Diagnostic value of antibody responses to multiple antigens from *Mycobacterium tuberculosis* in active and latent tuberculosis

Muhammad Andrian Senoputra^{1,2}, Beata Shiratori^{1,3,4}, Fakhrial Mirwan Hasibuan^{1,2}, Raspati Cundarani Koesoemadinata⁵, Lika Apriani⁵, Yugo Ashino^{1,3}, Kenji Ono⁶, Tetsuya Oda⁶, Makoto Matsumoto⁶, Yasuhiko Suzuki⁷, Bacht Alisjahbana⁵, Toshio Hattori^{1,3#}

¹Division of Emerging Infectious Diseases, Graduate School of Medicine, Tohoku University, 2-1 Seiryomachi, Aoba-ku, Sendai, 980-8574 Miyagi, Japan, e-mail: beatabucekova@gmail.com, ya82@yahoo.co.jp, toshatto@med.tohoku.ac.jp

²Public Health Science Program, Faculty of Medicine, Universitas Padjadjaran, Jl. Eicjkman 38, Bandung, 40161 West Java, Indonesia, e-mail: arial_dabbuss@yahoo.com, an2_dri@rocketmail.com

³Division of Disaster-related Infectious Diseases, International Research Institute of Disaster Science, Tohoku University, 2-1 Seiryomachi, Aoba-ku, Sendai, 980-8574 Miyagi, Japan

⁴Japan International Corporation of Welfare Services, 2-3-20 Toranomom YHK Bldg. 4F, Toranomom, Minato-ku, 105-0001 Tokyo, Japan

⁵TB-HIV Research Center, Medical faculty, Padjadjaran University, Jl. Eicjkman 38, Bandung, 40161 West Java, Indonesia, e-mail: r.cundarani@gmail.com, likaaji@gmail.com, b.alisjahbana@gmail.com

⁶Microbiological Research Institute, Otsuka Pharmaceutical Co., Ltd., 463-10 Kagasuno, Kawauchi-cho, 771-0192 Tokushima, Japan, e-mail: onok@otsuka.jp, odate@otsuka.jp, m_matsumoto@research.otsuka.co.jp

⁷Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University,
North 20, West 10, Kita-ku, Sapporo, 001-0020 Hokkaido, Japan, e-mail:
suzuki@czc.hokudai.ac.jp

Corresponding author. Mailing address: Division of Disaster-related Infectious Diseases,
International Research Institute of Disaster Science, Tohoku University, 2-1 Seiryomachi, Aoba-ku,
Sendai 980-8574, Japan. Tel: +81-22-717-8220, Fax: +81-22-717-8221. E-mail:
toshatto@med.tohoku.ac.jp

*MAS and BS contributed equally to this work.

Running title: Antibody responses against *Mycobacterium tuberculosis* antigens

Abstract

We investigated the antibody responses to ten prospective MTB antigens, and evaluated their ability to discriminate between latent (LTBI) and active pulmonary tuberculosis (TB). Our results indicate that plasma levels of anti-ACR, anti-LAM, anti-TDM-M, and anti-TBGL antibodies were higher in patients with active TB, compared to those in the LTBI and control subjects. No differences in the antibodies were observed between the control and LTBI subjects. Antibodies against the glycolipid antigens could not distinguish between *Mycobacterium avium* complex (MAC)-negative TB patients and MAC-infected LTBI individuals. The most useful serological marker was antibodies to α -crystallin, with MAC-negative TB patients having higher titers than those observed in MAC-positive LTBI and control subjects. Our data indicate that antibody to α -crystallin is promising target for the serological diagnosis of patients with active TB patients. When dealing with anti-glycolipid antibodies, MAC co-infection should always be considered in serological studies.

Keywords: tuberculosis, tubercular-glycolipid, mycolic acid, lipoarabinomannan, α -crystallin

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1. Introduction

One of the key challenges of controlling tuberculosis (TB) is the proper diagnosis and treatment of both active TB and latent TB infection (LTBI). The detection of antibodies to *Mycobacterium tuberculosis* (MTB) in a patient's serum is simple, applicable in various settings, and provides extremely rapid results [1]. The optimal serological tests must avoid cross-reactivity with bacillus Calmette–Guérin (BCG), environment mycobacteria, and non-mycobacteria antigens. Furthermore, there is demand for a test that can discriminate between active TB and LTBI with consistent performance in different populations. Despite, the protective role of antibodies in TB infection and their usefulness as diagnostic markers are controversial issues, several MTB cell wall proteins and highly antigenic glycolipids are promising candidates for serodiagnosis of TB infection [2]. A number of studies in humans and animal models have demonstrated that anti-MTB titers depend on the state of infection, and that they are correlated with the degree of mycobacterial burden [1, 3]. As the production of antibodies to certain antigens differs during latent infection and active disease, it is an accepted fact that a wide spectrum of humoral responses depend upon the stage of TB [4].

