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Case Report

Comparison TLR2 and TLR4 serum levels in children with pulmonary and extrapulmonary tuberculosis with and without a Bacillus Calmette-Guérin (BCG) scar

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ABSTRACT

The formation of a scar after *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccination influences the effectiveness of protection against *Mycobacterium tuberculosis* (MTB) infection. The innate immunity plays a critical role both in the pathophysiology of tuberculosis (TB) and BCG vaccination protection mechanism. Parts of innate immunity: macrophages, dendritic cells, and neutrophils, have microbial recognition surface receptors called Toll-like receptors (TLR) 2 and 4. The objective of this study is to compare the serum levels of TLR2 and TLR4 in BCG-vaccinated pediatric patients with pulmonary and extrapulmonary TB. This cross-sectional study included children aged less than 18 years old with contracted TB disease and had received BCG vaccination. The subjects were recruited by convenience sampling from both outpatient and inpatient care at Bhakti Medicare and Jakarta Islamic Hospital, from November 2018 to December 2019. Serum TLR2 and TLR4 levels measured using ELISA of the two groups of subjects: children with pulmonary TB (PTB) and extrapulmonary TB (EPTB), were then compared. The presence of BCG scars was included in the analysis. Independent T-test, ANOVA test, and Kolmogorov-Smirnov normality tests on the SPSS program were used to statistically analyze the results. Serum TLR2 and TLR4 levels were higher in EPTB group, but the difference was not significant (TLR2 $p = 0.758$ and TLR4 $p = 0.646$, respectively). Subjects with BCG scars in both groups have significantly higher serum TLR2 and TLR4 levels than those without BCG scars in the EPTB group (EPTB $p = 0.001$ and $p = 0.004$, respectively); (PTB $p < 0.001$ and $p < 0.001$, respectively). BCG vaccination and MTB infection stimulate better innate immune response in EPTB than in PTB and serum TLR2 and TLR4 levels in those with BCG scars were higher when compared to those without BCG scars.

1. Introduction

Tuberculosis (TB) is caused by bacteria (Mycobacterium tuberculosis) that most often affect the lungs. Tuberculosis is curable, preventable, and is the 10th leading cause of morbidity and mortality in the world [1]. Tuberculosis often occurs in children, who are more susceptible, especially those who are in close contact with bacteriologically confirmed TB patients [1–6]. Children also have a greater risk for

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developing severe TB. The clinical manifestations of pulmonary tuberculosis (PTB) are cough, fever, weight loss and night sweats. However, patients with extrapulmonary TB (EPTB) develop atypical symptoms depending on the location of the infection, which may delay diagnosis and treatment and lengthening hospital stays [1,2,7,8].

Bacillus Calmette-Guérin **(**BCG) vaccine offers effective protection against *Mycobacterium tuberculosis* (MTB) infection for up to 10–15 years, and vaccination in children can protect against miliary TB and TB meningitis [9,10]. The protective effect is influenced by the timing of vaccine administration, prior exposure to environmental MTB, especially in TB endemic areas, and age. BCG vaccination can also prevent primary TB infection, but it cannot prevent reactivation of the disease. Therefore, latent TB is a potential source of infection [9–12].

The innate immune response is initiated immediately when the intracutaneous *Mycobacterium bovis* in Bacillus Calmette-Guerin (BCG) vaccine is injected, as macrophages and dendritic cells (DC) in the epidermis and neutrophils stimulates the upregulation of cytokines and chemokines [13,14]. In infections by MTB that enter the lungs via inhaled air, alveolar macrophages recognize the pathogen through recognition receptors called pattern recognition receptors (PRRs). Various microbes possess pathogen-associated molecular pattern (PAMP) receptors that are recognized by pattern recognition receptors (PRRs) through cell wall components that subvert different microbes [14]. TLRs are very important PRRs of immune system required to initiate an effective innate immune response at an early stage of infection and inflammation [14–16]. Monocytes/Macrophages, Dendritic Cells (DC), and neutrophils have Toll-like receptor (TLR) 2 and 4 at their surfaces. TLR2 recognizes bacteria and parasite which have glycolipids and peptidoglycans, while TLR4 recognizes bacteria which has more specific lipopolysaccharide [13–16]. The purpose of this study is to compare serum TLR2 and TLR4 protein levels in paediatric patients suffering from PTB and EPTB considering the presence of BCG scars.

