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The assessment of cytokines in Quantiferon supernatants for the diagnosis of latent TB infection in a tribal population of Melghat, India

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Summary The tuberculin skin test (TST) and interferon-gamma release assays (IGRA), namely, the QuantiFERON-TB Gold test (QFT), remain the standard immunological diagnostic tools for latent tuberculosis (TB) infection (LTBI). However, the sub-optimal detection rates of both of these tests are major impediments in recognizing the population at risk. This study was aimed at evaluating additional cytokines besides interferon-gamma (IFN-γ) as biomarkers for improving LTBI diagnosis in the tribal population of Melghat, India. Seventy-four close TB contacts were stratified by QFT and TST results into: (i) QFT+/TST+ ($n=26$), (ii) QFT+/TST− ($n=12$), (iii) QFT−/TST− ($n=35$) and (iv) QFT−/TST+ ($n=1$) groups. A panel of cytokines (IL-6, IL-10, TNF-α and IL-2R) was then evaluated in antigen-stimulated QFT cell-free culture

Abbreviations: BCG, Bacille Calmette Guerin; BMI, body mass index; CFP-10, 10 kDa culture filtrate protein; CIIMS, Central India Institute of Medical Sciences; ELISA, enzyme-linked immunosorbent assay; ESAT-6, 6 kDa early secretory antigenic target; IFN-γ, interferon-gamma; IGRA, interferon-gamma release assay; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-10, interleukin-10; IL-12, interleukin-12; IL-18, interleukin-18; LTBI, latent TB infection; *M.tb*, *Mycobacterium tuberculosis*; MAHAN, Meditation Addiction Health AIDS Nutrition; NFHS, National Family Health Survey; PPD, purified protein derivative; PTB, pulmonary tuberculosis; QFT, QuantiFERON-TB Gold Test; TB, tuberculosis; TNF-α, tumor necrosis factor-α; TST, tuberculin skin test; UNICEF, United Nations Children's Fund.

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supernatants using IMMULITE-1000, an automated immunoassay analyzer. Cytokine estimation showed significantly higher levels of IL-6 in the QFT+/TST+ group, while significantly higher levels of IL-10 were found in the QFT−/TST− group. Correlation analysis identified a positive correlation between IL-6 and the QFT response ($r = 0.6723$, $P < 0.0001$), while a negative correlation was seen between QFT and IL-10 expression ($r = -0.3271$, $P = 0.0044$). Similarly, IL-6 was positively correlated with TST levels ($r = 0.6631$, $P < 0.0001$), and conversely, a negative correlation was found between TST and IL-10 expression ($r = -0.5698$, $P < 0.0001$). The positive and negative predictive values of IL-6 were found to be 92.59 and 93.33%, respectively, and the positive and negative predictive values of IL-10 were 96.55 and 91.18%, respectively. No significant impact of the demographic characteristics on cytokine positivity was observed. Our preliminary results suggest that the evaluation of additional cytokines in QFT cell-free culture supernatants may be valuable for the identification of LTBI. Combining IL-6 and IL-10 with QFT and/or TST could markedly improve the detection accuracy of LTBI. Our observations require investigation in larger well-characterized cohorts along with follow-up studies to further confirm the study outcome.

Introduction

Despite worldwide efforts to minimize the burden of tuberculosis (TB), developing countries, such as India, continue to account for nearly 2.2 million cases out of the global incidence rate of 8.7 million TB cases [1]. One of the major contributing factors for the high incidence of TB in India is the prevalence of latent TB infection (LTBI) [2]. LTBI significantly contributes to the pool of active TB cases at a later stage, especially in high risk TB environments [3]. The identification and prompt diagnosis of LTBI is therefore required to substantially reduce the risk of developing active disease [4,5]. Commercially available tools for screening populations at high risk for TB include the tuberculin skin test (TST) and more recently developed interferon-gamma release assays (IGRAs), such as the QuantiFERON-TB Gold test (QFT; Cellestis Limited, Victoria, Australia) [6]. These tests represent indirect markers of *Mycobacterium tuberculosis* (*M.tb*) exposure and measure the cellular immune response against TB infection [5]. However, the sub-optimal sensitivity of QFT and the low specificity of TST remain critical issues in the identification of LTBI [7].

Although reports indicate interferon-gamma (IFN-γ) to be a key cytokine that is involved in the cell mediated immune response against TB infection [8], its measurement alone is not adequate for the accurate diagnosis of LTBI [9]. The protective and pathologic response of a host to *M.tb* is complex and multifaceted, involving many components of the immune system [10,11]. Numerous cytokines have pleiotropic and regulatory effects that participate in the host defense and in inflammatory and tissue repair processes. These cytokines are classified into two types: pro-inflammatory cytokines, which include tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), interleukin-12 (IL-12), interleukin-18 (IL-18), and anti-inflammatory cytokines, consisting of interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-10 (IL-10) [12,13]. Both types of secreted cytokines mutually regulate and inhibit each other's functions and their balance and dynamic changes control or predict the clinical manifestations of active disease [14]. The identification of such cytokines in the whole blood of exposed subjects previously stimulated with TB antigens may thus be useful for the accurate and reliable diagnosis of LTBI.

