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Regulation network of serum cytokines induced by tuberculosis-specific antigens reveals biomarkers for tuberculosis diagnosis

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ABSTRACT. In this study, we identified potential serum biomarkers for the diagnosis of active tuberculosis (TB) and screening for latent TB infections (LTBIs). Peripheral blood samples from 40 healthy individuals, 40 patients with TB, and 40 LTBI individuals were stimulated with the TB-specific antigens ESAT-6 and CFP-10. Human inflammatory cytokine arrays were used to detect the expression of inflammatory cytokines. Cytokines with significant changes were screened to construct a cytokine regulation network. The levels of the cytokines CCL1 (I-309), CXCL9 (MIG), IL-10, IL-6, CSF2, CSF3, IL-8, IL-1α, IL-7, TGF-β1, CCL2, IL-2, IL-13, and TNFα were significantly upregulated in the active TB group. The levels of CCL3, IL-1β, CCL8, IFNγ, and CXCL10 were significantly increased in the TB groups compared to those in the healthy control group. sTNF RII was upregulated in the LTBI group. CCL4 and MIP1d were significantly increased in all groups.The upregulated cytokines were mainly found in the

IFNγ and IL-1α regulatory networks. Importantly, we found that CXCL10 (IP-10), CCL3, CCL8, and IL-1β may be more suitable than IFNγ for active or latent TB infection screening. Furthermore, we found that levels of CCL1 (I-309), CXCL9 (MIG), IL-10, IL-6, CSF2, CSF3, IL-8, IL-1α, IL-7, TGF-β1, CCL2, IL-2, and IL-13 after TB antigen stimulation may help distinguish between active and latent TB.

Key words: Cytokine regulation network; Inflammatory cytokines; Interferon-gamma; Tuberculosis

INTRODUCTION

Tuberculosis (TB) is currently the most serious infectious disease through out the world. According to the 2014 Global Tuberculos is Report, the number of people infected with *Mycobacterium tuberculosis* (MTB) has reached one-third of the world's population, meaning that nearly 2 billion people have latent TB infections. Furthermore, approximately 5-10% of latent TB infections become active over the lifetimes of the infected individuals. According to statistical data from the National Centers for Disease Control and Prevention (China), the number of people who died of TB in 2012 was 250,000, which is two times higher than the number of deaths from all other infectious diseases combined. Therefore, TBis a significant threat to human health, and we continue to lack success ful methods to control it.

The most effective method of TB control is early diagnosis and intervention (Abouda et al., 2015). Currently, the commonly used test for the clinical diagnosis of active TB and screening for latent TB infection is the interferon-gamma release assay (IGRA), which quantitatively detects mycobacterium tuberculosis-specific T cells in peripheral blood using an enzyme-linked immunospot assay (ELISpot) to determine whether an individual has been infected with TB. IGRA is a highly sensitive and specific method for detecting TB infection. However, it cannot distinguish between latent and active TB infections. Furthermore, IGRA is expensive and complicated to perform (Wagner et al., 2008; Dheda et al., 2009). While the detection of serum IFNγ may be an alternative method for TB diagnosis, the serum concentrations of IFNγ are often too low for easy detection (Lalvani and Millington, 2008). Therefore, researchers are looking for more suitable biomarkers of TB infection that are inexpensive and easy to detect.

As such, the following methods are currently being used to search fornew TB biomarkers: cytokine array analyses of the sera from patients with active or latent TB infections compared to those from healthy individuals (Weiner et al., 2012; Liu et al., 2013); microarray analyses of the supernatants of centrifuged peripheral blood after stimulation with TB-specific antigens from patients with active or latent TB infections compared to that from healthy individuals (Frahm et al., 2011; Kellar et al., 2011; Murthy et al., 2011; Borgstrom et al., 2012; Skogstrand et al., 2012; Anbarasu et al., 2013; Armand et al., 2014; Essone et al., 2014); and extraction and culture of leukocytes from the peripheral blood of patients with active or latent TB infection and healthy individuals, followed by analysis of the differences insecreted cytokines before and after stimulation with TB-specific antigens (Rivera-Ordaz et al., 2012). However, consistent results have not been obtained using the aforementioned methods because the number of cytokines analyzed is often small and the types of assays usedto detect them vary between studies; thus, a more systematic approach for analyzing all cytokines is needed.