2. Materials and methods

The research design was cross-sectional study in children with PTB and EPTB (locations of infection other than the lungs, such as the pleura, lymphatic glands, central nervous system, musculoskeletal system, gastrointestinal system, and pericardium) attending inpatient and outpatient services at Bhakti Medicare and Jakarta Islamic Hospital from December 2018 to November 2019. The subjects were recruited with convenience sampling method and the inclusion criteria were new cases of TB in children aged 0–18 years, vaccinated with BCG, not taking anti TB agent, contactable by researchers until the end of treatment at the same hospital, and whose parents signed an informed consent. The exclusion criteria were congenital disorders, any chronic disease (such as renal failure, diabetic mellitus**,** hypertension based on history taking and physical examination), immunocompromised state (currently receiving steroid or chemotherapy or positive for HIV infection), and drug-induced hepatitis due to anti-TB agents. General symptoms in subject were cough, fever, weight loss, lymphadenopathy, and a history of contact with TB patients.

The diagnosis of TB was confirmed and scored by a paediatrician. The Indonesian Paediatric TB Scoring System was created as one of the diagnostic approaches of TB in children, particularly in resource-limited healthcare facilities. Parameters assessed in the scoring system include cough, fever, TB contact history, chest x-ray imaging, tuberculin skin test (TST), nutritional state, lymph node involvement, and the bone or joint swelling. A total score of 6 or more is an indication of TB. Nutritional status is assessed based on body weight and height [2].

History of BCG vaccination was confirmed through anamnesis, health card record, and BCG scar formed at musculus deltoid of upper right arm found in physical examination. The schedule for BCG vaccine administration was form birth to 2 months of age, in which 0.05 ml of BCG vaccine (Bio Farma) was injected intradermally at musculus deltoid of upper right arm.

The tuberculin test (TST) is a test in which 0.1 ml PPD RT 23– 2TU produced by Biofarma was injected intracutaneously in the volar part of the right forearm. Reaction were assessed after 48–72 h by measuring the horizontal diameter of the induration formed at the injection site; diameter $>$ 10 mm is considered as a positive result. Tuberculin PPD RT 23 (2TU) is a purified protein derivative, prepared from a culture of seven selected strains of Mycobacterium tuberculosis. It is a clear liquid, uncoloured to light yellowish. One dose (0.1 ml) contains 2 Tuberculin Units, that equal to 0.04 μg of Tuberculin PPD RT 23 [2].

A complete blood laboratory and chest x-ray radiography were carried out. GeneXpert is a molecular test using the "real time" PCR method for quick diagnosis of TB but it is not available at the location of this study [17]. After the diagnosis of TB was confirmed, serum TLR2 and TLR4 protein levels were checked before anti-TB agent's administration.

2.1. Examination of TLR2 and TLR4 by ELISA

Two ml of venous blood samples were collected. The samples were immediately centrifuged at 3,000 rpm for 10–15 min to separate blood cells and serum. The serum was placed into an Eppendorf tube and stored in a freezer at –20 °C and sent to the Laboratory of the Medical Faculty of Hasanuddin University Makassar, Indonesia for ELISA analysis.

First, 100 μL diluent assay containing a protein buffer was added to each well. Subsequently, 100 μL of standardized fluid containing recombinant human serum TLR from a specific KIT (Human TLR2 ELISA Kit Catalog No: LS-F12773; Human TLR4 ELISA Kit Catalog No: LS-F22086, and Human CD4 ELISA Kit Catalog No: LS-F6263, LSBio, USA) or a diluted sample of patient serum was added to each well.The samples were incubated for 2 h at room temperature, each well was rinsed with sterile phosphate-buffered saline (PBS) for four times. Then 200 μL horseradish peroxidase (HRP)-Conjugated Streptavidin-was added to each well and the plate covered with an aluminum foil cover and incubated for 30 min at room temperature in the dark. The wells were again washed four times using sterile PBS. Next, 200 μL substrate solution containing 3,3′, 5,5′ - tetramethylbenzidine (TMB) was added to each well, and samples were incubated for 20 min at room temperature in the dark. Finally, 50 μL stopping solution containing H_2SO_4 was added to each well to stop the reaction. Samples were read using ELISA Reader 270 (Biomerieux, France) in a wavelength of 450 nm within 30 min. The concentration of TLR2 and TLR4 was recorded in units of ng/ml.

