International Journal of Infectious Diseases 40 (2015) 39–44

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Dynamics of immune parameters during the treatment of active tuberculosis showing negative interferon gamma response at the time of diagnosis

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ARTICLE INFO

Article history:
Received 7 August 2015
Received in revised form 15 September 2015
Accepted 25 September 2015

Corresponding Editor: Eskild Petersen, Aarhus, Denmark.

Keywords:
Interferon gamma release assay
False-negative result
Sensitivity
Interleukin 1 receptor antagonist
Interleukin 2
Interferon gamma-induced protein 10

SUMMARY

Objectives: In the performance of interferon gamma release assays (IGRA) for the diagnosis of tuberculosis (TB) infection, false-negative results are a major obstacle. In active TB patients, treatment-dependent changes of the negative test results remain unknown.
Methods: The treatment course of 19 smear-positive/culture-confirmed TB patients who had IGRA-negative results by QuantiFERON-TB in-tube (QFT-IT) method at the time of diagnosis (month 0) in a previous study, were monitored in the present study. Blood was further collected at months 2 and 7, and the concentrations of 27 immune molecules were measured in the plasma supernatants remaining after performing the IGRA, using a suspension array system.
Results: After initiating treatment, eight of the 19 QFT-IT-negative patients showed positive conversion, whereas the remaining 11 (58%) did not; the interferon gamma (IFN-γ) response was restored to levels higher than 1 IU/ml in only three of the eight patients with positive conversion. Plasma concentrations of interleukin 1 receptor antagonist, interleukin 2, and interferon gamma-induced protein 10 remained low after *Mycobacterium tuberculosis*-specific antigen stimulation at months 2 and 7 in the continuously QFT-IT-negative group, whereas the parameters were elevated only in the transiently QFT-IT-negative group.
Conclusions: It was demonstrated that a majority of active TB patients showing negative IGRA results did not regain sufficient levels of immune responsiveness despite successful treatment.

1. Introduction

The interferon gamma release assay (IGRA) is currently used as one of the representative tests to diagnose tuberculosis (TB) infection.[1] In this test, the cellular response to *Mycobacterium tuberculosis* is assessed by measuring the interferon gamma (IFN-γ) released from peripheral blood lymphocytes after stimulation with *M. tuberculosis*-specific antigens.[1]

The QuantiFERON-TB Gold In-Tube test (QFT-IT) is a commercially available IGRA based on the ELISA method; it has a sensitivity of 78–83% and specificity of 98–100%.[1] This imperfect sensitivity causes difficulties in ruling out TB infection, particularly when the

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http://dx.doi.org/10.1016/j.ijid.2015.09.021

prevalence of TB infection is high, and the low negative predictive value of the test may reduce the chance of a possible therapeutic intervention.

To assess the sensitivity of the QFT-IT, patients with bacteriologically proven active TB disease have often been recruited as surrogates for individuals with latent TB infection. The IGRA results also provide a clue to suspect active TB disease clinically.

Although weakened immunity in severe TB may affect the test results,[2] treatment-dependent changes of the negative IGRA results with a very low IFN-γ response have not been investigated fully.

This study group has recently reported the results of a cross-sectional study on the sensitivity of the QFT-IT method in Hanoi, Vietnam, and demonstrated that aging, emaciation, HIV co-infection, and a particular HLA genotype, DRB1*07:01, lowered the sensitivity of the test in active pulmonary TB patients.[3] In the present study, 19 of the 24 patients who showed false-negative results at the time of diagnosis were monitored. Further analysis of the treatment response and dynamics of immune parameters was performed, with the measurement of the concentrations of various cytokines and chemokines in the plasma supernatants remaining after use in the IGRA assay.

