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Diagnostic performance of plasma cytokine biosignature combination and MCP-1 as individual biomarkers for differentiating stages *Mycobacterium tuberculosis* infection

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SUMMARY

Objective: We aimed to identify plasma cytokine biomarkers that differentiate the infection stages of *Mycobacterium tuberculosis* (MTB).

Methods: This study included a total of 227 subjects consisting of active tuberculosis (ATB) patients, latent tuberculosis infection (LTBI) individuals, and healthy controls (HC). We analyzed the expressions of 38 plasma cytokines in the discovery cohort to identify the biosignatures for differentiating MTB infection states, area under the curve (AUC) were used to evaluate the diagnostic efficiency. The AUC of unique plasma biomarker was confirmed in the validation cohort.

Results: In the discovery cohort, the AUC of the 8-marker biosignature (eotaxin, MIP-1α, MDC, IP-10, MCP-1, IL-1α, IL-10, and TNF-α) in diagnosing ATB was 1.0. The sensitivity and specificity of the 5-marker biosignature (IP-10, MCP-1, IL-1α, IL-10, and TNF-α) in diagnosing LTBI were 94% and 81.25%, respectively. The AUC of the 3-signature biosignature (eotaxin, MDC, MCP-1) in differentiating ATB from LTBI was 0.94, with the sensitivity and specificity of 87.76% and 91.84%, respectively. Moreover, among all the single cytokine biomarkers, MCP-1 exhibited the highest AUC in diagnosing ATB (0.98) and differentiating ATB from LTBI (0.91). In the subsequent validation cohort analysis, we identified that the AUC of MCP-1 in diagnosing ATB and differentiating ATB from LTBI were 0.97 and 0.89, respectively, which was generally consistent with the results of the discovery cohort.

Conclusion: Cytokine levels in plasma can be used as biosignatures to diagnose ATB. The cytokine concentrations vary during the different stages of MTB infection, which might serve as biomarkers in differentiating ATB from LTBI. Future studies with a larger population and data from multiple institutions are needed to validate our findings.

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by MTB that spreads through the respiratory system and mainly invades lung tissues. According to the WHO Global Tuberculosis Report (2017), TB is the top leading cause of death from a single infectious

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agent.[1] It is estimated that about one-third of the world's population is infected with MTB.[2] Infected patients may experience asymptomatic elimination, latent infection, or active pulmonary and extrapulmonary tuberculosis. About 5–10% of individuals with LTBI progress to ATB in their lifetime .[2,3]

Diagnosis of TB status is challenging due to its diverse clinical forms and outcomes.[3] Current laboratory TB diagnostic methods include acid-fast staining, bacterial culture, and PCR amplification.[4] MTB culture is the gold standard for TB diagnosis, while its main limitation is the long response time taken. Identification of the acid-fast bacilli in sputum is the most widely used diagnostic test for ATB, but sputum smear microscopy has low sensitivity. PCR for MTB is typically based on the WHO-recommended Xpert MTB/RIF method, which is limited by its low sensitivity in smear negative or in HIV-TB co-infected subjects.[5,6] Recently, the interferon-gamma (IFN-γ) release assay (IGRA) has been broadly applied to diagnose MTB infection. However, IGRA is also unable to differentiate latent from active TB.[7]

MTB-induced immune response is complicated and not fully understood, which hinders the development of new immunological approaches to detect TB. Studies on cytokines have enhanced our understanding on the immune response: pro-inflammatory cytokines (such as IFN-γ, TNF-α and IL-1), anti-inflammatory cytokine (such as IL-4 and IL-10), regulatory cytokines (such as IL-13 and IL-17) and chemokines (such as MIP-1, IP-10 and MCP-1) are involved in the development of TB.[3,8] We sought to determine whether other cytokine responses or combinations thereof, could improve the performance of IFN-γ in IGRA. We hypothesized that combinations of multiple cytokines could be more sensitive for MTB infection than a single immune marker, and potentially distinguish individuals with ATB disease from persons with LTBI.

Materials and methods

Ethics statement

The study was conducted between July 2014 and January 2017 at Daping Hospital of Army Medical University (Third Military Medical University), Chongqing, China. The protocol of this study was approved by the ethics committee of Daping Hospital. Written informed consent was obtained from all participants.

Study subjects

The discovery cohort consisting of a total of 149 study subjects, including 50 patients with ATB, 49 subjects with LTBI, and 50 healthy controls. In an independent validation cohort, 28 ATB patients, 24 LTBI subjects, and 26 healthy controls were enrolled. The inclusion criteria for each participant group were as follows. All participants were adults ($\geq$ 18 years of age). ATB patients were diagnosed according to World Health Organization criteria, on the basis of clinical symptoms, positive sputum smears for acid-fast bacilli and/or MTB positive cultures, and confirmed by chest X-ray (CXR). Actually, ATB individuals were all newly diagnosed patients with active pulmonary tuberculosis and with no record of previous active tuberculosis disease. LTBI subjects were individuals who had history of close contact with ATB patients, and displayed positive results of IGRA (T-SPOT.TB), but showed no signs or symptoms of ATB disease and had negative cultures for MTB. Healthy controls were individuals who had no history of close contact with TB patients, and displayed normal chest radiograph, negative T-SPOT.TB and TST and no clinical symptoms of ATB. Exclusion criteria comprised: subjects with pregnancy and complications such as renal or liver disorders, HIV infection, cancer, autoimmune diseases, allergic diseases, diabetes mellitus and those receiving immunosuppressive therapy (such as glucocorticoids) or having received immunomodulator or anti-TB therapy. Each subject donated a 10 ml heparinized and 2 m1 EDTA-treated peripheral-blood sample. Heparinized blood samples were used to accomplish T-SPOT.TB assay within 24 h. The EDTA-blood samples were centrifuged within 30 min of collection, and plasma was stored at −80 °C until further cytokines analysis.

T-SPOT.TB assay

All participants underwent T-SPOT.TB assay (Oxford Immunotec Ltd, UK) according to the manufacturer's instructions. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml heparinized blood samples over Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and resuspended in the AIM V medium (Gibco, Grand Island, NE, USA) at the concentration of 2.5×10^5 per 100 µl. The PBMCs suspension was incubated overnight at 37 °C with specific TB antigens, ESAT-6 and CFP-10, in an enzyme-linked immune sorbent spot assay. The spots were counted after enzyme-linked antibody and substrate were added. More than six spots of either antigen were considered as positive, while six or less spots with both antigens were considered as negative.

Cytokine measurement by the multiplex cytokine assay system

The concentrations of 38 cytokines, including EGF, FGF-2, eotaxin, TGF-α, G-CSF, Fit-3L, GM-CSF, fractalkine, IFN-α2, IFN-γ, GRO, IL-10, MCP-3, IL-12p40, MDC, IL-12p70, IL-13, IL-15, sCD40L, IL-17A, IL-1RA, IL-1α, IL-9, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β and VEGF, in plasma were measured using a Human Cytokine/Chemokine Magnetic Bead Panel (HCYTMAG-60K-PX38, EMD Millipore Corporation, Billerica, MA, USA) by liquid chip Luminex xMAP technique. The manufacturer's protocol was followed for the multiplex bead assays. The concentration of 38 markers used for the standard curve in the Luminex assay ranged from 3.2 to 10,000 pg/ml.

ELISA

Plasma concentration of MCP-1 was determined using the Human CCL2/MCP-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), with 10 pg/ml being the minimum detectable dose. In this ELISA kit, the proprietary capture monoclonal antibody and the detection polyclonal antibody were both raised against natural and recombinant human MCP-1. Samples, reagents, and buffers were prepared according to the manufacturer's manuals. The concentration of MCP-1 used for the standard curve in the ELISA assay ranged from 31.3–2000 pg/ml.

Chest radiographic severity score

ATB patients of validation cohort were classified regarding the severity of disease according to pulmonary radiographic images using a double blind test and classified as minimal (stage 1, score 1), moderate (stage 2, score 2) and advanced disease (stage 3, score 3), as described by Abakay et al.[9] Lesions were considered minimal if there was no evidence of cavitation and the lesions were of slight to moderate density located above the second chondrosternal junction. Moreover, the lesions must only involve a segment of one or both lungs, and the combined extent of the lesions must be smaller than the volume of a single lung. Disseminated lesions of slight characterized moderate disease to moderate density in one or both lungs, and the collective lesion volume cannot exceed that of a single lung. In moderate disease, the lesions may also be densely packed so to appear confluent, but areas of high-density lesions cannot take up more than one third of the volume

Table 1
Baseline characteristics of the discovery cohort and validation cohort.