2.2. Statistical analysis

Statistical analysis was done by the SPSS v.23 program, using descriptive and bivariate analysis with *chi-square,* normality test of numerical data with Kolmogorov-Smirnov, *independent t-test*, and ANOVA test. Statistical tests were significant if p-value $<$ 0.05.

2.3. Ethics statement

This study received a recommendation for ethical approval from the Health Research Ethics Committee of the Hasanuddin University Hospital for Medicine, Indonesia with number 371/UN4.6.4.5.31/PP36/2019 on May 15, 2019.

3. Results

The research subjects were 69 children with new TB cases who met the inclusion and exclusion criteria. There were 57 PTB subjects (82.6%) and 12 EPTB subjects (17.4%), 47 subjects (68.1%) had BCG scars while 22 subjects (31.9%) did not have BCG scars. Among all subjects, 55.1% were under 6 years old, 56.5% had nutrition deficiency, 49.3% had a history of TB contact, and 55.1% had a positive TST. Among the 12 subjects with EPTB, 8 had a BCG scars (66.6%), 8 were female (66.6%),

Independent t-test.

Table 1
Bivariate analysis of the characteristics of the subjects based on diagnosis group.

Respondent Characteristics		Total (n = 69)	%	TBP (n = 47)	TBEP (n = 12))	p value
Gender	Female	33	47.8	25	8	0.151
	Male	36	52.2	32	4	
Age	< 6 years	38	55.1	37	1	0.000
	6–18 years	31	44.9	20	11	
Nutritional Status	Deficient	39	56.5	32	7	0.889
	Good	30	43.5	25	5	
History of TB Contact	None	35	50.7	30	5	0.490
	Exist	34	49.3	27	7	
TST	Negative	31	44.9	27	4	0.374
	Positive	38	55.1	30	8	
BCG Scar	Negative	22	31.9	18	4	0.906
	Positive	47	68.1	39	8	

Chi-square test.

11 were over 10 years old (91.6%), 7 had nutrition deficiency (58.3%), 7 had a history of TB contact (58.3%), and 8 had a positive TST (66.6%). There were 7 subjects suffering from pleural abnormalities, 1 with TB meningitis, 1 with TB spondylitis, and 3 in the lymph nodes. Statistical analysis with chi-square shows no significant differences except between age characteristics ($p < 0.001$) (Table 1).

There were 47 subjects, 68.1% with BCG scar and 31.9% without BCG scar. Bivariates analysis considered no significant differences between female and male ($p > 0.05$) (Table 2). Overall, mean serum of TLR2 and TLR4 levels are higher in EPTB subject than PTB subject, but there is no statistically significant difference ($p > 0,05$) (Table 3**).** Among 12 EPTB patients, mean serum of TLR2 and TLR4 levels are higher in patients with BCG scars than without BCG Scars and statistical analysis considered a significant difference ($p < 0.05$) (Table 4). Among 57 PTB patients, mean serum of TLR2 and TLR4 levels are higher in patients with BCG scars than without BCG scars and statistical analysis considered a significant difference ($p < 0.001$) (Table 5). Statistical analysis between serum levels of TLR2 and TLR4 with subject characteristics (gender, age , nutrition status, history of TB contact) considered no significant differences ($p > 0.05$) (Figs. 1–6**).**

Fig. 1 presented that Box plots comparison of TLR 2 serum levels (a) and comparison of TLR4 serum levels (b) based on age classification, shows no significant difference ($p > 0.05$). The boxes represent the inter-quartile range (25–75% of the sample); the line in the middle is the median (50%). The dots are the outliers as the whiskers extend up to 1.5 times the inter-quartile range. Fig. 2 illustrated are box plots comparison of TLR2 serum levels (a) and comparison of TLR4 serum level (b) according gender classification, shows no significant difference ($p > 0.05$). Fig. 3 shown box plots comparison of TLR2 serum levels (a) and comparison of TLR4 serum level (b) based on nutrition shows no significant difference ($p > 0.05$). Fig. 4 present is Box plots comparison of TLR2 serum levels (a) and comparison of TLR4 serum level (b) based on history of TB contact shows no significant difference ($p > 0.05$).