Our study population included latently infected subjects from the malnourished tribal region of Melghat, Maharashtra, India. Melghat is a hilly forested area located on the outskirts of the Amravati District of Maharashtra with a population of 0.3 million. The TB prevalence in the region is estimated to be nearly 32% and is one of the leading causes of death in the economically productive age group of 16—40 years [15]. According to the reports of United Nations Children's Fund (UNICEF), National Family Health Survey (NFHS) and Meditation Addiction Health AIDS Nutrition (MAHAN), Melghat also has the highest number of malnutrition cases in India [16]. The presence of malnutrition has been widely recognized as a risk factor for the development of tuberculosis disease in those who are infected. The reason for increased susceptibility to disease is attributed to malnutrition-induced

immunodeficiency, which affects the entire gamut of immune responses [17]. The prevalence of TB along with poor health status and limited resources are the key risk factors making the population more susceptible to latent infection. The early detection of LTBI among the tribal population is therefore required for proper treatment to be initiated and to minimize the risk of progression to active disease.

With the underlying aim of improving diagnoses and identifying novel biomarkers for LTBI, we carried out multiple cytokine analyses in antigen stimulated QFT cell-free culture supernatants from malnourished latent TB infected subjects of Melghat. Our primary objective was to evaluate the response of multiple cytokines in QFT cell-free culture supernatants for the effective screening and improved diagnosis of LTBI.

Materials and methods

Ethics statement

The study was approved by the Institutional Ethics Committee of the Central India Institute of Medical Sciences (CIIMS), Nagpur, and that of the Meditation Addiction Health AIDS Nutrition (MAHAN) Trust, Amravati, Maharashtra, India, and is in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Written consent forms were collected from each participant after a detailed oral explanation about the study.

Study design and participants

This was a prospective cohort study carried out over a period of 2 years in camps organized from ten different villages in the Melghat region of Maharashtra, India, between May 2009 and June 2011. A total of 224 participants were enrolled based on the information available from the Tribal Health Research Centre, Dharni run by the MAHAN Trust. Participants were screened using requisite inclusion and exclusion criteria. Primarily, participants were recruited from families having at least one sputum-positive pulmonary tuberculosis patient (PTB) living in the same household for at least 2 months prior to the start of anti-TB medication, who had continual exposure to TB and who were at high risk of infection.

Detailed information from all participants was obtained through a structured questionnaire concerning the risk factors for exposure to *M.tb*, the duration of exposure and the type of contact with the TB patient. Baseline characteristics, such as age, gender, weight, height, occupation, education and behavioral factors, were also recorded. Other information, such as any prior TST and the presence of infections or illnesses experienced in the last 3 months, was also documented. The BCG vaccination status was recorded based on the examination of the BCG scar on the left forearm.

Malnourishment among the study participants was determined by the information collected from the questionnaires regarding dietary uptake, physical attributes and BMI values.

A total of 42 participants refused to give blood and were excluded from the study. The remaining 187 participants who matched the inclusion criteria were selected for the study. Amongst them, participants with an earlier history of TB or who had been previously treated with anti-TB medication ($n = 57$) and pregnant women ($n = 10$) were excluded from the study. Children younger than 10 years ($n = 13$) were also excluded to avoid the impact of BCG reactivity on the TST. The exposure period of the participants was characterized as moderate based on living with active TB patients for 2—3 months and high for those living with active TB patients for more than 3 months. Sixteen participants with exposures of less than 1 month were not included in the study, while 12 participants whose QFTs and/or TSTs were not performed were also excluded from the study. Thus, a total of 74 participants with exposure periods varying from 3 months to 1 year were included in the present study as shown in Fig. 1. LTBI testing in all participants was performed 10—12 weeks after their last exposure to an active case.

All participants were negative for HIV infection and were not receiving any immunosuppressive drugs. A control population was recruited from the same villages. All of the control cases selected for the study were TST, sputum and culture negative; had no clinical or bacteriological features of TB; had normal chest radiographs with no history of anti-TB medications; and had normal BMI values.

The tuberculin skin test (TST) and the QuantiFERON-TB Gold test (QFT-G)

The TST was performed by the Mantoux method using 10 tuberculin units (TU) of purified protein derivative (PPD) (Span Diagnostics, India). The TST was administered intradermally by a certified technician and read after 48—72 h. In accordance with the National Tuberculosis Control Program, an induration of at least 10 mm was considered positive. Prior to performing the TST, 10 mL of venous blood were drawn into two heparin-containing tubes to perform the QFT

