RESEARCH ARTICLE

More significance of TB-IGRA except for the diagnose of tuberculosis

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Objective: Tuberculosis (TB)-interferon gamma release assay (IGRA) test has the characteristics of short time, high specificity, and high sensitivity, but it lacks the correlation research between TB-IGRA test results and body's immune cells, disease progression and prognosis, which is explored in this study.

Design: A retrospective study was carried out on positive TB-IGRA patients who were infected with TB and diagnosed at our hospital from January 2014 to June 2015. The TB-IGRA, routine blood test, T-cell subgroup data were collected for statistical analysis.

Results: TB-IGRA results were in positive proportion to the lymphocytes, CD4⁺T cells and CD4⁺CD28⁺T cells, whereas negative to the Treg cells. Patient with unilateral pulmonary lesion had higher TB-IGRA than those with bilateral pulmonary lesions. After the stimulation of TB-specific antigen, the proportion of CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺T Tcells were both increased and the CD4⁺IFN- γ ⁺T had positive correlation with the value of TB-IGRA.

Conclusions: IFN- γ was tested with TB-IGRA in patients with TB by the specific TB T cells and correlated with the lymphocytes, while the lymphocytes also closely related to the host's anti-TB immunity and disease outcome. Hence the result of TB-IGRA could reflect the specific anti-TB immunity ability of the host, disease progression and prognosis. This study further expands the application scope of TB-IGRA technology in the diagnosis of TB and lays a foundation for clinical practice to understand the immunity state of the patients with TB and the application of auxiliary clinical immunity regulators.

KEYWORDS

Mycobacterium tuberculosis, TB-IGRA, γ -interferon

1 | INTRODUCTION

Pulmonary disease caused by *Mycobacterium tuberculosis* infection is a major public health concern around the world and there were about

9.6 million new records of tuberculosis (TB) in 2014. China is a high-burden country of TB, ranking the second place in TB incidence and the first place in absolute quantity.¹ Recently, the prevention and control of TB is facing huge challenge because of the reduction in TB control measures, the decline of BCG vitality, the prevalence of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome, the inappropriate application of anti-TB drugs, and the gene mutations of *M. tuberculosis*, etc.^{2,3} WHO proposed the control strategy of TB in 2014, and the development of new techniques for early diagnosis of TB was high on the WHO's agenda.

Abbreviations: Alb, albumin; ALT, alanine aminotransferase; ASTA, aspartate aminotransferase; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; FSC, forward scatter; GLU, blood glucose; IGR, interferon gamma release assay; LYM, lymphocytes; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin-P; SSC, side scatter; TB, tuberculosis; TP, total protein.

Jun-Chi Xu and Ze-Yi Li contributed equally to this work.

At present, there are a lot of diagnosis methods for TB. Sputum smear may diagnose *M. tuberculosis* infection, but the detection rate is low; cultivation of the *M. tuberculosis* can confirm the disease, but it takes a long time; tuberculin skin test will cause larger damage to human body and there is a certain risk.⁴ Because of the lack of a better way for rapid diagnosis, it is very important to develop an early accurate diagnosis of TB.

Interferon gamma release assay (IGRA) is a detection method developed based on this.⁵ It indirectly indicates the *M. tuberculosis* infection by detecting the change of the immune system to *M. tuberculosis* infection. The theory of TB-IGRA is that, the originally-sensitized T cells may produce a large amount of IFN- γ when the human body is stimulated by the same TB antigen. TB-IGRA detects the IFN- γ in the whole blood or the IFN- γ released by mononuclear cell which is isolated from the whole blood under the stimulation of specific antigen, to determine whether patients are infected with *M. tuberculosis*.^{5,6} Currently, there are two mature TB-IGRA technologies around the world: one is enzyme-linked immunosorbent assay (ELISA), which detects the IFN- γ released by T cells in the whole blood after stimulation with specific antigen of *M. tuberculosis* once again, represented by QuantiFERON-TB-Gold approved by FDA (USA) in 2004⁷; the another is called enzyme-linked immune-spot assay (ELISpot), which isolates the mononuclear cells from the whole blood, cultures them in the in vitro environment, and detects the quantity of T cells that secrete IFN- γ under stimulation of specific *M. tuberculosis* antigen, and T-SPOT developed by the Oxford University is on behalf of this kind of method.⁸ The two methods have similar principles, but slightly different in detective technology and concrete operation. They both adopt early secreting antigen target (ESAT-6, RMW of 6000) and culture filtrate protein (CFP-10, RMW of 10 000), the specific antigens encoded by gene of RDI area of *M. tuberculosis*.^{9,10} Compared with the *M. tuberculosis* sputum smear, cultivation of *M. tuberculosis*, and tuberculin skin test, TB-IGRA with higher specificity, higher sensitivity, obviously less cross-reaction and shorter test time, is in a special position in early diagnosis, prevention and control of TB.

