Comparative Analysis of Cytokine mRNA Expressions in Human Tissues With Mycobacterium Tuberculosis Infection

Sung-Bae Park
Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan

Heechul Park
Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan

Yoon-Sung Choi
Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan

Ji Young Park
Department of Internal Medicine, Kosin University Gospel Hospital, Busan

Dongsup Lee
Department of Biomedical Laboratory Science, Hyejeon College, Hongsung

Jungho Kim
Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan

Hee-Kyung Chang
Department of Pathology, Kosin University College of Medicine, Busan

Sunghyun Kim (shkim0423@cup.ac.kr)
Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan

Research Article

Keywords: Tuberculosis, Mycobacterium tuberculosis, mRNA expression, Cytokine, Human FFPE tissue

Posted Date: January 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-146530/v1

Abstract

Background: One of the widely used diagnostic methods for *Mycobacterium tuberculosis* (MTB) infection is the acid-fast bacilli staining of formalin-fixed paraffin-embedded (FFPE) tissues; however, this method cannot discriminate between MTB and nontuberculous mycobacteria (NTM) species. Moreover, confirming tuberculosis (TB) using FFPE tissue specimens may be difficult owing to their low bacterial load. In addition, interference in molecular diagnostic assays, including polymerase chain reaction (PCR), may occur owing to fragmentation and genomic DNA cross-linkage in FFPE tissues formed during formalin fixation or paraffin-embedding procedures. Therefore, we aimed to investigate whether an automated molecular diagnostic method based on PCR-reverse blot hybridization assay can discriminate between human MTB-positive and -negative FFPE tissues and to compare the relative mRNA expression levels of various host immune markers between MTB-infected and uninfected human tissues using quantitative reverse transcription (qRT) PCR. A total of 52 human FFPE tissue samples from various regions of the body, including the lungs, lymph nodes, tendons, colon, and appendix, were collected and used for the molecular identification of *Mycobacterium* species and analysis of cytokine mRNA expression.

Results: *IFN-γ, TNF-α, IP-10, CXCL9, CXCL11*, and *GM-CSF* mRNA expression levels in MTB-infected tissues were significantly higher than those in uninfected samples. Additionally, the differences in the mRNA expression levels of *IFN-γ, CXCL9*, and *GM-CSF* between MTB-infected and uninfected tissues were statistically significant were statistically significant ($p < 0.05$). Correlation curve analysis indicated that the mRNA expression of *IFN-γ* was inversely proportional to that of *IP-10* and that the mRNA expression levels of *IFN-γ, TNF-α, CXCL9, CXCL11, GM-CSF*, and *TNFR* were proportional and well-correlated. Furthermore, to establish marker profiles for detecting MTB infection, the statistically significant expression levels of three markers were combined. We confirmed that the combined profile of *IFN-γ, CXCL9*, and *GM-CSF* expression levels was statistically significant ($P < 0.001$).

Conclusions: Although the mRNA expression patterns of host immune markers may vary according to MTB infection status, these patterns may be highly correlated and can be simultaneously used as an additional indicator for diagnosing TB in human tissue samples.

Background

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB) and remains a serious health problem worldwide [1]. According to a World Health Organization report in 2018, the Republic of Korea had the highest prevalence and mortality rate for TB among countries in the Organization for Economic Cooperation and Development [2]. Moreover, cases of multi-drug-resistant and extensively drug-resistant TB are rapidly increasing, leading to increased difficulties in efficient TB treatment and control [3].

Globally, one of the most widely used diagnostic methods for detecting MTB infection is the acid-fast bacilli (AFB) staining of formalin-fixed paraffin-embedded (FFPE) tissues; however, this method is affected by some factors: FFPE tissues may contain relatively low bacterial load, and granulomas may be caused by other kinds of microbes, including other bacterial and fungal species such as *Cryptococcus* species and *Listeria monocytogenes*. Moreover, AFB stains cannot discriminate between MTB and nontuberculous mycobacteria (NTM) infections [4,5]. Alternative diagnostic tools to detect MTB and simultaneously identify mycobacterial species in tissue samples are therefore needed to improve TB diagnosis and treatment [6].

Molecular diagnostic methods, including polymerase chain reaction (PCR) and real-time PCR, have recently been used to effectively diagnose and identify mycobacterial species in various types of clinical samples [4]; however, interferences in molecular diagnostic assays may occur owing to fragmentation and genomic DNA (gDNA) cross-links in FFPE tissues formed during formalin fixation or paraffin-embedding procedures [7].

