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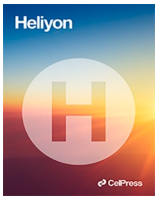

Research article

Potential biomarkers for evaluating the BCG vaccination response based on humoral immunity

Yan-Qing Chen [a,d,1], Shu-Hui Cao [b,d,1], Xin-Yu Yang [c,1], Yi Liu [d], Chuan-You Li [c,d,*]

[a] Department of Sentience and Technology, Beijing Geriatric Hospital, Beijing, China
[b] Department of Laboratory Center, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing, China
[c] Beijing Center for Disease Prevention and Control, Beijing, China
[d] Department of Bacteriology and Immunology, Beijing Key Laboratory on Drug-Resistant Tuberculosis Research, Beijing Tuberculosis and Thoracic Tumor Research Institute/Beijing Chest Hospital, Capital Medical University, Beijing, China

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ABSTRACT

Background: The current prophylactic tuberculosis vaccine Bacille Calmette-Guérin (BCG), was derived in the 1920s, but the humoral immune responses induced by BCG vaccination have not been fully elucidated to date. In this study, our aim was to reveal the profiles of antibody responses induced by BCG vaccination in adults and identify the potential biomarkers for evaluating the BCG vaccination response.

Methods: Proteome microarrays were performed to reveal the serum profiles of antibody responses induced by BCG vaccination in adults. ELISA was used to validate the potential biomarkers in validation cohort (79 healthy controls and 58 BCG-vaccinated subjects). Then combined panel was established by logistic regression analysis based on OD values of potential biomarkers.

Results: Multiple antigens elicited stronger serum IgG or IgM antibody responses in BCG vaccinated subjects than healthy subjects at 12 weeks post BCG vaccination; among the antigens, Rv0060, Rv2026c and Rv3379c were further verified using 137 serum samples and presented the moderate performance in assessment of the BCG vaccination response by receiver operating characteristic analysis. Furthermore, a combined panel exhibited an improved AUC of 0.923, and the sensitivity and specificity were 77.59 % and 91.14 %, respectively. In addition, the antibody response against Rv0060, Rv2026c and Rv3379c was related to the clinical background to a certain extent.

Conclusions: The novel antigens identified in our study could offer better knowledge towards developing a more efficacious vaccine based on humoral immune responses, and they could be potential biomarkers in assessments of BCG vaccination responses.

1. Introduction

Tuberculosis (TB) remains a major public health problem in many parts of the world, with nearly a quarter of the world's human population thought to be latent infected with the pathogenic *Mycobacterium tuberculosis* (MTB) [1]. The World Health Organization

* Corresponding author. Dongguang, Hutong, No. 5, Xicheng District, Beijing, China.
E-mail addresses: hailaer-324@163.com (Y.-Q. Chen), lichuanyou6688@hotmail.com (C.-Y. Li).
[1] These authors contributed equally to this work.

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estimates that approximately 10.6 million new TB cases and 1.4 million TB deaths among HIV-negative people occurred in 2021 [2]. Bacille Calmette-Guérin (BCG) is the only approved and available TB preventative vaccine, and it is produced using a live attenuated strain of *Mycobacterium bovis* (*M. bovis*), a relative of MTB [3]. The BCG vaccine has been used widely for over 100 years to against children TB and disseminated TB diseases. However, the BCG vaccine shows highly variable efficacy in protecting against adult pulmonary TB [4]. Therefore, a more effective vaccine is needed.

Since MTB is a facultative intracellular pathogen, previous studies on the immunologic response to MTB infection mainly focused on T cells and macrophages. In contrast, the role of B cells in the pathophysiology of MTB infection has been relatively overlooked [5]. While increasing compelling evidence demonstrates that antigen-specific humoral immunity also plays a critical role in protecting against MTB infection. For example, antibodies targeting high activity binding peptides (HABPs) can inhibit MTB entry into U937 cells [6]. Monoclonal antibodies against lipoarabinomannan (LAM), HBHA, MPB83 and 16 kDa α-crystallin can improve the outcomes of MTB infection in mice [7]. Moreover, the passive transfer of immune serum from humans to mice or from mice to mice has led to protective immunity against MTB [8–10]. Furthermore, a deficit of B cells or humoral immunity results in increased host susceptibility [11]. In addition, intradermal BCG vaccination elicits IgG and IgM responses to multiple MTB antigens [12], and the arabinomannan-containing conjugate vaccine also elicits an antibody response that reduces susceptibility to MTB infection [13]. Consequently, based on these findings, the induction of antibody-mediated humoral immunity should be considered an option in TB vaccine development strategies.

Antigen-specific antibodies are not only a key element in vaccine development but could also be potential biomarkers for evaluating responses following BCG vaccination. Conventionally, the response to BCG vaccination was evaluated by whether a BCG scar developed or the tuberculin skin test (TST) measuring the reaction to a purified protein derivative of MTB (PPD) [14], which is often used as an indicator of BCG vaccination efficacy. However, not all populations develop a scar [15], and the TST also has some limitations; it requires two visits and cross reacts with many nontuberculous mycobacteria, and the concept of a positive result ranges from 5 mm to 15 mm [16]. Thus, the identification of potential biomarkers based on humoral immunity is useful to design a novel method for assessing responses to BCG vaccination. In our paper, we used MTB proteome microarrays to reveal the serum profiles of IgG and IgM antibodies against almost all the MTB antigens at the systemic level in BCG-vaccinated subjects and healthy controls (HCs). On these basis, we used a larger number of samples from individuals with different clinical characteristics to further verify the three potential antigens, Rv0060, Rv2026c and Rv3379c, by ELISA.

2. Materials and methods

2.1. Study population

In 2014, we enrolled a total of 174 subjects in our paper and divided into two sets: one was the discovery set comprising of 18 HCs and 19 BCG-vaccinated subjects, and the other was the validation set comprising of 79 HCs and 58 BCG-vaccinated subjects. Their demographic and clinical characteristics were presented in Table 1. HCs were characterized by an absence of any BCG scar and radiographic signs suggestive of TB disease, as well as both a negative response to the TST (<5 mm, TB-PPD, Beijing Xiangrui Biological Products Co., Ltd.) and T-SPOT test (T-SPOT. TB, Oxford Immunuotec). BCG-vaccinated subjects were defined as HCs 12 weeks after vaccination, and need to meet the following conditions at the same time: positive response to TST (≥5 mm), presence of BCG scar,

Table 1
The demographic and clinical characteristics of the subjects.