Characteristics	Discovery cohort			Validation cohort		
	HC	LTBI	ATB	HC	LTBI	ATB
Number	50	49	50	26	24	28
Age (years)	32.7 ± 22.3	35.2 ± 14.8	34.2 ± 12.8	41.2 ± 9.1	44.1 ± 11.8	41.2 ± 13.6
Gender: male, *n* (%)	27 (54)	28 (57.1)	19 (38)	14(53.8)	13 (54.2)	15 (53.6)
TSPOT.TB: positive, *n* (%)	0 (0)	49 (100)	50 (100)	0(0)	24 (100)	28 (100)
TST: positive, *n* (%)	0 (0)	40 (81.6)	50 (100)	0(0)	20 (83.3)	28 (100)
TB culture: positive, *n* (%)	NA	0 (0)	39 (78)	NA	0(0)	24 (85.7)
Sputum smear: positive, *n* (%)	NA	0 (0)	50 (100)	NA	0(0)	28 (100)

Note: Data were expressed as mean ± standard deviation, Not Applicable (NA).

of one lung. Moreover, the total diameter of cavitations cannot exceed 4 cm in patients with moderate disease. If lesions were found to be more extensive than moderate disease, then it was considered advanced disease.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Data were expressed as the mean ± standard deviation or median (interquartile range), depending on the data characteristics. For parametric test, independent sample t test was employed for the comparison between groups while one-way analysis of variance (ANOVA) with Bonferroni was used for comparison among groups. As for non-parametric test, comparisons were made using either by Mann–Whitney *U* test for comparison between 2 groups or by Kruskal–Wallis test with Dunn's multiple comparisons for multiple groups, categorical variables (frequencies) were compared with $\chi 2$ statistics or Fisher exact test. Diagnostic performance of each cytokine was assessed by calculating the area under the receiver operating characteristic (ROC) curve. Optimal cut-off values were selected based on the Youden's index, defined as the largest difference between the sensitivity and 1-specificity, taken over all points on the ROC curve. The AUC was compared by using paired *z* test (DeLong test) in MedCalc software (version 14.8.1; Broekstraat, Mariakerke, Belgium) to reveal diagnostic performance differences between two indicators. Correlation analysis was carried out using the Spearman test. Values of $p < 0.05$ were considered as statistically significant.

Additionally, the cytokine profile of each individual was assessed to determine individual immune biosignatures. Initially, all data were used to calculate the global median value of each marker, which was used as a cut-off point to classify each individual as "low" or "high" producers of a given immune marker. Data were organized in gray-scale diagrams to calculate the frequency of high producers for each clinical group. Relevant data (≥ 50%) was highlighted in both bold and underline format. Radar charts were performed in Microsoft Excel (Microsoft Office 2010, Las Vegas, USA) to characterize the overall frequency of individuals with high levels of each marker for each study group, showing potential immune biomarker signature.

Finally, to understand how these markers could stratify our groups, HemI (Heatmap Illustrator, version 1.0) software package was used to plot the heatmap for log2 transformed values of plasma levels.[10]

Results

Demographic characteristics of the study population

The characteristics of the study population are summarized in Table 1. In the discovery cohort, mean age of healthy controls, latent TB subjects and ATB patients was 32.7 y (range 18–72 y), 35.2 y (range 23–68 y) and 34.2 y (range 21–63 y). Seventy-eight percent of the ATB group was positive for MTB culture, and nearly 82% of the LTBI group was positive for TST test. As for the validation cohort, mean age of healthy controls, latent TB subjects and ATB patients was 41.2 y (range 24–54 y), 44.1 y (range 19–57 y) and 41.2 y (range 20–66 y). Almost 86% of the ATB group was positive for MTB culture, and 83% of the LTBI group was positive for TST test. No significant inter-group differences were observed with respect to age and gender of discovery cohort and validation cohort ($p > 0.05$). Furthermore, there was no statistical difference between the baseline data of the two cohorts ($p > 0.05$).

Plasma cytokine profile and its dynamic change response to TB infection in the discovery cohort

To sum up, of the thirty eight cytokine indicators examined in each group, two indicators (Flt-3L and IL-4) were excluded from statistical analyses because their median levels were below the detection level. Twenty eight indicators (EGF, FGF-2, TGF-α, G-CSF, GM-CSF, fractalkine, IFN-α2, IFN-γ, GRO, MCP-3, IL-12p40, IL-12p70, IL-13, IL-15, sCD40L, IL-17A, IL-1RA, IL-9, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, MIP-1β, TNF-β and VEGF) did not differ among the three groups (data not shown). While the levels of eotaxin, IL-10, MDC, IL-1α, IP-10, MCP-1, MIP-1α and TNF-α in ATB and/or LTBI populations were significantly higher or lower than the healthy controls (Fig. 1 and Table 2, $p < 0.05$), indicating that they may be used as biomarkers to identify MTB infection subjects from healthy controls.

To fully characterize the immune profile of patients at different stages of TB, we analyzed the 8 indicators mentioned above of each subject. We compared cytokine production by individual donors to the global median for the entire cohort of each marker (Fig. 2). High producers, with production higher than the global median, are depicted with a black square and those with a production lower than the median are depicted as a white square. Healthy control group presented frequencies of high producers lower than 50% for almost all of the analyzed markers, while in LTBI group and ATB group, respectively, more than half of the indicators and almost all markers had frequencies higher than 50%. For instance, IL-10, IP-10, IL-1α, TNF-α, MDC, eotaxin and MCP-1 were at respective frequencies of 50%, 64%, 78%, 96%, 68%, 68% and 96% for ATB patients.

Identification of cytokine to distinguish ATB patients from healthy controls

To visualize the ability of cytokines to discriminate between ATB, LTBI and HC groups, heatmaps were plotted for the various cytokines in ATB versus HC; LTBI versus HC and ATB versus LTBI and compared the differentially expressed cytokines following adjustment for multiple comparisons. As shown in Fig. 3A, the cytokines that are differentially expressed in ATB and HC groups are eotaxin, IL-10, MDC, IL-1α, IP-10, MCP-1, MIP-1α and TNF-α.