Fig. 5 shown Box plots comparison of serum levels of TLR2 in subjects based on BCG Scar formation and TB diagnosis. The mean of TLR2 protein level shows significant difference between PTB_Scar(+)(32.560 ± 8.994), EPTB_Scar(+) 33.855 ± 6.817), PTB_Scar(-)(11.829 ± 5.592), EPTB_Scar(-) (13.977 ± 7.139) with p value of < 0.001. Fig 6 presented Box plots of Serum levels of TLR4 in subjects based on BCG Scar formation and TB diagnosis. The mean of TLR4 protein level shows

Table 2
Bivariat analysis of the characteristics of gender based on BCG Scar.

Characteristics		Total (n=69)	BCG scar positive (n=47)	% 68,1	BCG scar negative (n=22)	% 31,9	P value
Gender	Female	33	25	75,8	8	24,2	0,192
	Male	36	22	33,3	14	66,7	

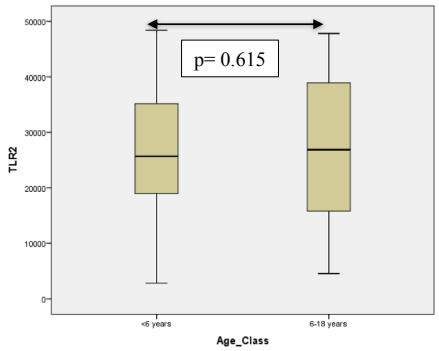
Table 4
Serum levels of TLR2 and TLR4 in EPTB subjects based on BCG scars.

Toll like receptors		EPTB Diagnosis		p value
		BCG Scar Positive (n = 8)	BCG Scar Negative (n = 4)	
TLR2 (ng/ml)	Mean (SD)	33.855 (6.817)	13.976.50 (7.139)	0.001
	Min-max	23.505–42.080	4.553–21.477	
TLR4 (ng/ml)	Mean (SD)	16.147 (3.100)	9.511(2.543)	0.004
	Min-max	10.998–19.820	6.677–12.560	

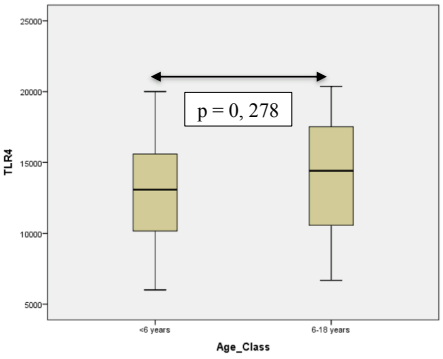
Independent t-test.

Table 5
Serum levels of TLR2 and TLR4 in PTB subjects based on BCG scars.

Toll like receptors		PTB Diagnosis		p value
		BCG Scar Positive (n = 39)	BCG Scar Negative (n = 18)	
TLR2 (ng/ml)	Mean (SD)	32.560 (8.994)	11.829 (5.592)	0.000
	Min-max	17.823–48.404	2.822–23.208	
TLR4 (ng/ml)	Mean (SD)	15.417 (2.720)	8.904 (1.594)	0.000
	Min-max	10.687–20.379	6.003–11.702	

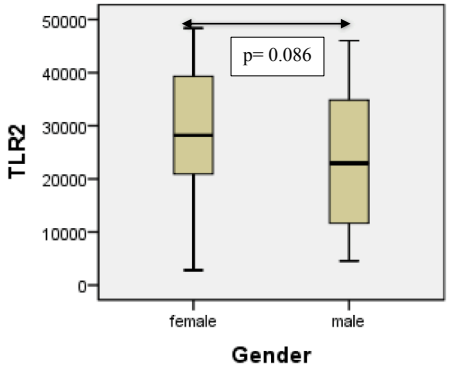


a. Box plots for Serum levels of TLR2 in subjects based on age classification

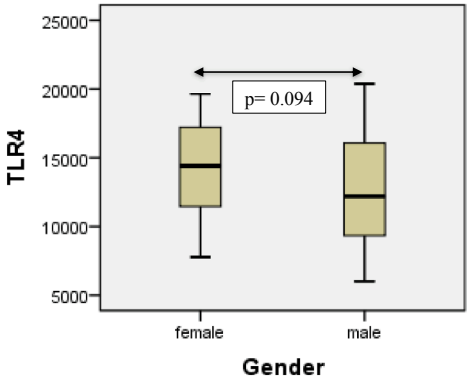


b. Box plots for Serum levels of TLR4 in subjects based on age classification

Fig. 1. A Comparison of TLR2 serum level B and Comparison of TLR4 serum level based on age classification.