Characteristics	Discovery set		Validation set	
	HCs	BCG- vaccinated	HCs	BCG-vaccinated
Total number	18	19	79	58
Median age (range), years	37 (21–49)	37 (21–49)	31 (19–49)	32 (18–49)
24 and under, number	2	2	19	14
25 to 34, number	5	5	27	17
35 to 44, number	6	6	23	19
45 and over, number	5	6	10	8
Sex, female/male	13/5	14/5	41/38	39/19
TST test				
Induration <5 mm, number	18	0	79	0
Induration ≥5, <10 mm, number	0	6	0	11
Induration ≥10, <15 mm, number	0	9	0	25
Induration ≥15 mm, number	0	4	0	22
BCG Scar				
Presence of scar, number	0	19	0	58
Absence of scar, number	18	0	79	0
T-SPOT result				
Positive, number	0	0	0	0
Negative, number	18	19	79	58
No Immunosuppression	18	19	79	58
HIV positive	0	0	0	0

HCs, healthy controls.

negative response to T-SPOT test, and absence of radiographic signs suggestive of TB disease. The TST test was performed by professionals with intradermal injection of 0.1 mL TB-PPD into the front 1/3 of the volar of the subject's left arm. Injection methods and reading strictly follow the "Tuberculin Skin Test Instruction Manual"of China. The BCG vaccine used in this study was provided by Shanghai Institute of Products Co., Ltd., and the strain used for vaccine production is D_2PB302 derived from Danish 823 strain. According to the People's Republic of China Health Industry Standard, the TST-positive results of BCG-vaccinated subjects were further categorized as generally positive with indurations $\geq$5 mm and $<$10 mm, moderately positive with indurations $\geq$10 mm and $<$15 mm, and strongly positive with indurations $\geq$15 mm. In addition, all subjects were not infected with HIV or had a history of immunosuppressive therapy. In either set, there were no significant differences in age and sex between the HCs and BCG-vaccinated subjects.

Venous blood samples were collected on an empty stomach from the abovementioned HCs at the time of enrollment, and the BCG-vaccinated subjects at 12 weeks after vaccination, using heparin-vacuum blood collection tube (BD Company, USA), and then followed by serum separation at 3000 r/min for 10 min at room temperature and aliquoting into cryopreservation tubes. All serum was stored at −80 °C before use.

2.2. MTB proteome microarray and data analysis

The MTB proteome microarrays (BCBIO, Guangzhou, China) comprised 3829 MTB H37Rv antigens and 433 CDC1551 (pathogenic strain) antigens with GST tags produced from the *S. cerevisiae* expression system. Each antigen was printed in duplicate on polymer slides (polymer slide H, CapitalBio). In addition, human IgG and IgM positive controls and BSA negative controls were also printed in duplicates on the polymer slides. Microarrays were performed in our paper to evaluate the serum IgG and IgM antibody responses to MTB antigens. Briefly, the proteome microarrays were preincubated in blocking buffer [3 % BSA in 1 × TBST (Tris-buffered saline with 0.1 % Tween 20), pH 7.4] for 10 min and gently shaken at room temperature. The blocking buffer was removed, new blocking buffer was added, and gently shaken at room temperature for 1 h. The blocking buffer was discarded and the microarrays were probed with 3 ml of individual serum samples at a dilution rate of 1:50 in 1 × TBST. The microarrays were incubated at room temperature with a gentle shake for 3 h, washed with TBST 5 times, and incubated with 3 ml of secondary antibodies containing Cy3-labeled goat anti-human IgG (1:1000; Jackson Laboratory, PA, USA; catalog:109-165-008) and Cy5-labeled anti-human IgM (1:1000; Jackson Laboratory, PA, USA; catalog:709-605-073). Shaken gently in the dark at room temperature for 45 min. Then microarrays were washed with TBST 5 times, with water 5 times, and dried in a SlideWasher (CapitalBio, Beijing, China). GenePix 4200A (Molecular Devices, CA, USA) was used to scan IgG at 532 nm or IgM at 635 nm.

The microarray data were processed and extracted with GenePix Pro 6.0 software (Molecular Devices, CA, USA) and analyzed as previously described [17]. In brief, the background calibration and quantile normalization of the raw fluorescence data were processed by the ''limma'' package for the R programming language. These normalized data were subjected to further statistical analysis. The differentially responsive antigens were defined on the basis of a p-value of 0.05 using the Bioconductor package in R3.0. The fold change was calculated and presented as $\log_2$ values with the antibody response ratios of BCG vaccinated subjects to healthy controls. A heatmap was generated using the "pheatmap" package in R3.1.0.

2.3. ELISA

The commercial MTB antigens Rv0060, Rv2026c and Rv3379c were purchased from BCBIO. In brief, 96-well flat-bottom plates were coated with 100 μl of antigens at 2.5 μg/ml in coating buffer (carbonate-bicarbonate, pH 9.6) and kept at 4 °C overnight. After being washed 3 × 5 min with PBST (phosphate-buffered saline, 0.05 % Tween 20, pH 7.4), the plates were blocked with blocking buffer (5 % skim milk in PBS) at room temperature for 2 h, followed by 3 × 5 min washes with TBST. Then, 100 μl individual serum samples at a 1:100 dilution in TBST were added to each well in duplicate, and negative controls were also included. Following an incubation at room temperature for 1.5 h and 3 × 5 min washes with PBST, 100 μl of goat anti-human IgG- or IgM-conjugated horseradish peroxidase at dilutions of 1:30000 or 1:10000, respectively, in PBST was added to each well and incubated at room temperature for 1 h. After 3 × 5 min washes with PBST, 100 μl of TMB substrate (BD, NJ, USA) was added to each well at room temperature for a 5–10 min, then 50 μl of 2 M H_2SO_4 was added to terminate the reaction. The optical density was recorded at 450 nm (OD_{450}) with an automated microplate reader (Perlong, Beijing, China).

2.4. Statistical analysis

GraphPad Prism V5.0 (GraphPad Software, CA, USA) and SPSS 17.0 software (IBM, NY, USA) were used for the statistical analyses. Chi-square test was used for the differences between categorical variables, Mann-Whitney test was used for the differences between two continuous variables, and Kruskal-Wallis test was used for the differences between three or more continuous variables. A P value of less than 0.05 was defined statistically significant. The area under the curve (AUC) of the potential biomarkers was evaluated by receiver-operating characteristic (ROC) curve analysis, and the sensitivity and specificity were calculated based on the optimal cutoff obtained by the largest Youden index. The combined antigen panel was acquired by logistic regression analysis with a stepwise procedure with an F-value probability of 0.05 for inclusion and 0.10 for exclusion.