2. Methods

2.1. Study subjects and IGRA

From July 2007 to March 2009, whole blood was collected from 504 adult patients in Hanoi, Vietnam, who had smear-positive/culture-confirmed pulmonary TB and a history negative for TB treatment. The blood was collected in heparinized tubes before anti-TB treatment was initiated (month 0).[3] The patients were tested with a commercially available ELISA-based IGRA (QFT-IT; Cellestis, Victoria, Australia), as reported previously.[3] Plasma supernatants were separated at 4000 rpm for 15 min (Model 2010; Kubota Co., Tokyo, Japan) and stored at −80 °C until measurement. The cut-off value to interpret the QFT-IT results was set at 0.35 IU/ml, as per the manufacturer's instructions.

In the present study, further blood samples for QFT-IT and other tests were collected and served for analysis at two more time points: after the initial phase of treatment (month 2) and close to the end of treatment (month 7). Positive conversion of the IGRA was defined by a negative result at month 0 and positive result(s) at month 2, month 7, or both time points.

2.2. Clinical data collection

The extents of cavitary lesions and infiltrates were also semi-quantitated by the grading method.[4] *M. tuberculosis* isolates were analyzed by single nucleotide polymorphism (SNP) and spoligotyping methods.[5]

2.3. Treatment course

Following the national standard regimen at that time, all patients received an 8-month course of the anti-TB treatment regimen 2S(E)HRZ/6HE, which was commonly administered during the study period in Vietnam.[5]

2.4. Immune analyte profiling by Bio-Plex assay and adiponectin ELISA assay

Immune molecules released into the plasma after TB antigen stimulation were estimated from their concentrations after a 16- to 24-h incubation with TB-specific antigens (TBAg) minus those with no antigens (Nil) obtained from the QFT-IT method. Their concentrations were determined using a human 27-plex assay (14 cytokines: interleukin (IL)-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, tumor necrosis factor alpha (TNF-α); seven chemokines: eotaxin, IL-8, IFN-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory proteins MIP-1α and MIP-1β, RANTES; and six growth factors: IL-7, fibroblast growth factor (FGF) basic, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor VEGF) (Bio-Plex Suspension Array System; Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. All samples, standards, and controls were run in duplicate and manipulated in accordance with the manufacturer's protocol. Samples were diluted in a 1:4 volume ratio with the sample diluent and incubated for 30 min at room temperature; they were then agitated at 300 rpm to be captured with antibody-coupled magnetic beads. Following three washes in a Bio-Plex Pro Wash Station, the samples were incubated with biotinylated detection antibodies and agitated at 300 rpm in the dark for 30 min at room temperature. Each captured analyte was detected by the addition of streptavidin–phycoerythrin and quantified using a Bio-Plex array reader. The fluorescence intensities in the samples and known standards were acquired and converted to the plasma concentrations of each analyte using the Bio-Plex 200 System software (version 6.0; Bio-Rad Laboratories). When the induction of immune molecules after TB antigen stimulation was below their detection limits, these molecules were excluded from subsequent analysis.

Total human adiponectin (low, middle, and high molecular weight) levels in plasma were also measured using the Quantikine Human Total Adiponectin/Acrp30 Immunoassay Kit (R&D Systems, Inc., Minneapolis, MN, USA). The mean minimum detectable dose was 0.246 ng/ml.

2.5. Statistical analysis

Values including cytokine concentrations among groups were analyzed by Kruskal–Wallis tests with multiple comparisons for all pairs by Steel–Dwass method. The inequality of proportions among the groups was analyzed by Fisher's exact test. The statistical analysis was performed using Stata version 12 (Stata Corp, College Station, TX, USA) and JMP 9 (SAS Institute Inc., Cary, NC, USA). A p-value of < 0.05 was considered to be statistically significant. The Bonferroni correction was also used for multiple comparisons, when appropriate.

3. Results

3.1. Characteristics of the patients who completed the three-time blood collection stratified by IGRA-negative or positive result at month 0

After the cross-sectional study reported previously,[3] 19 of the 24 IGRA-negative patients with culture-confirmed active pulmonary TB at the time of diagnosis completed the three-time blood collection at months 0, 2, and 7; these patients were thus analyzed in the present study. The 351 patients who initially showed QFT-IT-positive results and completed the three-time blood collection were set as a reference. Samples showing indeterminate results before treatment were omitted from this analysis.