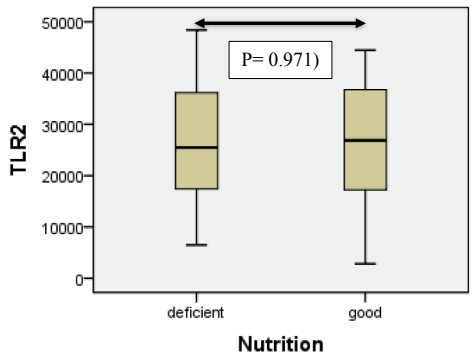


a. Box plots for Serum levels of TLR2 in subjects based on gender classification

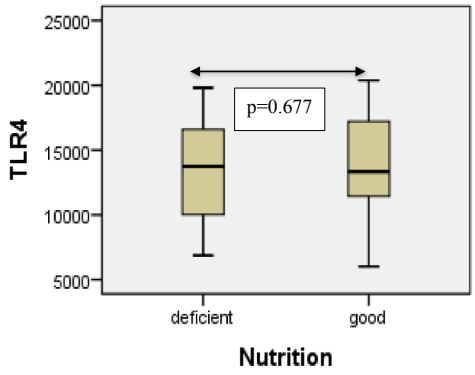


b. Box plots for Serum levels of TLR4 in subjects based on gender classification

Fig. 2. A Comparison of TLR2 serum level B and Comparison of TLR4 serum level based on gender classification.



a. Box plots for Serum levels of TLR2 in subjects based on nutrition



b. Box plots for Serum levels of TLR4 in subjects based on nutrition

Fig. 3. A Comparison of TLR2 serum level B and Comparison of TLR4 serum level based on age classification.

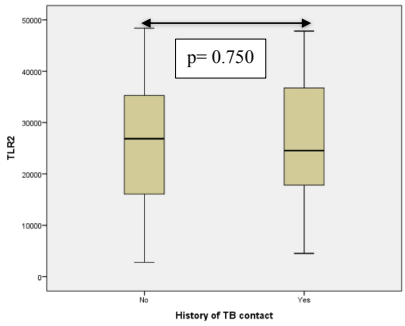
significant difference between PTB_Scar(+)(15.417 ± 2.720), EPTB_Scar (+) 16.147 ± 3.100), PTB_Scar(-)(8.904 ± 1.594), EPTB_Scar(-) (9.511 ± 2.543) with p value of < 0.001

4. Discussion

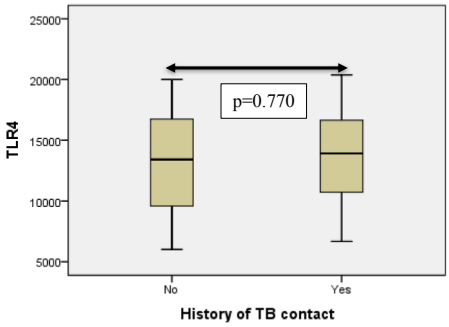
Diagnosis of TB in children is challenging because the signs and symptoms are often atypical. Children also typically have difficulty in expelling sputum for the microbiological examination. In contrast, TB diagnosis in adults is relatively easier, because the signs and symptoms are typical and they're supported by characteristic signs in the chest x-ray and it is easier for an adult to produce sputum to be tested for MTB [5,17]. According to the 2019 Global Tuberculosis Report, new cases of EPTB in Southeast Asia was 15%, higher than the world average 14%, 11% of all TB cases in Indonesia, mostly in children above 5–10 years old [18–20].

The World Health Organization recommends BCG vaccination to prevent severe forms of TB disease, treat latent TB in children living with HIV and children less than 5 years of age who have a history of contact with confirmed TB cases. The protective effectiveness of the BCG vaccination is 60–80% and decreases with age, especially in TB endemic areas. The protective effectiveness depends on the population, the area where TB is endemic, prior exposure to environmental mycobacterium, and age at vaccination. Most EPTB patients are over 5 years old, so the protective effect of vaccination is reduced [2,10,11].

