Infection and Drug Resistance

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ORIGINAL RESEARCH

Increased Expression of IL-10 in Peripheral Blood Mononuclear Cells Correlates with Negative Interferon-γ Release Assay Results in Culture-Confirmed Tuberculosis Patients

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Introduction: Interferon-γ release assays (IGRAs) can have high false-negative rates for active tuberculosis (TB) cases. Here we investigated factors, including potential anti-inflammatory mechanisms, that contributed to false-negative IGRA results.

Methods: We established two cohorts. In the first cohort, we reviewed IGRA results for confirmed TB cases diagnosed in our hospital in 2018. Cases with false-negative IGRA results were analysed to identify factors contributing to false-negative results. In the second cohort, we prospectively studied IL-10 expression levels in peripheral blood mononuclear cells (PBMCs) of IGRAs-positive and IGRAs-negative TB cases after antigenic stimulation to correlate IL-10 expression with IGRAs results.

Results: Of 1232 culture-confirmed TB cases, 1124 produced true-positive IGRA results and 108 had false-negative IGRA results. Multivariate logistic regression analysis identified glucocorticoid use and extrapulmonary TB as independent risk factors for false-negative IGRA results. Notably, IL-10 expression of the IGRA-negative group was significantly up-regulated as compared to that of the IGRA-positive group. The average cell supernatant IL-10 concentration of the IGRA-negative group was 4.77 pg/mL, a value that was statistically greater than the IGRA-positive group concentration (1.47 pg/mL, P = 0.007). After PBMCs pretreatment with BRD6989 (to enhance IL-10 secretion), average IFN-γ concentrations in cell supernatants from the IGRA-positive group significantly decreased from 59.73 pg/mL to 33.79 pg/mL (P = 0.011). By contrast, addition of AS101 (to inhibit IL-10 secretion) to false-negative group PBMCs led to an increase of average IFN-γ concentration in cell supernatants from 19.01 pg/mL to 45.10 pg/mL (P = 0.030), a result that was inversely correlated with IL-10 concentration.

Conclusion: Our data demonstrate that increased IL-10 secretion by PBMCs is inversely correlated with IGRA assay results in culture-confirmed TB patients. Glucocorticoids use and extrapulmonary TB are significantly associated with false-negative IGRA results. Combination testing to measure IL-10 secretion and IFN-γ release is recommended to improve IGRAs specificity.

Keywords: tuberculosis, interferon-γ release assay, interleukin 10

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB) complex, remains a global public health concern.[1,2] In 2019, approximately 10.0 million incident cases and 1.41 million deaths were attributed to TB worldwide, making it the leading cause of death from an infectious agent.[1] China has the third-highest TB

Received: 9 April 2021
Accepted: 1 July 2021
Published: 13 August 2021

burden globally, behind India and Indonesia.[1] Due to effective control strategies, the current incidence of TB in China has continually declined in recent decades.[3] Nevertheless, despite great achievements in TB control and prevention, only one-third of reported cases have been tested to confirm they are actual TB cases.[1,4] This high prevalence of unconfirmed TB cases has prompted clinicians to reevaluate the current diagnostic algorithm, while highlighting the need for novel and reliable diagnostic tests for early detection of TB cases.[5]

The interferon-γ release assay (IGRA), the predominant test used to diagnose latent TB infection, is based on detection of MTB-specific T cells that secrete IFN-γ.[6] In BCG-vaccinated populations, IGRAs provide superior specificity, as compared to tuberculin skin tests, for detecting past TB exposure.[7,8] Despite its limited utility for diagnosing active disease, IGRAs are still recommended in national guidelines of many countries as an alternative test for detection of active TB.[9,10] As an additional concern, negative IGRA results are often used to dismiss an active disease diagnosis even though results of previous observational studies suggest that approximately 8–19% of TB patient IGRA results are negative.[11] Consequently, inappropriate interpretations of false negative results will likely lead to misdiagnoses of cases as latent TB that are actually active TB cases. In turn, such diagnostic failures will lead to additional community transmission of TB and delayed therapeutic delivery to eliminate active infection in misdiagnosed patients with active disease.