Cord factor (trehalose 6, 6'-dimycolate [TDM]), the characteristic cell-wall component of MTB and other acid-fast bacteria, mediates the induction of immune reaction in early stages of TB infection. The detection of antibodies against TDM was proposed to be useful for rapid serodiagnosis of tuberculosis [5]. In addition, the tubercular-glycolipid antigen (TBGL) consists of a mixture of hydrophobic glycolipids (trehalose monomycolate, diacyl-trehalose, phenolic glycolipid, 2,3,6,6'-tetraacyl-trehalose-2'-sulfate, and 2,3,6-triacyl-trehalose) and TDM from the virulent mycobacterial strain H37Rv. Anti-TBGL IgG has been proposed as a useful serodiagnostic marker for active pulmonary tuberculosis in Japan, and Maekura et al. [6]. We also previously reported the usefulness of anti-TBGL assay in detection of latent TB infection in health care

workers in Philippines, however the assay usefulness was decreased in HIV individuals [7].

Furthermore, lipoarabinomannan (LAM) is another type of highly immunogenic cell wall-associated glycolipid, which consists of repeating arabinose and mannose units that are linked to a phosphatidylinositol moiety [8]. Serological analysis of anti-LAM antibody titers showed promising results for differentiating of individuals with and without active TB, however failed to diagnose LTBI in asymptomatic individuals [9].

Among MTB's protein-based antigens, the antigen 85 (Ag85) complex represents a major portion of MTB's secreted proteins, and is thought to be crucial for growth in hypoxic conditions that lack of nutrition, such as in granulomas [10]. In addition, it has recently been suggested that mycobacterial DNA-binding protein 1 (MDP-1) is involved in growth regulation by controlling glycolipid biosynthesis and cell wall biogenesis [11], and plays an important role in the stationary phase of TB infection [12]. Moreover, an in vitro study has demonstrated that the growth of MTB under hypoxic conditions results in the up-regulation of a 16 kDa α -crystallin (ACR) homologue [13]. Recent study serological study showed the elevation of anti-Ag85A and anti-MDP-1 antibodies in individuals with history of past TB infection, and high anti-ACR titers in active and past TB patients [14]. Heparin-binding hemagglutinin adhesin (HBHA) is a surface-associated protein that induces high levels of anti-HBHA antibodies in most patients with active TB, although not in healthy subjects with LTBI, BCG-immunized subjects, or healthy controls [15].

As the composition of the cell wall of MTB and other non-tuberculous mycobacteria, including MAC, share the similar antigens, it is logical to presume that the serological tests based on measuring humoral response to cell wall antigens may lack their specificity. Recently, IgA antibody against GPL core was found to reliably differentiate MAC disease from the diseases caused by *M. kansasii*, MAC contamination, pulmonary TB, bacterial pneumonia, and other non-infectious pulmonary diseases. Moreover, diagnostic performance of anti-GPL core antibody

yield high sensitivities and specificities to discriminate between MAC and TB infection in various geographical regions [16].

Based on this information, we designed this study to evaluate the humoral immune responses to **ten prospective protein and glycolipid** mycobacterial antigens in patients with TB, as well as in LTBI and healthy subjects, in order to determine the antigens' suitability for inclusion in serodiagnostic tests.