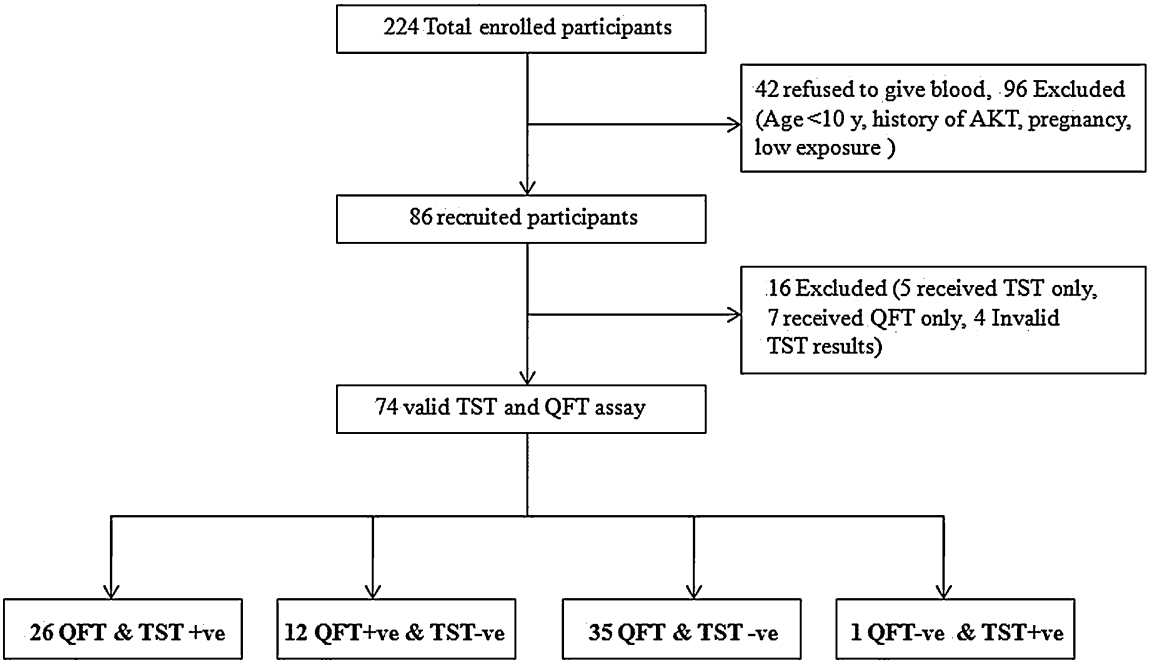

Figure 1 Study participation diagram. The figure presents the inclusion/exclusion criteria adopted for the recruitment of the 74 participants. TST-tuberculin skin test with a cut-off point of at least 10 mm; QFT— QuantiFERON-TB Gold with a cut-off point of at least 0.35 IU/ml.

assay. The QFT was performed according to the manufacturer's instructions. One tube contained only heparin as a negative control (unstimulated tube), and the other tube contained a peptide cocktail simulating ESAT-6, CFP-10 and TB7.7 proteins. The tubes were incubated for 20—24 h, after which the plasma was harvested and the concentration of IFN-γ was measured by an ELISA protocol. The IFN-γ values were calculated after subtracting the unstimulated value, and a cut-off value of 0.35 IU/mL was selected according to the manufacturer's instruction. The remaining plasma samples were stored at $-80\,^{\circ}C$ for later analyses of cytokines.

Cytokine estimation in antigen-stimulated QFT cell-free culture supernatants

The concentrations of additional cytokines (interleukin-6, interleukin-10, interleukin-2 receptor and tumor necrosis factor-α) secreted in response to antigen stimulation were determined in the QFT cell-free culture supernatants from all 74 exposed participants as well as healthy controls using the IMMULITE-1000 Immunoassay System (Siemens Healthcare Global), an automated microbead-based analyzer that makes use of chemiluminescent technology for analyte detection. In brief, plasma samples from the study participants were incubated with monoclonal antibodies coated on polystyrene beads for 30 min; thereafter, a polyclonal anti-antibody labeled with alkaline phosphatase was added before another 30-min incubation. Unbound conjugate was washed away by axial centrifugation and the chemiluminescent substrate added. After 10 min, light emission was measured and a four-parameter logistic model was used to calculate the sample concentration from the standard curves. All of the reagents were supplied in the kit, and the assays were performed using the manufacturer's instructions. The manufacturer's package insert states the upper and lower limits of detection for cytokines in plasma, which are based on the variability in the signal response observed in a very large number of non-reactive patient samples.

Statistical analysis

The frequencies (percentage) of the demographic, behavioral and clinical factors were measured on a nominal scale. Statistical analysis was performed using the MedCalc statistical software (version 10.1.2.0). Individual cytokine concentrations were compared in the groups using Student's *T* test with two-tailed probability, and a difference of $P < 0.05$ was considered to be significant. Test concordance was assessed using the kappa (κ) statistic. The

correlation between cytokines and the QFTs/TSTs was assessed using Pearson's two-tailed correlation test. PPV and NPV were calculated using a diagnostic test evaluation (2 × 2 table) (MedCalc 10.1.2.0) with healthy control subjects as the gold standard. GraphPad Prism 5 (version 5.03) was used to generate plots. Bivariate and multivariate logistic regression analyses were performed with SPSS software (version 22.0). A parsimonious regression model was derived using Wald's criterion for variable selection. The resulting model fitness was evaluated by referring to the Hosmer—Lemeshow test. Odds ratios along with 95% confidence intervals were obtained for each factor, and the model fitness was evaluated using a log-likelihood ratio test.

Results

Out of 224 exposed participants, 74 were included in the final analysis. The demographic and clinical characteristics of the study participants are shown in Table 1. Among the 74 participants, there were 40 males and 34 females, with a mean age of 34.43 years (range 12—65 years). Forty-four of the study participants (59%) were in the 18—40 years age group. The BCG vaccination status was confirmed only in 22 (30%) participants. Sixty-one percent of the participants were malnourished. Thirty-eight (51%) of 74 participants had a positive QFT result, whereas 27 (36%) participants had a TST greater than the positive threshold. There was a good strength of agreement between the QFT and TST assays (61/74, 82.43%, $\kappa = 0.651$). Based on the QFT and TST results, participants were categorized into the following four groups, (i) QFT+/TST+ ($n = 26$), (ii) QFT+/TST− ($n = 12$), (iii) QFT−/TST− ($n = 35$) and (iv) QFT−/TST+ ($n = 1$).