Several risk factors for false negative IGRA results have been reported, such as advanced age, glucocorticoid therapy and low peripheral lymphocyte counts.[11,12] Even so, a proportion of active patients without apparent risk factors have negative IGRA results, raising the possibility that a certain immunological mechanism in fact exists that dampens IFN-γ secretion by host cells. Such a mechanism may alter the balance between different types of key cytokines during TB infection and disease stages[13] or invoke production of multiple anti-inflammatory cytokines that could inhibit secretion of IFN-γ.[14,15] We therefore hypothesise that upregulation of certain anti-inflammatory cytokine(s) may be associated with false IGRA results in TB patients. To test our hypothesis, we conducted a retrospective study to investigate factors affecting IGRAs performance and also investigated potential anti-inflammatory mechanisms associated with false negative results. These results should provide a foundation on which to improve the currently used diagnostic algorithm for detecting active TB cases from IGRA results.

Materials and Methods

Setting

This study was conducted in the Beijing Chest Hospital, a national TB-specialised hospital. Two separate cohorts were included in our analysis. In the first cohort, we retrospectively collected 2403 patients with symptoms suggestive of TB from January 2018 to December 2018. Only laboratory-confirmed TB cases with valid IGRA results were included in the final analysis. Laboratory-confirmed TB cases were defined as individuals with either a positive aetiological or pathological result.[16] Detailed patient information, including demographic data, clinical diagnosis, laboratory examination findings, treatment complications and medication history, were obtained from electronic medical records. These data were subsequently analysed to identify risk factors associated with false-negative IGRA tests. In the second cohort, culture-confirmed TB patients with false-negative IGRA results were subjected to additional testing to reveal underlying mechanisms responsible for the absence of lymphocyte-derived IFN-γ release; these results were paired with control results obtained from culture-positive TB patients with positive IGRA results. Patients undergoing immunosuppression due to malignancy, solid organ transplantation or immunosuppressive treatment were excluded. All procedures were conducted in accordance with the Declaration of Helsinki. This study was approved by the Ethic Committee of Beijing Chest Hospital affiliated with Capital Medical University. Patients provided written informed consent prior to enrollment.

Cell Culture

Ten milliliters of whole blood were collected from each patient. Peripheral blood mononuclear cells (PBMCs) were separated as previously reported. Next, PBMCs (10×10^6 cells/mL) were cultured in RPMI 1640 (1×, Corning, Manassas, USA) supplemented with 10% foetal bovine serum and 2 mM L-glutamine. Next, each plate was incubated at 37°C with 5% CO_2. After stimulation of PBMCs with peptides of TB-specific antigens ESAT-6 and CFP-10 (Oxford Immunotec, Oxfordshire, UK) for 24 h, culture supernatant and PBMCs were separately collected by centrifugation (3000 rpm, 25°C, 20 min) before they were analysed to determine cytokine expression patterns. In addition, PBMCs from IGRA-positive and IGRA false-negative

groups were pretreated with BRD6989 (5 μM) (Selleck, Shanghai, China) and AS101 (0.5 μg/mL) (AbMole, Shanghai, China) for 24 h followed by stimulation with TB-specific antigens. After antigenic stimulation, supernatants and PBMCs were collected for further analyses.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from PBMCs using TRIzol reagent (Ambion North America, USA) then RNA concentrations were determined via spectrophotometry. Next, 1 μg of RNA was reverse transcribed with cDNA Synthesis SuperMix (Yeasen Biotech Co., Ltd., Shanghai, China) following the manufacturer's instructions. IL-10, TGF-β, and IL-4 mRNA levels were determined via real-time PCR using SYBR Green Master Mix (Yeasen Biotech, Shanghai, China). Primers were synthesised by a commercial primer synthesis company as follows: human IL-10, sense (5′-TCAAGGCGCATGTGAACTCC-3′) and antisense (5′- GATGTCAAACTCACTC ATGGCT-3′); human TGF-β, sense (5′-GCGGTACCTGA ACCCGTGTT-3′) and antisense (5′-GTCAATGTACAG CTGCCGCAC-3′); human IL-4, sense (5′-CCAACTG CTTCCCCCTCTG-3′) and antisense (5′-TCTGTTACG GTCAACTCGGTG-3′); and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense (5′-GGAG CGAGATCCCTCCAAAAT-3′) and antisense (5′- GGCT GTTGTCATACTTCTCATGG-3′).[17,18] For mRNA analysis, RT of total RNA was performed using random primers (Promega); analysis of GAPDH mRNA was conducted in a separate PCR reaction for each sample as a loading control for use in normalising PCR results based on a constant amount of template cDNA across samples. Ct values were converted to fold changes in expression (2-ΔΔCt values) following normalisation based on housekeeping gene levels.

