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Correlation of Regulatory T Cells Percentage and Lung Tuberculosis in Children

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ABSTRACT

Introduction: The prevalence of TB in Indonesia reaches 1.59 new cases per 1000 population and 27.3% are pediatric patients. Currently in Indonesia, the incidence of TB in children is still high, even though the *Bacille Calmette Guerin* (BCG) vaccination has been a mandatory immunization program since 1977. The role of T-Regulatory Cell (Treg) in the Mtb infection is very complicated.
Method: This observational study using cross sectional analyzed quantity of Treg Cells in the circulation of pediatric TB and non-TB patients. Measurement of circulatory Treg cell percentage was performed using Flow Cytometry.
Result: We found that pediatric TB patients have significantly higher that were almost two-fold higher Treg cell percentage compared to non-TB patients (3.51% vs. 1.86%; $p < 0.001$). It is concluded that there is an increase in the percentage of Treg cells in children with pulmonary TB compared to children who didn't had TB infection.
Conclusion: This finding provides an understanding of the complex mechanism of Treg cells in children with pulmonary tuberculosis, so that it can become the basis for the development of management of pediatric pulmonary TB and reduce morbidity and mortality of children due to pulmonary TB.

Keywords: tuberculosis; children; T cells; T reg
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INTRODUCTION

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (MTB).[1] The prevalence of TB in Indonesia reaches 1.59 new cases per 1000 population and 27.3% are pediatric patients. Currently in Indonesia, the incidence of TB in children is still high, even though the *Bacille Calmette Guerin* (BCG) vaccination has been a mandatory immunization program since 1977.[2] Several factors that pose a risk of developing childhood pulmonary TB disease include exposure to positive adult TB contacts. children, weak immune system, low social and economic conditions, population density, malnutrition and environmental hygiene. In general, microscopic acid-fast bacili (AFB) staining and bacteria culture through sputum are considered as gold standard to diagnosis TB. But in some cases, especially in children, diagnosis often based on complaints and symptoms, chest X ray, tuberculin skin test, and contact with adult TB patients; commonly known as pediatric TB Scoring System. However, currently many serological markers are being developed from blood to urine.[3,4]

The main mechanism of transmission of pulmonary TB in children is through direct droplet mechanism. Because it is an intracellular pathogen, the body's main mechanism of immunity defense against *Mtb* infection is through the mechanism of cellular immunity (Th1). *Mycobacterium tuberculosis* germs have the ability to weaken the immune system by shifting the activation of immune cells from cellular, namely Th1 to Th2, namely humoral. This is due to the effect of stimulating the proliferation of Treg cells by the *Mtb* infection. Treg cells are one of the immune devices, most of which are subtypes of $CD4^+$T helper cells. Treg cells are suppressive against the cellular immune system.[5]

Despite the various controversies about Tregs, these immunological markers have been shown to have a higher number in Peripheral Blood Mononuclear Cells (PBMC) from pediatric pulmonary TB patients compared to children who didn't had TB infection, so modulation of them may still provide preventive and therapeutic benefits given the lack of research in this area. So that further research is needed to understand the mechanism of the Treg cell complex in children with pulmonary tuberculosis as the basis for developing the management of pediatric pulmonary TB, reducing morbidity and mortality rates for children due to pulmonary TB. This study wanted to find out whether there is a difference in the percentage of Treg cells in children with and without pulmonary TB also whether there is a relationship between them.

METHODS

Study Setting

The study was conducted in the Department of Pediatric, Saiful Anwar Hospital, Malang, Indonesia. All procedures had been reviewed and approved by The Health Research Ethics

Commission of Saiful Anwar Malang Hospital with Ethical Clearance Register Number: 400/174/K.3/302/2020. Before the blood samples being collected, informed consent had been distributed to the patient's parents.

Eligibility

Patients eligible to participate in this research aged 1-18 years old [6,7]. We distributed the participants into 2 groups i.e pediatric TB patients (TB^+) and pediatric non-TB patients (TB^-). Each group consisted of 29 participants [9]. Exclusion criteria is the subject did not suffer from other lung infections, HIV infection, immunodeficiency conditions, allergic disease, worm infections nor malnutrition. Blood samples were collected via phlebotomy from accessible vein in cubital fossa (Median cubital or cephalic vein) for 3 cc from each participant. Blood samples were collected in an EDTA vacutainer.