BCG scar are formed in 52–93% of children after BCG vaccination and the mortality ratio was greater in those who did not have BCG scars [19]. Vaccination of BCG offers more protection in children who were vaccinated in the neonatal period, especially during the first and second years after the vaccine [19,20]. A systematic review reported that BCG vaccination could protect children from MTB infection and from



a. Box plots for Serum levels of TLR2 in subjects according History of TB Contact



b. Box plots for Serum levels of TLR2 in subjects according History of TB Contact

Fig. 4. A Comparison of TLR2 serum level B and Comparison of TLR4 serum level based on History of TB contact.

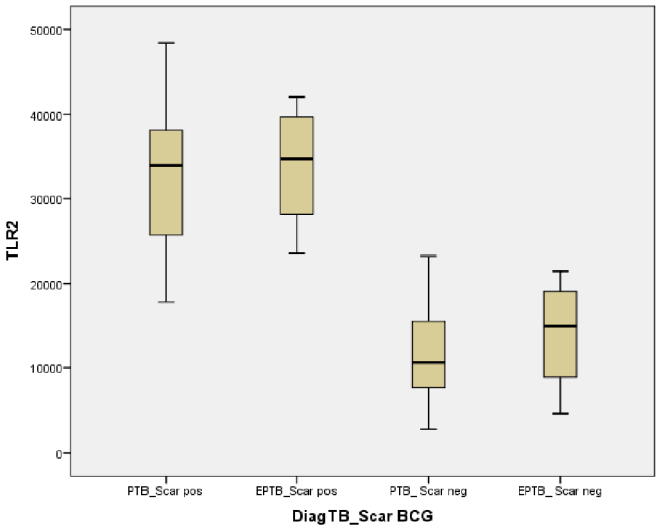


Fig. 5. Box plots of Serum levels of TLR2 in subjects based on BCG Scar formation and diagnosis TB.

developing TB disease [20,21]. Research on infants with low birth weight in endemic areas, where vaccination is carried out earlier, showed that the mortality rate fell more during the neonatal period than at 6 and 12 months of age [21]. Environmental mycobacterial exposure before BCG vaccination reduces the protective effect of BCG against PTB. BCG vaccination is effective in preventing miliary TB and TB meningitis in infants and children. There is no evidence that BCG strains influence the protective effect [22].

From all subjects, 47 subjects (68.1%) have BCG scar, consisting of 39 PTB subjects (82,98 %) and 8 EPTB subjects (17.02%) with various main complaints at the time of diagnosis. Coughing, night sweats and

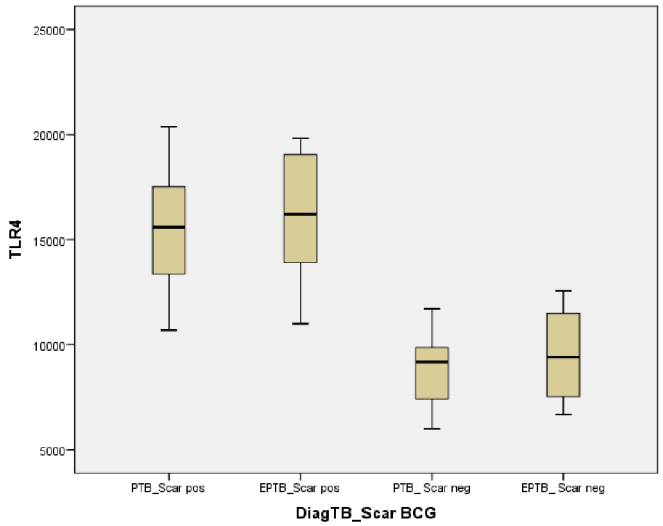


Fig. 6. Box plots of Serum levels of TLR4 in subjects based on BCG Scar formation and diagnosis TB.

weight loss are rare among patients with EPTB, most of whom have atypical symptoms. When the diagnosis was confirmed, the clinical symptoms already severe, and require longer hospitalisation and treatment [17,23,24]. In this study there were no children under 5 years with miliary TB and TB meningitis, and the economic status of the subject's parents included middle and low class. EPTB incidence in children is related to country of origin, immune disorders such as HIV, drug resistance, and complication (progressive clinical manifestation, causing sequelae or death) [23–27]. In a non-endemic area reported miliary TB disease mostly in infants, followed by TB lymphadenitis and TB meningitis in preschool children and pleural effusions and bone TB in older children. Poverty, country of origin, and limitations in access to health services play a role in TB meningitis cases [24].