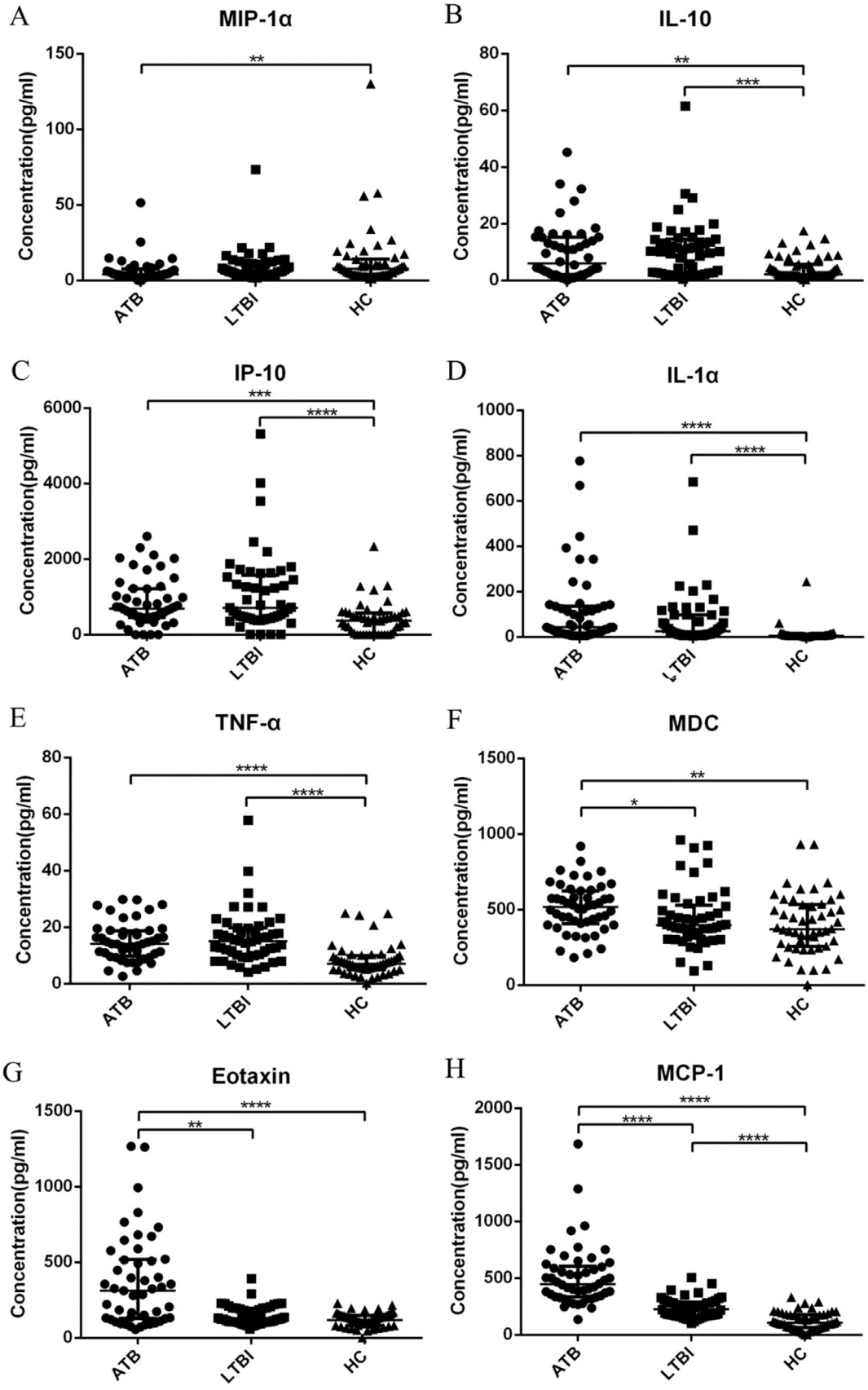

Fig. 1. Comparison of cytokine levels in discovery cohort. Graphs are included for the levels of the eight cytokine measured, including MIP-1α (A), IL-10 (B), IP-10 (C), IL-1α (D), TNF-α (E), MDC (F), eotaxin (G) and MCP-1 (H). Each dot represents the values obtained from individual subjects ($n = 50$, 49 and 50 for ATB, LTBI and HC groups, respectively). Median values (horizontal bar within each group) and error bars (interquartile range) are shown. Horizontal lines indicate a statistically significant difference between groups. *P* values were calculated using the Kruskal–Wallis test with Dunn's multiple comparisons. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

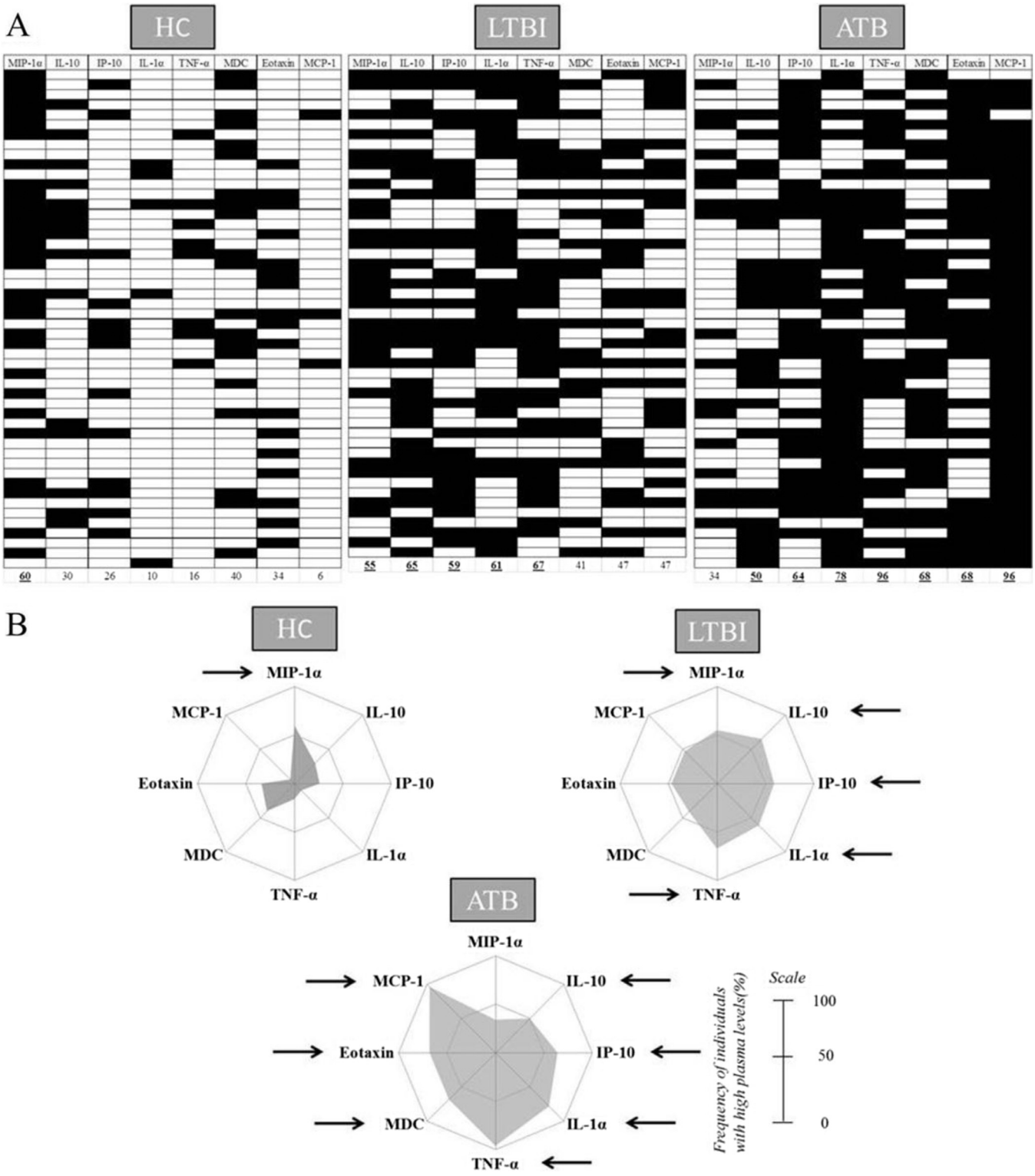

Fig. 2. Representation of a possible global signature of tuberculosis. (A) This diagram was designed using the value of the global median of each soluble mediator as the cutoff point to mark each individual as "low" or "high" regarding their expression levels of a given analyte. Those marked in black correspond to values higher than the overall median of the respective analyte, and blanks are those with less than the median values. Below each table are shown the frequency (%) of individuals with high levels of mediators while the relevant data (≥ 50%) are in bold and underlined format. (B) Radar charts summarize the signature of the mediators analyzed in pulmonary tuberculosis, relative to control and latent subjects, wherein each axis shows the proportion of subjects with high levels of a given mediator. The relevant data (≥ 50%) are indicated by arrows (→). ATB: active TB patients, LTBI: latent TB infected subjects, HC: healthy controls.

Table 2
Cytokine levels in ATB, LTBI and HC groups from the discovery cohort.

Cytokines	Group			p value		
	ATB ($n=50$)	LTBI ($n=49$)	HC ($n=50$)	ATB vs LTBI	ATB vs HC	LTBI vs HC
Eotaxin	312 (129.5–518)	130 (105.3–196)	118.7 (82.34–148.6)	< 0.01	< 0.0001	NS
IL-10	6.06 (2.03–15.29)	10.23 (2.65–14.54)	2.08 (1.95–5.68)	NS	< 0.01	< 0.001
MDC	517 (405.5–624.4)	397 (311.9–529.1)	369.4 (256.2–533.1)	0.04	< 0.01	NS
IL-1α	42.84 (15.6–136)	24.72 (8.67–97.31)	5.62 (5.62–9.12)	NS	< 0.0001	< 0.0001
IP-10	688.4 (453.1–1217)	713 (430.3–1570)	374 (34.52–574.3)	NS	0.0001	< 0.0001
MCP-1	447.1 (335.4–605.7)	227 (167.7–289.4)	105.5 (64.05–179.1)	< 0.0001	< 0.0001	< 0.0001
MIP-1α	3.96 (3–7.53)	6.57 (3.15–11.34)	7.87 (3.84–14.22)	NS	< 0.01	NS
TNF-α	14.11 (9.83–18.73)	15.07 (10.07–19.78)	7.04 (4.97–9.98)	NS	< 0.0001	< 0.0001

Note: Data were expressed as median levels (pg/ml) and interquartile range of cytokines measured in different subjects, p values were calculated using the Kruskal–Wallis test with Dunn's multiple comparisons, No Significance (NS).