In the current study, TB-specific antigens were used to stimulate the peripheral blood of

patients with active or latent TB infections and healthy individuals, and high-throughput cytokine microarrays were used to detect the expression of cytokines after stimulation. The results were combined with reported potential TB biomarkers to construct a cytokine regulation network of all potential markers, which was used to identify cytokines that could be superior to IFNγ for the rapid diagnosis of TB and for distinguishing betweenlatent and active TB infections.

MATERIAL AND METHODS

Subjects

In the present study, 120 participants aged 8-30 years who had been hospitalized or undergone a physical examination between February 2012 and February 2014 at the Guangzhou Women & Children Medical Center and Putian No.1 People's Hospital were enrolled. In total, 120 peripheral blood samples were collected from the 120 enrolled participants (40 healthy controls, 40 individuals with latent TB infection (LTBI), 40 patients with active TB. The 40 healthy controls did not have any radiological or clinical signs of TB and were also negative for TB by tuberculin skin testing (TST) (< 5 mm) and an IFNγ ELISpot against the TB-specific antigens culture filtrate protein-10 (CFP-10) andearly secretory antigen target-6 (ESAT-6). Individuals who had an allergic reaction to TST, serious malnutrition, malignancy, or immunodeficiency such as congenital immunodeficiency or HIV, as well as those receiving immunosuppressive therapies were excluded. The 40 individuals with LTBI were selected on the basis of a positive TST (> 10 mm) and an IFNγ ELISpot response against CFP-10, ESAT-6, or both in the absence of diagnostic criteria for TB. The 40 participants with active TB disease were selected from our affiliated tuberculosis hospital and were definitively diagnosed on the basis of the standard diagnostic criteria for pulmonary tuberculosis (Palou Llaudet, 1964). The samples were gender-matched. The probability of a person coming into contact with MTB increases over his/her lifetime; therefore, it was considered more reliable to deem a younger TST-negative and IFNγ ELISpot-negative individual as TB-negative than an older individual with the same test results. Therefore, the age of the healthy control group in this study was lower than those of the LTBI and TB group. This study was reviewed and approved by the local Ethics Committee (Guangdong Center for Tuberculosis Control). Written informed consent was obtained from each participant before study enrollment, and for child participants, was obtained from the guardians on their behalf.

Sample collection

Heparin sodium-containing anticoagulant tubes were used to collect 3 mL of peripheral blood from each participant, and each blood sample was divided into 3 parts. The samples were stimulated with TB-specific antigens overnight, and the supernatant or plasma was collected. The samples were stored at -80°C for future use. A double-blind method was followed while performing the microarray analyses. The researchers and sample information collectors were separated.

TB serum biomarker screening with human cytokine arrays

Fresh blood (1 mL) was stimulated with TB-specific antigens (ESAT-6 and CFP-10) or PBS (negative control) for 20 h at 37°C. Then, centrifugation was performed at 4000 *g*, and the plasma was collected. A commercial quantitative immuno-microarray (Quantibody Human

Cytokine Array 1, RayBiotech, Inc., Norcross, GA) was used to measure the concentrations of 40 cytokines in the plasma samples. The manufacturer protocol was followed, and the results were analyzed using the RayBiotech Q Analyzer program. Briefly, after 30 min of incubation with sample diluent, the glass chips were washed, and each well was overlaid with 100 µL diluted sample. After overnight incubation at 4°C and extensive washing, the detection antibody was added for 1 h and then washed away. AlexaFluor 555-conjugated streptavidin was then added for 2 h at room temperature. The signals (Cy3; 555 nm excitation, 655 nm emission) were scanned and extracted using a Genepix 4000B laser scanner (Axon Instruments, Foster City, CA, USA). The total amounts and concentrations of the biomarkers were evaluated in this study. The concentrations, expressed in pg/mL, were calculated using RayBiotech Q Analyzer software against a standard curve determined for each biomarker. The total amount of each biomarker was determined in pg by multiplying the concentration by the sample volume.

IFNγ ELISA

IFNγ enzyme-linked immunosorbent assay (ELISA) kits were purchased from Hygeianey [Mycobacterium Tuberculosis-Specific Cell Mediated Immune Response Detection Kit (ELISA), Wuhan, China]. The assay was performed following the manufacturer protocol. Briefly, 1 mL fresh blood was stimulated with TB-specific antigens (ESAT-6 and CFP-10), PBS (negative control), or lectins (positive control) for 20 h at 37°C. Then, centrifugation at 4000 *g* was performed, the plasma was collected, and IFNγ concentration was measured using a human IFNγ quantitative ELISA kit. The results are summarized in Table 1.

