The hypothesis of association was based on the ability of Ipr1 to induce apoptosis by regulating the transcriptional pathways in macrophages in response to intracellular pathogens. In addition, the presence of structural motifs like the SAND domain in SP110 and the presence of isoforms SP110a and SP110b made it a lucrative target, where a change in base can either hamper its DNA binding ability or result in alternative splicing, leading to more isoforms.

Confounding results and high false negatives can be found in a population-based genetic dataset if not checked for any underlying structure (stratification) in the study population. For the present data any bias induced by population stratification was ruled out of the study population and we obtained a fairly homogenous population for this study. Therefore, these data could be treated as representative of the north Indian population. Moreover, the non-split number of patients (n = 142) has sufficient statistical power of analysis, reducing the chances of spurious associations.

The exploration of tuberculosis disease association with SP110 variants has been taken up by many researchers. A study by Tosh et al. [15] indicated the association of 2 loci, rs2114592 and rs398464, in up to 365 families of smear-positive TB patients from the Gambia, Republic of Guinea, and Guinea-Bissau. They used a family-based design to explore disease association. At the same time, two community-based reports by Szeszko et al. [17] and Thye et al. [16] did not indicate any association between the SP110 polymorphism and tuberculosis patients from the Russian Federation and Republic of Ghana, respectively. Another report by Babb et al. [18] in a community-based study design from South Africa also did not observe any association with susceptibility to PTB. In the present study, consistent with earlier community-based reports, we also did not observe any association between SP110 variants and susceptibility to PTB in north Indians.

Surprisingly, when the association of the susceptibility of SP110 variants was probed with extrapulmonary tuberculosis, namely cervical tubercular lymphadenopathy, in a pilot study, we for the first time detected an allelic association in 3 SNPs in the SP110 gene. The variants were observed to confer susceptibility to LNTB with the T allele of rs6436915 conferring a 3-fold risk, the C allele of rs1427294 demonstrating a 5-fold risk, and the C allele of rs1346311 demonstrating a 7-fold risk.

A permutation analysis of allelic p values and Bonferroni correction for multiple testing identified rs1427294 as the locus that conferred a 5-fold risk toward development of LNTB in north Indians. All associated variants were in the intronic region and might have a role in alternative splicing and hence gene regulation. It is possible that these variants might not directly influence susceptibility but do so in conjunction with other factors or polymorphisms present in linkage disequilibrium. Since this is the first report indicating such a possibility, further study on a larger number of samples would validate these observations.

The present report for the first time has indicated an association of SP110 variants with LNTB, a form of extrapolmonary tuberculosis, and not PTB among north Indian patients.

Acknowledgments

The authors are indebted to all patients and volunteers for participating in this study. The support of the Medical Superintendent and staff at Rajan Babu Institute of Pulmonary Medicine and Tuberculosis, Kingsway Camp, New Delhi (India), and the Chest Clinic, Lok Nayak Hospital, New Delhi, for help in sample collection is duly acknowledged. The authors also thank CSIR for providing a Junior Research Fellowship to Abhimanyu.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.humimm.2011.03.014.

References