3. Results

3.1. Identification of antigens with differential antibody responses between BCG-vaccinated subjects and HCs using MTB proteome microarrays

To study the antibody responses to MTB antigens after the subjects were vaccinated with BCG, proteome microarrays comprising 4262 MTB antigens were detected using sera from 18 HCs to 19 BCG-vaccinated subjects in the discovery set. The results revealed that the IgG and IgM antibody responses against multiple antigens were significantly higher in the BCG-vaccinated subjects than in the HCs based on a p value of less than 0.05. Based on the values of fold change, the top 20 antigens with stronger immunogenicity were selected, which could be recognized by either the IgG or IgM antibody (Tables 2 and 3). Of note, five antigens, namely Rv2735c, Rv0060, Rv3379c, Rv2026c, and Rv3368c, were recognized by both the IgG and IgM antibodies. According to the NCBI GENE database, the annotations of the five antigens are presented in Table 4. In addition, the five antigen-specific IgG and IgM antibody responses in the serum of each individual from the BCG vaccination subjects and HCs are shown as a heatmap (Fig. 1A and B), and the antibody response to the same antigen exhibited person-to-person variations in both BCG-vaccinated subjects and HCs.

3.2. Validation of candidate antigens using ELISA

To verify the levels of IgG and IgM antibodies against Rv0060, Rv2026c and Rv3379c, we performed an ELISA using additional sera from 79 HCs to 58 BCG-vaccinated subjects in the validation set (Table 1). The results showed that the levels of three antigen-specific IgG (Fig. 2A) and IgM antibodies (Fig. 2B) were all significantly higher in the BCG-vaccinated subjects than in the HCs, which is consistent with the results of the proteome microarrays.

3.3. Performance of the three antigens in assessments of the BCG vaccination response

Traditionally, BCG scarring and TST tests for evaluating the BCG vaccination response have some limitations. Rv0060-, Rv2026c- and Rv3379c-specific antibody responses were markedly increased in BCG-vaccinated subjects compared to the HCs (Fig. 2), suggesting that these antigens may have potential uses as biomarkers for assessing the response to BCG vaccination. The performance of each antigen in assessing the vaccination response was evaluated by ROC analysis. The result displayed that the levels of IgG antibody against Rv0060 had the best AUC of 0.8788 (95 % CI: 0.8221–0.9355, cutoff: 0.3083, $p < 0.0001$, Fig. 3A) with a sensitivity of 89.66 % (95 % CI: 78.83%–96.11 %) and specificity of 70.89 % (95 % CI: 59.58%–80.57 %). The IgG antibody against Rv3379c also showed a better AUC of 0.8661 (95 % CI: 0.8075–0.9247, cutoff: 0.3175, $p < 0.0001$, Fig. 3E), with a sensitivity of 91.38 % (95 % CI: 81.02%–97.14 %) and specificity of 70.89 % (95 % CI: 59.58%–80.57 %). Moreover, the IgM antibodies against Rv2026c, Rv3379c and Rv0060 showed moderate AUCs of 0.7867 (95 % CI: 0.7068–0.8665, cutoff: 0.3035, $p < 0.0001$, Fig. 3D), 0.7560 (95 % CI: 0.6758–0.8362, cutoff: 0.2658, $p < 0.0001$, Fig. 3F), and 0.7537 (95 % CI: 0.6709–0.8365, cutoff: 0.7128, $p < 0.0001$, Fig. 3B), respectively. However, the IgG antibody against Rv2026c had the lowest AUC of 0.6431 (95 % CI: 0.5505–0.7356, cutoff: 0.1540, $p = 0.0043$, Fig. 3C), with

Table 2
The antigens with differential response to IgG antibody between BCG-vaccinated subjects and HCs.

No.	Rv Number	BCG Number[a]	IgG fluorescence intensity (Average ± SD)		Fold change[b]	P-value[c]
			HCs (n = 18)	BCG-vaccinated (n = 19)		
1	Rv2735c	Mb2754c	6.67 ± 1.16	8.09 ± 2.00	0.28	0.0100
2	Rv0195	Mb0201	6.39 ± 0.77	7.57 ± 0.89	0.24	<0.0001
3	Rv0060	Mb0061	6.43 ± 0.81	7.58 ± 0.78	0.24	0.0002
4	Rv3379c	Mb3413c	6.16 ± 0.52	7.26 ± 0.61	0.24	<0.0001
5	Rv3297	Mb3325	6.20 ± 0.82	7.27 ± 0.97	0.23	0.0013
6	Rv2572c	Mb2602c	6.32 ± 0.76	7.40 ± 0.99	0.23	0.0007
7	Rv3430c	Mb3460c	6.57 ± 0.69	7.66 ± 0.54	0.22	<0.0001
8	Rv1822	Mb1853	6.63 ± 0.69	7.72 ± 0.47	0.22	<0.0001
9	Rv2026c	Mb2051c	7.24 ± 1.39	8.44 ± 2.19	0.22	0.0308
10	Rv3368c	Mb3403c	6.22 ± 0.73	7.23 ± 0.78	0.22	0.0001
11	Rv2986c	Mb3010c	6.14 ± 0.70	7.13 ± 0.92	0.22	0.0004
12	Rv3495c	Mb3525c	6.42 ± 0.86	7.46 ± 1.00	0.22	0.0015
13	Rv2672	Mb2691	6.58 ± 0.65	7.59 ± 0.80	0.21	0.0003
14	Rv0400c	Mb0406c	6.40 ± 0.56	7.34 ± 0.90	0.20	0.0005
15	Rv3322c	Mb3351c	5.96 ± 0.62	6.82 ± 0.82	0.19	0.0030
16	Rv2150c	Mb2174c	6.39 ± 0.59	7.31 ± 0.59	0.19	<0.0001
17	Rv1870c	Mb1901c	6.43 ± 0.52	7.35 ± 0.59	0.19	<0.0001
18	Rv2835c	Mb2859c Mb2860c	6.44 ± 0.50	7.36 ± 0.61	0.19	<0.0001
19	Rv3203	Mb3228	6.63 ± 0.95	7.57 ± 1.48	0.19	0.0233
20	Rv3485c	Mb3515c	6.50 ± 0.74	7.40 ± 0.64	0.19	0.0004

[a] BCG number is equivalent to the Rv number according to the TubercuList database (http://tuberculist.epfl.ch/).
[b] Fold change refers to the Log_2 value of the ratio of the IgG antibody level of BCG vaccinated to that of HCs.
[c] P-value was acquired by Mann-Whitney tests.

Table 3
The antigens with differential response to IgM antibody between BCG-vaccinated subjects and HCs.