2. Materials and Methods

2.1. Subject selection and sampling

We recruited 117 consenting subjects who visited the Primary Health Centre and Latent TB Clinic at Padjadjaran University, Bandung, **Indonesia**. Patients with **culture-confirmed TB**, and with a chest radiology-confirmed diagnosis, were included in the active TB group (n = 16); none of these patients had taken medication within the past 2 weeks. The LTBI group was comprised of 66 participants who had documented exposure to a patient with confirmed TB, and who exhibited positive responses to the tuberculin skin test (TST), interferon-gamma release assay (IGRA) test, or both tests in the absence of clinical or radiographical signs of TB. **All the IGRA negative TST positive LTBI subjects (n = 12) had the BCG vaccination in their childhood, however the TST positivity is considered to reflect LTBI status due to their documented close contact with active TB patient.** Thirty-five healthy control subjects (HC) were recruited from the staff and students of the Medical Faculty of Padjadjaran University; these subjects had no TB-related symptoms, and exhibited **negative IGRA and TST findings**. Negative HIV status was confirmed using the HIV-1/2 enzyme-linked immunoassay (ELISA) (Abnova Corporation, Taipei, Taiwan) and the New Lav Blot 1 (Bio-Rad, Tokyo, Japan). Each subject provided their informed written consent to participate

in the various testing procedures, and the study's design was reviewed and approved by the Health Research Unit Ethical Committee University of Padjadjaran and Tohoku University.

QuantiFERON®-TB Gold In-Tube test (QFT-GIT) was performed according to the manufacturer's instructions (Cellestis, Carnegie, Australia). Blood samples for other assays were collected in tubes with EDTA, and the plasma was aspirated and stored at -80°C . After the whole blood sample was drawn, the patients underwent the TST using the Mantoux technique with 0.1 mL of purified protein derivate (PPD RT 23, Biofarma Bandung, Indonesia). Induration was evaluated within 48–72 h by an experienced investigator, and positive results were defined as an induration diameter of ≥ 10 mm.

2.2. Anti-TBGL IgA and IgG ELISA

The concentrations of anti-TBGL immunoglobulin A (IgA) and IgG were measured using an ELISA kit (Kyowa Medex Co., Ltd., Tokyo, Japan) as described previously [6].

2.3. Plasma IgA and IgG

Quantitative measurement of the plasma IgA and IgG concentrations was performed using an ELISA kit for human IgA and IgG (Mabtech AB). All procedures were performed based on the manufacturer's protocol as described previously [17], and with dilutions of 1:100,000 (IgA) and 1:1,000,000 (IgG).

2.4. Preparation of protein and glycolipid antigens

The genes encoding HBHA, LAM-binding protein (Rv0679c), Ag85A, Ag85B, ACR, and MDP-1 were isolated via PCR amplification from the genomic DNA of MTB H37Rv, and primers were designed to incorporate restriction sites for cloning. The amplified DNA sequences were cloned into the pET-42(+) vector (Novagen), and the various vectors were expressed using *Escherichia coli* BL21 (DE3) (Novagen). The recombinant proteins were purified via immobilized

metal ion affinity chromatography using a nickel-sepharose high performance column, according to standard protocols. The purity of the protein was analyzed by SDS-PAGE. The purified LAM (Nacalai tesque, Kyoto, Japan) was isolated from MTB AoyamaB. The purified trehalose di-methoxy-mycolate (TDM-M) and trehalose di-keto-mycolate (TDM-K) were semi-synthesized by chemically introducing methoxy-mycolic acid and keto-mycolic acid, respectively, to the 6, 6' positions on trehalose.

2.5. Detecting antibodies to TB antigens

The ELISA test was optimized with regard to antigen concentration and plasma dilutions. To detect antibodies to the various TB antigens, 96-well ELISA microplates (Corning, # 2592) were coated with 0.4 µg of HBHA, 1.4 µg of Ag85A, 0.6 µg of Ag85B, 0.1 µg of ACR, 0.8 µg of MDP-1, 0.4 µg of Rv0679c, 0.01 µg of LAM, 0.3 µg of TDM-M, and 0.3 µg of TDM-K per well. Microplates were then allowed to stand overnight at 4°C. The antigen-coated plates were then blocked with a solution of bovine serum albumin, and the assay was performed using the following steps. The plasma samples were diluted 1:100, 100 µl of the diluted sample were added into each well, and the wells were sealed and incubated overnight at 2–8°C. On the following day, after repeated washing steps, 100 µl of diluted protein G-horse radish peroxidase solution (Acris, Herford, Germany) was added to each well. The sealed plates were then incubated for 2 h at room temperature. Next, the plates were washed and 100 µl of tetramethylbenzidine substrate (Dojindo, Kumamoto, Japan) was added into each well, and the plates were then incubated in the dark for 10 min at room temperature. To stop the reaction, 100 µl of 0.5 M sulfuric acid was added to each well, and the absorbance was read immediately at 450 nm.