TB-IGRA had become a major method in clinical diagnosis of *M. tuberculosis* infection at present. However, there is a lack of relevant study about the correlation between TB-IGRA and body's anti-TB immunity memory and the course of illness. Therefore, further research and investigation are needed to explore whether the strength of TB-IGRA test results is associated with the host's anti-TB immune strength. In this study, the systematical analysis was performed to analyze the correlation between TB-IGRA and anti-TB immunity, expand the application scope and improve the value of TB-IGRA.

2 | MATERIALS AND METHODS

2.1 | Study participants

A retrospective study was performed on all adult patients hospitalized for diagnosis of TB for whom a QuantiFERON-TB-Gold assay had been performed in The Fifth People's Hospital of Suzhou between January 1, 2014 and June 30, 2015. We included totally 135 patients,

of them 100 were men and 35 were women. The mean age was 44.4. All identified cells of *M. tuberculosis* from the patients were of human type. The patients who were co-infected with HIV or treated with immunosuppressive therapy or chemotherapy before QuantiFERON-TB-Gold assay were excluded.

The recruited participants were classified into two groups: low IGRA group (n=67) and high IGRA group (n=68). Table 1 depicts age, lymphocyte cell count, T-cell subest, GLU, Alb, TBIL, TP, ALT, and AST.

2.2 | QuantiFERON-Gold In-Tube assay

The conditions of blood samples were fasting and before 9 AM. Three heparin tubes were collected: (i) negative control tube (NIL tube), (ii) antigen tube (AG tube; contained a coating of specific antigens of *M. tuberculosis* [ESAT-6, CFP-10, TB 7.7], which came into contact with the patient's T cells in the blood sample), and (iii) positive control tube containing phytohemagglutinin-P (PHA) (MIT tube). The concentration of IFN- γ secreted by the cells was measured by ELISA. The results were measured in IU/mL and interpreted in accordance with the manufacturer's recommendations as negative, positive, or indeterminate.

2.3 | Flow cytometric analysis

Monoclonal antibodies labeled with fluorochrome, CD3-APC, CD4-Percp, CD28-PE, and CD25-FITC; were purchased from BD Pharmingen (San Diego, CA, USA) and IFN- γ -FITC; was purchased from Cell Signaling Technology (Danvers, MA, USA). For each test to be performed, 50 mL of fresh heparinized whole blood of the patients or healthy donors was incubated with indicated antibodies (10 mL) for 15 minutes, then lysed with FACSTM lysing solution; (BD Biosciences, San Jose, CA, USA), subsequently washed with phosphate-buffered saline, fixed and eventually detected by BD FACSAria with BD FACSDiva software support.

2.4 | Data collection

The following data were collected: age, sex, and biological data (lymphocyte cell count, T-cell subest, blood glucose [GLU], albumin [Alb],

TABLE 1 General information of the 135 patients

	Low IGRA group	High IGRA group	P values
Age (years)	49.40 $\pm$ 2.435	39.91 $\pm$ 2.170	.0042
Total protein (g/L)	69.28 $\pm$ 0.980	68.34 $\pm$ 0.946	.4908
Globulin (g/L)	32.96 $\pm$ 0.854	30.98 $\pm$ 0.640	.0653
Albumin (g/L)	35.77 $\pm$ 0.865	37.36 $\pm$ 0.796	.1792
ALT (U/L)	18.18 $\pm$ 3.278	23.13 $\pm$ 5.508	<.0001
AST (U/L)	18.81 $\pm$ 1.573	22.75 $\pm$ 2.980	.5067
FBG (mmol/L)	5.97 $\pm$ 0.326	5.70 $\pm$ 0.200	.4373

total protein [TP], alanine aminotransferase [ALT], and aspartate aminotransferase [AST]).