No.	Rv Number	BCG Number	IgM fluorescence intensity (Average ± SD)		Fold change[a]	P-value[b]
			HCs (n = 18)	BCG-vaccinated (n = 19)		
1	Rv2026c	Mb2051c	8.32 ± 2.45	10.63 ± 2.81	0.35	0.0118
2	Rv0390	Mb0396	8.30 ± 1.28	10.08 ± 2.10	0.28	0.0020
3	Rv2284	Mb2305	6.95 ± 1.19	8.35 ± 1.18	0.26	0.0007
4	Rv2735c	Mb2754c	7.33 ± 1.49	8.62 ± 1.94	0.23	0.0149
5	Rv1109c	Mb1139c	6.67 ± 1.30	7.73 ± 1.13	0.21	0.0033
6	Rv3591c	Mb3622c	7.24 ± 1.03	8.38 ± 1.24	0.21	0.0030
7	Rv0060	Mb0061	6.71 ± 1.30	7.76 ± 0.91	0.21	0.0056
8	Rv1472	Mb1507	6.62 ± 1.00	7.62 ± 0.89	0.20	0.0019
9	Rv3783	Mb3812	7.56 ± 1.24	8.69 ± 0.93	0.20	0.0030
10	Rv0771	Mb0794	7.40 ± 1.01	8.50 ± 1.09	0.20	0.0025
11	Rv2077c	Mb2102c	6.41 ± 1.18	7.36 ± 0.99	0.20	0.0056
12	Rv2907c	Mb2931c	6.62 ± 0.87	7.57 ± 1.22	0.19	0.0066
13	Rv0010c	Mb0010c	10.37 ± 2.26	11.84 ± 2.17	0.19	0.0341
14	Rv3379c	Mb3413c	6.91 ± 0.94	7.88 ± 0.95	0.19	0.0017
15	Rv2786c	Mb2809c	7.33 ± 0.63	8.34 ± 0.70	0.19	0.0001
16	Rv2395	Mb2416 Mb2417	7.75 ± 0.91	8.76 ± 1.06	0.18	0.0025
17	Rv1283c	Mb1314c	7.25 ± 1.00	8.19 ± 1.42	0.18	0.0201
18	Rv3452	Mb3482	7.74 ± 0.96	8.74 ± 1.19	0.18	0.0072
19	Rv3368c	Mb3403c	6.78 ± 0.93	7.65 ± 0.74	0.18	0.0030
20	Rv1016c	Mb1044c	7.82 ± 0.72	8.83 ± 0.99	0.17	0.0005

[a] Fold change refers to the Log_2 value of the ratio of the IgM antibody level of BCG vaccinated to that of HCs.
[b] P-value was acquired by Mann-Whitney tests.

Table 4
Five antigens recognized by the IgG and IgM antibodies.

Rv Number	BCG Number	Annotation
Rv2735c	Mb2754c	Hypothetical protein
Rv0060	Mb0061	Antitoxin essential for bacterial viability
Rv3379c	Mb3413c	Probable 1-deoxy-D-xylulose-5-phosphate synthase dxs2
Rv2026c	Mb2051c	Universal stress protein family protein
Rv3368c	Mb3403c	Possible oxidoreductase

Annotations are acquired from the NCBI GENE database (https://www.ncbi.nlm.nih.gov/gene/).

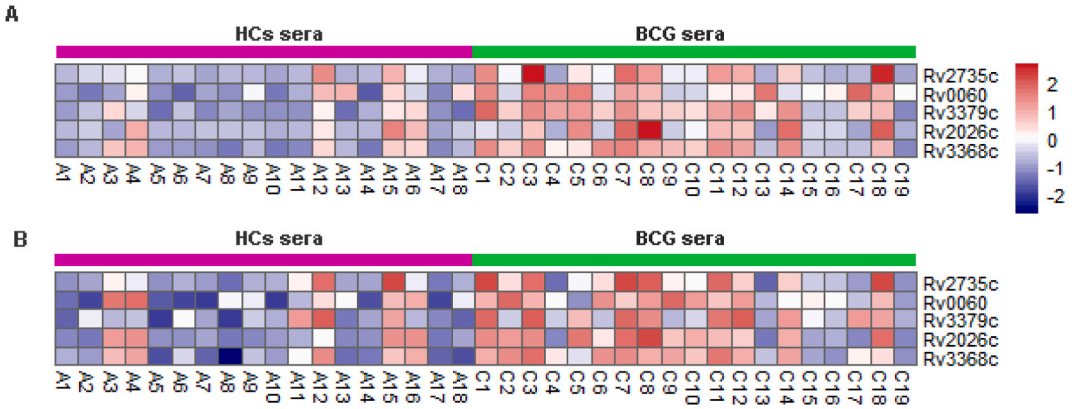

Fig. 1. Heatmap of antibody responses against five antigens. (A) IgG reactivity, (B) IgM reactivity. The heatmap presents the antibody responses in each individual serum from the BCG-vaccinated subjects and the HCs to each of the five antigens targeted by IgG and IgM simultaneously. Each row denotes one antigen, and each column denotes one serum sample. The color spectrum ranges from red to white to blue, indicating reactivity from high to low. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the highest sensitivity of 96.55 % (95 % CI: 88.09%–99.58 %) but the lowest specificity of 32.91 % (95 % CI: 22.75%–44.40 %).

Since antigens combination may enhance the assessment ability, logistic regression analysis was employed, which revealed that IgG antibody against Rv0060 and Rv3379c and IgM antibody against Rv3379c contributed the most to assessing the BCG vaccination response. Therefore, a combined panel = −8.149 + 5.480 X1+11.263 X2+5.349 X3 was established (X1: IgG against Rv0060, X2: IgG