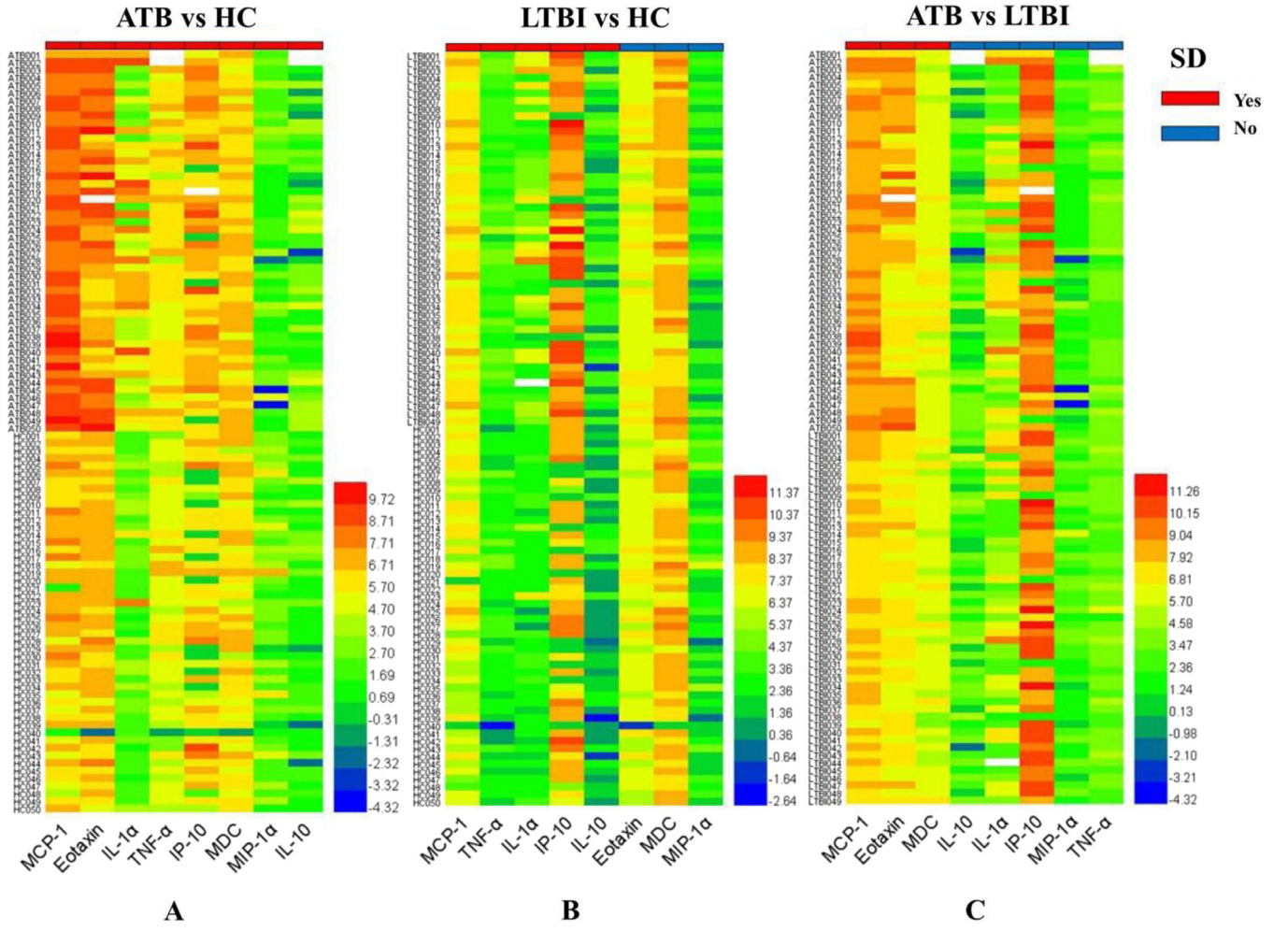

Fig. 3. The expression pattern of immunological markers determines a segregation profile for MTB infected subjects. Heatmaps of log2 transformed plasma levels of cytokines for ATB vs HC, LTBI vs HC and ATB vs LTBI were shown in panel A, B, C, respectively, in which each row stands for a sample and each column stands for a cytokine. High levels of cytokines are indicated in red and low levels of cytokines are indicated in blue, the white boxes are missing data. The annotation bar indicates if there is a significant difference (SD) between two groups or not for each cytokine.

Among them, the expression level of MIP-1α in ATB group was lower than that in HC group, while eotaxin, IL-10, MDC, IL-1α, IP-10, MCP-1 and TNF-α in ATB group were higher than those in HC group (Fig. 1, Table 2, $p<0.05$). ROC analysis was carried out to compare the values of these eight cytokines and their combinations in active TB diagnosis. The AUCs were 0.98 (MCP-1), 0.88 (IL-1α), 0.82 (TNF-α), 0.79 (eotaxin), 0.74 (IP-10), 0.68 (MDC and MIP-1α), 0.66 (IL-10) and 1.00 (combination) (Fig. 4A, Table 3), indicating that eight indicator combination is more conducive to the detection of ATB than single cytokine. The cut-off values of the eight cytokines in distinguishing between ATB and HC were 242.9 pg/ml (MCP-1), 15.43 pg/ml (IL-1α), 10.56 pg/ml (TNF-α), 200.3 pg/ml (eotaxin), 631.4 pg/ml (IP-10), 389.3 pg/ml (MDC), 7.76 pg/ml (MIP-1α), and 9.50 pg/ml (IL-10), respectively (Table 3). The results of paired z-test of AUC showed statistically significant bigger AUC for MCP-1 than other seven biomarkers (Fig. 4B). Furthermore, combining the eight markers does not improve the performance as compared to MCP-1 ($z=1.53$, $p=0.13$),