Table 1 Demographic and clinical characteristics of population under study.

Characteristics	Level	No. (%)
Age (years)	<18	10 (14)
	18—40	44 (59)
	>40	20 (27)
Gender	Males	40 (54)
	Females	34 (46)
Malnourished	Yes	45 (61)
	No	29 (39)
BCG	Yes	22 (30)
	No	52 (70)
Smoking	Yes	11 (15)
Alcohol consumption	Yes	7 (9)
Tobacco chewing	Yes	16 (22)
QFT Results	Positive (>0.35 U/ml)	38 (51)
	Negative (<0.35 U/ml)	36 (49)
TST results	Positive (>10 mm)	27 (36)
	Negative (<10 mm)	47 (64)

Characteristics of 74 subjects exposed to *M. tuberculosis* and at risk for LTBI are listed in the table. Percentages in different categories are indicated in parentheses.

The comparison of the cytokine levels in different study groups

Cytokine levels, in addition to IFN-γ, were estimated in the whole blood of study participants after stimulation with TB-specific antigens. The concentration of each cytokine was determined using the IMMULITE-1000 Immunoassay System. The levels of cytokines in different groups stratified by the QFT and TST results are represented as box plots in Fig. 2. Among the pro-inflammatory cytokines, significantly higher levels of IL-6 were observed in the QFT+/TST+ group (median: 507.6 pg/ml) compared to the QFT−/TST− group (median: 230.9 pg/ml) ($P = 0.0015$). However, no significant difference was observed in the levels of TNF-α and IL-2R between different groups. In contrast, significantly higher levels of anti-inflammatory cytokine IL-10 were found in the QFT−/TST− group (median: 28.1 pg/ml) compared to the QFT+/TST+ group (median: 10.1 pg/ml) ($P = 0.0013$). Cytokine estimation was also performed in the antigen-stimulated QFT cell-free culture supernatants of healthy control subjects from the same population. In comparison with exposed individuals, the control population had considerably lower cytokine levels. IL-6 levels were significantly higher in the QFT+/TST+ ($P < 0.0001$) group and in QFT−/TST− group ($P = 0.001$) compared to the control group. Similarly, IL-10 was significantly higher in the QFT+/TST+ group ($P = 0.007$) and in the QFT−/TST− group ($P < 0.0001$) compared to the control group. Table 2 presents the mean concentrations of the four cytokines in the antigen-stimulated QFT cell-free culture supernatants of exposed and healthy control subjects.

The correlation between cytokine expression and the magnitude of the QFT/TST responses

To determine the diagnostic specificity of cytokines, we further analyzed the correlation between the magnitude of the QFT and TST responses with the cytokine levels for individual patients. Fig. 3 shows the correlation between the concentrations of cytokines in plasma from latent TB infected individuals and their QFT and TST responses. We observed a positive correlation