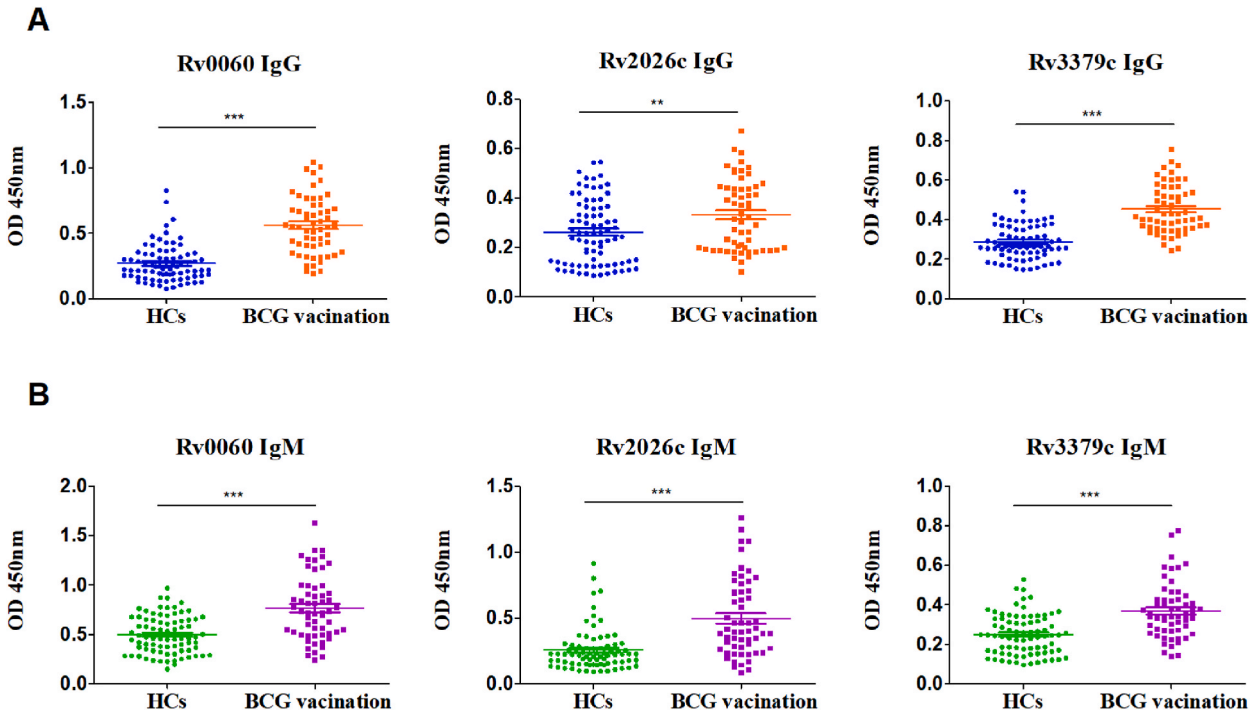

Fig. 2. IgG and IgM levels against Rv0060, Rv2026c and Rv3379c in BCG-vaccinated subjects and HCs assayed by ELISA. (A) IgG levels, (B) IgM levels. Two-sided p values obtained by Mann-Whitney test, (**) $p < 0.01$; (***) $p < 0.001$; and bars, median values.

against Rv3379c; X3: IgM against Rv3379c). In addition, the panel indeed showed an improved AUC of 0.923 (95 % CI: 0.8804–0.9655, cutoff: 0.5258, $p < 0.0001$, Fig. 3G), with a sensitivity of 77.59 % (95 % CI: 64.73%–87.49 %) and significantly increased specificity of 91.14 % (95 % CI: 82.59%–96.36 %).

3.4. Correlation of antibody reactivity with clinical characteristics

To investigate the factors affecting antibody responses, clinical information was collected, including participant sex, age, and the degree of TST responses. In the HCs, except for the levels of IgG against Rv0060 and Rv2026c, the levels of IgG against Rv3379c and IgM against Rv0060, Rv2026c and Rv3379c were all correlated with gender, and females exhibited higher antibody levels than males (Fig. 4A). For antibody responses in the HCs, age was not an influential factor (Fig. 4B). In BCG-vaccinated subjects, IgG and IgM reactivity against Rv0060 and IgG reactivity against Rv3379c showed higher antibody levels in females than males (Fig. 5A). Similar to HCs, age also did not affect the antibody responses in BCG-vaccinated subjects (Fig. 5B). Moreover, the degree of TST-positive responses did not exhibit a significant association with IgG (Fig. 6A) and IgM (Fig. 6B) antibody reactivity. These results indicate the complexity and heterogeneity of antibody reactivity against different antigens, which is why the combination of antigens exhibit better assessment efficacy than single antigens.

4. Discussion

To date, the significance of B cells or humoral immunity responses to BCG vaccination has been unclear [18]. Our research aimed to reveal the serum profiles of IgG and IgM antibodies for subjects with BCG vaccinations at the systemic level employing MTB proteome microarrays comprising 4262 antigens, most of which are equivalent to antigens of *M. bovis* made for BCG. Our results showed that a variety of antigen-specific IgG or IgM levels were higher in BCG-vaccinated subjects than in HCs. Among these, five antigens, namely Rv2735c, Rv0060, Rv3379c, Rv2026c, and Rv3368c, were recognized by both the IgG and IgM antibodies. It has been found that Rv0060 is an antitoxin in MTB, functions along with its cognate toxin Rv0059, to mediate reversible DNA ADP-ribosylation [19]; Rv2026c is an universal stress proteins [20]. However, whether the above 5 antigens have immunoprotective effect against TB needs to be further studied.

Previously, Brown et al. explored antigen-specific antibodies by employing ELISA with multiple different mycobacterial antigens, including recombinant MPT63 (Rv1926c), MPT6 (Rv1980c), KatG (Rv1908c), MPT51 (Rv3803c), ESAT-6 (Rv3875), MTC28 (Rv0040c), CFP-10 (Rv3874), HBHA (Rv0475), TbDP, 14 kDa protein and 38 kDa protein expressed in *Escherichia coli* and two purified preparations of LAM and Ag85; however, the LAM-reactive IgG responses were significantly increased in BCG-vaccinated subjects compared with a PPD-negative control group, and the other antigens were not statistically significant after the subjects received the

