

Discrimination between Active and Latent Tuberculosis Based on Ratio of Antigen-Specific to Mitogen-Induced IP-10 Production

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Mycobacterium tuberculosis is the major causative agent of tuberculosis (TB). The gamma interferon (IFN- γ) release assay (IGRA) has been widely used to diagnose TB by testing cell-mediated immune responses but has no capacity for distinguishing between active TB and latent TB infection (LTBI). This study aims to identify a parameter that will help to discriminate active TB and LTBI. Whole-blood samples from 33 active TB patients, 20 individuals with LTBI, and 26 non-TB controls were applied to the commercial IFN- γ release assay, QuantiFERON-TB Gold In-Tube, and plasma samples were analyzed for interleukin-2 (IL-2), IL-6, IL-8, IL-10, IL-13, tumor necrosis factor-alpha (TNF- α), IFN- γ , monokine induced by IFN- γ (MIG), interferon gamma inducible protein 10 (IP-10), interferon-inducible T cell alpha chemoattractant (I-TAC), and monocyte chemoattractant protein 1 (MCP-1) by using a commercial cytometric bead array. The *Mycobacterium tuberculosis* antigen-specific production of most of the assayed cytokines and chemokines was higher in the active TB than in the LTBI group. The mitogen-induced responses were lower in the active TB than in the LTBI group. When the ratio of TB-specific to mitogen-induced responses was calculated, IL-2, IL-6, IL-10, IL-13, TNF- α , IFN- γ , MIG, and IP-10 were more useful in discriminating active TB from LTBI. In particular, most patients showed higher IP-10 production to *Mycobacterium tuberculosis* antigens than to mitogen at the individual level, and the ratio for IP-10 was the strongest indicator of active infection versus LTBI with 93.9% sensitivity and 90% specificity. In conclusion, the ratio of the TB-specific to the mitogen-induced IP-10 responses showed the most promising accuracy for discriminating active TB versus LTBI and should be further studied to determine whether it can serve as a biomarker that might help clinicians administer appropriate treatments.

Mycobacterium tuberculosis, the major causative agent for tuberculosis (TB), is among the most successful human pathogens, infecting approximately 8.6 million people and leading to 1.3 million deaths each year (1). It is estimated that 2 billion people live with latent TB infection (LTBI) and are therefore a potential source of active TB (2, 3). Identifying LTBI is necessary in order to reduce the risk of development of the disease, while diagnosis of active TB can enable rapid treatment and disease control. To this end, diagnostic biomarkers that can accurately indicate disease status are needed (4, 5).

There is presently no diagnostic gold standard for LTBI. Until recently, the tuberculin skin test (TST) involving the intracutaneous injection of purified protein derivative (PPD) into the forearm was the only available method for diagnosing LTBI. However, PPD cross-reacts with nontuberculous mycobacteria as well as with *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine and has poor sensitivity in immunocompromised patients (6). The interferon gamma (IFN- γ) release assay (IGRA) has been widely used in clinical practice and public health policy for TB diagnosis (7). Commercial IGRAs such as the QuantiFERON-TB Gold In-Tube test (QFT-GIT) measure responses to *M. tuberculosis*-specific antigens, including early secretory antigenic target-6 (ESAT-6), culture filtrate protein 10 (CFP-10), and TB7.7 antigens, and discriminate between a *M. tuberculosis* infection and an immunity-induced response to BCG vaccination (8, 9). The IGRA therefore seems to be useful for the diagnosis of TB in individuals who have been recently vaccinated with BCG and those who are

immunocompromised. However, IGRAs have only shown mediocre results in the latter group and are also unable to discriminate between active TB and LTBI (10).

In addition to IFN- γ , many cytokines and chemokines have been investigated as potential biomarkers for *M. tuberculosis* infection and disease status (11–18). The levels of several cytokines, including interleukin-6 (IL-6), IL-10, IL-15, chemokine (C-X-C) motif ligand (CXCL)/interferon gamma-inducible protein 10 (IP-10), and monocyte chemoattractant protein 2 (MCP-2), were significantly higher in TB patients than in healthy controls (7, 11, 18–21); although these findings suggest important roles for these factors in disease pathogenesis, they are not sufficient for diagnosing

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active as opposed to latent infections. Several studies have also suggested that biomarker combinations such as IFN- γ –tumor necrosis factor alpha (TNF- α), IFN- γ –IL-2, IFN- γ –IL-4, and IL-15–MCP-1 might be more sensitive than single markers (18, 22–24). However, a better biomarker to improve the sensitivity and specificity in discriminating between active TB and LTBI is still needed.