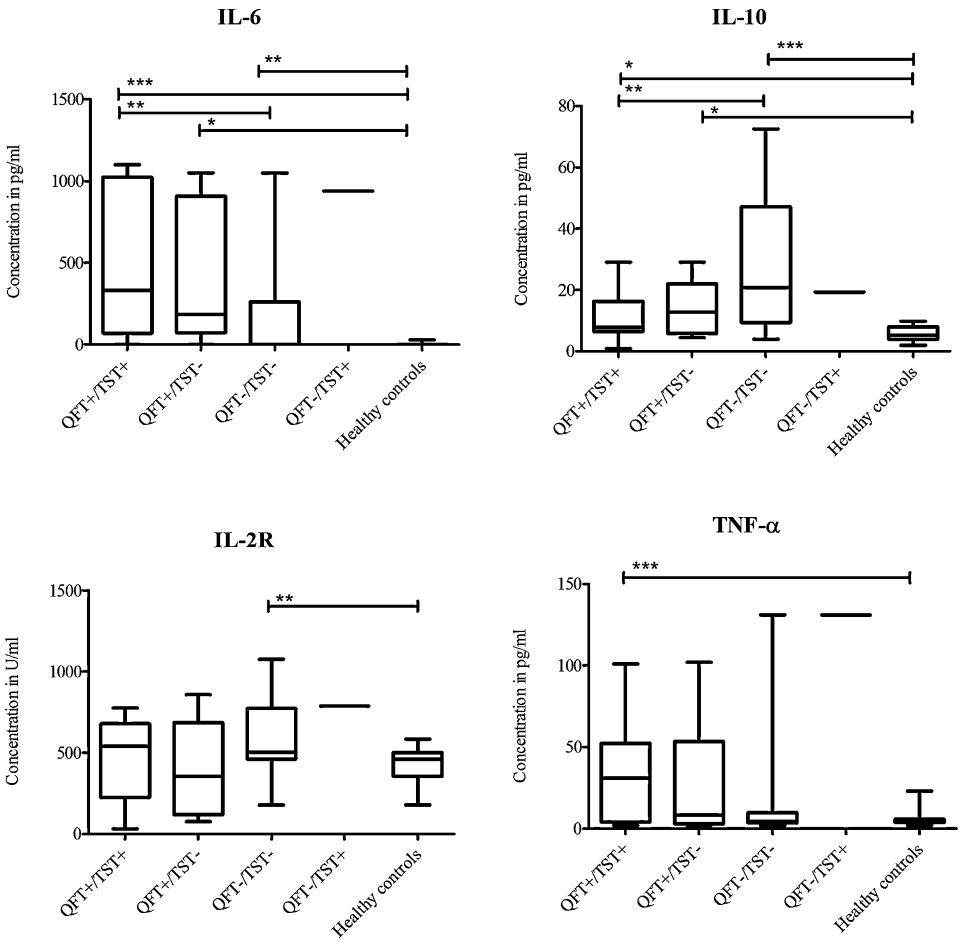

Figure 2 Responses of four cytokines to specific *M.tb* antigens. The box plots represent the cytokine levels of IL-6, IL-10, IL-2R and TNF-α in the antigen-stimulated QFT cell-free culture supernatants of the different study groups stratified by the QFT and TST results as well as in healthy control subjects. The median concentration of each biomarker is shown in each panel. Horizontal lines indicate a statistically significant difference between groups. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

Table 2 Median concentrations of cytokine production in different study groups.

Cytokine	QFT+/TST+ ($n = 26$)	QFT+/TST− ($n = 12$)	QFT−/TST− ($n = 35$)	QFT−/TST+ ($n = 1$)	Healthy controls ($n = 16$)
IL-6 (pg/ml)	507.6***,#	426*	230.9**,#	939	3.8
IL-10 (pg/ml)	10.9*,†	14*	28.1***,†	19.3	5.8
IL-2R (U/ml)	463.4	413.8	593.1**	788	424.5
TNF-α (pg/ml)	31.2***	27.6	12.5	131	5.74

Cytokine responses to combination of *M.tb* antigens (ESAT-6, CFP-10 and TB7.7) are listed in the table. Cytokine concentrations were estimated in antigen stimulated QFT cell-free culture supernatants by IMMULITE-1000 Immunoassay system. Statistical significance was determined by Student's *T*-test. All experimental groups were compared against healthy control group. *P* values indicated as $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$ were considered statistically significant.

[#] $P = 0.0015$ for QFT+/TST+ vs QFT−/TST− group.

[†] $P = 0.0013$ for QFT+/TST+ vs QFT−/TST− group.

between the expression levels of IL-6 ($r = 0.6723$, $P < 0.0001$) and the QFT response, while we observed a negative correlation between QFT and IL-10 expression ($r = -0.3271$, $P = 0.0044$) (Fig. 3a and b). Regarding the TST response, we observed similar results wherein the expression of IL-6 was positively correlated with the TST levels ($r = 0.6631$, $P < 0.0001$). On the other hand, a negative correlation was observed between the TST and IL-10 expression ($r = -0.5698$, $P < 0.0001$)

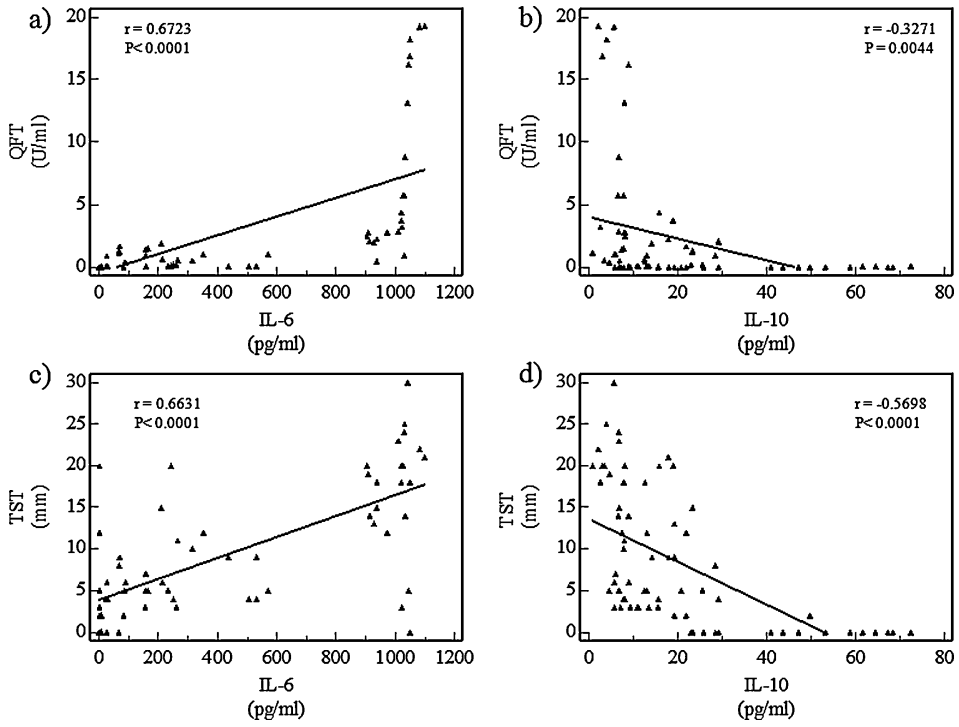

Figure 3 The correlation analysis between the QFT/TST results and cytokine expression: regression graphs show the correlation of the magnitude of the QFT/TST levels and the expression of different cytokines in the QFT supernatants of 74 exposed individuals from Melghat.

(Fig. 3c and d) Regression analysis between TNF-α, IL-2R expression and the QFT/TST responses did not identify a statistically significant correlation (Fig. S2).