As expected from the results of the previous report,[3] increasing age, low body mass index (BMI) at the time of diagnosis, and the HLA-DRB1*07:01 allele were observed more frequently in the 19 QFT-IT-negative patients than in the 351 QFT-IT-positive

patients before treatment (**Supplementary Material**, Online Resource 1). HIV co-infection was nearly significantly associated with QFT-IT-negative results ($p = 0.0541$). In addition, it was possible to combine QFT-IT results with *M. tuberculosis* strain genotypes in 90% of these patients. Approximately 60% of the *M. tuberculosis* strains were of the Beijing genotype in this population, according to the previous results of SNP analysis and spoligotyping.[5] Non-Beijing strains, particularly EAI strains, were predominant in the QFT-IT-negative group as compared to the QFT-IT-positive group (52.9% vs. 18.3%; $p = 0.0004$; **Supplementary Material**, Online Resource 1).

3.2. Treatment course of the patients stratified by IGRA-negative or positive result at month 0

During treatment, QFT-IT IFN-γ values (TBAg minus Nil) of the 19 patients who showed QFT-IT-negative results at month 0 remained relatively low (Table 1).

The number of patients with a positive sputum smear was relatively small at month 7, indicating that a majority of the patients in both groups had been treated successfully; only one of 19 (5.3%) and five of 351 (1.4%) patients were regarded as treatment failure in the QFT-IT-negative and positive groups, respectively, at month 0, and this difference was not significant (data not shown). The extent of cavitary lesions and infiltrates on chest X-ray at month 0 and month 7 were also not different between the QFT-IT-negative and positive groups (Table 1).

3.3. Characteristics of the IGRA-negative patients at month 0 stratified by the absence or presence of positive conversion during treatment

Eight of the 19 QFT-IT-negative patients showed positive conversion at month 2 or month 7, whereas the remaining 11 (58%) did not. Background information, such as age and low BMI, was further compared between these two subgroups, but no particular characteristics were statistically significant (Table 2). Only three of the eight patients with positive conversion showed IFN-γ values higher than 1 IU/ml at month 2 (1.0, 2.2, and 3.2 IU/ml) and month 7 (1.8, 5.2, and 10.0 IU/ml). These patients did not carry any of the DRB1*07:01 alleles (data not shown), whereas all of the patients carrying the HLA-DRB1*07:01 allele in the QFT-IT-negative group showed IFN-γ values lower than 1.0 IU/ml throughout the treatment period (data not shown).

During treatment, sputum smear and chest X-ray findings were not significantly different between the subgroups with and without positive conversion. A trace of cavitary lesions was not observed in any of the five patients without positive conversion, but was observed in three of the six patients with positive conversion (**Supplementary Material**, Online Resource 2), although this difference was not statistically significant ($p = 0.18$).

3.4. Treatment-dependent changes in immunological parameters in the blood from patients with IGRA-negative results before treatment

The concentrations of immune molecules induced by *M. tuberculosis* antigen-specific antigens were analyzed in patients

Table 1
Treatment response of the patients stratified by QFT-IT-negative or positive result at month 0