2.6. Identification of MAC infection

Anti-GPL-core IgA antibodies were evaluated using the CapiliaTM MAC Ab ELISA (Taunslaboratories, Inc., Shizuoka, Japan), according to the manufacturer's recommendations as

described previously [18]. The plasma samples were diluted by a factor of 41 and 5 different concentrations of the calibrators were added to the designated wells as standards. Based on the manufacturer's recommendations, positive test results were defined as readings of ≥ 0.7 U/mL.

2.7. Data analysis

For the statistical analyses, comparisons for more than two groups were performed using Kruskal-Wallis analysis, while comparisons between two groups were performed using Mann-Whitney U analysis. Differences with a p-value of <0.05 were considered statistically significant. To evaluate the various markers for active TB, we used logistic regression analysis, with Spearman's test to assess the correlations. ROC analysis of studied antibodies was performed to distinguish between active TB and LTBI group. Cut off value was selected based on the best sensitivity, specificity and likelihood ratio for each antibody. All analyses were performed using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (version 6.00, GraphPad Software, Inc., CA, USA).

3. Results

3.1. Study participants

The demographic characteristics, laboratory findings, and MAC infection status of the subjects are summarized in Table 1. The TB patients were mostly men, while the HC and LTBI groups contained mostly women. The TB patients were slightly younger than the subjects in the other two groups. Although the white blood cell (WBC) and lymphocyte counts were similar in the LTBI and HC groups, the TB group had significantly higher WBCs compared to the HC group. We observed increases in the number of neutrophils, platelets, and monocytes and significantly lower lymphocyte counts in the TB group, compared to those observed in the LTBI and HC groups.

Table 1

Characteristics of study participants.

	HC (n = 35)	LTBI (n = 66)	TB (n = 16)
Demographic data			
Sex, % male	37	17 §****; †*	75 ¶*
Age, median (range)	35 (18 - 54)	41 (18 - 54) §*	29 (18 - 50)
Laboratory findings			
WBC (10 ³ /μl)	7.4 (4.9 - 12.2)	8.15 (3.4 - 98)	9 (5.3 - 76) ¶*
Platelet (10 ³ /μl)	272 (156 - 465)	312.5 (139 - 602) §*; †*	370 (222 - 547) ¶**
Neutrophil % ^	60 (49 - 73)	60 (38 - 75) §**	68.5 (40 - 82) ¶*
Lymphocyte % ^	30 (19 - 43)	30 (12 - 54) §**	20.5 (10 - 44) ¶*
Monocyte % ^	7 (4 - 11)	7 (2 - 11) §***	9 (6 - 12) ¶**
Plasma IgA (ng/ml)	27.22 (12.50 - 14.42)	29.81 (3.53 - 98.79)	32.56 (9.32 - 200)
Plasma IgG (ng/ml)	19.04 (14.42 - 31.92)	18.39 (0.72 - 47.22)	20.45 (12.35 - 60.51)
TB close contact			
Yes, %	40	76 †***	-
No, %	60	24	-
Anti-GPL IgA (U/mL)	0.179 (0.000-2.837)	0.174 (0.065-6.021)	0.165 (0.101-3.640)
GPL positivity (%)	14	13	6

† = HC versus LTBI; ¶ = HC versus TB; § = LTBI versus TB; * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. The number of participants for ^ is as follows; HC = 33; LTBI = 55; TB = 16. **Anti-GLP IgA is the specific marker of MAC infection.**

However, there were no inter-group differences in the levels of plasma IgA and IgG. Among the 66 LTBI subjects, 76% had been in close contact with patients who had active TB, such as household contacts or healthcare workers. The remaining 24% of the LTBI subjects were subjects who were initially recruited as healthy controls, but subsequently exhibited a positive

QuantiFERON®-TB result. We observed MAC infection in more than 10% of the HC and LTBI subjects, and in 6% of the TB subjects; no significant inter-group differences in the anti-GPL IgA titers were observed.