Several different immune markers such as cytokines, chemokines, immune regulators, and immune stimulating factors have recently been shown to play important roles in hosts infected with MTB [8]. In a study, tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine, was reportedly implicated in not only the proliferation and differentiation of immune cells but also in inflammatory processes, including apoptosis of MTB-infected cells [9]. In another study, interferon-gamma (IFN-γ) and antigen-specific T cells reportedly controlled MTB infection [10]. In addition, interleukin 2 (IL-2) levels have been found to be significantly higher in patients with active TB than in healthy control groups [11], and IL-4 has also been reported to act as an anti-inflammatory cytokine in cases of TB infection [12]. Furthermore, C-X-C motif chemokine 9 (CXCL9; a monokine induced by IFN-γ) levels in the plasma of patients with active pulmonary TB are higher than that in the plasma of healthy individuals [8]. CXCL10 (an IFN-γ–inducible protein of 10, IP-10) levels have also been found to be elevated in patients with TB lymphocytic pleural effusion [13], and CXCL11 (an IFN-γ inducible T cell alpha chemoattractant) levels have been shown to be significantly increased by stimulation with an MTB antigen [14]. Importantly, research has also shown that the mRNA levels of these immune markers can be precisely measured in blood immune cells to distinguish among individuals with active TB, those with latent TB infection (LTBI), and healthy controls [15]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a biomarker that can be used to differentiate between persons with active TB and QuantiFERON TB Gold In Tube (QFT-GIT) test-positive persons [16]. Higher levels of plasma CCL11 (eotaxin) and TNF receptor (TNFR) have also been associated with loss of $CD4^+$ T cells [17].

Here, we aimed to investigate whether an automated molecular diagnostic method based on PCR-reverse blot hybridization assay (REBA) can discriminate between human MTB-positive FFPE tissues and MTB- and NTM-negative FFPE tissues and to compare the mRNA expression levels of various host immune markers in MTB-infected human tissues using quantitative reverse transcription PCR (qRT-PCR) TaqMan probe assays. We also attempted to overcome the limitations of conventional DNA testing by targeting

small amounts of fragmented DNA from MTB-infected FFPE tissues, and we present here an evaluation of the usefulness of this diagnostic method.

Results

Demographics

The demographics of the participants are shown in Table 1. The mean age of the participants from whom samples were collected was 51.7 years (range 17–87) and the male-to-female ratio was 2.05:1 (67.3% to 32.7%). FFPE tissues were obtained from various regions of the body, including the colon (*n* = 14, 26.9%), lungs (*n* = 13, 25.0%), ileum (*n* = 7, 13.6%), lymph nodes (*n* = 6, 11.6%), skin (*n* = 3, 5.8%), ears (*n* = 2, 3.8%), and appendix, bronchus, finger joint, kidney, testis, tendon, and soft tissue (each *n* = 1, 1.9%).

Mycobacterial infection pattern

REBA Myco-ID® assay was performed to investigate mycobacterial infections; the results are shown in Table 2. Among the 52 samples, 11 samples (21%) were MTB-positive, 20 (39%) were NTM-positive, 8 (15%) were MTB- and NTM-double positive, and 13 (25%) were negative.

Gene expression profiling of host immune markers in MTB-infected and uninfected tissue samples

Of the 25 target genes assessed, the gene expression levels of *CXCL9*, *CXCL10*, *CCL2*, *CCL3*, *CCL4*, *CCL11*, *IL1β*, *IL-2R*, *TNFR,* and *IL-6R* were higher in MTB-infected tissues than in uninfected tissues; however, the mRNA expression levels of *CXCL9*, *CCL2*, *CCL3*, *CCL4*, *IL1β,* and *IL-6R* varied in MTB-infected tissues, even though they exhibited higher mRNA expression levels. The mRNA expression levels of *IFN-γ*, *TNF-α*, *IL-2*, *IL-4*, and *CXCL11* reportedly increased upon infection with MTB based on previous studies; however, the differences in the mRNA expression levels of *IL-6* and *IL-8* between MTB-infected and uninfected tissues were not significant (Table 3).

mRNA expression patterns in samples according to MTB infection

Shown in Figure 1 are the mRNA expression levels of MTB-positive and MTB-negative tissues. The mRNA expression levels of *IFN-γ*, *TNF-α*, *CXCL9*, *CXCL10*, *CXCL11*, and *GM-CSF* were relatively higher in MTB-positive tissues than in MTB-negative tissues. In particular, *IFN-γ*, *CXCL9*, and *GM-CSF* were significantly higher ($p < 0.05$) in MTB-positive tissues than in MTB-negative tissues. *IL-2* and *CCL11* mRNA expression levels were lower in MTB-positive tissues than in MTB-negative tissues; however, the mRNA expression levels of *IL-2R* and *TNFR* were not different between MTB-positive tissues and MTB-negative tissues. *IL-4* mRNA expression levels were not detected owing to low levels. To establish marker profiles for detecting MTB infection, the expression levels of three markers whose differences between MTB-positive and MTB-negative tissues were statistically significant were combined and validated. The differences in the

combined profile of *IFN-γ*, *CXCL9*, and *GM-CSF* expression levels discriminated between MTB-positive and -negative tissues and was also statistically significant ($P < 0.001$).

Correlation curve analysis patterns of mycobacterial infection

The correlation curve analysis was based on the expression levels of *IFN-γ*, which is known to specifically increase in host tissues with MTB (Figure 2). The mRNA expression levels of *IFN-γ* were significantly correlated with those of *TNF-α, CXCL9, CXCL11, GM-CSF,* and *TNFR*; however, there was no correlation between the mRNA expression levels of *IFN-γ* and *IL-2* or *IL-2R*. Interestingly, the mRNA expression levels of *IFN-γ* and *CXCL10* were inversely proportional.