The current study was designed to develop a new biomarker for the diagnosis of different stages of *M. tuberculosis* infection. Participants were recruited following approval of the protocol by the ethics review committee. Interestingly, during our study, we found that the method for calculating the ratio of IP-10 levels in response to TB antigens and mitogen might be more sensitive in discriminating patients with active TB from individuals with LTBI or healthy controls without TB than that for measuring the concentrations of TB antigen-induced IP-10 or any other cytokines and chemokines that we have tested in this study.

MATERIALS AND METHODS

Study population. Participants were recruited from November 2010 to October 2012 following approval of the protocol by the Severance Hospital Ethics Review Committee (institutional review board [IRB] no. 4-2010-0213). All study subjects gave informed consent for the use of the samples obtained. The diagnosis of active pulmonary TB was based on all clinical, radiological, microbiological, and pathological results. Active TB was confirmed by culture of *M. tuberculosis* from respiratory specimens or by the presence of caseating granulomas in lung tissue. Patients with lymphocyte-predominant exudative effusions and adenosine deaminase levels of >40 IU/liter or those with a high likelihood of active TB based on clinical and radiological results were included if their conditions improved after antituberculosis treatment. In detail, among a total of 33 active TB patients, 25 patients were diagnosed as having active TB based on positive cultures of *M. tuberculosis* and 2 patients were diagnosed based on pathological results, which showed chronic granulomatous inflammation and positive PCRs. The remaining 6 patients were diagnosed based on the clinical and the radiological information. Since their chest computed tomography scans showed typical centrilobular nodules on their upper lungs, which resolved after a full-course of anti-TB treatment, they were finally classified as having active TB. The LTBI group consisted of household members with positive TST (>10 mm), who had been in close contact and lived with a patient with microbiologically confirmed active pulmonary TB for longer than 1 month (25). None of the household contacts showed the clinical symptoms or chest radiographic findings indicative of TB. The non-TB control group consisted of healthy adults with a negative TST, who were free of TB symptoms and did not have any contact with active pulmonary TB patients. All control subjects had normal chest X-ray results.

TST. Skin tests were performed using RT-23 tuberculin (Statens Serum Institute, Copenhagen, Denmark). The induration was measured 48 to 72 h later, and the cutoff for a positive result in immunocompetent subjects was 10 mm, according to the Korean TB guideline (25).

IFN- γ determination by QFT-GIT assay. The IFN- γ release assay was performed using the QFT-GIT assay kit. Briefly, 1 ml whole blood was drawn into the three QFT-GIT tubes precoated with saline (control), *M. tuberculosis*-specific antigens (ESAT-6, CFP-10, and TB7.7), or mitogen and incubated for 20 h at 37°C. After centrifugation, the supernatant was collected and stored frozen at –20°C until the IFN- γ concentration was determined by an enzyme-linked immunosorbent assay (ELISA) using a QFT Gold kit. The results were calculated using the manufacturer's QFT-GIT software. With the QFT Gold kits, we used 7 kinds of tubes with different lot numbers (80181, 80221, 80231, 80261, 80321, 80401, and 80451).

Multiplex analysis of cytokine production. Levels of IL-2, IL-6, IL-8, IL-10, IL-13, TNF- α , IFN- γ , monokine induced by IFN- γ (MIG), IP-10,

TABLE 1 Characteristics of the study subjects for the multicytokine assay

Characteristic ^a	Result for ^b :		
	Controls (n = 26)	LTBI subjects (n = 20)	Active TB patients (n = 33)
Age (median [range]) (yrs)	25 (22–54)	44 (22–60)	30 (20–63)
Sex (male/female)	12/14	4/16	19/12
Presence of a BCG scar (n [%])	14 (53.8)	18 (90)	21 (63.6)
Positive TST (n [%])		20 (100)	3 (75) (29 ND ^c)
Pulmonary TB diagnosis			
Positive AFB culture (n [%])			25 (75.8)
Positive QFT-GIT result (n [%])	0 (0)	11 (55)	33 (100)

^a AFB, acid-fast bacilli; BCG, bacille Calmette-Guérin; QFT-GIT, QuantIFERON-TB Gold In-Tube test; TST, tuberculin skin test.