ELISA

Concentrations of IL-10 and IFN-γ in cell culture supernatants were quantified using an ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. All experiments were performed in triplicate.

Statistical Analysis

Data analysis was performed using IBM SPSS Statistics 25. Categorical variables were compared using Pearson's Chi-square test or Fisher's exact test, while continuous variables were compared using Student's *t*-test or Mann Whitney *U*-test, as appropriate. The cutoff for statistical significance was $P < 0.05$. Independent risk factors for false-negative results were identified by means of a multivariate logistic regression model with forward stepwise selection.

Results

Participants

A total of 2403 patients were enrolled who presented with symptoms suggestive of active TB and sought care at Beijing Chest Hospital between January 2018 and December 2018. Of these patients, 1817 were diagnosed as active TB cases, including 1533 (84.37%) culture-confirmed TB cases and 284 (15.63%) clinically diagnosed TB cases. Among 1533 culture-confirmed TB cases, 301 were excluded from analysis, either because no IGRA was conducted or because a completed IGRA yielded an indeterminate result. Finally, 1232 patients were included in the analysis. Of the 1232 patients with culture-confirmed TB, 1124 (91.2%) had true-positive IGRA results, while the remaining 108 (8.8%) cases had false-negative IGRA results (Figure 1).

The 1232 patents included in our final analysis had a median age of 50 (IQR 30–62) years and 856 of them were male. More than 72.4% of patients were new TB cases, while 77.6% (957) of patients had pulmonary TB, 3.9% (47) had extrapulmonary TB and 18.5% (228) had concurrent pulmonary TB and extrapulmonary TB. The most noted complication was diabetes (21.3%), followed by tumour (3.8%) and immune dysfunction (2.8%) (Table 1).

Risk Factors for False-Negative IGRA Results

Comparisons of demographic and clinical characteristics of patients with true-positive and false-negative IGRA results are listed in Table 1. Univariate analysis revealed that older age, immunosuppression, use of glucocorticoids and use of metformin were significantly associated with false-negative IGRA results. Results of multivariate logistic regression analysis (Table 2) indicated that glucocorticoid use [aOR: 3.568; 95% confidence interval (CI): 1.240–10.268], extrapulmonary TB [aOR: 2.952; 95% CI: 1.401–6.22], respiratory disease [aOR: 2.878; 95% CI: 1.043–7.936] and tumour [aOR: 2.215; 95% CI: 0.995–4.929] were independent risk

