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The correlation of Foxp3 + gene and regulatory T cells with scar BCG formation among children with Tuberculosis

Farsida [a,1], Mochammad Hatta [b,1], Ilhamjaya Patellongi [b], Prihantono [b], Rahmini Shabariyah [a], Rahma Ayu Larasati (Laras) [a,*], Andi Asadul Islam [b], Rosdiana Natzir [b], Muh. Nasrum Massi [b], Firdaus Hamid [b], Andi Dwi Bahagia [b]

[a] Faculty of Medicine, Universitas Muhammadiyah Jakarta, Indonesia
[b] Faculty of Medicine, Universitas Hasanuddin Makassar, Indonesia

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ABSTRACT

Tuberculosis infection causes a complex immunological response, where interactions between the pathogen and the host are unique, making it difficult to treat and control this disease. According to WHO, an estimated 1 million children became ill with TB, and 233,000 children died of TB in 2017. Bacillus Calmette-Guérin (BCG) vaccines continue to be the only vaccines to prevent Tuberculosis (TB). Studies suggesting the association of BCG scar with decreased childhood mortality in developing countries have rekindled the interest in BCG scar. However, the direct effect of the BCG scar remains unknown. We examined 76 cases in this study. All Subjects were diagnosed with Tuberculosis. BCG scars were examined directly when physical examination at the BCG vaccination site was performed. Tuberculin Skin Test was performed with 0.1 ml purified protein derivative (PPD) solution (5TU PPD/0.1 ml) injected intradermally. We examined the FOXP3 gene by real-time PCR and the level of Treg byELISA. The comparison of the mean Treg gene expression and the Treg protein content was higher in the positive scar group than in the negative scar group. It shows that Treg plays a role in the Tuberculosis during its active phase development. Treg protein levels were higher in the combination of positive TST and scar. It shows that BCG scarring is an essential marker of a well-functioning immune system. Cheap and straightforward initiatives like early BCG vaccinations, monitoring BCG scarring, and revaccinating scar-negative children could have an enormous immediate impact on global child survival.

1. Introduction

Tuberculosis is a disease caused by the Mycobacterium Tuberculosis (Mtb). For thousands of years, humans have been infected with Mtb. This infection causes a complex immunological responses, where interactions between the pathogen and the host are unique, making it difficult to treat and control this disease. People of low economic backgrounds often contract the disease, thus worsening social and economic conditions. Although TB is a treatable infectious disease, it still has a high effect on mortality. TB is responsible for one in four deaths in the 19th century because of its chronic progressive transmission nature and prolonged treatment. Besides, the emergence of multi-drug resistant TB and the TB epidemic - putting a significant burden on society.

According to WHO, an estimated 1 million children became ill with TB, and 233,000 children died of TB in 2017. However, the actual burden of TB in children is likely higher, given the challenge in diagnosing childhood TB. Although overdiagnosis does occur in some settings, underdiagnosis is the rule in most TB-endemic areas, where young children can only access TB care via referral hospitals. Only 23% of the estimated 1.3 million children under five years who are eligible received preventive therapy in TB households in 2017. Ending the TB epidemic is a target under the Sustainable Development Goals that requires implementing a mix of biomedical, public health targets, socioeconomic interventions, and research and innovation [1]. Understanding the natural history of TB is fundamental to appreciate the variable vulnerability, and the diverse spectrum of disease observed in children.

Bacillus Calmette-Guérin (BCG) vaccines continue to be the only vaccines in use for the prevention of Tuberculosis (TB). The use of BCG

* Corresponding author.
E-mail address: dr.larasatirahma@gmail.com (R.A. Larasati (Laras)).
[1] These authors contributed equally to this work.

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in routine infant vaccination programs (estimated coverage at 90%) is estimated to globally prevent 117,132 TB deaths per birth cohort in the first 15 years of life [1]. However, BCG affords variable efficacy against pulmonary TB. There has been much debate regarding the advantages and disadvantages of BCG since its first introduction. Key elements of the debate surrounding BCG's effectiveness have included safety, loss of tuberculin sensitivity as a diagnostic tool, and, especially, the broad range of BCG effectiveness against TB. Studies suggesting the association of BCG scar with decreased childhood mortality in developing countries have rekindled BCG scar's interest. But the direct effect of BCG scar still unknown [2–4].