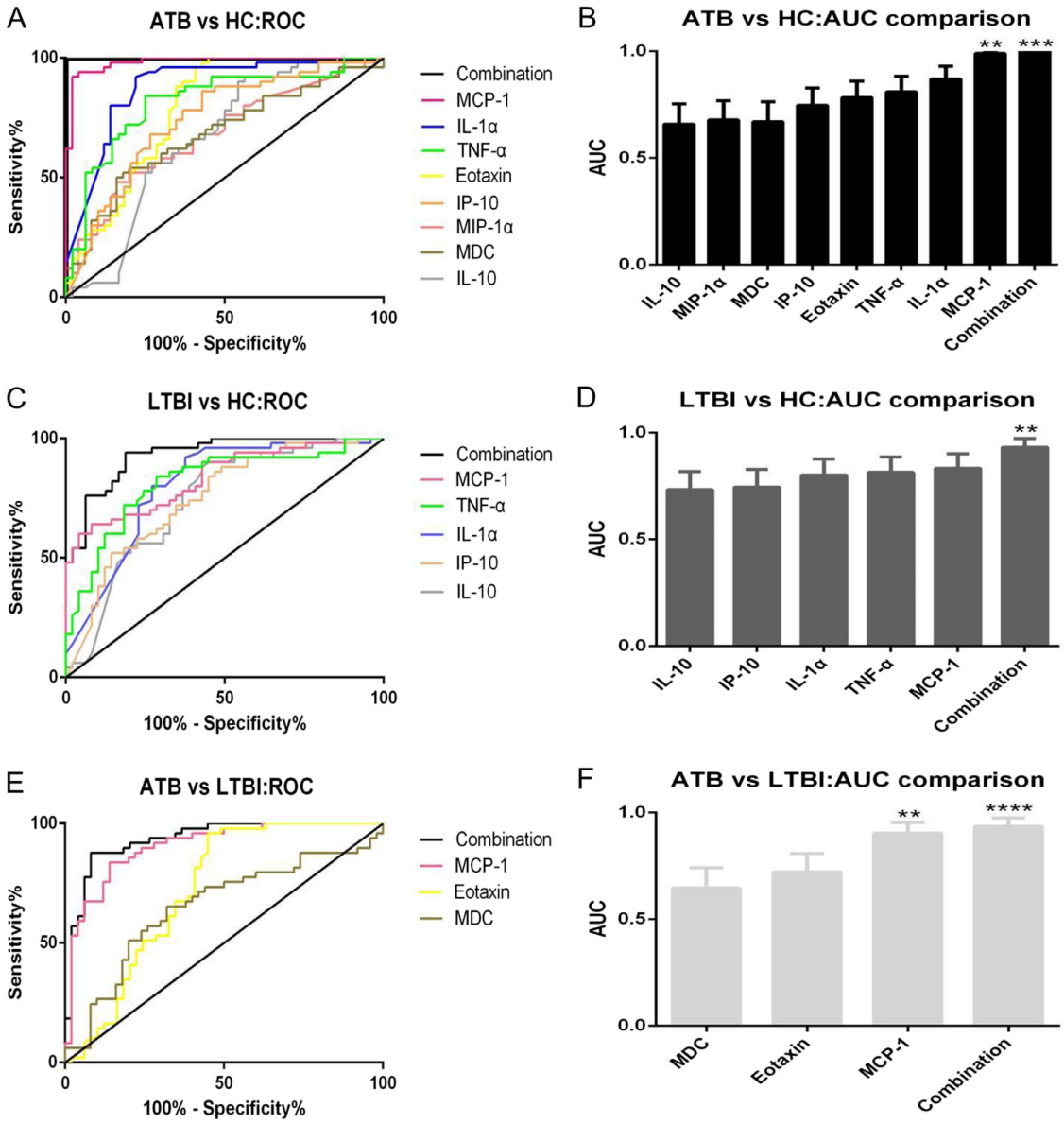

Fig. 4. Receiver operator characteristic curves and its AUC pairwise comparison of cytokines in the discovery cohort. ROC analyses depicting the accuracy of single marker and combinations of markers in distinguishing ATB and HC (A), LTBI and HC (C), ATB and LTBI (E). (B, D, F) The comparison among curves was done with Delong method, p value was calculated by comparing differences between AUCs of each two cytokines, and error bars represent the upper limit of 95% confidence interval. (B)*** Compared to 8 single cytokines (MCP-1, IL-1α, TNF-α, eotaxin, IP-10, MDC, MIP-1α and IL-10), all $p < 0.001$; ** Compared to 7 single cytokines (IL-1α, TNF-α, eotaxin, IP-10, MDC, MIP-1α and IL-10), all $p < 0.01$. (D) ** Compared to 5 single cytokines (MCP-1, TNF-α, IL-1α, IP-10 and IL-10), all $p < 0.01$. (F) **** Compared to eotaxin and MDC, all $p < 0.0001$; ** Compared to eotaxin and MDC, all $p < 0.01$.

Table 3
Diagnostic performance of each cytokine as individual biomarker for ATB and LTBI diagnosis in the discovery cohort.

Cytokines	ATB vs HC AUC (cut-off, sensitivity, specificity, p)	LTBI vs HC AUC (cut-off, sensitivity, specificity, p)	ATB vs LTBI AUC (cut-off, sensitivity, specificity, p)
MIP-1α	0.68 (7.76 pg/ml, 52%, 80%, < 0.01)	–	–
IL-10	0.66 (9.50 pg/ml, 90%, 45.83%, < 0.01)	0.74 (9.15 pg/ml, 88%, 57.14%, < 0.0001)	–
IP-10	0.74 (631.4 pg/ml, 86%, 57.14%, < 0.0001)	0.75 (620.6 pg/ml, 84%, 55.1%, < 0.0001)	–
TNF-α	0.82 (10.56 pg/ml, 84%, 75%, < 0.0001)	0.82 (11.08 pg/ml, 84%, 71.43%, < 0.0001)	–
IL-1α	0.88 (15.43 pg/ml, 92%, 78%, < 0.0001)	0.80 (15.43 pg/ml, 92%, 62.5%, < 0.0001)	–
MCP-1	0.98 (242.9 pg/ml, 94%, 96%, < 0.0001)	0.84 (133.4 pg/ml, 60%, 95.92%, < 0.0001)	0.91 (312.7 pg/ml, 83.67%, 86%, < 0.0001)
MDC	0.68 (389.3 pg/ml, 54%, 80%, < 0.01)	–	0.65 (448 pg/ml, 65.31%, 68%, < 0.01)
Eotaxin	0.79 (200.3 pg/ml, 96%, 59.18%, < 0.0001)	–	0.72 (254.9 pg/ml, 95.92%, 55.1%, < 0.001)
Combination	1.00 (NA, 100%, 100%, < 0.0001)	0.93 (NA, 94%, 81.25%, < 0.0001)	0.94 (NA, 87.76%, 91.84%, < 0.0001)

Note: P value was calculated as compared to the baseline area of 0.5, cut-off values were the concentrations of cytokines corresponding to the maximum Youden index (YI). The combination was actually the prediction probability, which is obtained by logistic regression analysis of multiple cytokines and then the ROC curve of combination was made by using the prediction probability. Since the prediction probability itself had no concentration unit, the cut-off values were not show.

therefore, MCP-1 is a plasma biomarker which is more sensitive and specific than the other seven for distinguishing active TB patients from healthy controls.

Identification of cytokine to distinguish LTBI subjects from healthy controls

Heatmap analysis showed that the expression profiles of IL-10, IL-1α, IP-10, MCP-1 and TNF-α were significantly different in LTBI group as compared to HC group (Fig. 3B). Actually, the expression levels of these five cytokines in LTBI group were higher than those in HC group (Fig. 1B-1E, H, Table 2). ROC analysis showed that the AUCs were 0.84 (MCP-1), 0.82 (TNF-α), 0.80 (IL-1α), 0.75 (IP-10), 0.74 (IL-10) and 0.93 (combination) (Fig. 4C, Table 3). The cut-off values of the five cytokines in distinguishing between LTBI and HC were 133.4 pg/ml (MCP-1), 11.08 pg/ml (TNF-α), 15.43 pg/ml (IL-1α), 620.6 pg/ml (IP-10), and 9.15 pg/ml (IL-10), respectively (Table 3). Then AUC comparison was used to evaluate the diagnostic utility of cytokine (single and combination), which indicated combination had a significant higher AUC than single cytokine (Fig. 4D, $p < 0.01$), and there was no difference in AUC between single cytokine (Fig. 4D, $p > 0.05$). Therefore, IL-10, IL-1α, IP-10, MCP-1 and TNF-α could be used as plasma biomarkers for distinguishing latent TB infection subjects from healthy controls, and combining the five markers could significantly improve the specificity and sensitivity.

Identification of cytokine to distinguish ATB patients from LTBI participants

As the results showed in Fig. 1 and Table 2, the expression levels of eotaxin, MDC and MCP-1 in ATB group were significantly higher than those in LTBI group ($p < 0.05$). Consistent with this, heatmap analysis also showed that the expression profiles of these three cytokines were significantly different in ATB group as compared to LTBI group (Fig. 3C). ROC analysis showed that the AUCs were 0.91 (MCP-1), 0.72 (eotaxin), 0.65 (MDC) and 0.94 (combination) (Fig. 4E, Table 3), indicating that three indicator combination is more conducive to discriminate between ATB and LTBI than single cytokine. The cut-off values of the three cytokines in distinguishing between ATB and LTBI were 312.7 pg/ml (MCP-1), 254.9 pg/ml (eotaxin), and 448 pg/ml (MDC), respectively (Table 3). AUC comparisons showed statistically significant bigger AUC for MCP-1 than other two biomarkers (Fig. 4F). In addition, combining the three markers does not improve the performance as compared to MCP-1 ($z = 1.90$, $p = 0.06$), therefore, MCP-1 is a plasma biomarker which is more sensitive and specific than the other two for distinguishing ATB patients from LTBI participants.