Receiver operating characteristic (ROC) curve analysis of the mRNA expression levels of host immune markers

ROC curve analysis was performed to ensure that the results were clinically applicable (Figure 3). The area under the curve (AUC) was 0.696 ($p = 0.0414$) for *IFN-γ*, 0.625 ($p = 0.1934$) for *TNF-α*, 0.679 (p = 0.063) for *CXCL10* (IP-10), 0.562 (p = 0.516) for *IL-2R*, 0.598 (p = 0.312) for *IL-2*, 0.511 (p = 0.925) for *CCL11*, 0.736 (p = 0.015) for *CXCL9*, 0.677 (p = 0.068) for *CXCL11*, 0.696 (p = 0.044) for *GM-CSF*, and 0.580 (p = 0.412) for *TNFR*. Only the *p*-values pertinent to *IFN-γ* and *CXCL9*, among 10 host immune marker genes, were significant ($p < 0.05$); the AUCs of both were approximately 0.7. In addition, the *p*-value and the AUC of the combination of *IFN-γ, CXCL9*, and *GM-CSF* expression levels were 0.0016 and 0.7989, respectively.

Discussion

TB remains a serious global health problem [18]. NTM infections make the accurate diagnosis and treatment of TB challenging [19], and effective methods are needed to accurately identify different mycobacterial species. Therefore, we determined, using an automated molecular mycobacterial identification system, and analyzed the mRNA expression patterns of various host immune markers in tissues according to MTB infection status. Recent studies have shown that many cytokines play an important role in cell-mediated immune responses to MTB infections [20]. The gold standard methods for analyzing host immune markers in FFPE tissues are immunohistochemistry (IHC) staining and flow cytometry analysis in which target-specific antibodies and homogenized tissues are used; however, these methods require a large amount of samples to simultaneously detect multiple markers. The simultaneous detection of multiple host markers in a small amount of samples and analysis of mRNA expression levels using molecular diagnostic assays with higher analytical sensitivity and specificity than IHC and flow cytometry methods may improve the diagnosis of TB. Therefore, we aimed to profile the gene expression patterns of 11 host immune markers in MTB-positive and -negative FFPE tissues.

Gene expression patterns were profiled using QuantiGene® Plex assay (Thermo Fisher Scientific), and out of a total of 25 immune cytokine genes, the mRNA expression levels of *IFN-γ, TNF-α, IL-2, IL-2R, IL-4, CXCL9, CXCL10, CXCL11,* and *GM-CSF*, which are known to increase upon infection with MTB, were

higher than those in uninfected tissues. Specifically, *TNFR* and *CCL11* expression levels were significantly increased. Although the expression levels of *CCL2, CCL3, CCL4, IL1β*, and *IL-6R* were also significant, the differences among sample results were borderline significant and therefore were excluded.

Therefore, to definitively confirm the gene expression patterns of 11 host immune markers, we performed qRT-PCR analyses using TaqMan probes. In a previous study, *IFN-γ* and *CXCL9* mRNA levels were found to be significantly higher in individuals with active pulmonary TB than in healthy controls [14]. In another study wherein the GM-CSF levels between IGRA-positive and -negative cases were compared, the cytokine levels in IGRA-positive cases were noted to be significantly higher than those in negative cases [16]; our results are consistent with this finding in tissue samples with and without MTB infection. Furthermore, in our ROC curve analysis, the AUCs of *IFN-γ, CXCL9*, and *GM-CSF* were approximately 0.7. To establish marker profiles that can discriminate between MTB-positive and -negative tissues, two groups, each consisting of combined and validated statistically significant mRNA levels of three immune markers, were used. A combined profile of *IFN-γ, CXCL9*, and *GM-CSF* mRNA expression levels can discriminate between MTB-positive and -negative groups and was statistically significant ($P < 0.001$). With this, even when clinical molecular diagnostic assays such as PCR-REBA and nested PCR assays for detecting MTB indicate negative results, accurately diagnosing a clinical case of TB is possible by analyzing the gene expression patterns of select host immune markers. Additionally, even if DNA fragmentation occurs, the mRNA levels of cytokines remain unaffected.

Our study has some limitations. First, a small sample size was used; further research with a larger sample would provide improved significance to succeeding results. Second, the method used in this study is not easily applicable in clinical settings owing to the use of tissue samples from patients. Our analysis was conducted as a preliminary study of the gene expression patterns in peripheral whole-blood samples for the detection of TB. Further research on cytokine analysis using peripheral whole-blood samples in patients with extrapulmonary TB would be required.

Conclusion

The mRNA expression levels of the host immune markers *IFN-γ, TNF-α, CXCL9, CXCL10, CXCL11, IL-2R,* and *GM-CSF* were higher in MTB-positive tissues than in MTB-negative tissues. Specifically, increased mRNA expression levels of *IFN-γ, CXCL9,* and *GM-CSF* were statistically significant ($p < 0.05$), and ROC curve data indicated that the AUCs of the combination of these three target mRNA expression levels exceeded 0.7. These results suggest that when TB is suspected, gene expression analysis may be used to accurately diagnose the presence of TB.

Methods

Patients and clinical samples

A total of 52 human FFPE tissue samples were collected between 2011 and 2017 by the Department of Pathology of Kosin University Gospel Hospital in Busan, Republic of Korea. Clinical data indicated that five of the samples were positive and 11 were negative for MTB infection according to Ziehl-Neelsen AFB staining. The other 36 samples were not assessed using AFB staining.