Plasma and PBMC Isolation

The blood samples were mixed with Phosphate Buffer Saline (PBS) in 1:1 volume ratio and was homogenized using vortex. The mixture of blood samples and PBS was then put into a centrifuge tube containing Lymphocyte Separation Medium (LSM) and centrifuged at 100 rpm for 30 minutes. After centrifugation, layers of erythrocytes, granulocytes (PMN), ficoll hypaque, buffy coat rings (lymphocytes and monocytes/Peripheral Blood Mononuclear Cells [PBMCs]), plasma, and PBS were formed.

Cell Staining and Flow Cytometric Analysis

The cell pellets were incubated with cell surface marking antibodies (Anti Human FITC CD4 [BioLegend® Catalog Number: 317408] and Anti Human PE / Cy.5 CD25 [BioLegend® Catalog Number: 302608]), then washed by adding 500 µl of solution. Cell staining buffer, centrifuged at 40° C at 2500 rpm for 3 min. The supernatant was discarded, while the formed pellets were washed with 500 µl of fixation buffer solution (Catalog Number: 420201 from BioLegend®) containing serum from bovine calf and sodium azide, homogenized and centrifuged at 40° C at 2500 rpm for 3 minutes. The supernatant was removed, the pellets were fixed by adding 500 µl of fixation buffer solution then incubated for 20 minutes in the dark at room temperature.

After the incubation was complete, the solution was centrifuged at 4° C at 2500 rpm for 3 min. The supernatant was discarded, the pellets formed were washed with a permeabilization wash buffer solution, centrifuged at 4° C at a speed of 2500 rpm for 3 min. The supernatant was removed, then the cell pellets were ready to be stained with intracellular antibody Anti human FoxP3 (BioLegend®, Catalog Number: 320208) which has been diluted with a permeability wash buffer solution with a certain ratio (FOXP3 Fix / Perm Buffer set, BioLegend® Catalog Number: 421403).

Each solution was stained with 50 µl of the diluted antibody, then incubated in the dark at room temperature for 20 minutes. After incubation, it was washed by adding 500 µl of permeabilization wash buffer solution and centrifuged at 4° C at 2500 rpm for 3 min. The supernatant was removed and added to the cell staining buffer solution and homogenized. Then, it was transferred to the cuvette and counted using a flow cytometer.

Data Analysis

Treg Cells Percentage were compared between groups. The date underwent Saphiro-Wilk's Test of Normality, Levene's Test of Homogeneity of Variance, and Independent t-Test. To evaluate association between two variables, Person Chi Square, Continuity Correction, and Fisher Exact Test were performed. All analysis was performed in SPSS Version 24.0 for Mac OS.

RESULTS

Characteristic of Research Subjects

As many as 58 children were recruited to the study, stratified in 5 age groups of comparable sizes and reflective of the reported age-dependent susceptibility to TB disease (group 1: <3 yr n=3, group 2: 3-<7 yr n= 16 , group 3: 7-<13 yr n=7, group 4: 13-<14 yr n=2, group 5: 14-18 yr n: 1). The highest number of TB pediatric patients was in the group 2; 3 to 7 years old, followed by 7 to 13 years old group, and 0 to 3 years old group. In the TB^+ group, there were only one sample in 14 to 17 years old group.

Malnutrition, as measured by anthropometry in which for children < 5 yr referred to WHO curve and > 5 yr according to CDC. Total undernourished children were 55%. All children underwent Acid Resistance Bacilli Test that indicated negative for TB. The characteristics of research subjects are shown in Table 1.

Table 1. Characteristics of Participants

Characteristics	TB^+ (n=29)	TB^- (n=29)
Gender (n)		
Male	15/29	16/29
Female	14/29	13/29
Age (Year) (mean ± SD)	5,93 ± 3,83	4,52 ± 4,13
0-<3 Year	3/29	8/29
3-<7 Year	16/29	12/29
7-<13 Year	7/29	6/29
13-<14 Year	2/29	2/29
14-18 Year	1/29	1/29
Nutritional status (n)		
Good nutrition	13/29	12/29
Malnutrition	16/29	17/29
Mantoux test (n)		
Positive	23/29	0/29
Negative	6/29	29/29
Unchecked	0/29	0/29
Acid Resistance Bacilli Test (n)		
Positive	0/29	0/29
Negative	29/29	0/29
Unchecked	0/29	29/29