3.2. Levels of antibodies to mycobacterial glycolipid antigens and Rv0679c

As shown in Fig. 1, the TB patients had significantly higher plasma levels of anti-TBGL IgA and IgG, compared to the HC ($p = 0.005$) and LTBI ($p = 0.0028$) groups. No significant differences were observed for those antibodies when we compared the HC and LTBI groups. When each group was divided based on their MAC infection status, similar patterns were observed for the MAC-negative groups. However, we found significant differences in the anti-TBGL IgA and IgG titers when we compared the MAC-positive and MAC-negative subjects within LTBI group (Fig. 1A, B). Both anti-TDM-M and TDM-K antibody levels were significantly higher in TB patients, regardless of their MAC status, compared to those in the HC and LTBI groups. There were no significant differences in the anti-TDM titers when we compared the HC and LTBI groups (Fig. 1C, D). Similar to the other antigens, the TB patients had significantly higher plasma levels of anti-LAM IgG, compared to the HC ($p = 0.0023$) and LTBI ($p = 0.0013$) groups. Even when we excluded the MAC-positive subjects, the active TB group still had significantly higher anti-LAM titers, compared to the HC and LTBI groups (Fig. 1E). Furthermore, MAC-positive subjects in the LTBI group had higher anti-LAM titers compared to MAC negative subjects (Fig. 1E). Although the TB patients had higher anti-Rv0679c titers, there were no significant inter-group differences, even when MAC status was considered (Fig. 1F).

3.3. Levels of antibodies to mycobacterial protein antigens

No significant inter-group differences were observed for the anti-Ag85A titers (Fig. 2A). However, the anti-Ag85B titers were higher in the TB group, compared to those in the HC ($p = 0.036$) and LTBI ($p = 0.015$) groups; similar results were observed when we excluded MAC positive subjects (Fig. 2B). Among the responses to the protein antigens, anti-ACR titers exhibited the greatest discriminatory values, as the TB group had higher anti-ACR titers than the HC ($p < 0.0001$) and LTBI ($p < 0.0001$) groups; this relationship did not change when we excluded MAC-positive subjects (Fig. 2C). In addition, higher anti-HBHA and anti-MDP-1 titers were associated with MAC infection in the HC group, while the MAC-negative TB patients had higher anti-HBHA titers than the MAC-negative LTBI subjects (Fig. 2D, E).

3.4. The antibodies' discriminatory value

The results of our logistic regression model indicated that anti-ACR titers ($p < 0.001$) were the strongest independent predictor of TB status, followed by titers of antibodies to LAM ($p = 0.021$) and several other antibodies (anti-TDM-M, anti-TDM-K, anti-Ag85A, and anti-HBHA). To further evaluate the ability of each antibody to discriminate between TB and LTBI, we used an ROC analysis. As shown in Table 2, anti-ACR and anti-LAM titers had the greatest discriminatory value, with similar sensitivities, specificities, and likelihood ratios, although a minor difference was observed in their areas under the curve. The anti-TDM-M titers had a lower sensitivity and specificity than the anti-ACR and anti-LAM titers. The greatest specificity was provided by anti-TBGL IgG, although this provided a relatively low sensitivity.

Figure 2. Levels of antibodies to mycobacterial protein antigens. Anti-Ag85A (A), Anti-Ag85AB (B), anti-ACR (C), anti-HBHA (D), and anti-MDP-1 (E) titers were measured in HC, LTBI and TB groups and each group was divided based on MAC infection status.

Table 2

The antibodies' discriminatory value.

Antibody	AUC		Standard error		Cut-off		Sensitivity (%)		Specificity (%)		Likelihood ratio	
	All	MAC -	All	MAC -	All	MAC -	All	MAC -	All	MAC -	All	MAC -
anti-ACR	0.820	0.811	0.063	0.066	> 0.387	> 0.342	81.25	86.67	71.21	68.42	2.822	2.744
anti-LAM	0.754	0.769	0.078	0.086	> 2.570	> 2.570	81.25	80	71.21	75.44	2.822	3.257
anti-TDM-M	0.738	0.739	0.065	0.069	> 0.230	> 0.230	75.00	73.33	66.67	70.18	2.250	2.459
anti-TBGL IgG	0.737	0.751	0.079	0.084	> 2.812	> 2.812	62.50	60.00	81.82	87.82	3.438	4.886

AUC = area under the curve

3.5. Correlations between antibodies in LTBI and TB groups.

Next, we selected the antibodies with the greatest discriminatory value to study their relationship with the other antibodies and with the TB and LTBI subjects (Table 3A, B). In the LTBI group, anti-ACR titers were correlated with anti-Ag85A, anti-Ag85B, and anti-HBHA titers, although there was no correlation with the anti-glycolipid antibodies. Similarly, the anti-glycolipid antibodies were not correlated with the anti-protein antibodies (Table 3A).