2.5 | Statistical analysis

The data were analyzed by GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) and SPSS (SPSS Inc., Chicago, IL, USA), and presented as mean $\pm$ standard error of the mean (SEM). For comparisons of the collected data, nonparametric test (Mann-Whitney *U* test), Student's *t* test and the chi-square test were performed and two-tailed $P < .05$ was considered statistically significant. Nonparametric correlation test (spearman) was applied for analyzing the correlation between TB-IGRA and Lymphocytes (LYM)%, LYM, CD4⁺T(%), CD4⁺CD28⁺T(%) or CD4⁺CD25^{high}T(%). The bar in every group in figures represents the mean $\pm$ SEM.

3 | RESULTS

3.1 | Differences between the two groups in proportion and absolute value of peripheral blood leukocytes

Routine blood test showed proportions of neutrophils, lymphocytes, and monocytes were (75.75 $\pm$ 0.87)%, (13.43 $\pm$ 0.752)% and (9.603 $\pm$ 0.2977)% in low TB-IGRA groups, and their absolute values were (4.782 $\pm$ 0.2097) 10^6 /mL, (1.039 $\pm$ 0.052) 10^6 /mL and (0.6260 $\pm$ 0.029 48) 10^6 /mL; while proportions of neutrophils, lymphocytes, and monocytes were (69.32 $\pm$ 1.81)%, (21.33 $\pm$ 1.084)%, and (10.23 $\pm$ 0.48)% in high TB-IGRA groups, and their absolute values were (4.221 $\pm$ 0.1818) 10^6 /mL, (1.255 $\pm$ 0.062) 10^6 /mL, and (0.6176 $\pm$ 0.036 41) 10^6 /mL; Comparison between the two groups showed high TB-IGRA group had obviously higher ratio ($P < .0001$) and absolute value ($P = .0016$) of lymphocytes than those of low-value group, with statistical significant difference; while absolute value of neutrophils was lower in the high TB-IGRA group than that of low TB-IGRA group ($P = .0452$) (Figure 1).

3.2 | Positively correlation between the TB-IGRA and lymphocytes in patients with pulmonary tuberculosis

The result of TB-IGRA was positively correlated with the patients' lymphocyte percentage ($r = .4811$, $P < .0001$), and absolute value of lymphocytes ($r = .5333$, $P < .0001$) (Figure 2).

3.3 | Differences between the two groups in the subgroup of peripheral blood lymphocytes

Flow cytometry detection showed proportions of CD4⁺T cells, CD8⁺T cells, CD19⁺B cell, and CD56⁺NK cells of low TB-IGRA group were (33.16 $\pm$ 1.123)%, (30.86 $\pm$ 1.415)%, (11.11 $\pm$ 0.581)%, and (19.42 $\pm$ 1.207)%; while the proportions of CD4⁺T cells, CD8⁺T cells, CD19⁺B cell, and CD56⁺NK cells of high TB-IGRA group

were (38.05 $\pm$ 0.794)%, (29.77 $\pm$ 1.051)%, (12.45 $\pm$ 0.681)%, and (18.94 $\pm$ 1.052)%. Comparison on two groups found higher proportion of CD4⁺T cells in high TB-IGRA group than that in low TB-IGRA group ($P = .0005$). Further analysis on the subgroups of CD4⁺T cells revealed the proportion of CD4⁺CD28⁺T cells in high TB-IGRA group was higher than the cells of CD4⁺CD28⁺T in low TB-IGRA group ($P < .0001$). And at the same time, the proportion of CD4⁺CD25^{high}Treg cells in high TB-IGRA group was lower than that in low TB-IGRA group ($P < .0001$) (Figure 3).

3.4 | Weak correlation between the result of TB-IGRA and CD4⁺T cells, CD4⁺CD28⁺T cells and CD4⁺CD25^{high}T cells in patients with pulmonary tuberculosis

The result of TB-IGRA was positively correlated with CD4⁺T cells ($r = .3718$, $P = .0014$) and CD4⁺CD28⁺T cells ($r = .3445$, $P < .0001$), while negatively correlated with CD4⁺CD25^{high}T cells ($r = -.3465$, $P < .0001$) (Figure 4).

3.5 | Differences between the value of TB-IGRA and the gender and sputum smear

Table 2 indicates sex and sputum smear, the TB-IGRA high group has higher proportion of male ($P = .0027$) and lower positive rate ($P = .0017$) than TB-IGRA low group (Table 2). Furthermore, the time of sputum smear transformed into negative between the low IGRA group and high IGRA group had no statistical significance ($P = .6378$) but the median times of low IGRA group was 90 and high IGRA group was 60 (Figure 5A).