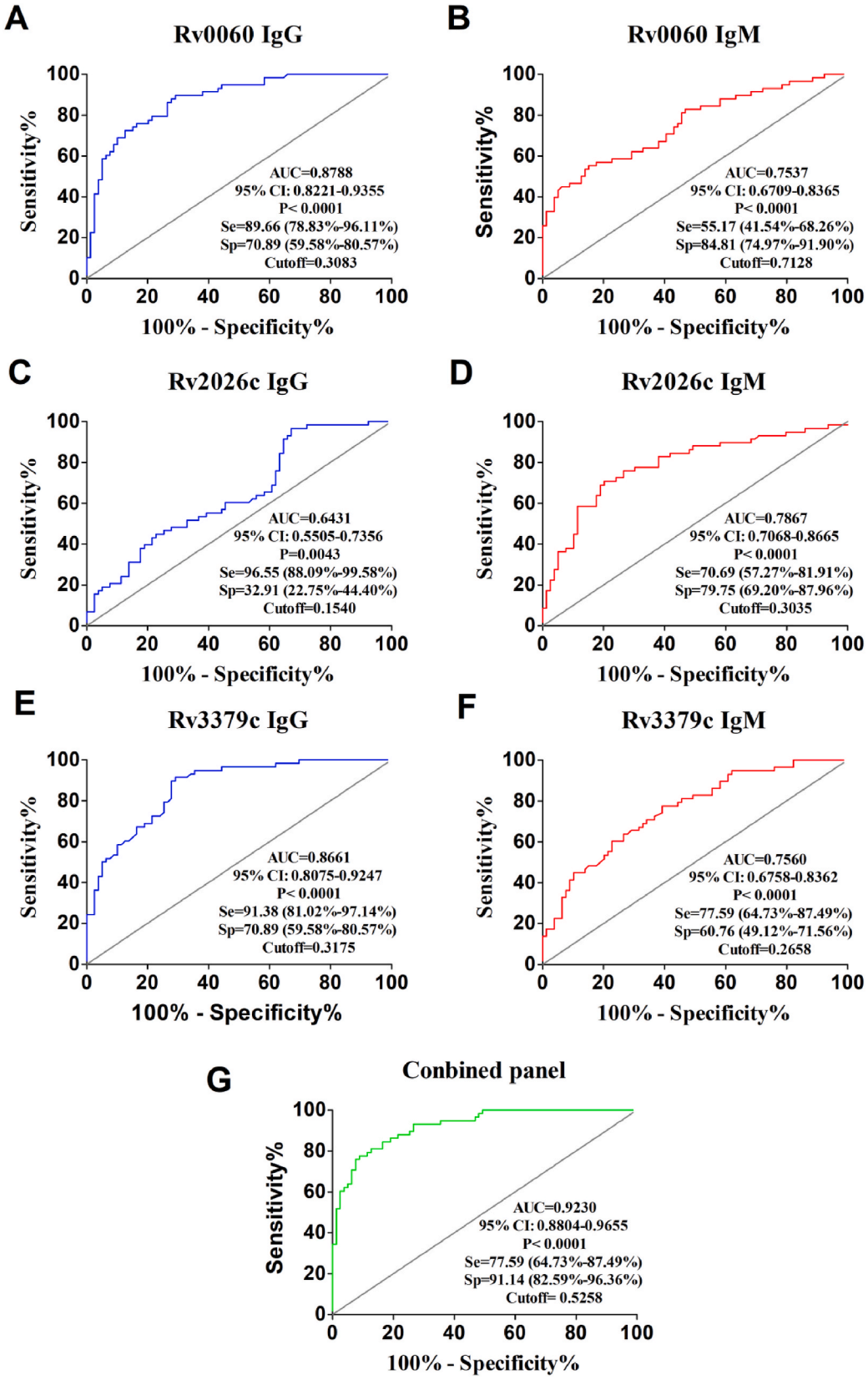

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Fig. 3. The performance of Rv0060-, Rv2026c- and Rv3379c-specific IgG and IgM responses in evaluations of BCG vaccination responses as analyzed by ROC curve analysis. (A) IgG against Rv0060, (B) IgM against Rv0060, (C) IgG against Rv2026c, (D) IgM against Rv2026c, (E) IgG against Rv3379c, (F) IgM against Rv3379c, and (G) combined antigen panel includes IgG against Rv0060, IgG against Rv3379c, and IgM against Rv3379c. 95 % CI, 95 % confidence interval; Se, sensitivity; and Sp, specificity.

BCG vaccination [21]. The top 20 antigens for the stronger IgG antibodies revealed in our study did not overlap with the results of Brown's study above, which also indirectly indicates the accuracy of our results. De Vallière et al. also reported a significant induction of the LAM-specific IgG response following both primary and secondary BCG vaccination in 10 HCs [12]. Furthermore, Turneer et al. also found that the PPD-specific IgM and IgG levels were also increased following BCG vaccination in 75 adults according to ELISA [22]. Considering the LAM and PPD with limitations in purification, the novel antigens identified in our study are easy to produce and purify as recombinant antigens and some of the antigens are now commercially available, which may provide a reference for developing a more efficacious vaccine based on humoral immune responses and could be potential biomarkers during the assessment of BCG vaccination responses.

BCG scarring and TST reactivity are commonly used methods for assessing BCG vaccinses but have some limitations. In comparison, serological methods based on the detection of antibody have many advantages, including convenient sampling, easy operation, rapid determination (results can be obtained within hours) and cheap costs [23,24]. Although serological tests have not been developed to assess BCG vaccination responses, a number of serological tests and commercial kits for TB identification have been developed [25,26]. Therefore, identifying reliable potential biomarkers for developing a novel method based on serological responses for evaluating BCG vaccination response is critical and encouraged considering the inadequacy of current assessment test. In our study, three potential antigens, Rv0060, Rv2026c and Rv3379c, were further verified by ELISA in other independent serum samples, and the results showed that the IgG and IgM antibody responses against the three antigens were consistent with the microarray results. Here, we first evaluated the possible use of Rv0060, Rv2026c and Rv3379c as potential biomarkers for assessing BCG vaccination responses using ROC analysis. The results displayed that the anti-Rv0060 IgG antibody had the highest AUC of 0.8788, a better sensitivity of 89.66 % and a moderate specificity of 70.89 %. To improve the assessment performance of a single antigen, logistic regression analysis was employed to develop a combined panel with an improved AUC of 0.923, a sensitivity of 77.59 % and a significantly increased specificity of 91.14 %. Since BCG-vaccinated subjects with negative TST results were not included in our study, further efforts are needed to establish serodiagnostics based on Rv0060, Rv2026c and Rv3379c.

We analyzed clinical characteristics, such as the gender, age, and the degree of TST responses, to determine whether they influenced antibody reactivity. We noted that anti-Rv3379c IgG levels and anti-Rv0060, Rv2026c and Rv3379c IgM levels were related with gender, and women displayed higher antibody levels than men. We assumed that higher antibody levels in women induced by BCG vaccination may provide better protective efficacy against MTB; in fact, men had higher TB rates (56 %) than women (32 %) in the 2020 global TB report [2]. Furthermore, the degree of TST-positive responses was not associated with IgG and IgM reactivity against Rv0060, Rv2026c and Rv3379c in our study; that is, TST may be a delayed-type hypersensitivity reaction to tuberculin antigen mediated by T cells [27]. Due to the limited sample size, the above speculation requires further large-scale verification. However, this study still has some limitations. For example, due to the lack of comparison with TB patients, it cannot be ruled out that the identified antigens in our study can distinguish TB patients from vaccinated-subjects; our paper only studies the antibody response 12 weeks after BCG vaccination, and lacks the test results at other times; the subjects are all adults, not infants, and different populations may have different responses to BCG; the HCs in this study were not necessarily those who had never received BCG vaccine, but they were negative in the TST test and T-SPOT test, so they were relative controls; the BCG strain used in our study was D_2PB302, It is unclear whether other BCG vaccines would result in the same results. Therefore, these issues are worthy of our further study.

In conclusion, we have used proteome microarrays to reveal that multiple antigens could elicit strong serum IgG or IgM response after vaccination in healthy adults, which may have potential value for better designing novel effective vaccines by considering the role of humoral immunity. Furthermore, Rv0060, Rv2026c and Rv3379c were further validated by ELISA and exhibited moderate performances when we assessed the BCG vaccination response, while the combined panel showed improved power compared with each single antigen, suggesting that these antigens may be potential biomarkers in developing an efficient serological test for the assessment of the BCG vaccination response.