Improved diagnostic performance by a combination of biomarkers

The participants with cytokine levels greater than the normal detection limit (5.9 pg/ml for IL-6, 9.1 pg/ml for IL-10, 710 U/ml for IL-2R and 8.1 pg/ml for TNF-α) were regarded as positive, and those below the detection limit were otherwise regarded as negative (Table 3). Of the panel of cytokines, IL-6 and IL-10 had the highest positivity rate. Fifty-two out of 74 (70%) participants were positive for IL-6, and 45 out of 74 (61%) were positive for IL-10. IL-2R and TNF-α had positivity rates of 23 and 43%, respectively; however, they were less significant than the QFT positivity rate of 51% and the TST positivity rate of 36%. We further assessed the diagnostic performance of all of the cytokines when combined with the QFT and the TST. In the QFT+/TST+ group, IL-6 had a 96% positivity rate. IL-6 had the highest positivity rate (91%) in the QFT+/TST− group as well. In the QFT−/TST− group, we observed a maximum positivity rate for IL-10 (80%), followed by a 43% positivity rate for IL-6.

Predictive values of cytokines in the detection of LTBI

To further evaluate the diagnostic utility of cytokines for LTBI, the positive and negative predictive values (PPV and NPV) were calculated for the individual cytokines in each group (Table 4). IL-6 had the highest positive and negative predictive values in the QFT+/TST+ group (92.59 and 93.33%, respectively). IL-10, on the other hand, had the highest positive and negative predictive values in the QFT−/TST− group (96.55 and 91.18%, respectively). TNF-α also had a significant PPV in the QFT+/TST+ group (88.89%). However, its negative predictive value was found to be considerably low compared to that of IL-6 and IL-10.

The association between demographic characteristics and cytokine responses

The association between cytokine positivity and the QFT/TST results was studied through bivariate analysis. Cytokine positivity was the outcome, and the concordant/discordant sets were treated as predictors (the QFT−/TST+ group was excluded from the

Table 3 Detection rates of individual cytokines and cytokines in combination with QFT and TST.

Cytokines	Normal Detection Limit	Total (%) ($n=74$)	QFT+/TST+ ($n=26$)	QFT+/TST− ($n=12$)	QFT−/TST− ($n=35$)	QFT−/TST+ ($n=1$)
IL-6 +ve	>5.9 pg/ml	52 (70)	25	11	15	1
IL-6 −ve	<5.9 pg/ml	22 (30)	1	1	20	0
IL-10 +ve	>9.1 pg/ml	45 (61)	9	7	28	1
IL-10 −ve	<9.1 pg/ml	29 (39)	17	5	7	0
IL-2R +ve	>710 U/ml	17 (23)	4	2	10	1
IL-2R −ve	<710 U/ml	57 (77)	22	10	25	0
TNF-α +ve	>8.1 pg/ml	32 (43)	16	6	9	1
TNF-α −ve	<8.1 pg/ml	42 (57)	10	6	26	0

Responders with cytokine level greater than the normal detection limit were regarded as positives and those below detection limit were regarded as negatives. Table shows the number of positive responders in each study group.

Table 4 Positive and negative predictive values of cytokines for LTBI in different groups.

Cytokines	Positive predictive value				Negative predictive value			
	QFT+/TST+	QFT+/TST−	QFT−/TST−	QFT−/TST+	QFT+/TST+	QFT+/TST−	QFT−/TST−	QFT−/TST+
IL-6	92.59%	84.62%	88.24%	33.33%	93.33%	93.33%	41.18%	100.00%
IL-10	85.00%	87.50%	96.55%	50.00%	46.88%	75.00%	91.18%	100.00%
IL-2R	57.14%	40.00%	76.92%	25.00%	37.14%	56.52%	34.21%	100.00%
TNF-α	88.89%	75.00%	81.25%	33.33%	58.33%	70.00%	35.00%	100.00%

Table shows the PPV and NPV of cytokines in those with LTBI categorized into different concordant/discordant sets. The predictive values were calculated using Diagnostic test evaluation (2 × 2 table) (MedCalc 10.1.2.0) with healthy control subjects as gold standard.

model because of the lack of a substantial number of individuals in the group). Negative concordance (QFT−/TST−) was considered to be the reference level. The association of risk factors (baseline characteristics) and the QFT/TST results with cytokine positivity was studied through multinomial multivariable logistic regression obtained in terms of the adjusted odds ratio, as shown in Table 5. The results indicate that the QFT/TST groups were significantly associated with cytokine positivity. For IL-6, the concordant and discordant groups (QFT+/TST+, and QFT+/TST−) had a significantly higher odds ratio of 33.33 (95% CI: 4.05—274.36) and 14.67 (95% CI: 1.7—126.39), respectively, compared to the QFT−/TST− group. Similar results were seen for TNF-α wherein the odds ratio was highest for the QFT+/TST+ group (4.62, 95% CI: 1.54—13.81). However, for IL-10 and IL-2R, there was a decrease in the odds ratio in the discordant and concordant groups compared to the reference group. The inclusion of demographic characteristics (gender, malnutrition, BCG status, smoking, alcohol consumption and tobacco chewing) in the multinomial multivariate regression model did not demonstrate a significant deviation in the odds ratios, with the exception of TNF-α, in which the adjusted odds ratio had a four-fold increase.