The diagnostic efficacy of MCP-1 in the validation cohort and the correlation between hematological indices and imaging-based TB severity

MCP-1 differentiates MTB infection states

Based on the results of the discovery cohort, only one candidate biomarker, MCP-1, was able to differentiate between any two groups among the ATB, LTBI, and HC groups; thus, it was selected for further validation in our study. Peripheral blood from 28 ATB patients, 24 individuals with LTBI, and 26 HC were collected (baseline data were showed in Table 1). EDTA-anticoagulant plasma was separated and evaluated for the expression of MCP-1 by ELISA. Results showed that the ATB group exhibited a significantly higher MCP-1 level compared to both the LTBI group and the HC group (ATB: 395.3 (322.7–534) pg/ml; LTBI: 204.9 (119.7–314.4) pg/ml; HC: 164.9 (136.2–200.1) pg/ml; ATB vs LTBI, $p < 0.0001$; ATB vs HC, $p < 0.0001$). However, MCP-1 concentrations were not significantly different between the LTBI group and the HC group (Fig. 5A, $p > 0.05$). Furthermore, ROC curve analysis was performed to evaluate the diagnostic efficacy of MCP-1. Results showed that the AUC of MCP-1 in differentiating ATB from HC was 0.97, with the sensitivity and specificity of 96.15% and 96.43%, respectively (Fig. 5B). The AUC of MCP-1 in differentiating ATB from LTBI was 0.89, with the sensitivity and specificity of 79.17% and 92.86%, respectively (Fig. 5C). These results were consistent with the MCP-1 data in the discovery cohort, suggesting that MCP-1 can be used as a single biomarker to differentiate the MTB infection stages.

MCP-1 is positively correlated with imaging-based TB severity but does not correlate with hematologic indices

Thus far, our current data had demonstrated that MCP-1 could be used to as a single biomarker to diagnose ATB and differentiate ATB from LTBI. Next, we evaluated the correlation between MCP-1 concentration in ATB patients and lung injury severity. A senior radiologist read and analyzed the chest X-ray images of the ATB patients. The lung injury severity was evaluated and scored to 3 levels based on the X-ray images. Spearman correlation analysis showed that MCP-1 plasma concentration positively correlated with the lung injury severity (Fig. 6). This finding suggested that the increase of plasma MCP-1 level in the ATB patients positively correlated with disease progression, and MCP-1 may serve as a prognostic marker for the severity of TB infection. Moreover, previous studies have reported that the counts of peripheral leukocytes, red blood cells, and platelet may affect the plasma/serum levels of cytokines.[11–14] Among the three groups in the validation cohort, the counts of neutrophils, lymphocytes, monocytes, and platelets were statistically different between groups (Table 4). However, Spearman correlation analysis showed that the counts of total or subtype white blood cells, red blood cells, or platelets were not correlated with the plasma concentration of MCP-1 (Table 5, $p > 0.05$), which indicated the differential levels of MCP-1 among MTB infection populations were not caused by differences of blood cells counts. The non-correlation between plasma MCP-1 level and the counts of peripheral nucleated cells, red blood cells, and platelets is an advantage of using plasma MCP-1 as a TB diagnosing biomarker.

Discussion

Accurate diagnosis of ATB disease and determine the stages of MTB infection are important to decrease transmission of TB, but current diagnostic tests are inadequate. Though IGRA are also unable to differentiate ATB from LTBI, the increased specificity and sensitivity of IGRA to diagnose MTB infection has been recognized. Multi-cytokine biosignatures may be the direction of diagnosis ATB and LTBI.

In this study, we evaluated the diagnostic efficacies of single and multiple plasma cytokines in diagnosing the stages of MTB infection among Chinese adult patients. The results showed that the plasma cytokine secretion characteristics can differentiate between the ATB, LTBI, and HC groups. Among the 38 plasma cytokines tested in the ATB, LTBI, and HC groups, we identified 8 biosignatures that differentiate the ATB group and/or the LTBI group from the HC group. In the subsequent independent analysis among the validation cohort, we focused on the biomarker MCP-1 that was capable of differentiating between any two groups among the ATB, LTBI, and HC groups.

In the discovery cohort (149 individuals), using the Luminex liquid chip, we identified 8, 5, and 3 cytokine biomarkers that were differentially expressed between the ATB and HC groups, the LTBI and HC groups, and the ATB and LTBI groups, respectively. As shown in the radar map and heat-map, the differential cytokines expression among the three groups could be used to differentiate between the different stages of TB infection. In the analysis of the ATB and HC groups, the AUC of a single cytokine in diagnosing

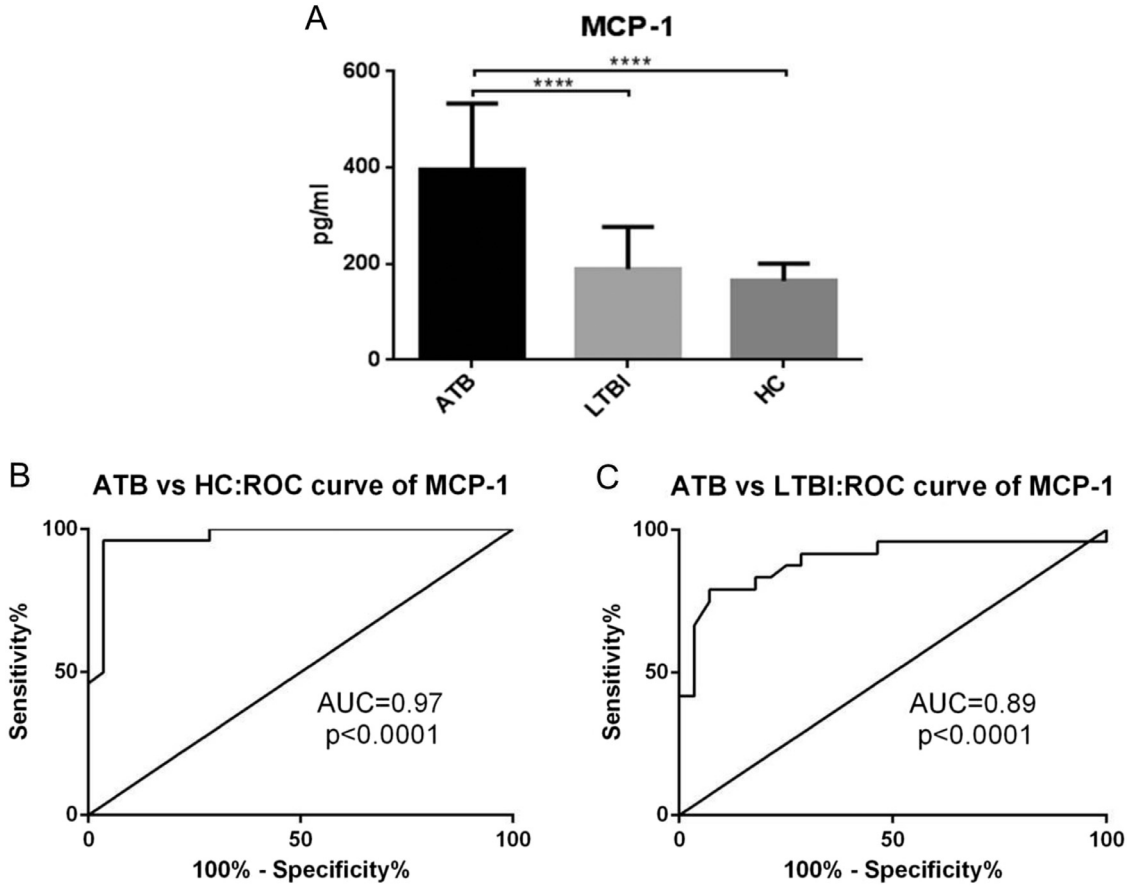

Fig. 5. MCP-1 in groups of validation cohort. (A) Comparison of plasma levels of MCP-1 among HC, LTBI, and ATB patients. Data were shown as Median ± Interquartile range, horizontal lines indicate a statistically significant difference between groups. The plasma MCP-1 was higher in ATB patients than that in LTBI subjects and healthy volunteers. (B) ROC curves of MCP-1 in distinguishing ATB patients from healthy controls (96.15% sensitivity and 96.43% specificity). (C) ROC curves of MCP-1 in differentiating ATB and LTBI (79.17% sensitivity and 92.86% specificity). AUC = area under the curve.

Table 4
Hematological parameters of the validation cohort.

Laboratory findings: median (interquartile range)	Group			*p* value		
	ATB ($n=28$)	LTBI ($n=24$)	HC ($n=26$)	ATB vs LTBI	ATB vs HC	LTBI vs HC
WBC 10^9/L	5.99 (4.93–8.07)	6.26 (5.23–8.29)	5.26 (4.91–6.27)	NS	NS	NS
Neutrophil 10^9/L	3.94 (2.57–5.54)	4.36 (3.07–6.43)	3.19 (2.79–3.66)	NS	NS	0.02
Lymphocyte 10^9/L	1.38 (0.99–1.74)	1.57 (1.05–1.85)	1.74 (1.50–2.07)	NS	0.02	NS
Monocyte 10^9/L	0.44 (0.30–0.54)	0.39 (0.27–0.62)	0.29 (0.23–0.33)	NS	0.01	0.02
Eosinophil 10^9/L	0.12 (0.08–0.30)	0.08 (0.02–0.32)	0.11 (0.08–0.13)	NS	NS	NS
Basophil 10^9/L	0.04 (0.02–0.05)	0.03 (0.01–0.04)	0.03 (0.02–0.04)	NS	NS	NS
RBC 10^{12}/L	4.21 (3.91–4.53)	4.32 (3.65–5.01)	4.69 (4.25–5.08)	NS	NS	NS
Platelet 10^9/L	257.0 (176.3–307.0)	231.0 (186.5–261.3)	197.0 (164.3–217.3)	NS	0.01	0.02

NS: No Significance.