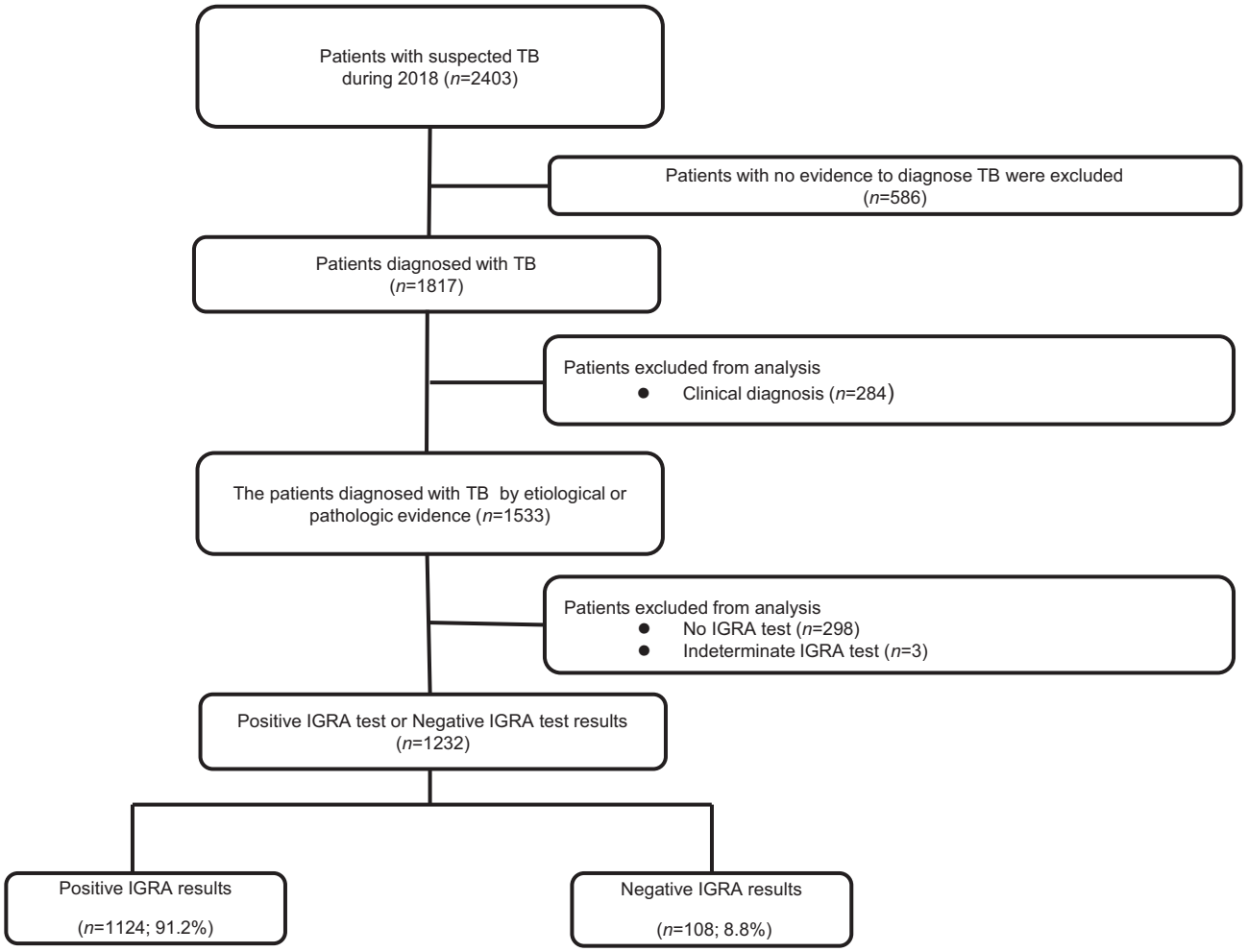

Figure 1 Flowchart of enrollment of patients with suspected tuberculosis.
Abbreviations: TB, tuberculosis; IGRA, interferon-γ release assay.

factors for false-negative IGRA results. By contrast, we identified no factors responsible for increased odds of testing positive via IGRA.

Transcript Levels of Anti-inflammatory Cytokine Genes in Blood Leukocytes

Detection of high concentrations of several anti-inflammatory cytokines was correlated with suppressed production of IFN-γ, such as IL-10, IL-4 and TGF-β.[14,15] In order to investigate whether expression of anti-inflammatory cytokines correlated with negative IGRA results in culture-positive patients, we analysed transcript levels corresponding to expression levels of anti-inflammatory cytokine genes in MTB-stimulated leukocytes. As shown in Figure 2, expression of IL-10 in the IGRA-negative group was significantly up-regulated as compared results for the IGRA-positive group. By contrast, no differences in mRNA levels of IL-4 and TGF-β were detected between the groups. Notably, the average concentration of IL-10 in cell supernatants of the IGRA-negative group was 4.77 pg/mL, which was statistically higher than the IGRA-positive group IL-10 concentration (1.47 pg/mL, $P = 0.007$).