This study is to get the correlation between regulatory T (T reg) cells in children with Tuberculosis. We looked for an association with the presence or absence of BCG scars and tuberculin test results. Regulatory T cells protect the host during infections by preventing collateral damage to the host's tissue from excessive inflammation induced by the immune response to the pathogen. The T reg cell's role in suppressing immunity to *Mtb*, which is the most important bacterial pathogen that establishes a persistent infection in humans in terms of morbidity and mortality, is only the early stage of an investigation.

2. Results

2.1. General characteristic of study population

Based on the research subject, male 40 (52.6%) make up most of the population. Most re those at the age of 0–5 years by 41 people (53.9%). Poor nutrition 44 people (54.8%). Patient with contact history with adult TB was 39 (51.3%). Most of the TST tests were positive (>10 mm) as many as 40 people (62.6%). Most of the scar marks were positive in 47 people (61.8%) Table 1.

2.2. The level of FOXP3 gene and Treg serum Based on scar Formation

The mean comparison of Foxp3 expression and Treg protein content was higher in the positive scar group than in the negative scar group Table 2, Fig. 1

2.3. The expression of FOXP3 gene and level of Treg based on TST Result

The mean comparison of the Foxp3 gene and the level of Treg was higher in the positive TST group Figs. 2 and 3.

The results of the comparison test showed that there was a significant difference in the mean protein content of Foxp3 expression, between the Scar (+) TST (+) (12,570 ± 1,508), Scar(+) TST(−) (10,580 ± 1,158), and Scar(−) TST(+) groups (8,936 ± 1,326). and Scar – mantox – (6,860 ± 1,098) with a significant value of p 0,000.

The test results on the mean Treg protein levels showed a significant difference between Scar (+) TST(+) (291,885 ± 20,780), Scar(+) TST (−) (261,277 ± 17,807), Scar (−) TST (+) (231,632 ± 23,359) and Scar (−) TST(−) (204,275 ± 17,912) with a p value of 0.000 Fig. 4.

Table 1
General characteristic of subjects.

Characteristic		N	%
Sex	Female	36	47,4%
	Male	40	52,6%
Age Group	0–5	41	53,9%
	6–12	24	31,6%
	12–18	11	14,5%
Nutritional Status	Malnutrition	44	57.9%
	Normal	32	42.1%
Contact History	No Contact	37	48,7%
	Positive Contact	39	51,3%
TST	less than10	36	47,4%
	>10	40	52.6%
Scar	+	48	63,2%
	–	28	36,8%

Table 2
The level of FOXP3 gene and Treg serum based on scar formation.

Parameter		N	Mean	SD	Min	Max
FoxP3 + Gene Expression	Scar –	29	7,790	1,582	5,330	10,804
	Scar +	47	11,681	1,679	9,191	14,815
level of Treg	Scar –	29	216,539	24,443	178,428	270,407
	Scar +	47	278,209	24,681	234,189	326,607

3. Discussion

3.1. Characteristic of study population

We conducted a study on 76 subjects consisting of 40 males and 36 females. All the subjects received the BCG vaccine in two months of age. All the subjects had been diagnosed with Tuberculosis and confirmed with scoring and thorax Rontgen. Most subjects (N: 41) were diagnosed with Tuberculosis in 0–5 years of age. Forty-seven of subjects has BCG scar in the deltoid area from one of their arm. Thirty-nine subjects had TB history in their family. Forty-four of the population study has malnourished nutritional status.

3.2. Correlation of BCG scar with tuberculosis

BCG scar reading has been used as an indicator of vaccine status and became standard practice for assessing BCG's protective effect in retrospective studies. The presence of a BCG scar has been an indication of the protection against TB. Nevertheless, more than fifty percent of the study population with BCG scar got infected with TB in this case.

However, scars may be present or not after the BCG vaccine. Scars may be absent due to administrating lower doses of vaccine in childhood, the difficulty of injecting the entire amount of vaccine and the relatively weak local immune response in young children, or the probability that they may even disappear with time [5–7].

Several explanations have been put forward concerning such variability, including the improper handling and administration of the vaccine, exposure to non-tuberculous mycobacteria (NTM) in the per equatorial region, low immunogenicity of the vaccine, concomitant malnutrition, and other infectious diseases as well as a genetically determined low immune response to the vaccine [8–10].