Ethics approval

Medical research on humans was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the ethics committee of the Beijing Chest Hospital affiliated with Capital Medical University (Beijing, China), and the approval number was 2013-16.

Informed consent

Informed consent was received from all individual participants in the study.

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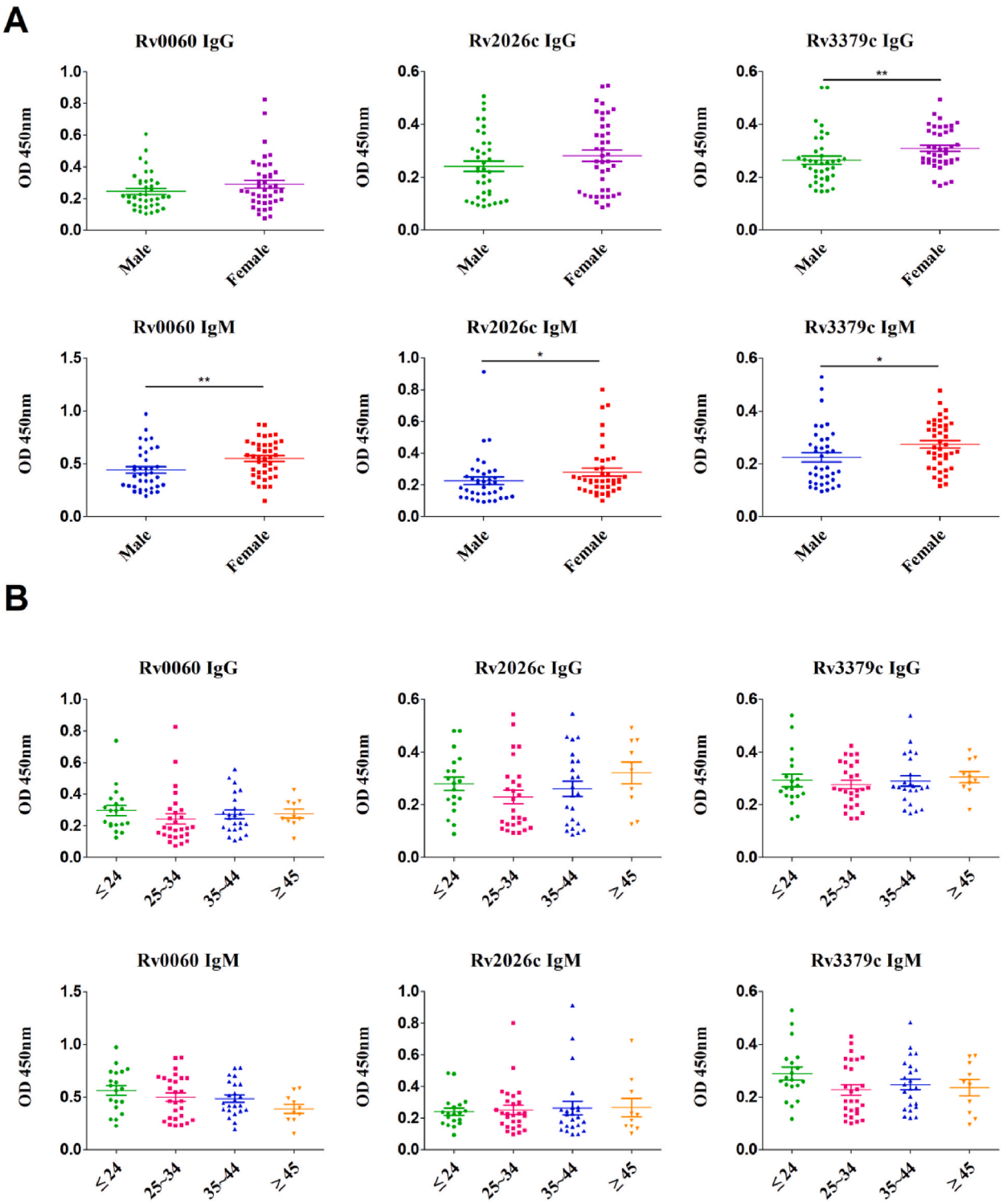

Fig. 4. Correlation between antibody responses and clinical information in HCs. (A) The levels of IgG and IgM against Rv0060, Rv2026c, and Rv3379c in males and females and (B) the levels of IgG and IgM against Rv0060, Rv2026c, and Rv3379c at different ages. Two-tailed p values by Mann-Whitney or Kruskal-Wallis test, (*) $p < 0.05$, (**) $p < 0.01$; horizontal lines, median values.