^b LTBI, latent tuberculosis infection; TB, tuberculosis.

^c ND, not determined.

I-TAG, and MCP-1 were measured in the supernatants described above with commercial cytometric bead array human cytokine and chemokine flex sets (BD Biosciences, San Jose, CA, USA) according to the manufacturer's recommendations.

Data analysis. Data were analyzed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA, USA). Variables that were not normally distributed were compared across and within groups using the nonparametric Mann-Whitney test. Antigen-dependent and mitogen-induced biomarker production values were measured by subtracting the concentration measured in the control tube from those measured in the antigen and mitogen tubes, respectively. The diagnostic performances of the antigen-dependent and mitogen-induced biomarker values were compared with a receiver operating characteristic (ROC) curve analysis to determine the area under the curve (AUC) and the optimal cutoff levels.

RESULTS

Characteristics of the study population. The study included 33 patients with active pulmonary TB, 20 subjects with LTBI, and 26 non-TB controls (Table 1). The LTBI group was slightly older than the other groups: median ages were 44 years (range, 22–60 years) for the LTBI group, 30 years (range, 20–63 years) for the active TB group, and 25 years (range, 22–54 years) for the non-TB control group. The male to female ratios were 4/16, 19/12, and 12/14, respectively, and BCG scars were seen in 18 (90%), 21 (63.6%), and 14 (53.8%) of the subjects, respectively. For the QFT-GIT assay, results for all of the non-TB controls were negative; positive results were observed in 11 (55%) subjects with LTBI and all 33 (100%) patients with active TB. We defined the LTBI group as TST-positive individuals who had been in close contact and lived with patients with microbiologically confirmed active pulmonary TB for longer than 1 month, according to the Korean TB guidelines (25).

Comparison of cytokine and chemokine levels in response to TB antigens or mitogen. To identify new biomarkers for detecting active TB and discriminating LTBI, the levels of 11 cytokines and chemokines were evaluated (Fig. 1). The baseline levels of all cytokines, as observed in unstimulated whole-blood samples from each group, were subtracted from the corresponding levels in TB antigen- and mitogen-stimulated samples.

The median levels of IL-2, IL-6, IL-13, MIG, IP-10, I-TAG, MCP-1, and IL-8 were higher in active TB patients than in non-TB