In the present study, the absence of scarring on those who received vaccination may have influenced the effectiveness. This is because the vaccinated children who did not develop a scar (if, for example, the vaccine no longer contained live BCG due to inadequate storage) may have been considered unvaccinated, thus generating a non-differential misclassification, which could minimize the protective effect of the vaccine.

Based on previous findings, the ability of BCG to induce scars may vary by sex, but the direct effect of sex still unknown [11]. There was also no significant difference between the age groups. Tuberculosis mostly affects adults in their most productive years. However, all age groups are at risk. Over 95% of cases and deaths are in developing countries. The observed effects of age, sex, and origin may reflect a combination of the effects of various host and microbial factors not included in the present study. All groups have the same risks. Age 0–5 have a longer time being contacted by their parents because they spent their activity at home. We knew that transmission rates due to direct contact with family are relatively high. In conjunction with recent findings, household-based contact interventions that include treatment of latent infections may prevent disease progression in young contacts of index patients in settings with a high tuberculosis burden [12–14].

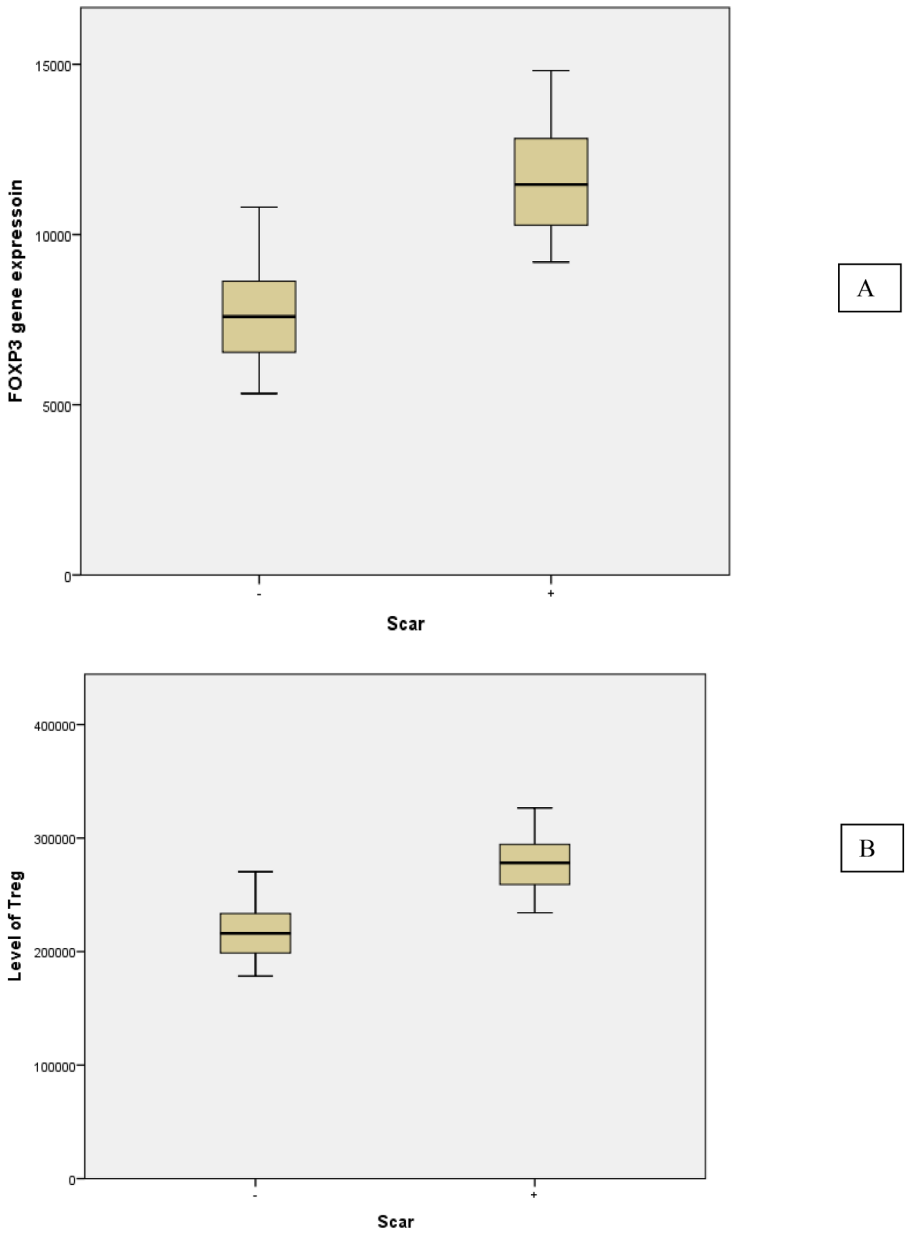

Fig. 1. A. Comparison of FOXP3 expression B. Comparison level of T Reg based on Scar formation.

Genetic and environmental factors also influence differences in the shape and size of scars. It is evidence in Lalor et al.'s research results in two populations that have genetic and microbial environmental differences between Malawi and England. In this study, the scar size that appeared after three months after BCG vaccination in the Malawian population was smaller than the median scar size in the UK population [9].