Genomic DNA extraction

gDNA extraction from FFPE tissue samples was performed using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the gDNA extracted from the FFPE tissues were measured using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), and the extracted DNA was stored at -20°C until further use.

Molecular identification of *Mycobacterium* species

To confirm MTB-positive and -negative FFPE tissues, we used REBA Myco-ID® Kit (YD Diagnostics, Yongin, Republic of Korea), for the molecular identification of *Mycobacterium* species, according to the manufacturer's instructions. A single PCR cycle was used for initial pre-denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 30 cycles at 65°C for 30 s, and one cycle at 72°C for 10 min. The amplified PCR products were then loaded into an HybREAD 480® System (YD Diagnostics), and all reactions were automatically performed by the instrument. After the processes were complete, the data were interpreted using an HybREAD 480® Scanner (YD Diagnostics).

Total RNA isolation

Total RNA isolation from human FFPE tissue samples was performed using PureLink™ FFPE Total RNA Isolation® kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The concentration and purity of isolated total RNA from FFPE tissues were measured using NanoDrop™ 2000 (Thermo Fisher Scientific), and the isolated RNA was then stored at -80°C until further use.

Reverse transcription (RT) reaction

RT reactions were performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) cDNA Synthesis kit (Thermo Fisher Scientific) and Random Hexamers (Invitrogen) according to the manufacturers' instructions. The mixture was initially heated at 65°C for 5 min and then incubated at 25°C for 10 min, 50°C for 50 min, and 70°C for 15 min. All reactions were performed using SimpliAmp Thermal Cycler (Life Technologies, Carlsbad, CA, USA). The final cDNA samples were stored at -20°C until further use.

Gene expression profiling by multiplex bead array

Gene expression profiling for targeting the mRNA (transcript) expression levels of 25 different host immune markers, including C-C motif chemokine ligand 2 (*CCL2*, monocyte chemoattractant protein 1, MCP1), *CCL3* (macrophage inflammatory protein 1-alpha, MIP-1α), *CCL4* (MIP-1β), *CCL11* (eotaxin), C-X-C

motif chemokine 8 (*CXCL8*, IL-8), *CXCL9* (MIG), *CXCL10* (IP-10), *CXCL11* (I-TAC), *GM-CSF, IFN-γ*, IFN-γ receptor 1 (*IFN-γR1*), *IL-1α* (IL-1F1), *IL-1β* (IL-1F2), *IL-2, IL-2Ra, IL-4, IL-5, IL-6, IL-6Ra, IL-10, IL-12, IL-17, IL-18, TNFRI*, and *TNF-α*, was analyzed using QuantiGene® Plex Gene Expression Assay (Thermo Fisher Scientific) and MAGPIX® multiplexing system (Luminex, Austin, TX, USA). All target gene expressions were normalized against three endogenous control genes encoding peptidyl-prolyl cis-trans isomerase B (PPIB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyltransferase 1 (HPRT1). The process was performed according to the manufacturer's instructions.

Quantitative RT-PCR TaqMan probe assays

qRT-PCR TaqMan probe assays (Roche Diagnostics, Mannheim, Germany and Applied Biosystems, Waltham, MA, USA) were performed to target the mRNA expression of multiple host immune markers ; qRT-PCR primer and TaqMan probe pairs were used to amplify the mRNA of Th1-type cytokines and receptors (*IFN-γ, TNF-α, TNFR, IL-2*, and *IL-2R*), Th2-type cytokine (*IL-4*), IFN-γ-induced chemokines (*CXCL9, CXCL10,* and *CXCL11*), chemotactic factor (*CCL11*), stimulating factor (*GM-CSF*), and internal reference protein (*GAPDH*).

Statistical analysis

All statistical analyses were performed using GraphPad Prism v. 5.00 (GraphPad Software, San Diego, CA, USA). Significant differences in the mRNA expression levels of MTB-positive and MTB-negative tissues, together with 90% confidence intervals (CIs), were calculated and compared using unpaired *t*-tests. Associations between the mRNA expression levels of IFN-γ and other cytokines were measured using Spearman's rank correlation coefficient (r). An ROC curve analysis was performed to confirm clinical applicability. A *p*-value of less than 0.05 was considered statistically significant.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the Kosin University Gospel Hospital (KUGH 2017-11-042). FFPE tissue samples were collected after obtaining informed consent for research with the approval of the Biobank of Kosin University Gospel Hospital and all experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The data analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sectors.

Authors' contributions

SK and HC designed the project and analyzed the data. SP and HP designed the paper, analyzed, wrote and drafted the paper. SP, HP, YC, JK, JP and DL collected the data and reviewed the paper critically. All authors read and approved the final paper.

Acknowledgments

This study was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) and was funded by the Ministry of Education (NRF-2016R1C1B108888 and NRF-2020R1C1C1007169) and the BB21 Plus Project in 2020.