3.6 | Influences of lesion distribution and pulmonary cavity of patients with pulmonary tuberculosis on TB-IGRA detection value

X-ray detection on the pulmonary lesions of patients with pulmonary TB showed the result of TB-IGRA was 234.2 $\pm$ 27.33 in unilateral lesion, but 169.1 $\pm$ 18.86 in bilateral lesions. Comparison found the result of TB-IGRA in unilateral lesion was higher than that of bilateral lesions ($P = .0105$). Analysis on pulmonary cavity found the result of TB-IGRA was 210.5 $\pm$ 18.99 in the non-cavity group, while 171.3 $\pm$ 28.98 in the cavity group, and there were no statistical differences between the two groups ($P = .3211$) (Figure 5B,C).

3.7 | The relationship between the value of TB-IGRA and CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺T cells

The study had detected the CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺T cells by flow cytometry which were stimulated by the TB-specific antigen (ESAT-6, CFP-10, TB 7.7) and found that the proportion of CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺T cells were both increased and the CD4⁺IFN- γ ⁺T cells had positive correlation with the value of TB-IGRA but the CD8⁺IFN- γ ⁺T were not (Figure 6).

FIGURE 1 Differences between patients with high TB-IGRA and low TB-IGRA in proportion and absolute value of peripheral blood leukocytes. (A, B) Statistical results for proportion and absolute value of neutrophils from high TB-IGRA group and low TB-IGRA group. (C, D) Statistical results for proportion and absolute value of lymphocytes from high TB-IGRA group and low TB-IGRA group. (E, F) Statistical results for proportion and absolute value of monocytes from high TB-IGRA group and low TB-IGRA group

FIGURE 2 Analysis of the correlation between the results of TB-IGRA and lymphocytes in patients with pulmonary tuberculosis. (A) Correlations between the results of TB-IGRA and the proportion of lymphocytes. (B) Correlations between the results of TB-IGRA and the absolute value of lymphocytes

4 | DISCUSSION

Pulmonary disease caused by *M. tuberculosis* infection is a major public health concern in the world. WHO points out in Global Tuberculosis Report in 2015 that there were about 9.6 million new records of TB in 2014,¹ and China is one of the 22 high-burden TB countries, ranking

the second place in TB incidence and the first place in absolute quantity (11%). Investigation shows that about 1/3 of the population in China have been infected by the *M. tuberculosis*.

The data of inpatients with TB in this study were collected from 2014 to 2015. We analyzed their routine blood test, T-cell subgroups and TB-IGRA. Statistical analysis found that there were statistically

FIGURE 3 Differences between high TB-IGRA group and low TB-IGRA group in subgroup of peripheral blood lymphocytes (A) Statistical results for proportion of CD4⁺T cell form high TB-IGRA group and low TB-IGRA group. (B) Statistical results for proportion of CD8⁺T cell form high TB-IGRA group and low TB-IGRA group. (C) Statistical results for proportion of CD19⁺B cell form high TB-IGRA group and low TB-IGRA group. (D) Statistical results for proportion of CD56⁺NK cell form high TB-IGRA group and low TB-IGRA group. (E) Statistical results for proportion of CD4⁺CD28⁺T cell form high TB-IGRA group and low TB-IGRA group. (F) Statistical results for proportion of CD4⁺CD25^{high}T cell form high TB-IGRA group and low TB-IGRA group

FIGURE 4 Analysis of the correlation between the results of TB-IGRA and CD4⁺T cells, CD4⁺CD28⁺T cells and CD4⁺CD25^{high}T cells in patients with pulmonary tuberculosis. (A) Correlations between the results of TB-IGRA and the proportion of CD4⁺T cells. (B) Correlations between the results of TB-IGRA and the absolute value of CD4⁺CD28⁺T cells. (C) Correlations between the results of TB-IGRA and the absolute value of CD4⁺CD25^{high}T cells

significant differences between low IGRA group and high IGRA group in proportion and the absolute value of lymphocytes. Further analysis found the proportions of peripheral CD4⁺T cells and CD4⁺CD28⁺T cells were obviously lower in low IGRA group than the high IGRA

group, while the proportion of CD4⁺CD25^{high}Treg cells in low IGRA group was higher than the high IGRA group. Correlation analysis also found that the strength of TB-IGRA was positively correlated with the proportion and absolute value of lymphocytes, the proportions