		Negative result before treatment ($n = 19$)		Positive result before treatment ($n = 351$)		p-Value
QFT-IT-positive result	Month 0	0	(0.0%)	351	(100.0%)	<0.0001
	Month 2	5	(26.3%)	312	(88.9%)	<0.0001
	Month 7	6	(31.6%)	300	(85.5%)	<0.0001
QFT-IT value (IU/ml)	Month 0	0.21	(0.05–0.25)	8.61	(3.46–14.9)	<0.0001
	Month 2	0.11	(0.03–0.52)	3.80	(1.15–11.64)	<0.0001
	Month 7	0.19	(0.02–0.79)	2.85	(0.88–8.82)	<0.0001
Positive sputum smear	Month 0	19	(100.0%)	351	(100.0%)	1.0000
	Month 2	4	(21.1%)	40	(11.4%)	0.2623
	Month 5	1	(5.3%)	6	(1.7%)	0.3107
	Month 7	1	(5.3%)	5	(1.4%)	0.3107
Chest X-ray						
Extent of cavity (number of affected zones of the lung field)[a]	Month 0					0.6753
	0	5	(27.8%)	105	(31.2%)	
	1	9	(50.0%)	174	(51.6%)	
	2	3	(16.7%)	46	(13.7%)	
	3	1	(5.6%)	8	(2.4%)	
	>4	0	(0.0%)	4	(1.2%)	
	Month 7					0.1304
	0	8	(72.7%)	234	(91.8%)	
	1	3	(27.3%)	19	(7.5%)	
	2	0	(0.0%)	1	(0.4%)	
	3	0	(0.0%)	1	(0.4%)	
	>4	0	(0.0%)	0	(0.0%)	
Extent of infiltration (number of affected zones of the lung field)[b]	Month 0					0.1723
	0	2	(11.1%)	16	(4.7%)	
	1	3	(16.7%)	81	(23.9%)	
	2	4	(22.2%)	117	(34.5%)	
	3	3	(16.7%)	73	(21.5%)	
	>4	6	(33.3%)	52	(15.3%)	
	Month 7					0.3157
	0	3	(27.3%)	106	(41.7%)	
	1	4	(36.4%)	99	(39.0%)	
	2	4	(36.4%)	33	(13.0%)	
	3	0	(0.0%)	11	(4.3%)	
	>4	0	(0.0%)	5	(2.0%)	

QFT-IT, QuantiFERON-TB in-tube.
[a] $n = 18$ and $n = 337$ at month 0; $n = 11$ and $n = 255$ at month 7.
[b] $n = 18$ and $n = 339$ at month 0; $n = 11$ and $n = 254$ at month 7.

Table 2
Characteristics of QFT-IT-negative patients at month 0 stratified by the absence or presence of positive conversion

		With no positive conversion during treatment ($n=11$)		With positive conversion during treatment ($n=8$)		p-Value
Sex	Male	11	(100.0%)	7	(87.5%)	0.4211
	Female	0	(0.0%)	1	(12.5%)	
Age at diagnosis, years		55.4	(37.4–67.0)	43.3	(30.8–49.0)	0.1167
Body mass index, kg/m^2		16.7	(15.8–19.1)	15.9	(13.1–17.8)	0.3637
Smoking habit	Smoker/ex-smoker	8	(72.7%)	6	(75.0%)	1.0000
	Non-smoker	3	(27.3%)	2	(25.0%)	
BCG vaccination[a]	No	1	(9.1%)	1	(12.5%)	1.0000
	Yes	1	(9.1%)	0	(0.0%)	
	Unknown	9	(81.8%)	7	(87.5%)	
Sputum smear	Scanty	2	(18.2%)	0	(0.0%)	0.7611
	+	4	(36.3%)	4	(50.0%)	
	++	3	(27.3%)	3	(37.5%)	
	+++	2	(18.2%)	1	(12.5%)	
HIV status	Negative	9	(81.8%)	7	(87.5%)	1.0000
	Positive	2	(18.2%)	1	(12.5%)	
White blood cell count $\times 10^9$/l		8.6	(5.3–12.0)	9.9	(9.5–18.8)	0.2151
Lymphocyte count $\times 10^9$/l		1.4	(0.8–2.0)	2.1	(1.7–2.5)	0.1345
Drug resistance	Isoniazid	1	(9.1%)	1	(12.5%)	1.0000
	Rifampicin	1	(9.1%)	0	(0.0%)	1.0000
	Streptomycin	2	(18.2%)	1	(12.5%)	1.0000
	Ethambutol	1	(9.1%)	0	(0.0%)	1.0000
HLA-DRB1*07:01 allele number	0	6	(54.5%)	6	(75.0%)	0.1852
	1	4	(36.4%)	0	(0.0%)	
	2	1	(9.1%)	2	(25.0%)	
MTB strains[b]	Beijing	1	(11.7%)	2	(25.0%)	0.5335
	EAI	6	(66.7%)	3	(37.5%)	
	Other	2	(22.2%)	3	(37.5%)	