References

1. Kaur G, Chander AM, Kaur G, Maurya SK, Nadeem S, Kochhar R, et al. A genomic analysis of *Mycobacterium immunogenum* strain CD11_6 and its potential role in the activation of T cells against *Mycobacterium tuberculosis*. BMC Microbiol. 2019;19:64.
2. World Health Organization. 2018. Global tuberculosis report.
3. Park YS, Lee CH, Lee SM, Yang SC, Yoo CG, Kim YW, et al. Rapid increase of non-tuberculous mycobacterial lung diseases at a tertiary referral hospital in South Korea. Int J Tuberc Lung Dis. 2010;14:1069-71.
4. Sun JR, Lee SY, Perng CL, Lu JJ. Detecting *Mycobacterium tuberculosis* in Bactec MGIT 960 cultures by Inhouse IS6110-based PCR assay in routine clinical practice. J Formos Med Assoc. 2009;108:116-25.
5. Lee HS, Park KU, Park JO, Chang HE, Song J, Choe G. Rapid, sensitive, and specific detection of *Mycobacterium tuberculosis* complex by real-time PCR on paraffin-embedded human tissues. J Mol Diagn. 2011;13:390-4.
6. Azov AG, Koch J, Hamilton-Dutoit SJ. Improved diagnosis of mycobacterial infections in formalin-fixed and paraffin-embedded sections with nested polymerase chain reaction. APMIS. 2005;113:586-93.
7. Amemiya K, Hirotsu Y, Oyama T, Omata M. 2018. Relationship between formalin reagent and success rate of targeted sequencing analysis using formalin fixed paraffin embedded tissues, Clinica Chimica

Acta, 488:129-34.

8. Kim S, Lee H, Kim H, Kim Y, Cho J, Jin H, et al. Diagnostic performance of a cytokine and IFN-γ induced chemokine mRNA assay after *Mycobacterium tuberculosis*-specific antigen stimulation in whole blood from infected individuals. J Mol Diagn. 2015;17:90-9.
9. Keane J, Balcewicz-Sablinska M, Remold H, Chupp G, Meek B, Fenton M, et al. Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. Infect Immun. 1997;65:298-304.
10. Mogues T, Goodrich M, Ryan L, LaCourse R, North R. The relative importance of T cell subsets in immunity and immunopathology of airborne *Mycobacterium tuberculosis* infection in mice. J Exp Med. 2001;193:271-80.
11. Sun Q, Wei W, Sha W. Potential role for *Mycobacterium tuberculosis* specific IL-2 and IFN-γ responses in discriminating between latent infection and active disease after long-term stimulation. PLoS One. 2016;11:e0166501.
12. Qi H, Sun L, Jin YQ, Shen C, Chu P, Wang S, et al. rs2243268 and rs2243274 of Interleukin-4 (IL-4) gene are associated with reduced risk for extrapulmonary and severe tuberculosis in Chinese Han children. Infect Genet Evol. 2014;23:121-8.
13. Santos A, Correa R, Ribeiro-Alves M, Soares A, Mafort T, Leung J, et al. 2018. Application of Venn's diagram in the diagnosis of pleural tuberculosis using IFN-γ, IP-10 and adenosin deaminase PLoS One. 2018;13:e0202481.
14. Chung W, Lee K, Jung Y, Kim Y, Park J, Sheen S, et al. Serum CXCR3 ligands as biomarkers for the diagnosis and treatment monitoring of tuberculosis. Int J Tuberc Lung Dis. 2015;19:1476-84.
15. Jeong Y, Hur Y, Lee H, Kim S, Cho J, Chang J, et al. Discrimination between active and latent tuberculosis based on ratio of antigen-specific to mitogen-induced IP-10 production. J Clin Microbiol. 2014;53:504-10.
16. Balcells M, Ruiz-Tagle C, Tiznado C, Garcia P, Naves R. Diagnostic performance of GM-CSF and IL-2 in response to long-term specific-antigen cell stimulation in patients with active and latent tuberculosis infection. Tuberculosis (Edinb.). 2018;112:110-9.
17. Gutierrez-Rivas M, Jimenez-Sousa MA, Rallon N, Jimenez JL, Restrepo C, Leon A, et al. High plasma levels of sTNF-R1 and CCL11 are related to CD4+ T-cells fall in human immunodeficiency virus elite controllers with a sustained virologic control, Front Immunol. 2018;9:1399.
18. Lee HJ, Ko HJ, Kim S, Jung YJ. Pasakbumin A control the growth of *Mycobacterium tuberculosis* by enhancing the autophagy and production of antibacterial mediators in mouse macrophages. PLoS One. 2019;14:e0199799.
19. Nasiri M, Dabiri H, Fooladi A, Amini S, Hamzehloo G, Feizabadi M. High rates of nontuberculous mycobacteria isolation from patients with presumptive tuberculosis in Iran. New Microbes New Infect. 2017;6:12-7.
20. Frahm M, Goswami ND, Owzar K, Hecker E, Mosher A, Cadogan E, et al. Discriminating between latent and active tuberculosis with multiple biomarker responses. Tuberculosis (Edinb.). 2011;91:250-6.