Expression of IL-10 Negatively Affected Secretion of IFN-γ

To investigate the potential inhibitory role of IL-10 on IFN-γ secretion, we pretreated PBMCs with BRD6989 (a small molecule that enhances IL-10 secretion) and AS101 (a small molecule that inhibits IL-10 secretion). As shown in Figure 3, after pretreatment with BRD6989, the average IFN-γ concentration in IGRA-positive group cell supernatants significantly decreased from 59.73 pg/mL to 33.79 pg/mL ($P = 0.011$). By contrast, addition of AS101 to PBMCs of the false-negative group led to an increase in

Table 1 Crude Association Between Negative IGRA Results and Confirmed TB Patient Characteristics

Variable	Positive IGRA n=1124	Negative IGRA n=108	Crude OR	*p* value
Age, y, median (IQR)	49(30–62)	52(36–63)	1.007(0.997–1.018)	0.161
Age≥60	339(30.16)	33(33.33)	1.158(0.761–1.762)	0.494
Sex				
Female	343(30.52)	33(30.56)	1.002(0.653–1.538)	0.993
Male	781(69.48)	75(69.44)	Reference	
Position				
PTB	882(78.47)	75(69.44)	0.624(0.404–0.962)	0.031
PTB+EPTB	205(18.24)	23(21.30)	1.213(0.747–1.970)	0.434
EPTB	37(3.29)	10(9.26)	2.998(1.447–6.211)	0.002
Treatment history				
New cases	818(72.78)	74(68.52)	1.228(0.802–1.882)	0.344
Retreated cases	306(27.22)	34(31.48)	Reference	
Complication	399(35.50)	44(40.74)	1.244(0.832–1.862)	0.287
Immune dysfunction	27(2.40)	7(6.48)	2.816(1.196–6.627)	0.013
Diabetes	242(21.53)	21(19.44)	0.880(0.535–1.447)	0.613
Tumor	39(3.47)	8(7.41)	2.226(1.012–4.893)	0.059
Hepatitis	27(2.40)	3(2.78)	1.161(0.346–3.891)	0.809
Respiratory disease	18(1.60)	6(5.56)	3.614(1.404–9.308)	0.005
Glucocorticoid use	13(1.16)	6(5.56)	5.027(1.871–13.507)	0.001
Metformin use	67(5.96)	7(6.48)	1.093(0.489–2.445)	0.828
Insulin use	125(11.12)	6(5.56)	0.470(0.202–1.093)	0.073

Abbreviations: IGRA, interferon-γ release assay; OR, odds ratio; IQR, interquartile range; PTB, pulmonary tuberculosis; EPTB, extrapulmonary tuberculosis.

Table 2 Multivariate Analysis of Association Between Negative IGRA Results and Confirmed TB Patient Characteristics

Variable	IGRA Result	
	a OR	*p*
EPTB	2.952(1.401–6.223)	0.004
Tumor	2.215(0.995–4.929)	0.050
Respiratory disease	2.878(1.043–7.936)	0.041
Glucocorticoid use	3.568(1.240–10.268)	0.018

Abbreviations: IGRA, interferon-γ release assay; a OR, adjusted odds ratio; PTB, pulmonary tuberculosis; EPTB, extrapulmonary tuberculosis.

average cell supernatant IFN-γ concentration from 19.01 pg/mL to 45.10 pg/mL (P = 0.030), showing that IFN-γ concentration was negatively correlated with IL-10 expression level.

Discussion

Diagnosing pulmonary TB remains challenging, due to the fact that traditional culture-based testing of approximately one-half of patients with active TB fails to produce positive test results.[1] Thus, IGRA diagnostic tests, which are based on MTB-specific immunological responses, should be used to confirm TB diagnostic results except in areas with high TB burden (as directed by the World Health Organisation, WHO).[10] Increasing concerns have been raised by clinicians with regard to the high proportion of false-negative IGRA results due to the low diagnostic sensitivity of this assay. In a retrospective study from the United States, 12.3% of TB patients had a negative result, thereby leading to higher mortality associated with delayed treatment.[19] Meanwhile, researchers in China have observed that 8.7% of microbiologically confirmed pulmonary TB patients yielded false-negative IGRA results.[20] Consistent with results of previous studies, we found that false-negative IGRA results were obtained for approximately one tenth of culture-confirmed pulmonary TB cases, aligning with WHO recommendations that IGRAs not be used to rule out active TB disease.[10] As mentioned above, this rule is especially important in regions with high TB burden, where a high proportion of patients have symptoms suggestive of TB but produce negative