In the TB group, the correlations among the anti-glycolipid antibodies were stronger than those in the LTBI group. As TDM contains the trehalose 6, 6'-dimycolate component, it was not surprising that anti-TDM-M and anti-TDM-K were strongly correlated with each other, and with anti-TBGL IgG in the LTBI and TB groups (Table 3A, B). In addition, anti-LAM titers were associated with the titers for anti-ACR and both anti-TDM antibodies. (Table 3B).

Table 3**A** Correlation of studied antibodies in LTBI group.

Antibody	anti-ACR		anti-TDM-M		anti-LAM		anti-TBGL IgG	
	r	p	R	p	r	p	r	p
anti-Ag85A	0.332	**						
anti-Ag85B	0.379	**						
anti-HBHA	0.353	**						
anti-TDM-M							0.642	**
anti-TDM-K			0.954	**			0.626	**
anti-LAM							0.374	**
anti-TBGL-IgA			0.358	**	0.402	**	0.436	**
anti-TBGL-IgG			0.642	**	0.374	**		

B Correlation of studied antibodies in TB group.

Antibody	anti-ACR		anti-TDM-M		anti-LAM		anti-TBGL IgG	
	r	p	R	p	r	p	r	p
anti-ACR					0.5	*		
anti-TDM-M					0.591	*	0.779	**
anti-TDM-K			0.988	**	0.571	*	0.821	**
anti-LAM	0.5	*	0.591	*				
anti-TBGL-IgG			0.779	**				
plasma IgA			0.691	**			0.682	**
plasma IgG			0.621	**				

* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

Discussion

ACR is considered a marker for MTB's dormant state. However, in our study, we found higher anti-ACR IgG titers in the group with active TB, compared to in the LTBI and HC groups. This finding is supported by those of another study, which reported significant difference in anti-ACR titers when they compared active TB cases with inactive TB and control cases [19]. In addition, our ROC curve demonstrated that anti-ACR titers had the greatest ability to discriminate between active TB and LTBI. As the ACR protein is mostly located on the inner site of the bacterial cell membrane, and is rarely observed in the outer cell wall [13, 20], it appears that the antigen is less accessible during LTBI, although the microenvironment and expression of ACR may differ during active TB. Furthermore, Balu et al. have recently demonstrated that anti-ACR antibodies play a protective role during passive immunotherapy for tuberculous infection in their experimental mouse models [21]. This finding support the idea that the anti-ACR antibodies reflect the TB activity, however the direct protective role needs to be evaluated in further studies.

We observed that anti-LAM IgG titers were significantly higher in patients with active TB, compared to those in the LTBI and HC groups. This finding is supported by several other studies that found higher levels of anti-LAM IgG in HIV-negative patients with TB, compared to controls with a positive TST result [22, 23]. The study of Perley et al. showed that anti-LAM titers were similar among healthy, latent and active TB subjects, however LTBI subjects had avidity intermediate between uninfected controls and active TB patient [24]. In the same study, the MAC positivity in studied groups was not clearly described, therefore the possible antibody cross reaction between species could not be excluded. MAC positive subjects of our study showed the tendency toward increased anti-LAM antibody titers, and exclusion of MAC positive subjects increased the discriminatory value of anti-LAM antibody. We used anti-GPL core IgA for identification of MAC

infection, however usage of different target antigens, such as bacterial lysate, may lead to variable results.