QFT-IT, QuantiFERON-TB in-tube; BCG, bacille Calmette–Guérin; MTB, *Mycobacterium tuberculosis*.
[a] Self-declaration.
[b] $n=9$ and $n=8$.

showing negative results at month 0 with positive conversion at months 2 or 7, in patients showing negative results at month 0 without positive conversion, and in 21 randomly selected patients who showed a QFT-IT-positive result at month 0, and then completed the treatment course.

Of the 27 immune analytes in the plasma supernatants, IL-1RA, IL-2, IFN-γ, and IP-10 showed differences in concentrations among the groups, even after Bonferroni correction; these immunological parameters were predominantly induced in the QFT-IT-positive reference group, whereas their concentrations in QFT-IT-negative subgroups were low (Figure 1). Although the induction levels of these immune parameters gradually decreased during treatment in the QFT-IT-positive group, significant differences remained between the QFT-IT-positive group and the continuously negative group without positive conversion. In the transiently negative group with positive conversion, levels of these immune parameters were restored to some extent during the course of treatment; concentrations of IL-1RA and IL-2 in the transiently negative group were significantly higher than those in the continuously negative group at month 7 (Figure 1). Concentrations of IL-10, known as a regulatory cytokine, were not significantly different between these two subgroups; adiponectin concentrations did not show a significant difference either (data not shown).

4. Discussion

The QFT-IT test results of Vietnamese patients with culture-confirmed active pulmonary TB enrolled in a cross-sectional study were recently reported,[3] and factors that possibly lower the sensitivity of this ELISA-based IGRA were identified: aging, emaciation, HIV co-infection, and a particular HLA genotype, DRB1*07:01.[3] In the present study, the IGRA-negative TB patients from the previous study underwent continued monitoring during treatment, and treatment-dependent changes in immune status were characterized by measuring the concentrations of cytokines and chemokines in the plasma supernatants.

IL-1RA, IL-2, and IP-10, as well as IFN-γ, were more or less inducible after TB antigen-specific stimulation (TBAg minus Nil) in active pulmonary TB. The induction levels were significantly lower in the continuously IGRA-negative group than in the IGRA-positive group, even after initiating treatment. This is possibly due to immune regulatory mechanism(s), including impaired antigen presentation through a particular HLA molecule or long-lasting T-cell anergy,[6,7] although immunoregulatory IL-10 levels relevant to the regulatory T-cells were not different between these groups.

The difference in cytokine/chemokine induction was gradually lost between the IGRA-positive group and the transiently IGRA-negative group. The immune dynamics in the patients in whom the immune response was restored may be consistent with those of an early report, which showed the suppression of IFN-γ production from CD4+ T-cells in response to TB-specific peptides in patients with severe pulmonary TB before treatment.[2] However, the IFN-γ response was fully restored (>1 IU/ml) during the treatment period in only three of the 19 patients (16%).

It has thus been demonstrated that IGRA-negative patients tend to have continuously low immune responsiveness despite successful treatment, measuring not only IFN-γ but also other immune molecules. These findings indicate that factors unaffected by treatment are important when negative IGRA results are interpreted in active TB.

IL-1RA is a natural antagonist of IL-1 produced by activated monocyte/macrophages, and blocks the binding of IL-1α and IL-1β to the type I IL-1 receptor, without exerting agonist activity. IL-1RA is elevated in the blood of patients with inflammation, and IL-1RA has been suggested as a marker for TB disease activity and response to treatment.[8] Increased levels of IL-1RA were found both in the lung epithelial lining fluid and in the blood from patients with active pulmonary TB.[9] IL-1 is not easily detected in the circulation during human disease, and IL-1RA may be a better maker than IL-1 as an indicator of ongoing inflammation.[10]