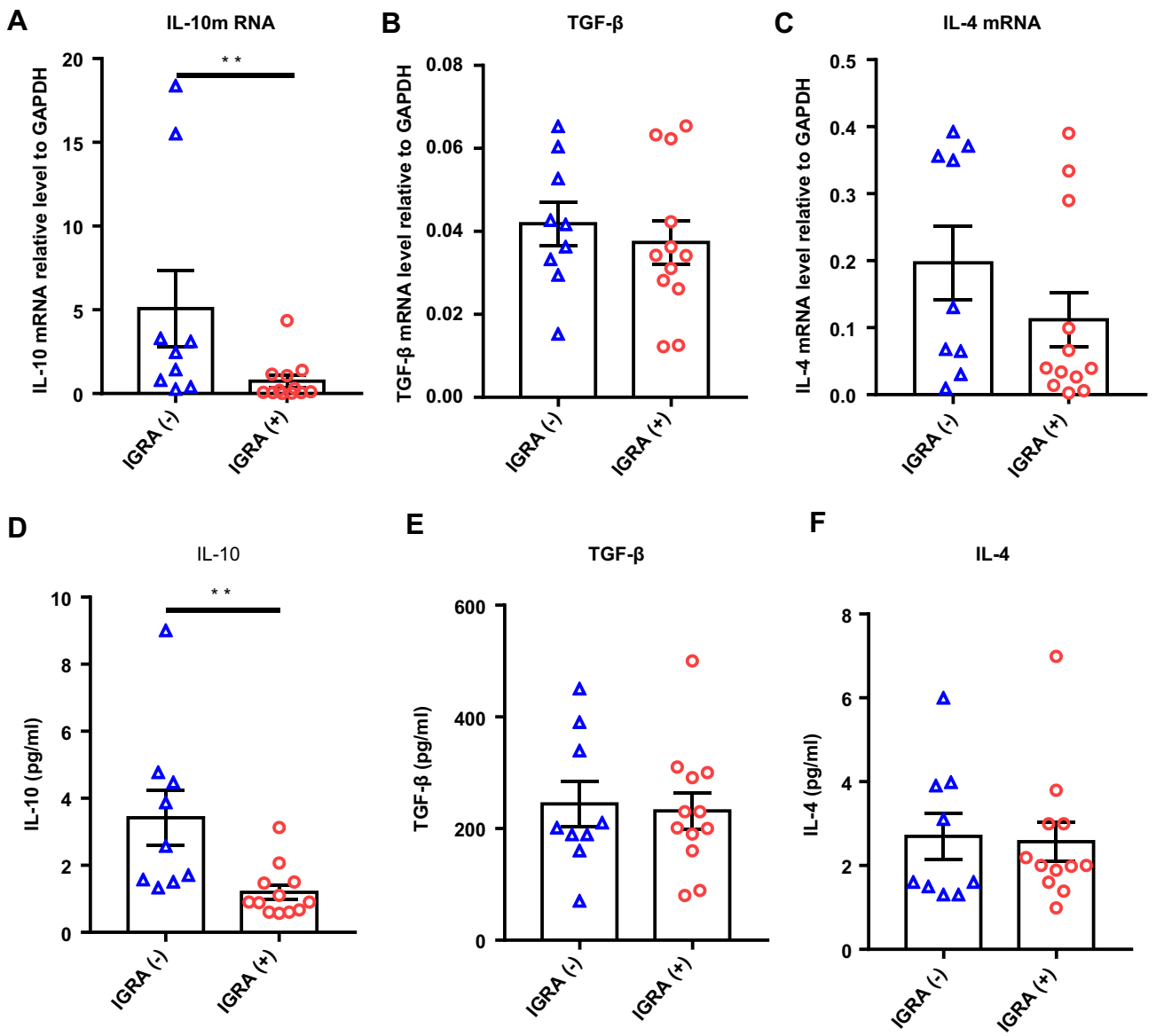

Figure 2 Up-regulated expression level of IL-10 in peripheral blood mononuclear cells from culture TB patients with negative IGRA. (**A**–**C**) The mRNA expression levels of IL-10 (**A**), TGF-β (**B**) and IL-4 (**C**) in PBMC stimulated with Mtb antigens; (**D**–**F**) The levels of IL-10 (**D**), TGF-β (**E**) and IL-4 (**F**) in supernatant of PBMC stimulated with Mtb antigens. Results are shown as means± SEM; *t*-test, $^{**}p<0.01$.