Discussion

Currently, IFN-γ is the only soluble immunological marker dedicated to LTBI screening. Because of the limitations associated with TST, several reports suggest the superior utility of the QFT over the TST for the diagnosis of LTBI [18]. However, the QFT results also exhibit dynamic variability. A single IGRA test may therefore fail to predict infection progression; however, a combination of IGRA tests with other complementary markers may provide a more accurate diagnosis or prediction of disease [19]. Moreover, in settings where TB infection is associated with other risk factors, such as malnutrition, additional biomarkers along with the existing tests for LTBI need to be evaluated for accurate diagnosis and treatment to be initiated. The tribes of Melghat are among one such population with a high prevalence of both LTBI and malnutrition. Due to the limitations associated with both of the existing diagnostic tests for LTBI, we evaluated a panel of cytokines in antigen-stimulated QFT cell-free culture supernatants for the LTBI diagnostic accuracy in a tribal population.

Several reports have established that malnutrition is a complex syndrome involving the dysfunction of the whole spectrum of immune

Table 5 Correlation between demographic characteristics and cytokine response in concordant/discordant sets obtained through bivariate and multivariable logistic regression analysis.

QFT/TST result	IL-6			IL-10		
	Positive/ total (%)	Unadjusted OR [95% CI]	Adjusted OR [95% CI][a]	Positive/ total (%)	Unadjusted OR [95% CI]	Adjusted OR [95% CI][a]
QFT−/TST−	15/35 (43%)	1.00	1.0	28/35 (80%)	1.00	1.0
QFT+/TST−	11/12 (92%)	14.67 (1.7–126.39)	19. 65 (1.596–241.796)	7/12 (58%)	0.35 (0.08–1.44)	0.29 (0.05–1.52)
QFT+/TST+	25/26 (96%)	33.33 (4.05–274.36)	33.29 (3.31–334.37)	9/26 (35%)	0.13 (0.41–0.42)	0.09 (0.02–0.38)
QFT/TST result	IL-2R			TNF-α		
	Positive/ total (%)	Unadjusted OR [95% CI]	Adjusted OR [95% CI][a]	Positive/ total (%)	Unadjusted OR [95% CI]	Adjusted OR [95% CI][a]
QFT−/TST−	10/35 (29%)	1.00	1.0	9/35 (26%)	1.00	1.0
QFT+/TST−	2/12 (17%)	0.50 (0.09–2.69)	0.53 (0.07–3.91)	6/12 (50%)	2.88 (0.74–11.27)	9.16 (1.33–62.89)
QFT+/TST+	4/26 (15%)	0.45 (0.12–1.65)	0.69 (0.16–2.97)	16/26 (62%)	4.62 (1.54–13.81)	15.04 (2.93–77.26)

The table represents the association of QFT/TST results with the cytokine response through bivariate analysis shown as unadjusted OR and after inclusion of demographic characteristics (Age, Gender, Malnutrition, BCG, Smoking, Alcohol consumption and Tobacco chewing) into multivariable regression model as adjusted OR.

[a] Adjusted for, Gender, Malnutrition, BCG, Smoking, Alcohol consumption and Tobacco chewing.

responses, thus increasing susceptibility to infection and disease. Apart from malnutrition, the type of exposure to an active TB case also influences the risk of latent infection. Household exposure due to prolonged physical contact or exposure within the community due to religious gatherings, cultural practices and recreational activities significantly affects the risk of TB exposure and the subsequent immune response against it [20].

To evaluate the diagnostic utility of cytokines, QFT and TST analyses were carried out, followed by cytokine evaluations of the antigen-stimulated QFT cell-free culture supernatants of all of the participants. The study population was then stratified on the basis of the QFT and TST results into concordant and discordant sets, and the results were then correlated with the selected panel of cytokines (TNF-α, IL-6, IL-10 and IL-2R). It was observed that in the QFT+/TST+ group, 25 of 26 (96%) subjects had a positive IL-6 response, suggesting a good correlation between the QFT/TST results and IL-6. The patterns of cytokine responses in the different groups also indicate that IL-6 is secreted in significantly higher concentrations in the QFT+/TST+ group compared to the QFT−/TST− group. Like IFN-γ, IL-6 is involved in the T helper type 1 (Th1) cellular immune response, which is involved in the clearance of *M.tb* [21]. IL-6 has multiple roles, including inflammation, hematopoiesis and the differentiation of T cells [22]. The anti-inflammatory cytokine IL-10, in contrast, had suppressed levels in 17 of the 26 (65%) subjects in the QFT+/TST+ group. On the other hand, the highest levels of this cytokine were observed in the QFT−/TST− group. IL-10 is involved in the Th2 response and exerts a negative influence on the immune response. IL-10 secreted by macrophages and T cells during infection and possesses macrophage deactivating properties including the down regulation of IFN-γ production [11,23]. Our results are consistent with recent findings suggesting that control of TB in the latent stage is not only associated with an increased expression of Th1 cytokines but also with the suppression of Th2 cytokines [10,24]. The balance between the two types of cytokines (pro- and anti-inflammatory) is reflected in the resultant host resistance against infection [25].

TNF-α mediates the early inflammatory response against pathogens and is produced by a variety of cells, including macrophages, lymphocytes, neutrophils, mast cells, and endothelial cells. It is considered to be necessary for the removal of bacteria from inflammatory lesions [26,27]. Consistent with earlier reports, we did find higher levels of TNF-α in the QFT+/TST+ group compared to the

other study groups, but the observed values were not significant [24].