The restoration of IL-2 production in the IGRA-negative group with positive conversion is consistent with previous reports:[11,12]

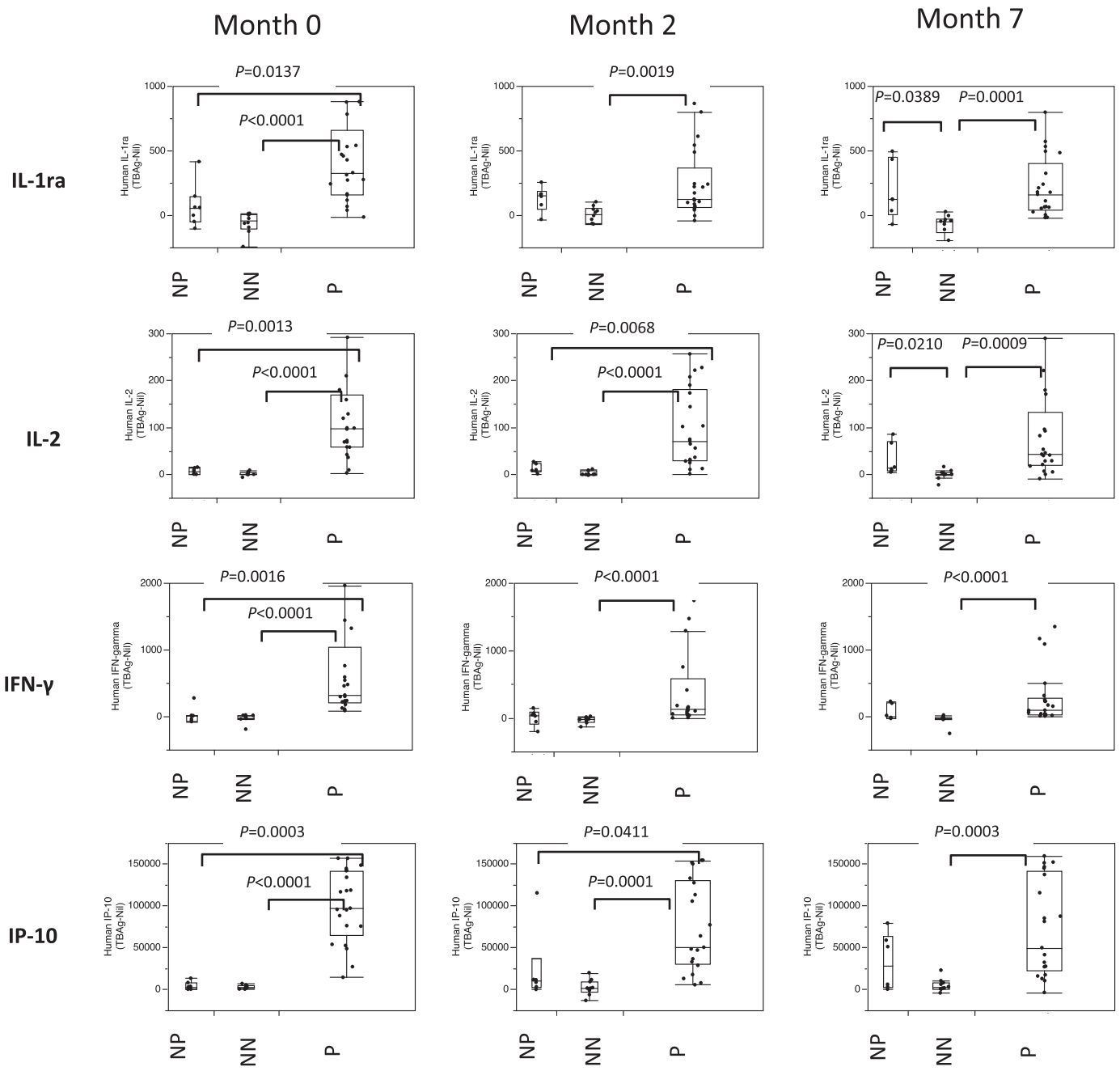

Figure 1. Immune analyte concentrations after TB antigen-specific stimulation at months 0, 2, and 7. Immune analyte concentrations (ng/ml) after TB antigen-specific stimulation (TBAg minus Nil) were compared among IGRA-negative groups at month 0 with positive conversion at months 2 or 7 (NP) and without positive conversion (NN), and the IGRA-positive reference group at month 0 (P). A significant difference in IL-1RA, IL-2, IFN-γ, and IL-2 concentrations was observed even after Bonferroni correction (uncorrected $p < 0.0005$ by Kruskal–Wallis test). All pairs were further compared by Steel–Dwass method, and their significant p-values are shown.

in patients with normal TB immunity, it is known that antigen-specific IFN-γ-only-secreting effector T-cells are predominant before treatment and dual IFN-γ/IL-2-secreting or polyfunctional T-cells with memory cell characteristics become predominant after starting treatment.[11,12] In the continuously IGRA-negative group, effector memory T-cells might have failed to expand for unknown reasons. Indeed, when the suspension array data were analyzed, low IL-2 induction in QFT-IT was observed together with the low IFN-γ response in the patients without positive conversion.

IP-10 is a chemokine expressed by antigen-presenting cells in response to IFN-γ, and IP-10-based tests are comparable with the IGRA response[13] before and during treatment.[14] This study group has previously reported that circulating adiponectin may be a marker for the severity of the disease,[15] but this parameter was not clearly associated with IGRA results in the present study.

Changes in clinical phenotypes relevant to treatment failure, such as chest radiographic lesions and smear-negative conversion, were not associated with the IGRA results at month 0. In the present study, the transiently IGRA-negative group with positive conversion did not show any clinical backgrounds distinct from those of the continuously IGRA-negative group without positive conversion, presumably because disease severity is further affected by the combination of other factors including patient age, patient BMI, and the bacterial burden.

Furthermore, *M. tuberculosis* strains of non-Beijing EAI genotype were frequently observed in the IGRA-negative group in this study. This may be partly confounded by the fact that non-Beijing strains are often observed in elderly people in Hanoi.[16] However, confirmation based on multivariable analysis was not performed because of the small sample size.