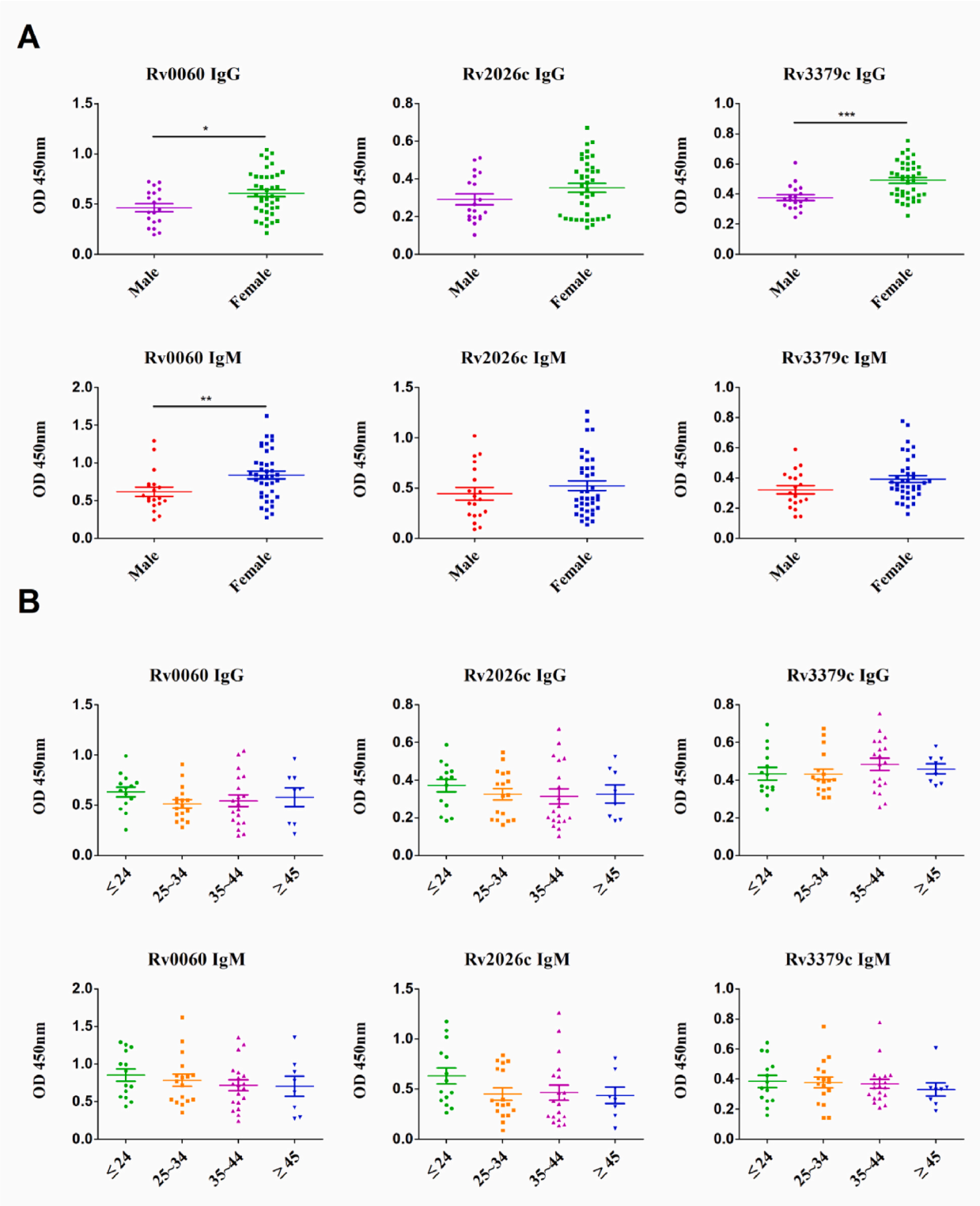

Fig. 5. Correlation between antibody responses and clinical information in BCG-vaccinated subjects. (A) The IgG and IgM levels against Rv0060, Rv2026c, and Rv3379c in males and females; (B) the IgG and IgM levels against Rv0060, Rv2026c, and Rv3379c at different ages. Two-tailed p values by Mann-Whitney or Kruskal-Wallis test, (*) $p < 0.05$, (**) $p < 0.01$; horizontal lines, median values.

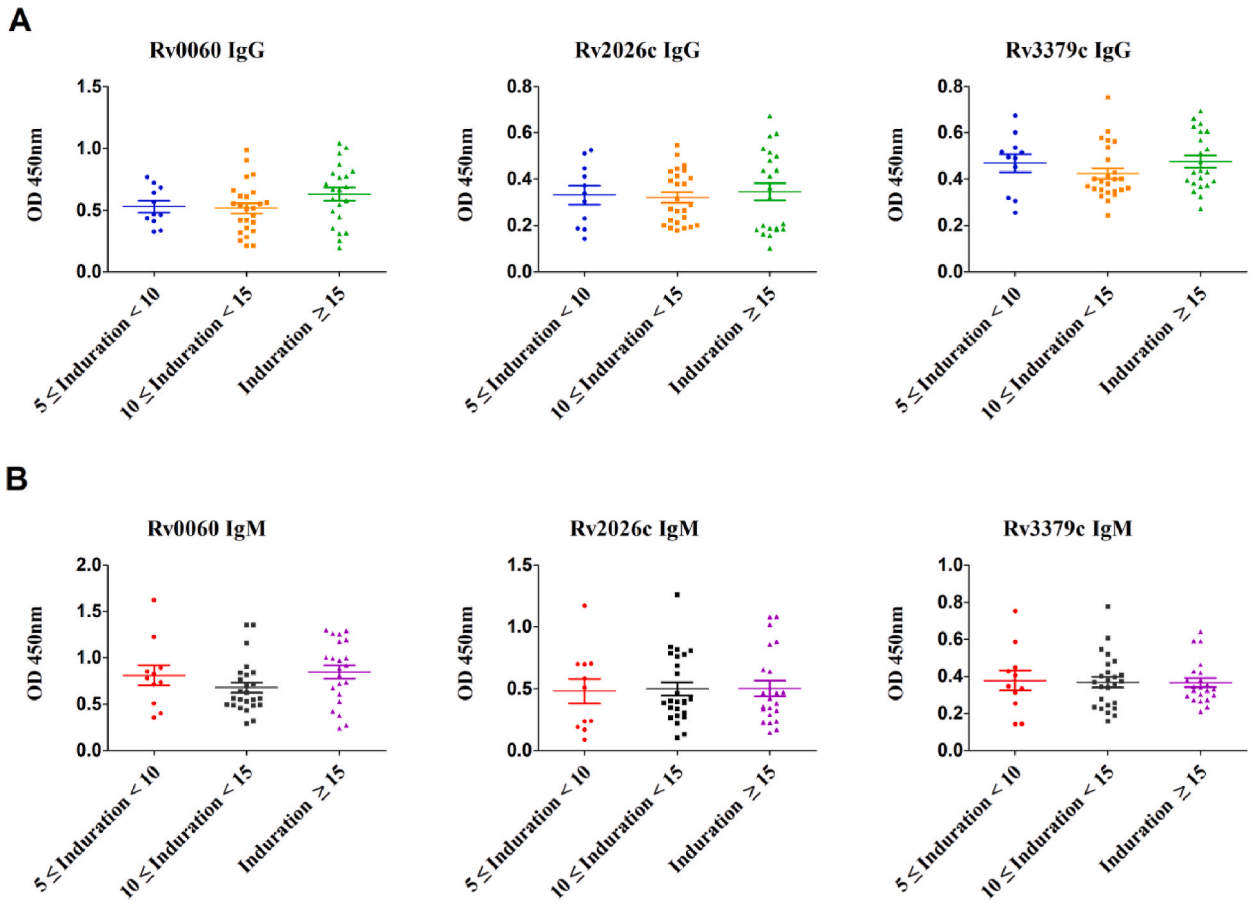

Fig. 6. Correlation between antibody reactivity and the degree of TST responses in BCG-vaccinated subjects. (A) IgG levels against Rv0060, Rv2026c, and Rv3379c at different indurations of TST-positive response, (B) IgM levels against Rv0060, Rv2026c, and Rv3379c at different indurations of TST-positive response. Two-tailed p values by Kruskal-Wallis test; horizontal lines, median values.

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CRediT authorship contribution statement

Yan-Qing Chen: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Shu-Hui Cao:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Xin-Yu Yang:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Yi Liu:** Supervision, Resources. **Chuan-You Li:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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