Table 1. IFNγ ELISA results after TB-specific stimulation.

PBS (IU/mL)	Lectins-PBS (IU/mL)	TB-PBS (IU/mL)	Result
≤8	Any	≥0.35 and ≥ PBS/4	TB positive
	≥0.5	<0.35	TB negative
	≥0.5	≥0.35 and < PBS/4	TB negative
	<0.5	<0.35	uncertain
	<0.5	≥0.35 and < PBS/4	uncertain
>8	Any	Any	uncertain

Statistical analysis

Data are presented as the means ± standard error of the mean (SEM). Statistical analyses were conducted using BRB-ArrayTools statistical software developed by Dr. Richard Simon and BRB-ArrayTools Development Team (Biometrics Research Branch, NCI, Bethesda, MD, USA). The significance analysis of microarrays (SAM) was used for class comparison and selection of the target cytokines. The cytokine regulation network was constructedusing EMBL String online. Differences between groups were analyzed using the two-tailed Mann-Whitney test. A P value of 0.05 or less was considered to indicate statistical significance.

RESULTS

Baseline data

The baseline characteristics of the three groups are summarized in Table 2. No significant

differences in gender (P = 0.567), ethnicity, or the outcomes of TST, IGRA, or LJ culture were found.

To screen for cytokines associated with active TB, the concentrations of 40 common plasma cytokines were detected after stimulation with TB-antigens using high-throughput protein microarrays (Figures 1A-D). The results obtained from the bloodof healthy individuals after stimulation with PBS was set as a reference, and the fold change and significant differences in the levels of each cytokine in the blood samples of the different groups after TB antigen stimulation were analyzed. The results are shown in Figures1E-G. The expression of IFNγ was significantly increased by TB antigen stimulation both in patients with active TB infections and those with LTBI (2.44-and 2.16-fold, respectively); however, the difference in IFNγ expression between these two groups was not significant (P = 0.231). In the healthy controls, TB antigen stimulation resulted in only a slight increase in IFNγ expression (1.25-fold, P= 0.044) (Figure 1A-G).

Table 2. Baseline characteristics of the three groups.

Characteristic	Healthy controls	Latent TB	Active TB
Total number	40	40	40
Gender			
Female	20	22	19
Male	20	18	21
Ethnicity	Han Chinese	Han Chinese	Han Chinese
Age (Mean years ± SEM)	16 ± 9.06	18.00 ± 10.35	18.47 ± 12.68
TST			
Positive	0	40	40
Negative	40	0	0
Unknown	0	0	0
IGRA			
Positive	0	40	40
Negative	40	0	0
Unknown	0	0	0
LJ Culture			
Positive	0	0	40
Negative	0	0	0
Unknown	40	40	0

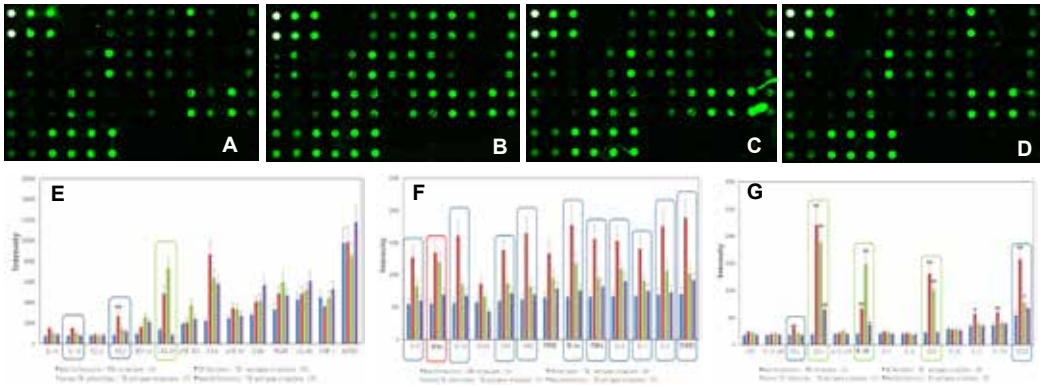

Figure 1. Human cytokine array analysis of serum cytokines induced by TB-specific antigens. (**A**) Healthy controls with PBSstimulation. (**B**) LTBI patients with TB-antigen stimulation. (**C**) Healthy individuals with TB-antigen stimulation. (**D**) TB patients with TB-antigen stimulation. The intensity of the signal was quantified with Genepix software, and the data are shown in graphs **E**, **F**, and **G** (data are presented as averages ± SD).