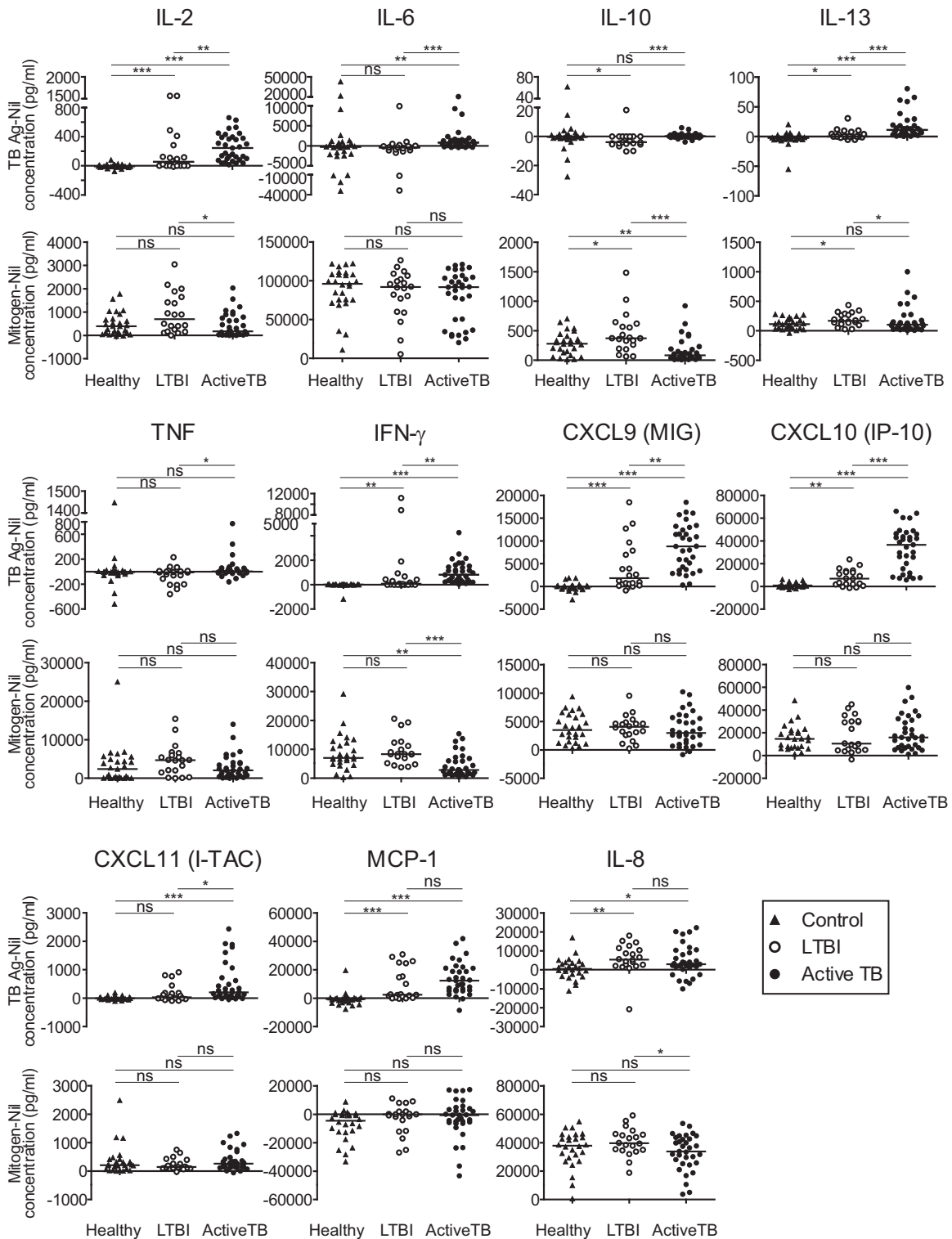


FIG 1 Cytokine levels in active TB and LTBI. Whole blood was stimulated with *M. tuberculosis*-specific antigens (ESAT-6, CFP-10, and TB7.7) or with mitogen for 24 h, and the levels of TB-specific and mitogen-induced cytokines and chemokines were measured in active TB patients, LTBI subjects, and non-TB healthy control subjects. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

controls, in accordance with IFN- γ responses measured by the QFT-GIT assay. In addition, IL-2, IL-6, IL-10, IL-13, TNF- α , MIG, IP-10, and I-TAG levels were higher in active TB patients than in LTBI subjects, which was also consistent with the IFN- γ

response. The levels of these factors differed significantly between samples from non-TB controls and active TB patients and between samples from active TB patients and LTBI subjects, implying that they are potentially useful biomarkers.

Ratio of TB-specific to mitogen-induced responses. While analyzing the responses after stimulation of whole-blood samples with TB antigens or mitogen, we found that the levels of IL-2, IL-10, IL-13, IL-8, and IFN- γ in response to mitogen were relatively lower in active TB patients than in LTBI individuals (Fig. 1). Even though this phenomenon had been previously observed in some studies (13, 26), it has not been seriously considered. Based on our finding, the ratio of TB antigen-specific to mitogen-induced nonspecific responses was therefore calculated in individual samples (Fig. 2A). Interestingly, the ratios for IL-2, IL-6, IL-13, TNF- α , MIG, IP-10, I-TAG, IL-8, and IFN- γ were markedly higher in active TB patients than in non-TB controls. In addition, the ratios for IL-2, IL-6, IL-10, IL-13, TNF- α , MIG, IP-10, and IFN- γ were higher in active TB patients than in LTBI individuals. The diagnostic performances of the biomarkers were evaluated using ROC curves. The AUC is widely recognized as a measure of the discriminatory ability of a diagnostic test; in general, ROC curves with an AUC of 0.8 are considered to have good diagnostic ability. The biomarkers with potential (AUC values of >0.8) are summarized in Table 2 and Fig. 3. ROC curves corresponding to the TB-specific responses of IL-13, IFN- γ , and IP-10 revealed AUCs of 0.8409, 0.75, and 0.8848, respectively (Table 2), which were statistically significant. However, the ratios of TB-specific to mitogen-induced responses were associated with higher AUC values (0.8909, 0.8318, and 0.9242, respectively) that were also statistically significant compared to those of the TB-specific responses (Table 2). Importantly, the AUC for the IP-10 ratio displayed the highest value among the AUCs for the tested parameters, including the TB-specific IP-10 response, indicating that the IP-10 ratio is the most valuable diagnostic marker to discriminate active TB versus LTBI. This tendency was still observed even though we used tubes with different lot numbers, indicating that our finding is not due to lot-to-lot differences (data not shown).