Tables

Table 1. Tissue sample sources

Characteristics	*n* (%)
Age (median = 51.7 SD ± 16.3; range = 17–87)	
≤ 39	11 (21.1)
40–59	21 (40.4)
≥ 60	20 (38.5)
Sex	
Male	35 (67.3)
Female	17 (32.7)
Locations	
Colon	14 (26.9)
Lung	13 (25.0)
Ileum	7 (13.6)
Lymph node	6 (11.6)
Skin	3 (5.8)
Ear	2 (3.8)
Bronchus	1 (1.9)
Testis	1 (1.9)
Tendon	1 (1.9)
Kidney	1 (1.9)
Appendix	1 (1.9)
Soft tissue (hand)	1 (1.9)
Finger joint	1 (1.9)
Total	**52 (100)**

Table 2. Molecular identification of *Mycobacterium* species using PCR-REBA

MTB infection	NTM infection	MTB & NTM infection	Negative	Total
n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
11 (21)	20 (39)	8 (15)	13 (25)	52 (100)

Table 3. mRNA expression levels of host immune markers measured using gene expression profiling assay

Target gene	Relative median fluorescent intensity (MFI) value (range)
IFN-γ	3.25 (0–6.4)
INFGR1	1.75 (0–4.4)
TNF-α	3.4 (0–4.6)
TNFRSF1A	21.9 (2.1–29.1)
IL-1A	2.3 (0–5.2)
IL-1β	4.45 (0–14.4)
IL-2	2.3 (0.8–7.5)
IL2RA	4.75 (0.7–8.9)
IL-4	2.65 (0–4.2)
IL-5	1.8 (0–2.5)
IL-6	0.7 (0–1.1)
IL-6R	7.4 (1.0–27.1)
IL-8	0.3 (0.1–0.5)
IL-10	2.75 (0.6–4.7)
IL-12A	3.35 (0–5.8)
IL-17A	2.1 (0.7–3.5)
IL-18	2.65 (0.2–4.6)
CXCL9	1.5 (0.1–24.8)
CXCL10 (IP-10)	4.4 (1.1–9.8)
CXCL11	2.1 (1–2.8)
CCL2	2.9 (0.6–44)
CCL3	4.85 (1.7–29.6)
CCL4	5.05 (0–22.4)
CCL11	10.45 (3.9–13)
CSF2	2.95 (1.4–3.9)