The QFT−/TST− group with TST indurations of <10 mm and QFT values <0.35 U/ml (generally observed in healthy controls) had a 43% positivity rate for IL-6 and an 80% positivity rate for IL-10, suggesting that in such cases, QFT is unable to detect infection, although effector T cells directed against TB antigens are present in these exposed subjects. This group is of interest because in the absence of any evidence of LTBI, we observed a positive cytokine response.

Although the QFT−/TST+ group was not included in the analysis due to the lack of sufficient data, the reason for the high TST and cytokine responses in this individual requires further consideration. One of the reasons for the observed discordance could be due to a false negative result caused by malnutrition-induced antigen-specific immunosuppression. An alternative explanation may be a false positive result due to nontuberculous mycobacteria or BCG vaccination. However, the fact that the individual from this category had up-regulated cytokine expression in response to antigen stimulation suggests that the individual was indeed infected with TB at some point in the past. Such discordance in the QFT and TST results has been previously reported [28].

Because the TST and IGRAs are the only diagnostics tools for LTBI, other cytokines that have expressions that are correlated with these test outcomes may be useful in the characterization of *M.tb* infection. We therefore evaluated the correlation between the magnitude of the QFT and TST response and the magnitude of the responses of our panel of cytokines. Correlation analysis suggested that the individuals with the largest TST results and the highest QFT values were the ones with the highest IL-6 and lowest IL-10 concentrations, indicating that these cytokines are important predictive markers of LTBI.

Furthermore, an optimal diagnostic tool should have the ability to discriminate between the target condition and health. This discriminative potential was quantified by evaluating the predictive values of each cytokine. Based on the results, IL-6 and IL-10 had the highest positive predictive values (PPV) (92.59 and 96.55%, respectively), indicating the high probability of having an infection in the subjects with positive results. Similarly, the high negative predictive values (NPV) of IL-6 and IL-10 (93.33 and 91.18%, respectively) signified the probability of not having an infection in the individuals with negative test results. These values thus demonstrated the high accuracy and specificity of the two cytokines in detecting the presence of infection in the exposed population.

The impact of demographic characteristics (age, gender, malnutrition, BCG vaccination status, smoking, alcohol consumption and tobacco chewing) on cytokine positivity was studied through multivariate regression analysis to determine if these factors influence the test outcomes. In our previous study, age was regarded as a strong predictor for QFT positivity [29] (QFT positivity was highest in >40 age group). Similarly, there are reports on the association between smoking and LTBI [30]. Such potential confounders or risk factors might therefore weigh upon the specificity of the test and influence the result. In the present study, however, we found no such impact of demographic characteristics on the test outcomes, indicating that these factors were not significant predictors of cytokine positivity and thereby validated the diagnostic specificity of the cytokines.

The panel of cytokines was also evaluated in healthy control individuals from the same population; however, in contrast, all cytokines were found to be within the normal detection limits when compared with the TB-exposed individuals.

The results of the present study have demonstrated that individuals without a history of tuberculosis and exposure to *M.tb* had a Th1 (IFN-γ, TNF-α and IL-6) and Th2 response similar to that found by Demissie, with relatively low IFN-γ production when compared to IL-10 [31,32]. There are few published reports in the literature on the production of IL-10 by household contacts of patients of tuberculosis. According to Olobo et al., these levels are due to the stimulation of mycobacterial antigens that induce IL-10 production by mononuclear cells [33]. IL-10 is probably involved in the natural defense against excessive pro-inflammatory responses generated by IL-6 and TNF-α [34]. Therefore, the simultaneous presence of IL-10 and IL-6 may provide protection to the household contacts of TB patients. Recently, John et al. also identified IL-6 and IL-10 as clinically relevant biomarkers that are required for the improved diagnosis, prevention and treatment of tuberculosis [35].

There are few reports on the evaluation of cytokines as biomarkers for *M.tb* infection in the Indian population. Dhanasekaran et al. evaluated the response of multiple cytokines for the diagnosis of LTBI in an exposed South Indian population [36]. Anbarasu et al. reported IL-6 to be a promising diagnostic marker in the Indian population [37]. In the present study, we report IL-10 to be a useful diagnostic biomarker of LTBI in the Indian population.

Although our results suggest important roles of IL-6 and IL-10 in LTBI diagnosis, further evaluation of these findings in a larger number of exposed individuals along with proper follow-up studies are needed. Such studies may further help us to establish the roles of both IL-6 and IL-10 in the prognosis of TB disease following therapeutic measures. Furthermore, an evaluation of additional T cell-specific cytokines in QFT supernatants may be helpful for improving and validating the existing diagnostic tests.

Conclusions

In conclusion, the measurement of multiple cytokines in response to TB infection can be useful for LTBI diagnosis in the tribal population of Melghat. Among the panel of cytokines, IL-6 and IL-10 appear to be the most important diagnostic markers of *M.tb* infection. An evaluation of such cytokines, in addition to IFN-γ, could markedly improve the performance of IGRAs in the effective screening of individuals with LTBI in the TB-exposed tribal population. Our observations require further investigation in larger well-characterized cohorts, in addition to follow-up, to further confirm the study outcome.

Authors' contributions

PRB, AAH and RSK acquired, analyzed and interpreted the data. HFD, GMT and RSK conceptualized and designed the study and critically revised the manuscript for important intellectual content. NPA, MSP and ARS contributed to the acquisition and analysis of the data. All of the authors approved the final version of the article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2015.02.003.

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