ATB ranged from 0.66 to 0.98, with the sensitivity of 52–96% and the specificity of 46–96%. The ROC and AUC analysis showed that the diagnostic efficacy of MCP-1 was significantly higher than that of the other 7 cytokines. Additionally, we demonstrated that the 8-biomarker combination may be used to diagnose ATB from HC, with the sensitivity and specificity of 100%. This suggested that the 8-cytokine combination could be used as a biosignature to differentiate between ATB and HC.

Differentiating ATB from LTBI is very challenging in laboratory diagnosis. Here we identified 3 biomarkers that differentiated ATB from LTBI, including MDC, eotaxin, and MCP-1. Of the three, MCP-1 exhibited higher diagnostic efficacy than the other two. Moreover, the 3-biomarker combination increased the sensitivity (88%) and specificity (92%) of differentiating ATB from LTBI, which are superior to these reports.[15–17] The results above demonstrated that the expression pattern of multiple plasma cytokines can be used as the biosignature to differentiate between ATB and LTBI.

In the discovery cohort, the ROC and AUC analysis showed that MCP-1 was the only biomarker that exhibited the highest diagnostic efficacy among all cytokines. MCP-1 also differentiated between different MTB infection stages (ATB, LTBI, and HC). To validate these results, we recruited a validation cohort comprising 78 individuals. Since the reliability, reproducibility and sensitivity of ELISA were comparable to Luminex method,[18,19] and based on cost considerations, we selected ELISA other than the Luminex technology to analyze the MCP-1 levels of the validation cohort. The results in the validation cohort were generally consistent with the results in the discovery cohort. In addition, we also discovered

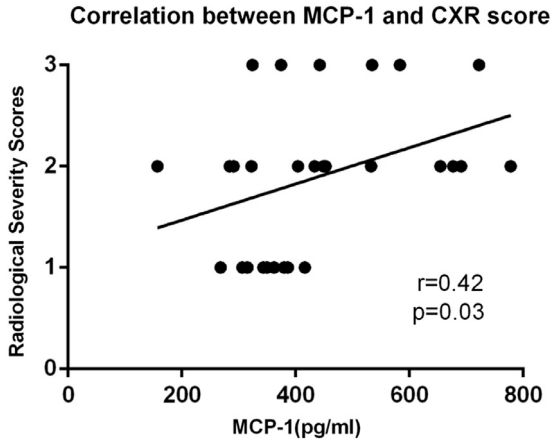

Fig. 6. Correlation between plasma levels of MCP-1 and lung injury in ATB patients of validation cohort. Lung injury of ATB patients was classified in a double-blind test and then divided into three grades. Images are representative of each grade – minimal (1), moderate (2) and advanced (3) disease. The level of MCP-1 was positive correlated with degree of lung injury in patients with active TB. Spearman's rank correlation coefficient, the corresponding p value, and the line of best fit are shown.

Table 5
Correlations of MCP-1 with hematological parameters in validation cohort.

Measurements	ATB: MCP-1 (pg/ml) $r(p)$	LTBI: MCP-1 (pg/ml) $r(p)$	HC: MCP-1 (pg/ml) $r(p)$
WBC 10^9/L	0.34(0.08)	−0.04(0.86)	−0.05(0.80)
Neutrophil 10^9/L	0.16(0.40)	0.01(0.94)	−0.17(0.40)
Lymphocyte 10^9/L	0.30(0.12)	−0.09(0.65)	0.17(0.40)
Monocyte 10^9/L	−0.03(0.88)	−0.14(0.51)	−0.02(0.92)
Eosinophil 10^9/L	0.27(0.17)	0.01(0.97)	−0.07(0.74)
Basophil 10^9/L	0.04(0.06)	−0.06(0.76)	−0.19(0.34)
RBC 10^{12}/L	0.32(0.09)	0.14(0.49)	0.21(0.31)
Platelet 10^9/L	−0.12(0.55)	0.06(0.76)	0.37(0.07)

$r(p)$: Spearman correlation coefficient (p value).

that the plasma MCP-1 level was not correlated with the count of leukocytes, red blood cells, or platelet in the peripheral blood, which further supports the advantages of using MCP-1 as a single TB diagnostic biomarker. Moreover, our data indicated that MCP-1 concentration was positively correlated with the imaging-based lung injury severity in ATB patients. This correlation is consistent with the results of Ganachari et al.,[20] wherein the high TB severity was associated with the high MCP-1 level. This suggests that MCP-1 may serve as a biomarker that monitors TB progression.

The 8 cytokines that differentiate between ATB, LTBI and HC included 5 chemokines (eotaxin, MDC, MIP-1α, IP-10, and MCP-1), 2 pro-inflammatory cytokines (IL-1α and TNF-α), and 1 anti-inflammatory cytokine (IL-10). All of the 8 cytokines are related to the TB development process.[8] Among them, MCP-1 is a key mediator in the inflammatory process as its signaling induces the production of many pro-inflammatory cytokines.[21] Furthermore, MCP-1 could induce the formation of granuloma and inhibits the spread of MTB.[22] Some publications have suggested that the 8 cytokines we studied here may be used as biomarkers for ATB and/or LTBI diagnosis and differential diagnosis. The results in our research were basically consistent with some reports,[16,17,23–28] while some data were not fully consistent with other published papers.[29–31] We speculate that the reasons may be related to the racial difference of the study subjects,[32] the type difference of plasma sample [29,33] and the results may also be affected by HC selection bias.[34]

Notably, IGRA have better sensitivity and specificity compared to traditional diagnosis methods.[35,36] However, neither clinical methods (such as IGRA), nor TST, differentiate ATB and LTBI,[37] thus no methods can validate the clinical TB diagnosis. The innovation of the present study was that we identified three plasma cytokines that differentiated ATB from LTBI, including MDC, eotaxin, and MCP-1. Of the three biofactors, MCP-1 exhibited the highest diagnostic efficacy, and differentiated ATB from HC. In addition, we demonstrated that plasma MCP-1 level was not correlated with the blood cell counts (Table 5). Therefore, MCP-1 could make up for the disadvantages of other clinical methods such as IGRA, and become a single plasma biomarker that diagnoses and differentiates ATB from LTBI.

This study has the following three limitations: first, the cytokines we screened may not be TB-specific biomarker. Because a control population (with lung diseases in whom TB disease is excluded) was lacking, other bacterial infection/inflammation may also induce the production of these cytokines by macrophages, dendritic cells, lymphocytes, NK cells, and other immune cells. Future studies are needed to include patients with other types of infection to assess whether these markers can differentiate TB from other bacteria-induced pneumonia. Second, the size of the study population was small. A future study with a larger size of study population is needed to validate our conclusion. Third, because the data from the discovery cohort demonstrated that MCP-1 was capable of differentiating the different stages of TB infection, we only tested the expression of MCP-1 in the validation cohort, which was also due to the high cost of ELISA testing. More cytokines will be validated in the future study with a larger sample size. Because of our limited knowledge of host immune responses to MTB infection and the resultant lack of adequate biomarkers for the different phases of infection, the mechanism of cytokines in pathogenesis of TB need to be discovery.