IGRA results that potentially lead to misdiagnoses. Thus, physicians should not eliminate TB as a possible diagnosis based only on a negative IGRA results but instead should take extra steps to evaluate and monitor cases at high-risk for false-negative IGRA results to confirm they do not have active TB.

Another important finding of our study is that increased PBMCs expression of IL-10 rather than IL-4 and TGF-β correlated with negative IGRA results. IFN-γ production is controlled by multiple cytokines secreted by immune cells.[14,15] An experimental study by Brooks et al revealed that IL-10 could abort T cell responses when present during antigen stimulation.[21] Therefore, we speculate that endogenous up-regulation of IL-10 inhibits development of effective memory responses by memory T cells, thereby suppressing IFN-γ production; this mechanism may be responsible for false-negative IGRA results obtained for active TB patients. Our findings have several important implications for the clinical management of TB patients. On one hand, the level of IL-10 in peripheral blood mononuclear cells could be used as a useful indicator for predicting the occurrence of false-negative IGRA results. The combined detection of IL-10 and IFN-γ could then serve as a strategy for improving the specificity of IGRA assays in widespread use. On the

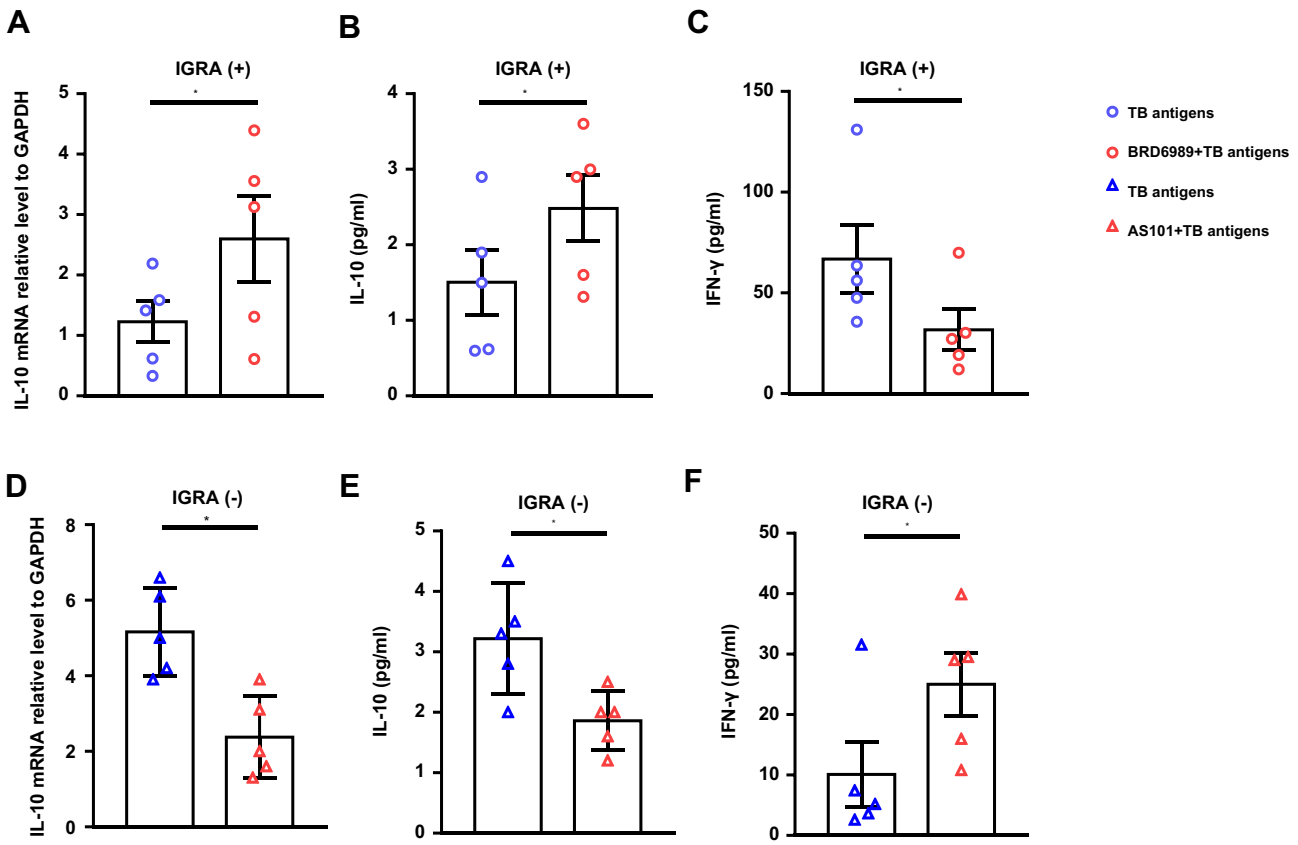

Figure 3 The change of IL-10 expression regulates the secretion of IFN-γ by PBMC. (**A**–**C**) The IL-10 mRNA expression levels (**A**) and the IL-10 (**B**), IFN-γ (**C**) levels in supernatant of PBMC stimulated with Mtb antigens after using BRD6989 pretreatment from IGRA positive TB group. (**D**–**F**) The IL-10 mRNA expression levels (**D**) and the IL-10 (**E**), IFN-γ (**F**) levels in supernatant of PBMC stimulated with Mtb antigens after using AS101 pretreatment from IGRA negative TB group. Results are shown as means± SEM; *t*-test, **p*<0.05.

other hand, given the importance of IL-10 for the regulation of memory T cells, homeostatic maintenance of the balance between IL-10 and IFN-γ may affect host defences to MTB,[13] thus leading to diverse TB patient prognoses and therapeutic outcomes. Nevertheless, further study is urgently needed to investigate the role of IL-10 in the pathogenesis and prognosis of TB disease.

Glucocorticoids have been widely used for the treatment of inflammatory and autoimmune diseases, due to their inhibitory effects on innate and cellular immune responses[22] and their function as a predominant negative regulator of IFN-γ production. As a consequence, prior use of glucocorticoids was found to be an independent risk factor for false-negative IGRA results, as demonstrated in numerous previous studies. For example, Richards et al revealed that glucocorticoids could drive human T cell differentiation to generate a phenotype with high IL-10 expression.[23] Although the exact reason remains unclear, we speculate that IL-10 may be a link between glucocorticoids use and IFN-γ production during cellular immune responses.