TABLE 2 Compares the positive rate of sputum smear or sex between low IGRA group and high IGRA group

	Low IGRA group		High IGRA group		χ^2	P values
	Positive (male)	Positive rate (male rate, %)	Positive (male)	Positive rate (male rate, %)		
Sputum smear	47	70.1	34	50.0	5.709	.017
Sex	44	65.7	56	82.4	4.890	.027

FIGURE 5 The time of sputum smear transformed into negative, influences of lesion distribution and pulmonary cavity of the patients with pulmonary tuberculosis on TB-IGRA detection value. (A) Statistical analysis the time of sputum smear transformed into negative between the low IGRA group and high IGRA group (B) Statistical analysis the differences between unilateral lesions and bilateral lesion group in the results of TB-IGRA. (C) Statistical analysis the differences between non-cavity group and cavity group in the results of TB-IGRA

of peripheral CD4⁺T cells and the proportions of CD4⁺CD28⁺T cells, while negatively associated with the proportion of CD4⁺CD25^{high}Treg cells. What is more, the study also found the TB-specific antigen could stimulate the CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺T cells and proved the CD4⁺IFN- γ ⁺T was the main factor of the value of TB-IGRA. The above results indicated that the strength of the TB-IGRA was closely related to the quantity and subgroup proportion of peripheral lymphocytes especially CD4⁺T lymphocytes in patients with TB. Existing study has confirmed that CD4⁺T lymphocytes played a very important role in host's anti-infection against *M. tuberculosis*.^{11,12} IFN- γ secreted by CD4⁺T lymphocytes is an important cytokine for body to fight with *M. tuberculosis*,^{13,14} and the Th1/Th2 ratio and proportion of CD4⁺CD25^{high}Treg cells in its subgroup had an important effect on the outcome and recurrence of TB.¹⁵⁻¹⁷ Combined with the results of this research and existing theoretical basis, it was considered that when infected by *M. tuberculosis*, the body inhibited the immune escape of TB by regulating the Th1/Th2 ratio, decreasing the proportion of CD4⁺CD25^{high}Treg cells in peripheral blood, and increasing the secretion of IFN- γ detected by TB-IGRA. Therefore, this study suggested the strength of TB-IGRA, which was closely correlated with the quantity of lymphocyte and the proportion of its subgroups, could more directly reflect the body's immune status.

Further research via combination of TB-IGRA results and TB condition found that TB-IGRA results of the patients with bilateral lesions were lower than unilateral lesions, and no differences were found

in analysis of intrapulmonary cavity and hospitalization stays. These results verified higher TB-IGRA meant the host could produce more IFN- γ , which resulted in stronger immunity ability of host against the immune escape of *M. tuberculosis*, so less pulmonary lesions were developed.

TB-IGRA, applied to clinical diagnosis of TB as a new technology, not only can indirectly reflect the *M. tuberculosis* infection in vivo, but also can indicate the activation of specific immune system, disease progression and prognosis. This study analyzed the correlation between TB-IGRA results and body's immune function and disease outcome from a new angle and found that the strength of the TB-IGRA was closely associated with the in vivo anti-TB immunity of patients with TB. It can more intuitively reflect the specific anti-TB immunity of the body relative to detection on the T-cell subgroup. This research further expanded the application scope of TB-IGRA in TB diagnosis, and laid a foundation for further clinical understanding of the immunity state of the patients with TB and the application of auxiliary immunity regulator.

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FIGURE 6 Analysis the relationship between the value of TB-IGRA and $CD4^+IFN-\gamma^+$ and $CD8^+IFN-\gamma^+$ T cells. (A) The gating strategies and representative results of IFN- γ expression in $CD4^+$ and $CD8^+$ T cells. (B) Statistical results for IFN- γ expression in $CD4^+$ T cells before and after tuberculosis-specific antigen stimulate. (C) Statistical results for IFN- γ expression in $CD8^+$ T cells before and after tuberculosis-specific antigen stimulate. (D) Correlations between the value of TB-IGRA and $CD4^+IFN-\gamma^+$ T cells. (E) Correlations between the value of TB-IGRA and $CD8^+IFN-\gamma^+$ T cells

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