The TB-specific IP-10 levels were higher in active TB patients than in LTBI subjects, whereas no differences were observed for mitogen-induced levels between the two groups. The TB-specific and mitogen-induced IP-10 levels were compared for each individual to investigate the reason that the IP-10 ratio showed a greater difference than the IP-10 level for TB antigen in discriminating active TB versus LTBI (Fig. 2B). Interestingly, most of the active TB patients showed a higher response to TB antigen stimulation but few to mitogen induction. However, healthy controls and LTBI subjects had lower TB-specific than mitogen-induced IP-10 levels, accounting for the significant difference in the IL-10 ratios between the groups.

The optimal cutoff values for the discrimination between active TB versus LTBI were determined by ROC curve analysis and are summarized in Table 3. The cutoffs for the median values of TB-specific IL-13, IFN- γ , and IP-10 were 0, 172.84, and 23780.88, respectively; these were able to discriminate 100%, 93.4%, and 69.7% of the true active TB patients (sensitivity) and 60%, 60%, and 100% of the true LTBI subjects (specificity), respectively. Cutoffs for the ratios of TB-specific to nonspecific responses were 0.0363, 0.0454, and 0.7906, respectively, and were able to discriminate 78.79%, 87.88%, and 93.34% of the true active TB cases and 85%, 75%, and 90% of the true LTBI cases, respectively. Unexpectedly, the IP-10 cutoff value of 0.7906 was able to distinguish between active TB and LTBI with 93.4% sensitivity and 90% specificity (Fig. 2C). Based on this optimal cutoff value, 31 of 33 active TB patients were positively identified, but none of the non-TB

healthy controls and only 2 of 20 LTBI subjects were positive. The 2 active TB patients who were identified as negative had lower TB-specific than mitogen-induced IP-10 levels, but no other unusual characteristics.

DISCUSSION

Commercial IGRAs such as the QFT-GIT are widely used to diagnose *M. tuberculosis* infection, but they cannot distinguish active TB disease from LTBI (10, 27). In the current study, several cytokines and chemokines were evaluated in active TB patients, LTBI subjects, and non-TB healthy controls for their discriminatory potential for the enhanced diagnosis of active TB infection compared to that of the conventional QFT-GIT assay. In accordance with previous findings, IL-2, IL-13, MIG, IP-10, and IFN- γ were present at higher levels in active TB patients than in LTBI subjects and non-TB healthy controls. In addition, IL-6 and IL-10, TNF- α , and I-TAG levels were higher in the active TB than in the LTBI group. However, these markers were unable to distinguish between the groups accurately. Moreover, single cytokines may only reflect inflammation; thus, mitogen-induced responses were assumed to reflect the subjects' immune status. When the ratio of the TB-specific to mitogen-induced responses was calculated, ratios for IL-13, IP-10, and IFN- γ were higher than the corresponding values for TB antigen-stimulated responses. In particular, the IP-10 ratio, when the optimal cutoff value was applied, identified 93.9% of the active TB and 90% of the LTBI subjects out of the TB-related subjects.