Collectively, we identified 8 cytokines that differentiate stages of TB infection (ATB and LTBI). Our study suggests that a test with these combined biomarkers can accurately diagnose ATB and LTBI. Our findings also revealed the potential value of MCP-1 in diagnosing ATB, differentiating ATB from LTBI, and evaluating the progress of ATB.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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References

1. World health organization, global tuberculosis report 2017, who press, geneva, 2017, available: http://www.Who.Int/tb/publications/global_report/en/.
2. Sharma S.K., Mohanan S., Sharma A. Relevance of latent TB infection in areas of high TB prevalence. *Chest* 2012;**142**:761–73.
3. O'Garra A, Redford P.S., McNab F.W., Bloom C.I., Wilkinson R.J., Berry M.P. The immune response in tuberculosis. *Annu Rev Immunol* 2013;**31**:475–527.
4. Chegou N.N., Hoek K.G., Kriel M., Warren R.M., Victor T.C., Walzl G. Tuberculosis assays: past, present and future. *Expert Rev Anti-Infect Ther* 2011;**9**:457–69.
5. Sohn H., Aero A.D., Menzies D., Behr M., Schwartzman K., Alvarez G.G., et al. Xpert mtb/rif testing in a low tuberculosis incidence, high-resource setting: limitations in accuracy and clinical impact. *Clin Infect Dis* 2014;**58**:970–6.
6. Al-Darraji H.A., Abd Razak H., Ng K.P., Altice F.L., Kamarulzaman A. The diagnostic performance of a single genexpert mtb/rif assay in an intensified tuberculosis case finding survey among HIV-infected prisoners in Malaysia. *PLoS One* 2013;**8**:e73717.
7. Sester M., Sotgiu G., Lange C., Giehl C., Girardi E., Migliori G.B., et al. Interferon-gamma release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. *Eur Respir J* 2011;**37**:100–11.

8. Domingo-Gonzalez R., Prince O., Cooper A., Khader S.A. Cytokines and chemokines in mycobacterium tuberculosis infection. *Microbiol Spectr* 2016;**4**.
9. Abakay O., Abakay A., Sen H.S., Tanrikulu A.C. The relationship between inflammatory marker levels and pulmonary tuberculosis severity. *Inflammation* 2015;**38**:691–6.
10. Deng W., Wang Y., Liu Z., Cheng H., Xue Y. Hemi: a toolkit for illustrating heatmaps. *PLoS One* 2014;**9**:e111988.
11. Torres Lde S., Okumura J.V., da Silva D.G., Belini Junior E., de Oliveira R.G., Mimura K.K., et al. Plasma levels of tgf-beta1 in homeostasis of the inflammation in sickle cell disease. *Cytokine* 2016;**80**:18–25.
12. Terashima T., Amakawa K., Matsumaru A., van Eeden S., Hogg J.C., Yamaguchi K. Bal induces an increase in peripheral blood neutrophils and cytokine levels in healthy volunteers and patients with pneumonia. *Chest* 2001;**119**:1724–9.
13. Hadley T.J., Peiper S.C. From malaria to chemokine receptor: the emerging physiologic role of the duffy blood group antigen. *Blood* 1997;**89**:3077–91.
14. Klinger M.H., Wilhelm D., Bubel S., Sticherling M., Schroder J.M., Kuhnel W. Immunocytochemical localization of the chemokines rantes and mip-1 alpha within human platelets and their release during storage. *Int Arch Allergy Immunol* 1995;**107**:541–6.
15. Biraro I.A., Kimuda S., Egesa M., Cose S., Webb E.L., Joloba M., et al. The use of interferon gamma inducible protein 10 as a potential biomarker in the diagnosis of latent tuberculosis infection in Uganda. *PLoS One* 2016;**11**:e0146098.
16. Shu C.C., Wu M.F., Hsu C.L., Huang C.T., Wang J.Y., Hsieh S.L., et al. Apoptosis-associated biomarkers in tuberculosis: promising for diagnosis and prognosis prediction. *BMC Infect Dis* 2013;**13**:45.
17. Luo J., Zhang M., Yan B., Zhang K., Chen M., Deng S. Imbalance of th17 and treg in peripheral blood mononuclear cells of active tuberculosis patients. *Braz J Infect Dis* 2017;**21**:155–61.
18. Carson R.T., Vignali D.A.. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *J Immunol Methods* 1999;**227**:41–52.
19. de Jager W., te Velthuis H., Prakken B.J., Kuis W., Rijkers G.T. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol* 2003;**10**:133–9.
20. Ganachari M., Guio H., Zhao N., Flores-Villanueva P.O. Host gene-encoded severe lung TB: from genes to the potential pathways. *Genes Immun* 2012;**13**:605–20.
21. Sawyer A.J., Tian W., Saucier-Sawyer J.K., Rizk P.J., Saltzman W.M., Bellamkonda R.V., et al. The effect of inflammatory cell-derived mcp-1 loss on neuronal survival during chronic neuroinflammation. *Biomaterials* 2014;**35**:6698–6706.
22. Slight S.R., Khader S.A. Chemokines shape the immune responses to tuberculosis. *Cytokine Growth Factor Rev* 2013;**24**:105–13.
23. Ragno S., Romano M., Howell S., Pappin D.J., Jenner P.J., Colston M.J. Changes in gene expression in macrophages infected with mycobacterium tuberculosis: a combined transcriptomic and proteomic approach. *Immunology* 2001;**104**:99–108.
24. Lienhardt C., Azzurri A., Amedei A., Fielding K., Sillah J., Sow O.Y., et al. Active tuberculosis in africa is associated with reduced th1 and increased th2 activity in vivo. *Eur J Immunol* 2002;**32**:1605–13.
25. Choi R., Kim K., Kim M.J., Kim S.Y., Kwon O.J., Jeon K., et al. Serum inflammatory profiles in pulmonary tuberculosis and their association with treatment response. *J Proteomics* 2016;**149**:23–30.
26. Mihret A., Bekele Y., Bobosha K., Kidd M., Aseffa A., Howe R., et al. Plasma cytokines and chemokines differentiate between active disease and non-active tuberculosis infection. *J Infect* 2013;**66**:357–65.
27. Zhang J.A., Liu G.B., Zheng B.Y., Lu Y.B., Gao Y.C., Cai X.Z., et al. Tuberculosis-sensitized monocytes sustain immune response of interleukin-37. *Mol Immunol* 2016;**79**:14–21.
28. Gutlapalli V.R., Sykam A., Tenali S.P., Suneetha S., Suneetha L.M. High levels of plasma interferon gamma and +874t/a gene polymorphism is associated with HIV-TB co-infection. *Hum Immunol* 2016;**77**:1264–70.
29. Yao X., Liu Y., Liu Y., Liu W., Ye Z., Zheng C., et al. Multiplex analysis of plasma cytokines/chemokines showing different immune responses in active TB patients, latent TB infection and healthy participants. *Tuberculosis* 2017;**107**:88–94.
30. Djoba Siawaya J.F., Chegou N.N., van den Heuvel M.M., Diacon A.H., Beyers N., van Helden P., et al. Differential cytokine/chemokines and kl-6 profiles in patients with different forms of tuberculosis. *Cytokine* 2009;**47**:132–6.
31. Yu Y., Zhang Y., Hu S., Jin D., Chen X., Jin Q., et al. Different patterns of cytokines and chemokines combined with ifn-gamma production reflect mycobacterium tuberculosis infection and disease. *PLoS One* 2012;**7**:e44944.
32. Coussens A.K., Wilkinson R.J., Nikolayevskyy V., Elkington P.T., Hanifa Y., Islam K., et al. Ethnic variation in inflammatory profile in tuberculosis. *PLoS Pathog* 2013;**9**:e1003468.
33. Fujisawa T., Fujisawa R., Kato Y., Nakayama T., Morita A., Katsumata H., et al. Presence of high contents of thymus and activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophage-derived chemokine in patients with atopic dermatitis. *J Allergy Clin Immunol* 2002;**110**:139–46.
34. Meenakshi P., Ramya S., Lavanya J., Vijayalakshmi V., Sumanlatha G. Effect of ifn-gamma, il-12 and il-10 cytokine production and mrna expression in tuberculosis patients with diabetes mellitus and their household contacts. *Cytokine* 2016;**81**:127–36.
35. Dai Y., Feng Y., Xu R., Xu W., Lu W., Wang J. Evaluation of interferon-gamma release assays for the diagnosis of tuberculosis: an updated meta-analysis. *Eur J Clin Microbiol Infect Dis* 2012;**31**:3127–37.
36. Li T., Bao L., Diao N., Sun F., Gao Y., Wong K.W., et al. Influencial factors of the performance of interferon-gamma release assays in the diagnosis of childhood tuberculosis. *Clin Exp Med* 2015;**15**:303–9.
37. Haas C.T., Roe J.K., Pollara G., Mehta M., Noursadeghi M. Diagnostic 'omics' for active tuberculosis. *BMC Med* 2016;**14**:37.