Biomarkers for active TB

Theoretically, after TB antigen stimulation, a good diagnostic marker for active TB would be significantly upregulated in patients with active TB infection, but would not be significantly changed in healthy people or in those withLTBI. Therefore, markers that are upregulated by TB antigen stimulation and show significant differences between the patients with active TB infections and those with LTBI and healthy controls are potential markers. However, to limit the number of potential markers identified, we chose a fold change cut-off value for upregulated cytokines. The IFNγ fold changes in the active TB and LTBI groups after TB antigen stimulation were 2.44 and 2.16, respectively. Therefore, cytokines with more than a 2-fold change after TB antigen stimulation were regarded as potential biomarkers. The following criteria for the screening of active TB-related cytokines were set: B/A>2 ($P < 0.05$, meaning the change must be significant); D/A ≤ 1.2 ($P > 0.05$, meaning the change should not be significant); and C/A ≤1.5 ($P > 0.05$, meaning the change should not be significant) where the variables are defined as A: healthy controls with PBS stimulation; B: active TB patients with TB antigen stimulation; C: LTBI with antigen stimulation; D: healthy controls with TB antigen stimulation. Only the following cytokines met the screening criteria: IL-1α, IL-2, IL-6, IL-7, IL-8, IL-10, IL13, CCL1 (I-309), CCL2, CSF2, CSF3, CXCL9 (MIG), TGF-β1, and TNFα. The results of the screening for biomarkers of active TB are shown in Table 3.

Table 3. Results of screening for biomarkers of active TB.

	D/A Fold change (P value)	B/A Fold change (P value)	C/A Fold change (P value)
CCL1/I-309	0.980675 (0.542)	2.061273 (0.023)	1.191427 (0.098)
CXCL9	1.200000 (0.222)	2.878799 (0.000)	1.424819 (0.066)
IL-6	1.089638 (0.154)	2.331404 (0.001)	1.504092 (0.056)
IL-10	1.200000 (0.432)	2.846600 (0.001)	1.508316 (0.134)
CSF3	1.191019 (0.442)	2.316416 (0.030)	1.444639 (0.122)
CSF2	1.121869 (0.195)	2.659569 (0.021)	1.327780 (0.145)
IL-1α	1.154403 (0.213)	2.712148 (0.000)	1.489933 (0.055)
IL-8	1.200000 (0.078)	2.278722 (0.011)	1.420574 (0.079)
IL-7	1.106920 (0.111)	2.081981 (0.001)	1.333387 (0.111)
IL-2	1.056429 (0.234)	2.533989 (0.000)	1.500067 (0.051)
TGF-β1	1.208361 (0.078)	2.701383 (0.000)	1.461406 (0.099)
CCL2	1.208944 (0.099)	3.188441 (0.000)	1.424508 (0.106)
TNFα	1.1556326 (0.122)	2.37459867 (0.002)	1.453060 (0.111)

Cytokines for TB screening

A good TB screening indicator should be able to detect active and latent TB infections simultaneously. Therefore, its expression should be greatly upregulated after TB antigen stimulation both in patients with active TB and those with latent TB infections, but not in healthy individuals. Thus, we specified the criteria of C/A>2, D/A ≤ 2, and B/A>2 for the identification of TB-related cytokines. We found that only CCL8, IFNγ, and CXCL10 met these criteria. However, although the D/A values for CCL3 and IL-1β were >1.2, the B/A and C/A values for those cytokines were much greater than 2. Thus, CCL3 and IL-1β were also considered potential cytokines for TB screening. The fold changes in these cytokines before and after TB antigen stimulation are summarized in Table 4.

Non-specific cytokines

We also found that many cytokines, including CCL4 and MIP1d, were significantly

upregulated by stimulation with TB antigens in both the healthy controls and patients with active or LTBI. Thus, these cytokines were considered to be non-specific.

Table 4. Results of screening for potential TB cytokines.