Figures

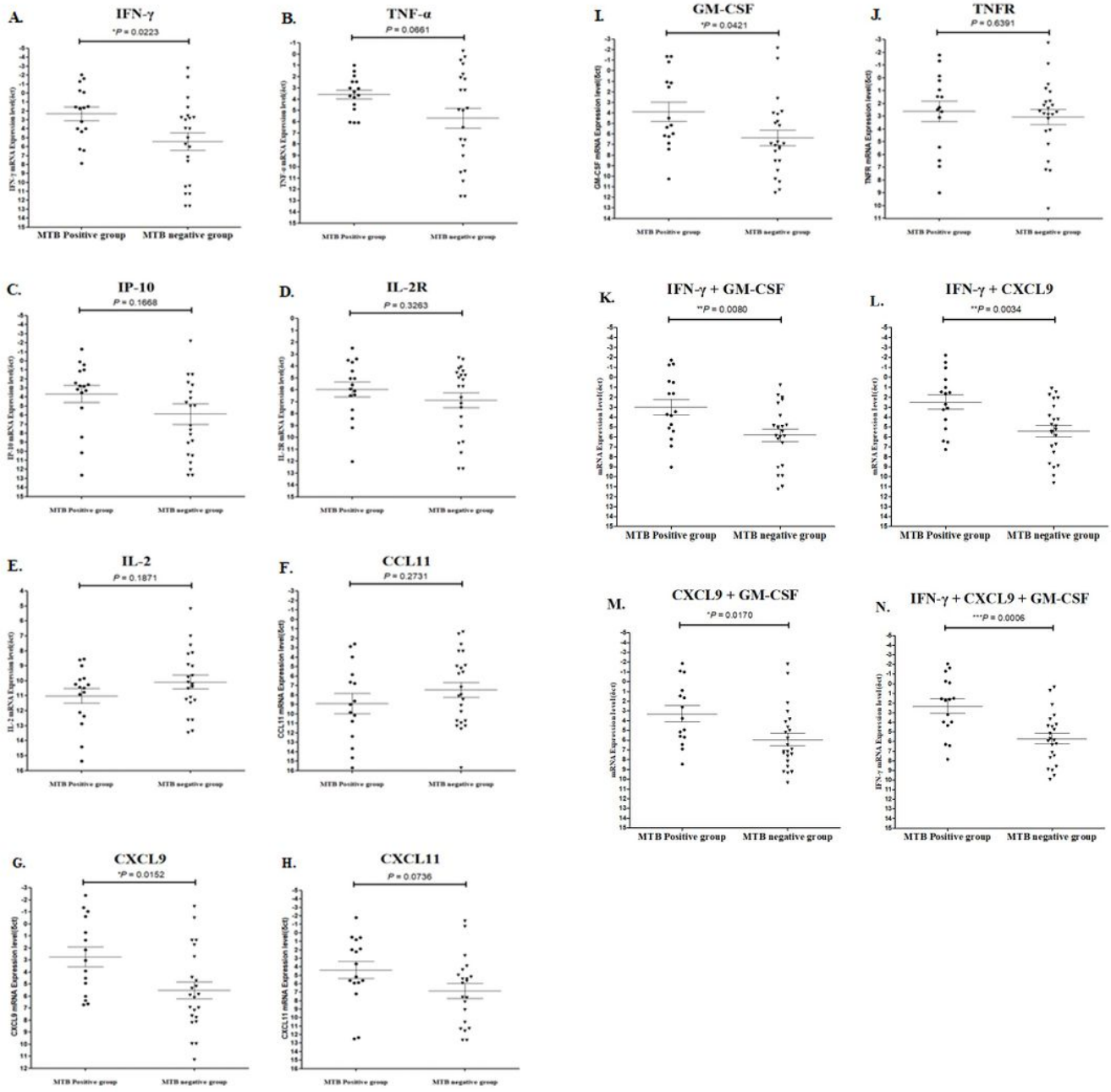

Figure 1

Comparison of the relative mRNA expression levels of host immune markers in tissues. A. Interferon-gamma (IFN-γ), B. Tumor necrosis factor-alpha (TNF-α). C. Interferon gamma-inducible protein 10 (IP-10). D. Interleukin-2 receptor (IL-2R). E. IL-2. F. CCL 11 (Eotaxin). G. Chemokine (C-X-C motif) ligand 9 (CXCL9).

H. CXCL11. I. Granulocyte-macrophage colony-stimulating factor (GM-CSF). J. TNFR. K. IFN-γ + GM-CSF. L. IFN-γ + CXCL9. M. CXCL9 + GM-CSF. N. IFN-γ + CXCL9 + GM-CSF.

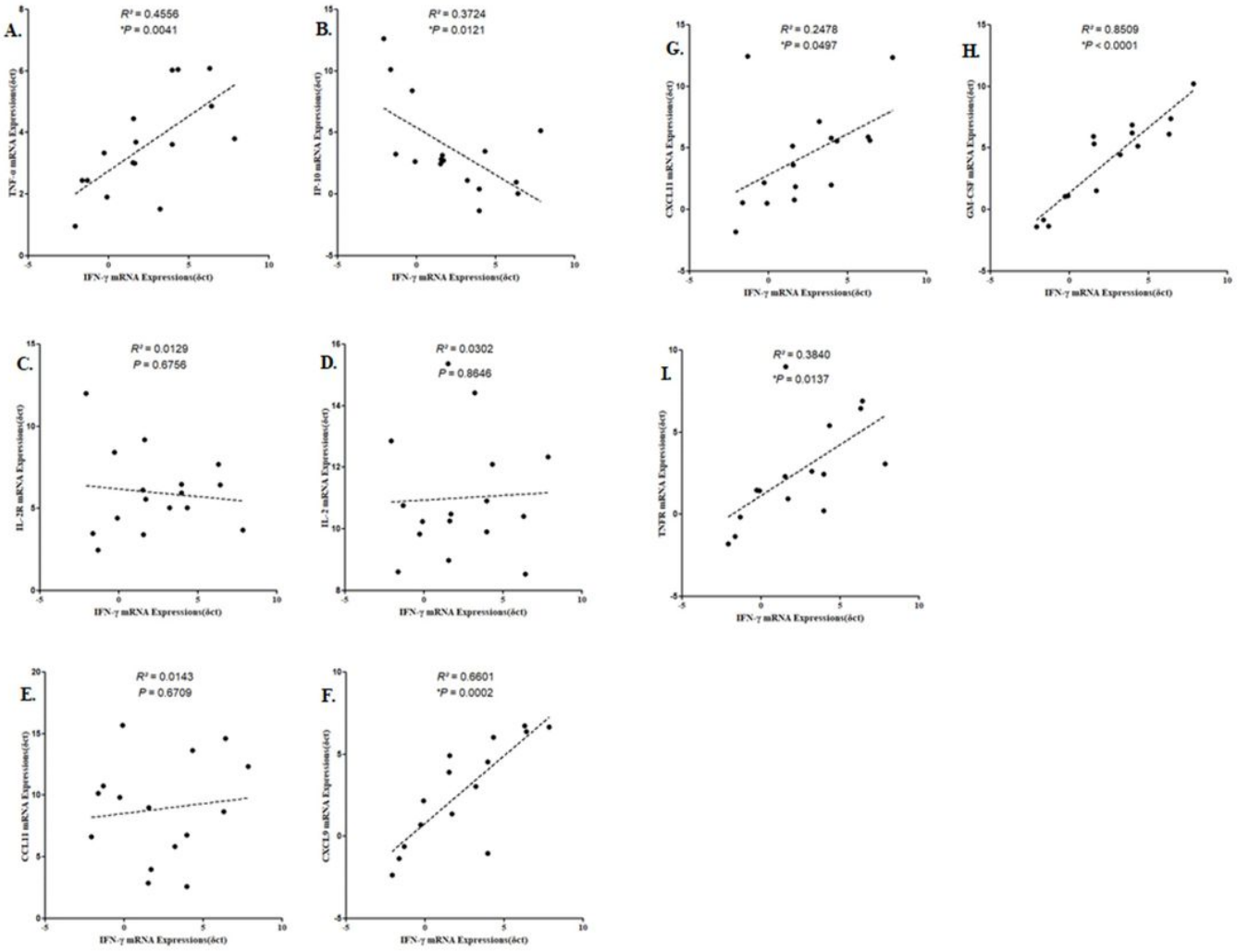

Figure 2

Correlation curve analysis of mRNA expression levels of IFN-γ and other host immune markers in tissues. A. Tumor necrosis factor-alpha (TNF-α). B. Interferon gamma-inducible protein 10 (IP-10). C. Interleukin-2 receptor (IL-2R). D. IL-2. E. CCL11 (Eotaxin). F. Chemokine (C-X-C motif) ligand 9 (CXCL9). G. CXCL11. H. Granulocyte-macrophage colony-stimulating factor (GM-CSF). I. TNFR.