3.3. Correlation level of Treg and FOXP3 with scar BCG

Reports are pointing out that Tregs could interfere with BCG-mediated protection against TB. Tregs are known to be induced by environmental mycobacteria, BCG, and M. tuberculosis infection. Pre-exposure of the host to environmental mycobacteria may induce Tregs against antigens, typical to environmental mycobacteria, BCG, and M. tuberculosis. Such sensitized Tregs may further be stimulated by subsequent vaccination with BCG and by M. tuberculosis infection. Thus, Tregs induced first by environmental mycobacteria may initiate the process leading towards downmodulation of the BCG vaccine's efficacy. However, there may be scanty pre-induced Tregs available for stimulation by BCG vaccination when there are no environmental mycobacteria. In Treg-attenuated, BCG-immunized mice, which were then infected with M. tuberculosis, the mycobacterial lung load was significantly [6,11,15].

To evaluate Treg's impact in protection against M. Tuberculosis, we evaluate the expression of Treg and foxp3 in children with Tuberculosis. There is a significant difference between the two groups. A group with scar positive has a bigger account in treg and foxp3. The Presence of scars after BCG vaccination has been used as an indicator of vaccination success and in estimating vaccine coverage. Indeed, the presence of a scar does not always correlate with protection against TB; however, it may reflect the efficacy BCGs against TB. Among BCG vaccinated individuals, having a scar is associated with further reduced mortality because of TB. Subjects with BCG scar have a higher amount of Treg and FOXP3 gene. It shows that Treg plays a role in the development of Tuberculosis in its active phase.