As another important finding of this work, extrapulmonary TB was associated with a false-negative IGRA result, as reported previously.[20] As an explanation for this association, recruitment of memory lymphocytes at extrapulmonary TB disease sites due to cell release of chemokines may result in decreased numbers of memory lymphocytes in peripheral blood that leads to false-negative IGRA results. A recent systematic review by Zhou et al revealed that body fluids outperformed blood when used as specimens for IGRA assays conducted for diagnosing extrapulmonary TB.[24] Our study results highlight the fact that a lower IGRA cut-off value may be clinically relevant for extrapulmonary TB patients, who have lower numbers of memory lymphocytes in peripheral blood as compared to other types of TB patients.

We must acknowledge several limitations of our study. First, considering that the IGRA assay was not conducted in

19.4% of culture-confirmed TB patients in our cohort, selection bias may have occurred that weakened the significance of our conclusion. Second, multiple population groups at high risk for false-negative IGRA results were not included in our analysis, including HIV-infected TB patients and young children with suspected TB, due to patient transfers to other hospitals. Further study is warranted to confirm our observations in these special populations. Finally, despite the observation of increased expression of IL-10 in the IGRA-negative group, we could not completely elucidate the mechanism whereby MTB triggered secretion of this anti-inflammatory cytokine.

In conclusion, our data demonstrate that increased expression of IL-10 in peripheral blood mononuclear cells correlates with negative interferon-γ release assay results in culture-confirmed TB patients. Older age and use of glucocorticoids are significantly associated with false-negative IGRA results. Thus, combined detection of IL-10 and IFN-γ could be used as a potential strategy for improving the specificity of widely used IGRA assays.

Ethical Approval

This study was approved by the Ethic Committee of Beijing Chest Hospital affiliated to Capital Medical University. Written informed consent was obtained from each patient.

Acknowledgments

We would like to thank members of the Department of Tuberculosis, Beijing Chest Hospital for their assistance in collection of clinical data.

Funding

This work was supported by the Beijing Hospitals Authority' Ascent Plan (DFL20191601), the National Natural Science Foundation (82072243) and the National Major Project(2018ZX10103001-004).

Disclosure

The authors declare that they have no competing interests.

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