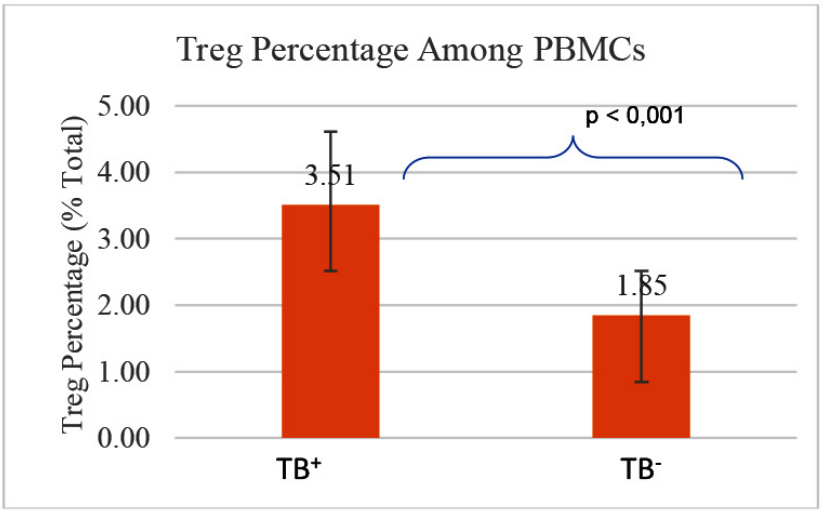

Figure 1. Comparison of Treg Percentage between TB and non-TB children. Mean Treg percentage was significantly higher in TB^+ patients compared to the TB^- patients ($p<0.001$, independent t-test).

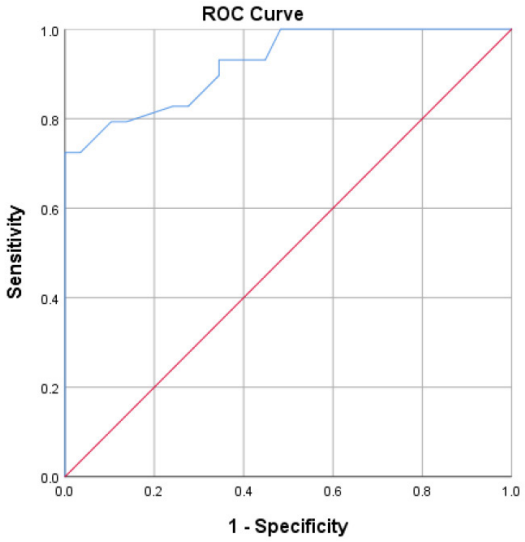

Figure 2. Receiver Operator Curve (ROC) of Treg percentage in Pediatric Pulmonary TB.

Elevated Treg Percentage in Pediatric Pulmonary TB

The Percentage of Tregs from PBMCs of TB^+ was significantly higher than the TB^-. Data on the percentage of Tregs are presented in Figure 1. The association between Treg percentage with incidence of Pediatric pulmonary TB also evaluated in the present study. Using representative plots in Figure 2, Treg cut off are chosen with optimal sensitivity and specificity. The cut off value for the percentage of Treg was 2.46%, with sensitivity 82% and specificity 72%. With association analysis, it can be concluded that there is a significant relationship between the incidence of pulmonary TB disease in children and an increase Treg percentage (p=0,000).

DISCUSSION

In this study, we examined the incidence of pediatric pulmonary TB associated with Treg cell percentage. Treg cell plays one of the pivotal roles for *Mycobacterium tuberculosis* to evading immune system. Treg cells are known for homeostasis and maintain the balance especially in Mtb infection. Although Treg cell protects lung tissue from destructive cellular immunity derived from Mtb invasion, but it also poses the risk of cellular immunity from failing to eliminate Mtb. This will lead an opportunity for Mtb to become a latent infection; while in childhood pulmonary TB disease to become severe until therapy failure.[8] We want to prove whether there is increasing of percentage of Treg cells in pediatric pulmonary TB disease, and whether there is relationship between them.

Based on the sex distribution, the prevalence of pulmonary TB disease in children was higher in boys (51.7%) than in girls (48.2%), differ only by one patient. This finding also supported by previous studies that shows prevalence and risk of pulmonary TB in children is not influenced by gender.[9,10] Every subject in this study also has negative AFB sputum smear. This is in accordance with the theory that pulmonary TB in children is pauci-bacillary which means the number of Mtb is low and likely undetectable. This often causes delays in diagnosing TB in children.[11] Hookworm manifestation are excluded from this study since it has been previously observed that there is increased Tregs percentage in patients with hookworm manifestation. Administration of deworming therapy such as albendazole to TB patients with worm coinfection has actually decreased the levels of Treg cells.[8] The majority (86.2%) of subject with pediatric pulmonary TB disease (+) were less than 10 years old. Another study conducted in Brazil performed between 145 children ranged between 1 and 18, also shows same result with 61.4% of pulmonary TB patients were children under 10 years old.[12] Meanwhile in Sudan, it's also reported that 74,9% of TB cases in children occurred under the age of 10.[9] This phenomenon could be explained by theory that said children who have been vaccinated with BCG undergoes a shifting of T cell composition. It is showed that naïve T cell will dominate, resulting memory T cells (CD27+CD45RA- and CD27-CD45RA+) outnumbered and not sufficient enough against TB.[7]