Several studies tried to identify potential biomarkers for diagnosing active TB and discriminating between active TB and LTBI. Wang et al. reported that the IL-2/IFN- γ ratio discriminated between active TB and LTBI with a sensitivity of 77.2% and a specificity of 87.2% (24). In addition, Frahm et al. reported that the combination of IL-15 and MCP-1 identified active TB and LTBI with a sensitivity of 83% and a specificity of 88% (18). In our study, we found that the IP-10 ratio of TB-specific to mitogen-induced responses displayed better sensitivity and specificity (93.9% and 90%, respectively) in identifying active TB from LTBI than other reported parameters. Therefore, the use of the IP-10 ratio might support an improved diagnosis of active TB disease when combined with the standard diagnostic methods, including microbiology, molecular tests, and clinical and radiological assessments. IP-10 (also known as CXCL10 or IFN- γ -induced protein 10) is a chemokine that is expressed at a high level in inflammatory diseases such as systemic lupus erythematosus, thyroid disease, acute coronary syndrome, and allergies (28–30). IP-10 is expressed in the bronchial epithelium (31) and in inflamed tissues (32) of active TB patients, who demonstrate upregulation of the helper T (Th) 1-type cytokine IFN- γ . IP-10 secretion may play an important role in recruiting activated T cells, which is necessary for protective immunity. In addition, chemokines and their receptors, including IP-10, contribute to the formation and maintenance of granulomas in TB (33). IP-10 plays a role in *M. tuberculosis* infection, and several studies have shown that antigen-stimulated IP-10 responses have a sensitivity similar to that of QFT-GIT for detecting active TB (12, 13, 34, 35). Our results also showed a higher level of IP-10 in active TB patients, consistent with previously published studies; IP-10 can therefore serve as a potential diagnostic biomarker.

Since there is no gold standard for diagnosing LTBI, the biomarker studies to discriminate LTBI from active TB, including