	D/A fold change (P value)	B/A Fold change (P value)	C/A Fold change (P value)
CCL3	3.400166 (0.000)	11.55941 (0.000)	9.892162 (0.000)
IL-1β	1.81988 (0.045)	3.332547 (0.000)	7.421621 (0.000)
CCL8	1.055971 (0.213)	5.863285 (0.000)	4.585284 (0.000)
CXCL10	0.584581902 (0.564)	3.466649909 (0.000)	5.20739568 (0.000)
IFNγ	1.204253 (0.231)	2.437919 (0.010)	2.161414 (0.011)

Relationships of cytokines in the network

A cytokine that is a good TB biomarker would be secreted directly by TB-specific T cells after TB-specific stimulation rather than by phagocytes or other non-specific T cells stimulated by the same antigens. Additionally, a good biomarker would not be regulated by other cytokines as that would make it non-specific and cause it to be easily affected by other diseases. With that in mind, we studied the regulation network of the 25 upregulated cytokines. As shown in Figure 2, these cytokines are mainly located in the regulation networks of the four cytokines IFNγ, IL-11, IL-1α, and IL-7. IL-1β, IL-6, IL-17A, IL-2, and SCF2 were in the middle of the regulation network and, therefore, may serve to amplify the signal of the network. CXCL9, CXCL10, IL-8, IL-15, CCL2, and CCl3 were located more down-stream in the regulation network, and therefore may perform their functions directly. IL-10 may act as a negative regulatorof this network as it negatively regulates several of the 25 upregulated cytokines, including IL-6, IL-8, IL-2, CSF2, CCL2, and CSF3. Additionally, IL-10 was positively regulated by CSF3. Accordingly, IL-10 and CSF3 levels may be balanced by the regulation of each other. Moreover, TGFβ, CCL4, CCL8, PDGFB, and OGN were not included in the regulation network of the 25 cytokines, and after antigen simulation, may be secreted by T cells, B cells, or phagocytes to exert their functions.

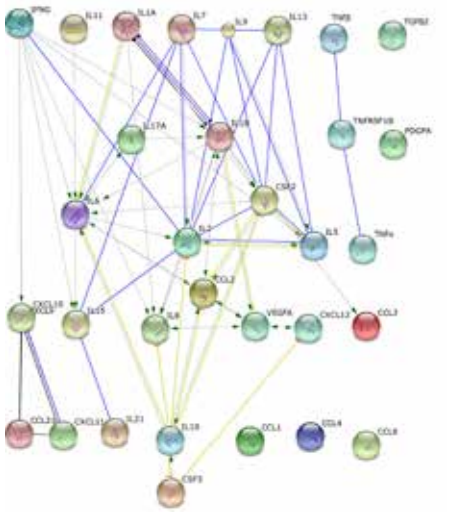

Figure 2. Relationships of cytokines in the regulation network.

DISCUSSION

Although biomarkers for the diagnosis of active TB and the screening of TB infection have been studied for a long time, no significant breakthroughs have been made. In the current study, high-throughput inflammatory factor microarrays were used for the detection of serum cytokines in the peripheral blood from patients with active TB, those with LTBI, and healthy individuals after stimulation with TB-specific antigens ESAT-6 and CFP-10. Furthermore, a regulation network of the cytokines whose expressions were upregulated was constructed. Importantly, we identified several biomarkers that may be suitable for the diagnosis of active TB and large-scale screening for TB infections.

In the present study, we found that the difference in the expression of IFNγ between the active TB and LTBI groups was not significant. Therefore, IFNγ cannot be used for the differential diagnosis of active and latent TB infections, whichis consistent with the results of a previous study (Wagner et al., 2008). Similar to IFNγ, IP-10 was upregulated in both active and latent TB infections, and there was a higher increasein the LTBI group than that in active TB patients. Thus, IP-10 may only be suitable for TB screening and not for the diagnosis of active TB. These results are in agreement with those of previous studies (Lighter et al., 2009; Holm et al., 2014), and are not surprising considering that IP-10 was found to be under the regulation of IFNγ in the regulation network constructed herein. However, the concentration of IP-10 in the serum was much higher than that of IFNγ, so IP-10 may be easier to detect. Furthermore, the changesin IP-10 levels were more significant than changes in IFNγ levels, so IP-10 could be used for the screening of TB in place of IFNγ. Additionally, we found that the expression levels of CCL8 (MCP-2), IL-1β, and CCL3 (MIP-1α) were significantly upregulated in both active and latent TB infections after TB-specific antigen stimulation. IL-1β has been reported to be greatly upregulated after TB antigen stimulation (Al-Attiyah et al., 2012), and the regulation network shows that it is regulated by IFNγ. We are the first to observe significant upregulation of MCP-2 and MIP1a after TB antigen stimulation. In particular, MCP-2 expression was almost unchanged in healthy individuals, but was increased 2.5-fold in active TB and LTBI. Therefore, MCP-2 and MIP1α can be used to screen for TB.