In this study, Treg cells were counted from PBMCs which often describes as profile of systemic immune cells. Beside of PBMCs, isolation of Treg cells can be obtained from various sites of Mtb infections, such as fluids from bronco-alveolar lavage (BAL), ascites, pericardial fluid, and pleural fluid. The expression of FOXP3 messenger ribonucleic acid (mRNA) and the frequency of CD4 + CD25HI T cells originating from local sites of Mtb infection were greater than those in the systemic circulation.[8]

Compared to control group, the mean percentage of Treg cells in pediatric pulmonary TB patients was significantly higher. This is in line with what Cardona *et al.* who stated that many previous studies

showed that there was an increase in the percentage of Treg cells in Mtb infection.[13] It has also been demonstrated that an increase in the percentage of Tregs is associated with the incidence of pediatric pulmonary TB. Treg can be induced by infection of Mtb, through switching the gene expression of macrophages. *Mycobacterium tuberculosis* polarize macrophage from M1 macrophages (classically activated macrophages) which play a role in cellular immunity and are bactericidal, into M2 macrophages which are a regulator of the immune system. M2 macrophages mediates tissue repair and immune escape of pathogens, resulting inhibition in the formation of tuberculous granuloma and decreased bactericidal activity.[14] Mtb *Mycobacterium tuberculosis* cell wall also contains lipoarabinomannan which further stimulate Treg cells to produce TGF-β that will induce Treg development by stimulating the expression of transcription factor FOXP3.[15]

The percentage of average Treg in the group of children with pulmonary TB in the present study was the highest at the age of 13-14 years. This is in line with the theory of immunity in children which states that cellular and hormonal immunity in adulthood is at the optimal response stage.[16] So that Mtb infection at this age will trigger an increase in the percentage of Treg cells in response to pulmonary TB infection.

However, Treg cells should not be used to predict the incidence of pulmonary TB because there is still lack of evidence about the consistency of the percentage of Treg cells at various ages. Although Treg cells act as immunomodulators against pulmonary TB infection and other disease in children, Treg cells percentage can also increase due to chronic exposure to environmental mycobacteria, BCG, worm infections, chronic infections, immunosuppressive drugs, malnutrition, atopic diseases and others.[17] In contrast, Xu *et al* (2016) reported the percentage of Treg cells in PBMC cultures was increased in patients who were negative acid-resistant bacteria in their sputum compared to the positive acid-resistant bacteria. However, their study did not compare sick patients with healthy controls, but they examined Treg percentages in the same subject when they were diagnosed with pulmonary tuberculosis and after the subject recovered with negative BTA sputum. In that condition, Treg response as post-inflammatory resolution due to Mtb infection, where Treg prevent excessive tissue destruction by suppressing Th17, IL-17, natural killer T like cell (NKT-cell like) and other various components of immune system.[18]

There are several limitations in this study. The cause-effect relationship cannot be distinguished whether pediatric pulmonary TB is the cause of the increased Treg cell percentage or vice versa. Several other factors that affect the percentage of Treg cells such as BCG immunization, previous exposure to environmental mycobacteria, parasitic infections, nutritional status and genetic polymorphisms were not evaluated in this study.[19,20] Further study with more specific Treg marker, secreted Treg cytokines, will provide more information about development and functional properties of Treg during pulmonary TB. The degree of severity of pulmonary TB also needs to determine association between Treg cell percentage and disease severity. Evaluating Treg percentage before and after TB therapy could be helpful to know the nature of Treg during TB infection.

CONCLUSION

It is concluded that there was an increase Treg cell percentage in pediatric pulmonary TB compared to children without TB. There was also an association between an increase in the percentage of Treg cell and incidence of pulmonary TB in children.

FUNDING

No funding was received.

CONFLICT OF INTEREST

The authors confirm that no conflict of interest exists with regard to this current work.

ETHICS CONSIDERATION

Ethics approval has been obtained from the Ethics Committee, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia prior to the study being conducted.

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