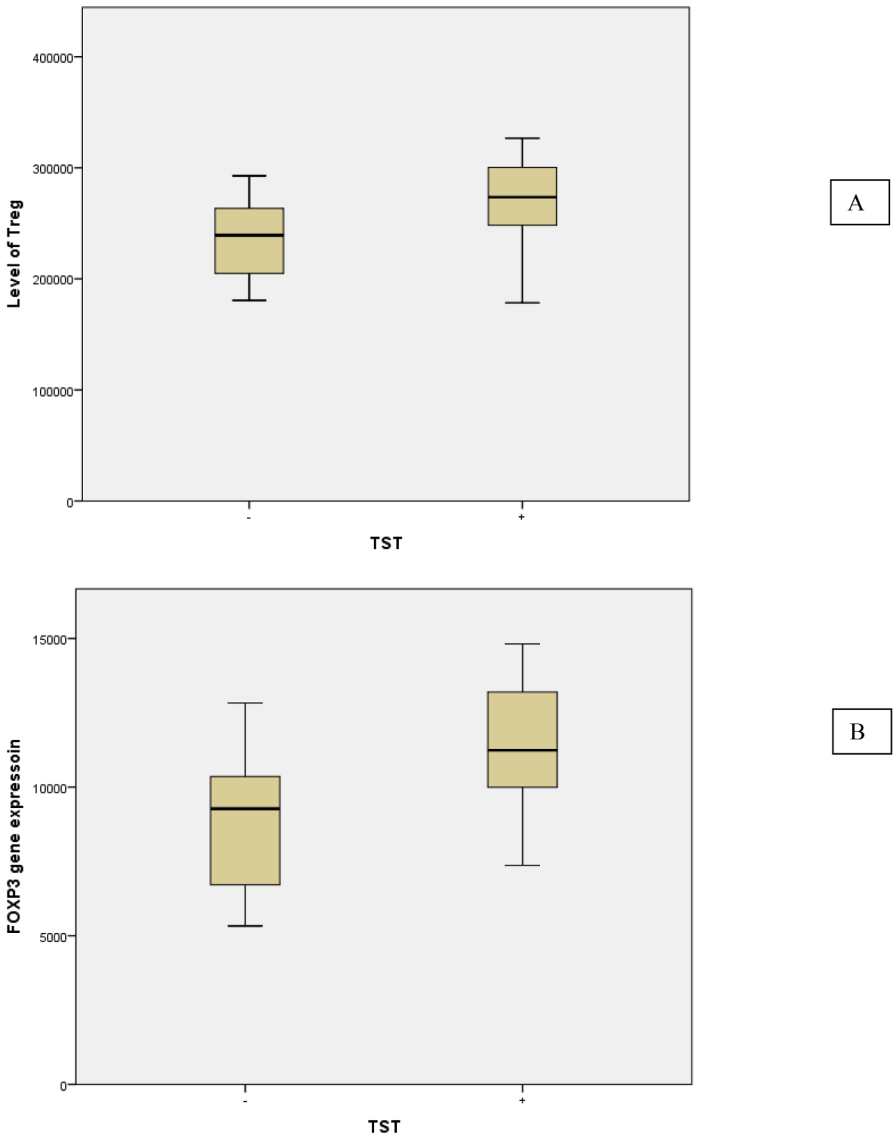

Fig. 2. The Expression of FOXP3 gene and Level of Treg based on TST Result.

3.4. Correlation level of Treg and FOXP3 with tuberculin SkinTest

Short of demonstrating viable organisms in body tissues and fluids, the tuberculin skin test is the only method of detecting M. tuberculosis infection in an individual. It is used in the diagnosis of Tuberculosis in individual patients and in epidemiological settings to measure the prevalence of tuberculous infection in populations [16,17].

We use >10 mm as positive results for this study. There are exact amounts between positive and negative results [18]. Twenty-one (50%) subjects have positive results. So it means 50% of subjects have false-negative results. The Mantoux test does not measure immunity to TB but the degree of hypersensitivity to tuberculin. There is no correlation between the size of the induration and the likelihood of current active TB disease, but the reaction size is correlated with the future risk of developing TB disease. The test has a low positive predictive value for the current active disease [19,20].

In this study, we did not examine the direct impact of T regulatory cells against TB. In this study, we see significant differences in the level of T reg between each Mantoux group. There is a higher level in the group with more significant TST results in diameter. Active TB is characterized by increased Treg and decreased Th1 responses, which contribute to the establishment of active infection. However, the roles of Treg and Th1/Th2/Th17 cells in latent TB have not been elucidated [21].

4. Materials and methods

4.1. The selection of study population

This Cross-Sectional study was carried out at the Bhakti Medicare Hospital, Cicurug West Java, Indonesia, after obtaining permission from the Institutional Ethics Committee. Our subjects are the infants recruited for the study from July to December 2019. The analysis of Treg and mRNA expression was held at The Molecular and Biology Laboratory Universitas Hasanuddin, Makassar South Sulawesi, Indonesia. Only those children whose parents consented to participate in the study were recruited. A total of 76 cases were included by the method of purposive sampling. All Subjects were diagnosed with Tuberculosis by anamnesis, physical examination, tuberculin skin test, and thorax Rontgen. The 2 ml of whole blood was taken from each subject. The blood is taken before any use of medication.