Serum levels of TLR2 were higher than TLR4 in both EPTB and PTB patients, furthermore, serum levels of TLR2 and TLR4 were higher in EPTB. Higher levels of TLR in EPTB can be contributed by the existence of primary and/or secondary lesions as sites of pathogen entry in EPTB, where some cases of EPTB may be accompanied by involvement of the lungs. Inflammation occurs in both location, inducing innate and adaptive immune responses [23,24]. *Toll Like Receptors* (TLR) 2 and TLR4 are microbial recognition receptors that are present in many cells such as monocytes/macrophages, mast cells, dendritic cells (DC), neutrophils, adipose tissue, B lymphocytes and T lymphocytes. TLR2 recognizes bacterial glycolipids and peptidoglycan and TLR4 is specific for bacterial lipopolysaccharide that is on the walls of MTB. Among the TLR families, TLR2, TLR4, TLR9 and their adapter molecules MyD88 play the most prominent role in initiating the immune response against TB. *Mycobacterium bovis* Bacillus Calmette -Guerin (BCG) is a live-attenuated vaccine that can influence the innate and adaptive immune responses to prevent and fight against TB infection [13,14,28].

There was no difference in serum levels of TLR2 and TLR4 in subjects based on characteristics (gender, age group, nutritional status or history of TB contact). TLRs have multiple roles in infection, inflammation, reproduction, development, autoimmunity, cancer, inflammation/allograft rejection and regeneration so that serum levels of TLR can be influenced by them [29,30]. TLR2 can recognize many ligands, including bacterial lipopeptides, fungal zymosan, parasitic and viral proteins. Gram-positive lipoteichoic acid (LTA) due to the formation of TLR2 heterodimers with two other TLRs, namely TLR1 or TLR6. TLR1/TLR2 heterodimers can recognize triacylated lipoproteins, whereas TLR2/TLR6 can recognize diacylated lipoproteins, therefore the TLR2 levels are higher than TLR4 [14,15,28,30]. The innate immune response, which was indicated by serum TLR2 and TLR4 levels, was

influenced by BCG scar post vaccination and infection site of MTB [30–32].

Race/ethnicity and pathogen strain can influence TLR2 and TLR4 responses to produce cytokine in MTB infection as differences was found in Filipinos, Chinese and White people [33]. However, the subjects in this study were limited to Indonesians only. Polymorphisms of TLR2 and TLR4 genes can influence the risk susceptibility, severity and outcome of pulmonary tuberculosis (PTB) infection.

The macrophage is a crucial cell in these events, as it is involved in phagocytosis and killing of mycobacteria as well as the initiation of adaptive T-cell immunity. An effective innate immune response will activate adaptive immune response through cross talk dendritic cells, as one of the antigen-presenting cells that is competent towards naive T lymphocytes induces an effector T lymphocyte response. There is an increase in Foxp3 + gene expression and serum Treg levels in children who have TB and have BCG scars, as a marker of adaptive immune response in TB diseases [34,35].

TLR 2 and TLR4 responses cannot be used as a parameter of effectiveness of the innate immune response to support clinical practice for diagnosis or prognosis TB disease, because they are influenced by various factors [29,30]. In addition, innate response only lasts for a short time before it is followed by the activation of adaptive immune response, which is more specific and has the potential to control MTB infection [14,29,30].

5. Conclusions

Serum TLR2 and TLR4 protein levels were significantly higher in EPTB than in PTB patients and in those with BCG scars than without BCG scars. The innate immune response after BCG vaccination provides protection from severe TB in children<5 years of age. It is necessary to further investigate TLR2 and TLR4 levels in healthy children who have been vaccinated with BCG to compare whether healthy children who have BCG scar have stronger immune response to TB infection. Serum level of TLR2 and TLR4 in children with BCG scars helps us understand the importance of scar tissue formation after each BCG vaccination. Several limitations in this study include the lack of data about involvement of pulmonary abnormalities in EPTB patients, unknown timing of vaccination, and no comparison of serum TLR level between healthy children and children with TB. To overcome this study limitation, better facilities in are needed to perform supporting examination such as histopathology test for other location of MTB infection.

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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