The HLA-DRB1*07:01 allele, an endogenous genetic factor, has previously been reported to be associated with QFT-IT false-negative results or a low IFN-γ response.[3] This tendency was shown throughout the treatment course in the present study, although it did not reach statistical significance, presumably due to the relatively small sample size.

These findings will provide insights into factors affecting QFT-IT false-negative results over a long term, although they may not be extrapolated to those of another IGRA, the T-SPOT.TB. Nevertheless, a variety of factors that potentially suppress the IGRA response should be investigated further and the test performance of the IGRA should be improved in future studies.

Acknowledgements

This work was supported by a grant from the Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), MEXT, Japan. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank Dr Nguyen Phuong Hoang, Dr Pham Thu Anh (Hanoi Lung Hospital), Dr Phan Thi Minh Ngoc, Ms Nguyen Thi Ha (NCGM-BMH Medical Collaboration Center), and all the healthcare staff of the relevant district TB centers for supporting the site implementation.

Ethics statement: The study was approved by the ethics committees of the Ministry of Health, Vietnam, National Center for Global Health and Medicine, and The Research Institute of Tuberculosis, JATA Japan. All participants were enrolled upon provision of written informed consent.

Conflict of interest: The authors have declared that no competing interests exist.

Author contributions: IM carried out the immunoassays, drafting the paper. NTLH participated in supervising the on-site implementation of the study. LTH and DBT carried out the immunoassays. LTL and PHT participated in conception, design and supervision of the study. VCC participated in supervising the on-site implementation of the study. MH participated in analysis and interpretation of the data. NKo and SS participated in conception and design of the study. KH and NH were responsible for technical transfer and supervision. NK was responsible for the conception, design and overall supervision of the study, analysis and interpretation of data, drafting the paper or substantially revising it. All authors read and approved the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.09.021.

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