BCG scars are examined directly when physical examination at the

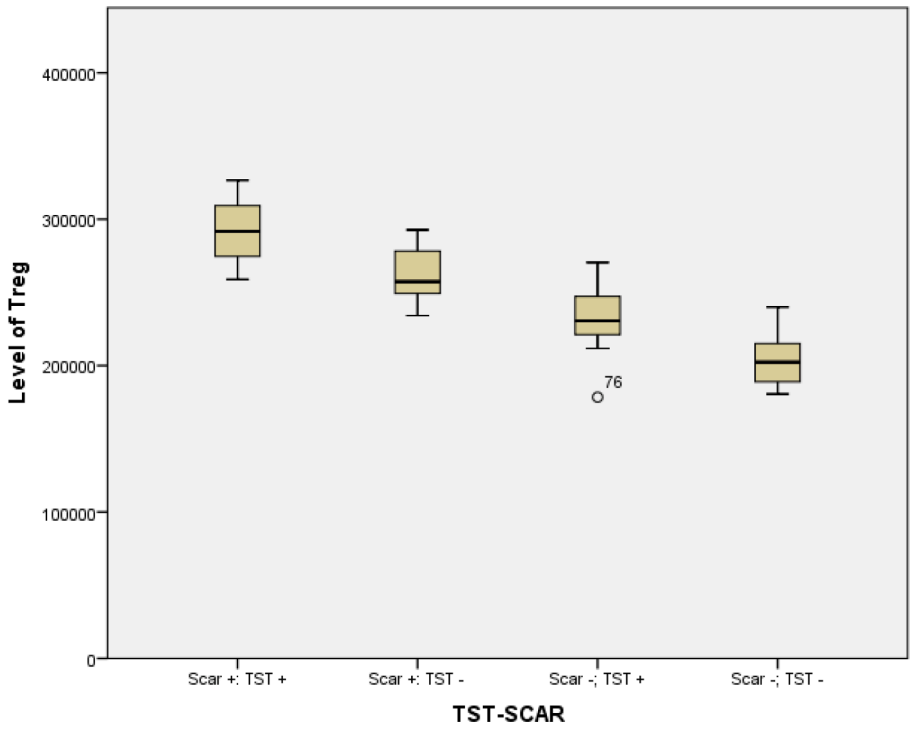

Fig. 3. The Comparison Level of T Reg Based on Scar Formation and TST Result.

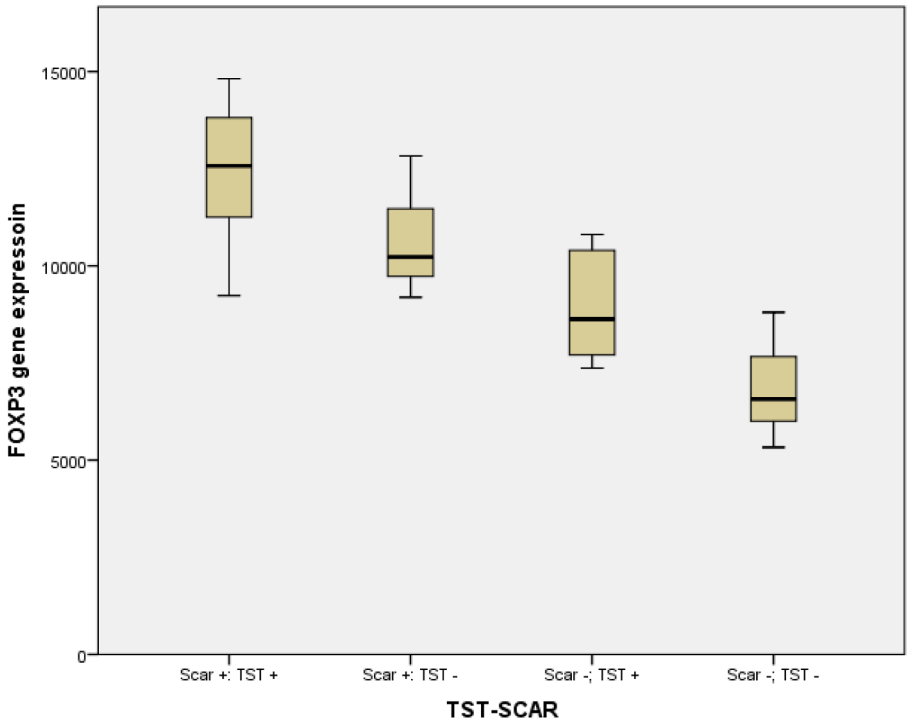

Fig. 4. The Comparison Of FOXP3 Expression Based on Scar Formation and TST Result.

BCG vaccination site is performed. Tuberculin skin test was done with 0.1 ml purified protein derivative (PPD) solution (5TU PPD/0.1 ml, SPAN diagnostic limited) injected intradermally with a 26–Gauge needle on the flexor aspect of the left forearm 2–3 in. below the elbow. Tuberculin skin test was read after 48–72 h by a ballpoint pen technique.