Our results revealed higher anti-TDM titers in the patients with active TB, compared to in the HC and LTBI groups, which could reflect the antigen load and accessibility in patients with active TB. This finding is in agreement with the findings of another study, which reported that anti-cord-factor IgG titers were significantly elevated in the sera of patients with active TB, and that these titers were useful for the early diagnosis of active pulmonary TB [25, 5]. Moreover, more than half of active TB patients were positive for anti-TDM at the onset of TB symptoms, and the antibody titers had tendency to decline after the initiation of treatment [5]. In addition, TDM is composed of a unique glycolipid that is comprised of trehalose and mycolic acid methyl esters, which differ in their composition among mycobacterial species [26, 27]. The study of Pan et al. showed that anti-cord factor IgG antibody in sera of TB patients most strikingly recognized methoxy-mycolic acid, whereas the keto-mycolic acid was recognized weakly [27]. Here, we also examined the methoxy- and keto-mycolate subclasses of TDM, and found the strong correlation between the anti-TDM-M and anti-TDM-K titers. Although MAC positive LTBI and TB subjects had slightly higher titers of anti-TDM antibodies compared to MAC negative subjects in the same group, the difference did not reach statistical significance. Different bacterial burden and localization of MTB may explain why we found significant difference in the anti-TDM-M and TDM-K IgG titers when we compared the LTBI and TB subjects. Thus, high titers of anti-TDM may serve as an indicator for TB reactivation.

Previous study has compared TDM alone and TDM that was mixed with other glycolipids and reported that the addition of the glycolipids improved the serological test's sensitivity [7]. As TDM and TBGL contain the trehalose 6, 6'-dimycolate component, it was not surprising that anti-TDM-M and anti-TDM-K were strongly correlated with each other, and with anti-TBGL IgG in the LTBI

and TB groups in this study. Moreover, by using ROC analysis, we found that anti-TDM-M and anti-TBGL IgG had similar area under the curve (AUC), but AUC of anti-TBGL IgG improved when MAC positive subjects were excluded. We also noticed that the average anti-TBGL titers and the proposed cut-off levels appear to vary according to severity of the disease and the origin of the studied subjects [6, 7, 28-31].

Previous studies have also reported that MTB expresses different immunodominant antigens during different states of infection [4], although it should be noted that the production of specific antibodies depends on several factors, such as the antigen's amount, accessibility, chemical structure, and its localization. Therefore, antibodies to latency-related antigens may not necessarily increase in the plasma, because the spatial location of the antigen may prevent contact with the host's immune system.

Several limitations for our study need to be mentioned. This cross-sectional study represents only small number of Indonesian TB patients. Also testing of all healthy and TB contacts by the combination of TST and IGRA method would maximise the chance of accurate diagnosis of LTBI. We also could not determine the protective role of studied antibodies and their correlation with successful treatment. Future research should also include the serodiagnosis of extrapulmonary TB, as well as patients co-infected with HIV. As the reliability of the results depends on the size of the study, it is necessary to confirm our findings in a larger-scale study.

The most common cause of pulmonary non-tuberculous mycobacterial disease is MAC infection [32], and cases of MAC co-infection in TB patients are occasionally reported [33, 34]. In addition, cross-reactivity for TDM antibodies has been reported in MAC and TB infections [35]. Therefore, to minimize any potential confounding, we evaluated antibodies that were specific for the GPL-core [18, 35, 36]. We found that only 1 of the 16 (6%) patients with active TB was MAC-positive, although the sputum cultures were confirmed to be negative for MAC infection in

all TB cases. As the HC and LTBI subjects did not have any symptoms that were suggestive of infectious disease, high anti-GLP-core titers in these groups may reflect previous or latent MAC infection. Nevertheless, excluding those subjects from each group did not noticeably change the discriminatory properties of the antibodies that we studied. However, it should be noted that none of the anti-glycolipid antibodies could sufficiently discriminate between MAC-negative TB patients and MAC-infected LTBI subjects. Furthermore, the anti-ACR titers were only minimally effected MAC infection, as the MAC-negative TB patients had higher titers than the MAC-positive LTBI subjects and the MAC-positive HC subjects. Therefore, future serological studies should consider the effect of previous or latent MAC infection in healthy and LTBI subjects and possible MAC co-infection in TB patients, especially when evaluating anti-glycolipid antibodies, and properly screen for MAC co-infection to avoid any potential confounding.

The World Health Organization has recommended that TB diagnostic tools for wide use should have sensitivities and specificities of >80% and >95%, respectively. Based on these criteria, none of our antigens could be used alone in a diagnostic test. However, although the antigens' discriminatory values did not meet the specified criteria, it is possible that combining several antigens may improve the diagnostic reliability of future tests.

Conclusion

Anti-ACR and anti-glycolipid antibodies are promising targets for improving the serological diagnosis of patients with active TB, and the increase of their titer may indicate the reactivation of LTBI.

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