We further report that the expression levels of CCL1 (I-309), CXCL9 (MIG), IL-10, IL-6, CSF2, CSF3, IL-8, IL-1α, IL-7, TGF-β1, CCL2, IL-2, IL-13, and TNFα were significantly different in active TB patients from those with LTBI and controls. Some of these cytokines have been previously identified in the context of TB. For example, I-309 was shown to be upregulated in DC cells after TB-specific antigen stimulation (Jang et al., 2008; Yu et al., 2012) as were CXCL9 (MIG) (Bai et al., 2011), IL-10 (Hasan et al., 2011), IL-6 (Zhang et al., 1994), IL-7, IL-8 (Nakaya et al., 1995; Fietta et al., 2002; Boggaram et al., 2013; Huang et al., 2014), and G-CSF (Higgins et al., 2008). IL-10 is ananti-inflammatory molecule that mainly plays a negative role in immune regulation (Boussiotis et al., 2000). Interestingly, in the regulation network constructed here, IL-10 was under the regulation of CSF2 and CSF3. Therefore, it may be a non-specific factor that induces aweak immune responseduring infections. Thus, IL-10 is not a TB-specific marker, but may be involved in mutual regulation with CSF3 and CSF2 to balance the levels of those 3 cytokines.IL-6 and IL-2 were in the middle of the network and were mainly regulated by IL-1α, IL-11, and IL-17. They also regulated the expression of other cytokines, and thus may have an intermediate amplification function in the regulation network. Alternatively, they may be non-specific cytokines that function as signal amplifiers.

The TNFα regulation network was independent from the network constructed here. However, a previous study showed that TNFα was upregulated after TB stimulation (Olobo et al., 2001).

Studies have demonstrated that TGF-β2 is upregulated after TB stimulation (Olobo et al., 2001; Li et al., 2012). The regulation network constructed herein showed that TGF-β2 was independent from the IFNγ signaling pathway; thus, it may have its own regulation network, and follow-up studies are needed to identify that network.

Although IFNγ, IL-1α, IL-7, and IL-11 were all at the upstream end of the regulation network, IFNγ expression was upregulated both in active and latent TB infections, whereas the expressions of IL-1α, IL-7, and IL-11 were only upregulated in active TB patients. Therefore, this regulation network may help distinguish between active and latent TB infections. Hence, it may be possible to distinguish active TB from LTBI by performing additional studies on these cytokines and on the combined detection of multiple cytokines.

Considering the mutual regulation of cytokines in the regulation network, IL-1α and IL-7 may be more valuable than IFNγ for the diagnosis of TB because they are at the upstream end of the network, and may be expressed by TB-specific T cells after TB-specific antigen stimulation. Of course, determining the source of IL-1α and IL-7 expression requires further study, and the sensitivity and specificity of these cytokines in the diagnosis of TB needs to be investigated in a larger study population.

According to Figure 2, IFNγis an important cytokine in this regulation network because many cytokines, including CXCL9, CXCL10, IL-1β, IL-6, and IL-15, were downstream of IFNγ. IFNγ may be a specific cytokine secreted by TB-specific T cells after TB antigen stimulation. Therefore, IFNγ is a very good specific biomarker, and the samemay be said for IL-1α and IL-7. Although CXCL10 (IP-10) has been reported by previous studiesto be closely related to TB (Ruhwald et al., 2008; Latorre et al., 2014) and can be used as a biomarker in the screening for TB, it is also under the regulation of IFNγ.

There are several limitations in the current study. First, the sample size was small. Second, the expressions of only 40 inflammatory factors were investigated, and thus more immune-associated factors need to be examined in the future to obtain more integrated regulation networks. Lastly, differences in cytokines between the groups before stimulation and differences in the same sample before and after stimulation were not excluded.

In conclusion, the current study revealed that there are mutual cellular regulations among different cytokines as seen in the regulation network. Moreover, we identified several new biomarkers that may be applied forTB screening and the diagnosis of active TB. Of particular importance, we provide evidence that the combined use of multiple indicators may one day raise the sensitivity and specificity of TB detection.

Conflicts of Interest

The authors declare no conflicts of interest.

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