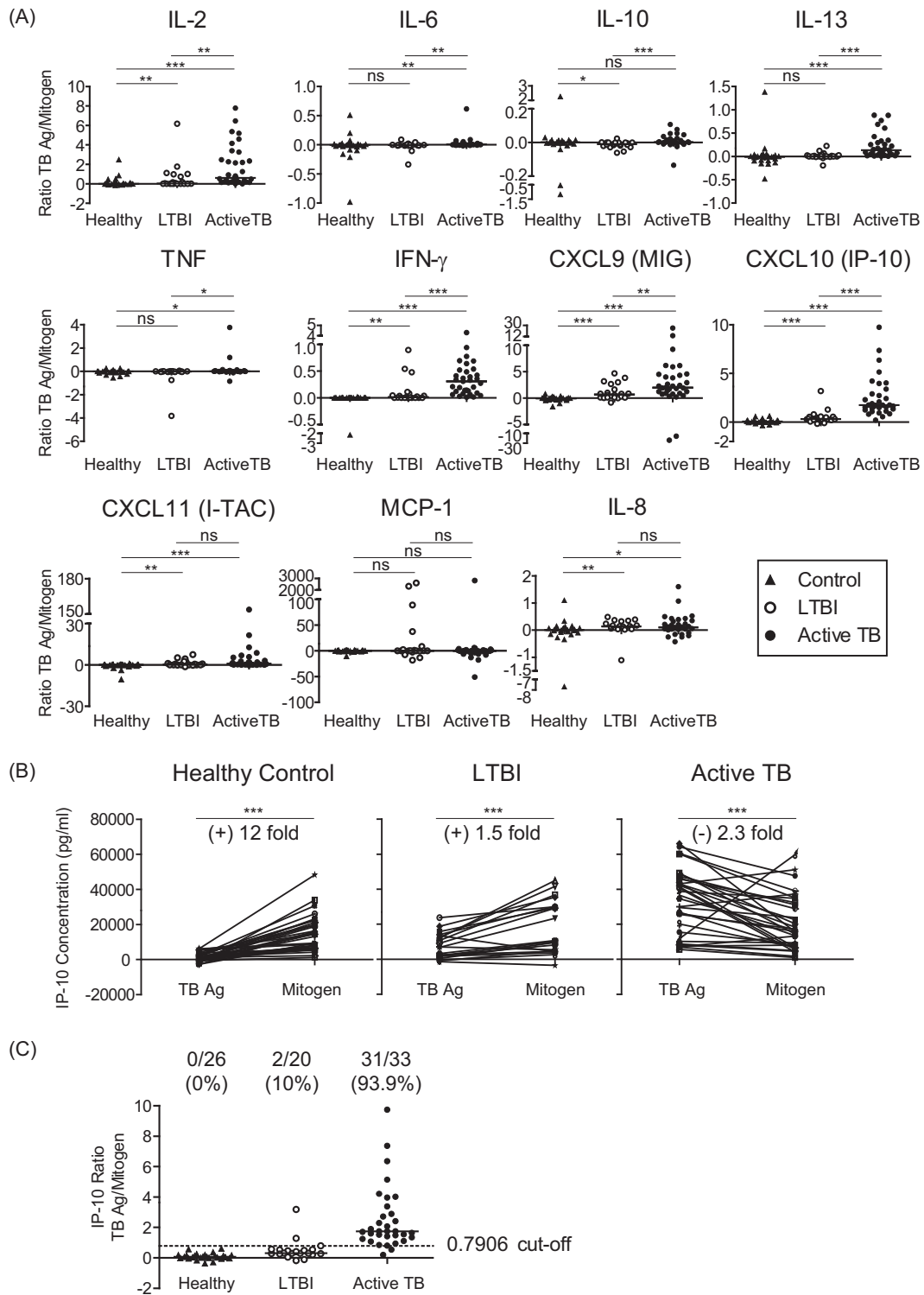


FIG 2 Ratio of TB-specific to mitogen-induced responses. Whole blood was stimulated with *M. tuberculosis*-specific antigens (ESAT-6, CFP-10, and TB7.7) or with mitogen for 24 h, and the levels of TB-specific and mitogen-induced cytokines and chemokines were measured in active TB patients, LTBI subjects, and non-TB healthy control subjects. (A) Ratios of TB-specific to mitogen-induced responses are shown. (B) Comparison of TB-specific and mitogen-induced IP-10 levels. (C) The optimal cutoff value and positive cases are indicated on the graph. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

our study, are often confronted with a difficulty in defining an LTBI group. In the current study, we defined the LTBI group as persons with no sign of active TB, who had close contact with infectious TB patients and positive TST according to the Korean

TB guideline (25). Since 45% of the LTBI individuals defined in our study were QFT negative, it would be worthwhile to compare the responses after dividing the LTBI group into those who were QFT positive and negative. Indeed, we found that the ratio of

TABLE 2 AUC values obtained from the receiver operating characteristic curve analysis

Analytical criterion ^a	Target ^b	AUC ^c	95% CI ^d	P
TB Ag	IL-13	0.8409	0.714–0.927	<0.0001
	IFN- γ	0.7500	0.612–0.859	0.002477
	IP-10	0.8848	0.767–0.956	<0.0001
TB Ag/mitogen ratio	IL-13	0.8909	0.775–0.960	<0.0001
	IFN- γ	0.8318	0.704–0.920	<0.0001
	IP-10	0.9242	0.818–0.979	<0.0001

^a TB, tuberculosis; Ag, antigen,^b IL-13, interleukin-13; IFN- γ , interferon-gamma; IP-10, interferon gamma-induced protein 10/chemokine (C-X-C) motif ligand-10.^c AUC, area under the curve.^d CI, confidence interval.