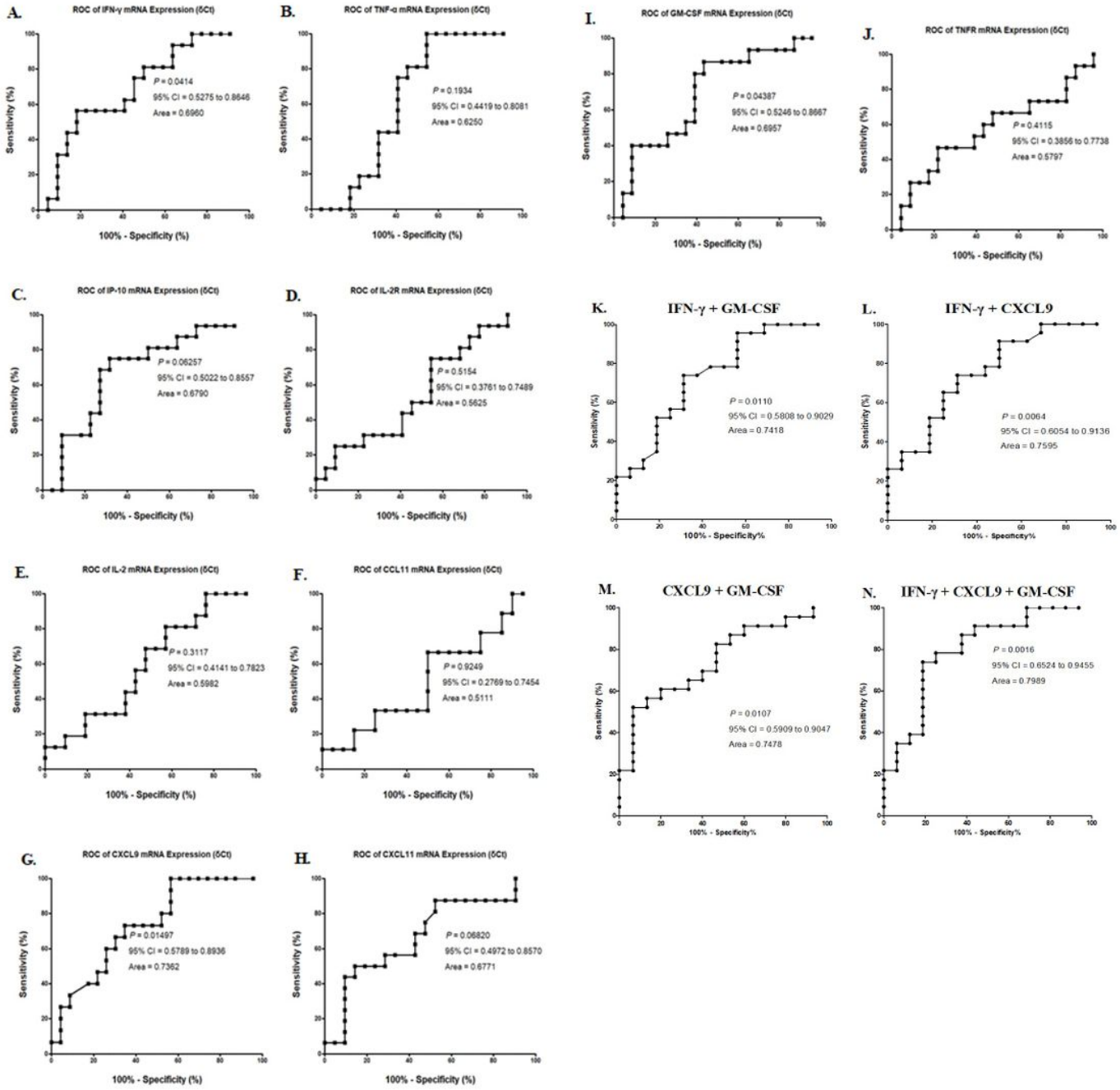

Figure 3

Receiver operating characteristic (ROC) curve analysis of host immune marker mRNA expression levels in tissues. A. Interferon-gamma (IFN-γ), B. Tumor necrosis factor-alpha (TNF-α). C. Interferon gamma-inducible protein 10 (IP-10). D. Interleukin-2 receptor (IL-2R). E. IL-2. F. CCL11 (Eotaxin). G. Chemokine (C-X-C motif) ligand 9 (CXCL9). H. CXCL11. I. Granulocyte-macrophage colony-stimulating factor (GM-CSF). J. TNFR. K. IFN-γ + GM-CSF. L. IFN-γ + CXCL9. M. CXCL9 + GM-CSF. N. IFN-γ + CXCL9 + GM-CSF