We followed national guidelines for the diagnosis of active TB cases. A TB case was considered to be a child with M. tuberculosis isolated from clinical specimens, or with the presence of symptoms, signs, and/or radiological images compatible with TB (when chest radiography was doubtful, thoracic computed tomography was performed), and/or a

positive TST (as defined previously), and who responded clinically to antituberculous chemotherapy. Close contact with a bacillary TB case was used as diagnostic support.

This study's exclusion criteria are HIV-positive or have a history of HIV parents, recent contacts of active tuberculosis cases, children with old healed TB, under immune-suppressive agents medication, and patients on long-term systemic corticosteroid therapy (>than six weeks).

4.2. The examination of FOXp3 gene by Real-time PCR

The process of examining the primer oligonucleotide specific gene for GAPDH as the primer: F: 5′-GAA GGT GAA GGT CGG AGT-3′ and R: 5′-GAA GAT GGT GAT GGG ATT TC-3′ as 'housekeeping gene' (internal control). Treg mRNA gene using a specific primer forward F: 5′-GGC ACT CCT CCA GGA CAG-3′ and R: 5′-GCT GAT CAT GGC TGG GCT CT-3′. Reaction conditions were designed as follows: 30 seconds at 95°C, and 40 cycles of denaturation at 95°C for 10 seconds followed by annealing at 60°C for 15 seconds and for 40 seconds. Real-time reverse transcription-PCR (QRT-PCR) process using one step Green QRT-PCR master mix kit. The data were analyzed with the PCR results using Bio-Rad CFX Manager 3.1 software (Biorad, USA) [22–25].

4.3. The examination of Treg with ELISA

The first step is to add 100 μL assay diluent containing buffer protein to each well. Next, 100 μL of standard liquid containing recombinant human protein Treg from the specified KIT or diluted sample from the patient's serum into each well was added. Furthermore, incubation was carried out for 2 h at room temperature. Flush the liquid in each well and wash it with sterile Phosphate Buffered Saline (PBS). This washing process is carried out four times in a row. Then 200 μL of the "conjugate" liquid containing "horseradish peroxidase" (HRP) streptavidin (HRP) was added to each well and covered with plastic cover and incubated for 2 h at room temperature. The liquid is sucked and then washed again as much as four times using a sterile PBS liquid. The next process was adding 200 μL of a substrate solution containing 3.3′, 5.5′ - Tetramethylbenzidine (TMB) to each well, and incubated for 20 min at room temperature. The microplate was kept in the dark to avoid light. After incubation, the reaction was stopped by adding 50 μL of a stopping solution containing H2SO4 into each well and reading it using ELISA Reader 270 (Biomerieux, France) with a wavelength of 450 nm in 30 min. Next, read CD25 concentrations in pg/ml units.

4.4. Statistic analysis

Statistical analysis used the Chi-square test to determine the association between BCG scar and tuberculin skin test and the association between BCG scar and various factors. The statistical testing results, a P value less than 0.05, were considered statistically significant in the relationship between the two variables. Statistical analysis was performed using Microsoft Excel and SPSS Statistical Software.

5. Conclusions

The expression of Treg gene mRNA was higher in the positive scar combination of positive Mantoux compared to other combinations. Treg protein levels were higher in the combination of positive Mantoux scar. BCG scarring is an important marker of a well-functioning immune system. Cheap and straightforward initiatives like early BCG vaccinations, monitoring BCG scarring, and revaccinating scar-negative children could have an enormous immediate impact on child survival worldwide.

CRediT authorship contribution statement

Farsida: Conceptualization, Funding acquisition, Writing - original draft. **Mochammad Hatta:** Conceptualization, Writing - original draft. **Ilhamjaya Patellongi:** Data curation, Methodology. **Prihantono:** Supervision. **Rahmini Shabariyah:** Resources, Project administration. **Rahma Ayu Larasati:** Project administration, Writing - review & editing. **Andi Asadul Islam:** Formal analysis, Validation. **Rosdiana Natzir:** Formal analysis, Validation. **Muh. Nasrum:** Formal analysis, Validation. **Firdaus Hamid:** Formal analysis, Validation. **Andi Dwi Bahagia:** Formal analysis, Validation.

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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