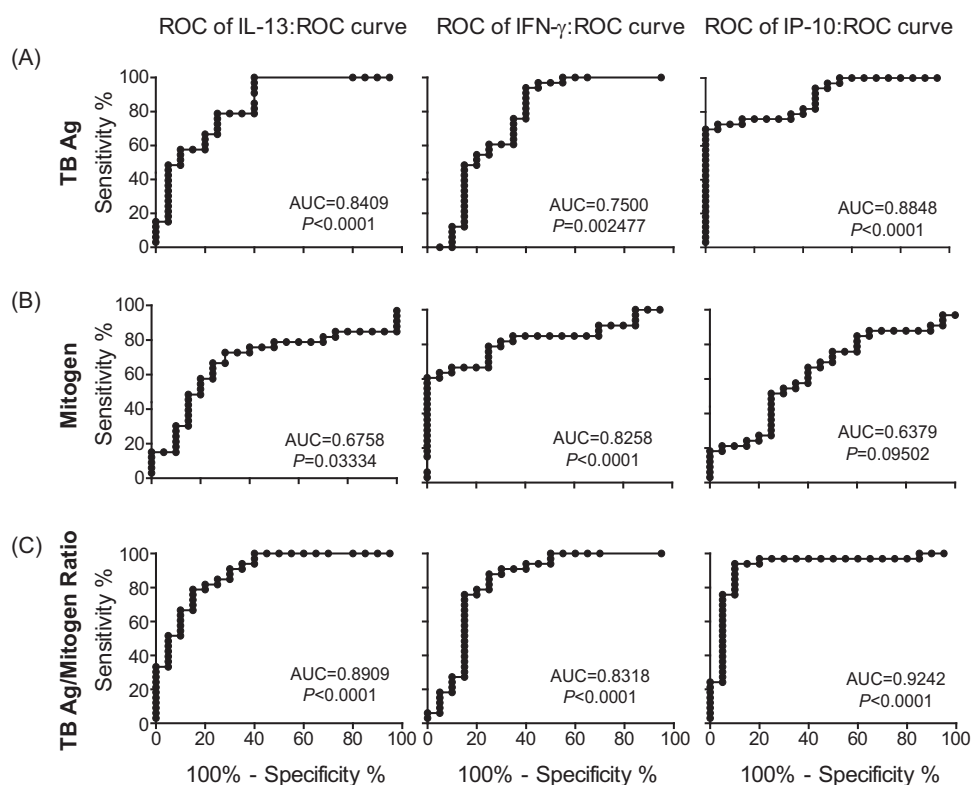
IP-10 in response to TB antigens and mitogen is higher in QFT-positive than in QFT-negative individuals, suggesting that the TB antigen-specific IFN- γ response and IP-10 production have a certain relation (see Fig. S1 in the supplemental material). However, although we restricted the LTBI group to the persons positive for both the TST and QFT, it was still obvious that the ratio of TB antigen-induced and mitogen-induced IP-10 productions is the most reliable parameter to discriminate between the LTBI individuals and active TB patients with high values for both sensitivity and specificity (see Table S1 in the supplemental material). These data indicate that the method using the IP-10 ratio might be applicable to help in the diagnosis for distinguishing active TB from LTBI, irrespective of the QFT response in defining the LTBI group.

TABLE 3 Optimal cutoff values with associated sensitivities and specificities to discriminate between LTBI and active TB

Analytical criterion ^a	Target ^b	Cutoff	Sensitivity (%)	Specificity (%)
TB Ag	IL-13	0	100	60
	IFN- γ	172.84	93.94	60
	IP-10	23,780.88	69.7	100
TB Ag/mitogen ratio	IL-13	0.0363	78.79	85
	IFN- γ	0.0454	87.88	75
	IP-10	0.7906	93.94	90

^a TB, tuberculosis; Ag, antigen,^b IL-13, interleukin-13; IFN- γ , interferon-gamma; IP-10, interferon gamma-induced protein 10/chemokine (C-X-C) motif ligand-10.

A potential diagnostic biomarker that is able to discriminate active TB from LTBI, depending on the IP-10 cutoff value, was identified in this study. This parameter reflects individuals' immune status, thereby increasing the detection rate and improving diagnostic accuracy. Based on the bias in sensitivity and specificity evidenced by the cutoff value, the ratio of TB-specific to mitogen-induced responses might be a reliable diagnostic biomarker for active TB. Although additional studies with a larger sample size are needed to confirm the clinical utility of this ratio, these findings present a promising method, which can discriminate between active TB and LTBI, to support the current diagnostic methods for TB. This potential biomarker might help the detection of patients with real active TB within 2 h with improved sensitivity and specificity, thereby improving the accuracy of diagnosis and advancing the time of therapy.

**FIG 3** ROC curves for TB-specific and mitogen-induced cytokine responses. The AUCs of TB-specific (A) and mitogen-induced (B) IL-13, IFN- γ , and IP-10 levels and the ratios of TB-specific to mitogen-induced responses (C) were compared between active TB patients and LTBI. The AUCs and the P values are indicated on the graphs.

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