The Open Microbiology Journal

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RESEARCH ARTICLE

Higher Treg FoxP3 and TGF-β mRNA Expression in Type 2 Reaction ENL (Erythema Nodosum Leprosum) Patients in *Mycobacterium leprae* Infection

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Abstract:

Background and Aim:

The pathology of leprosy is determined by the host immune response to *Mycobacterium leprae*. Almost 40% of patients with leprosy undergo immune-mediated inflammatory episodes such as type 1 reactions and Erythema Nodosum Leprosum (ENL or type 2 reactions). Regulatory T (Treg) is a subset of T cells that are involved in the immune response. Treg cells express Forkhead Box P3 (FoxP3), which plays a role in suppressing the immune response. FoxP3 may work alongside Transforming Growth Factor Beta (TGF-β) to down-regulate T cells responses, leading to the antigen-specific anergy associated with leprosy, whereas ENL occurrs mostly in multibacillary leprosy patients. Based on that, the aim of our study was to analyze Treg FoxP3 and TGF-β mRNA expression in type 2 reactions ENL with *Mycobacterium leprae* infection.

Methods:

Forty-nine newly diagnosed multibacillary (MB) leprosy patients attending the Dermatovenereology Clinic of Leprosy Subdivision, Sanglah General Hospital, Denpasar, Indonesia, were included in the study. The study group consists of 25 leprosy patients with ENL and 24 non-ENL leprosy patients. Twenty-five patients were included in the study as healthy controls. In this study, Treg FoxP3 and TGF-β mRNA expressions were identified with the Real-time PCR method. Analysis of Variant (ANOVA), Chi-square test and odds ratio (OR) calculation were used; $p<0.05$ was considered statistically significant.

Results:

The result of this study showed that the mean of Treg FoxP3 mRNA expression was 13.3 ± 2.9 on ENL leprosy patients, 11.6 ± 4.1 on non-ENL, and 9.3 ± 1.2 on healthy controls. The mean of TGF-β mRNA expression was 11.7 ± 2.7 on ENL leprosy patients, 9.5 ± 3.6 on non-ENL, and 9.3 ± 1.2 in healthy patients. Statistical analysis for Treg FoxP3 and TGF-β mRNA level between ENL, non-ENL patients and healthy control group showed significance at $p<0.05$.

Conclusion:

From this study, it was concluded that higher Treg FoxP3 and TGF-β mRNA expressions were found in type 2 reaction ENL patients with *Mycobacterium leprae* infection. The role played by Treg FoxP3 and TGF-β in type 2 reaction episodes can possibly provide a new target for the treatment of this still-challenging complication of leprosy. Further studies are required to determine the involvement of other cytokines in type 2 reaction ENL patients.

Keywords: *Mycobacterium leprae*, ENL, Treg, FoxP3, TGF-β, Leprosy patients.

Article History	Received: June 18, 2020	Revised: October 4, 2020	Accepted: October 7, 2020

1. INTRODUCTION

Leprosy, also known as Hansen's disease, is a chronic infectious disease caused by *Mycobacterium leprae*. During the reporting year, 210,758 new leprosy cases were detected globally. The trends showed an overall gradual decline from 265,661 in 2006 to 210,758 in 2015. But marginal increases in new cases were observed in 2015 in the South and East Asian Region from 154,834 (in 2014) to 156,118 (in 2015). South and East Asian Region accounted for 74% of the global new caseload [1].

The majority of countries with high new case detection rates are located in the African Region and South and East Asian Region. Indonesia reported 17,202 new cases, 8% of the global caseload in 2015. The proportion of multibacillary (MB) cases indicates the presence of advanced cases of leprosy and, indirectly, the magnitude of the infection in the community. This proportion was 60.2% globally. MB proportion among new cases in 2015 was 84.6% in Indonesia [2].

The clinical presentation and pathology of leprosy depend on the patient's immune response to *M. leprae*. Multi-drug therapy is considered a highly effective treatment for the infection. However, 40% of patients with leprosy experienced immune-mediated inflammatory episodes, for instance, type 1 reactions and type 2 (Erythema Nodosum Leprosum) reactions. ENL is mainly present in patients with a high bacterial index, 50% occurs in lepromatous leprosy (LL) patients and 5-10% in borderline lepromatous (BL) patients [3].

Cumulative ENL incidence varied from 0.2% to 4.6%, with an average of 1.2%. Studies in Indonesia and India revealed the incidence of ENL among MB cases between 1.0% and 8.9%, with an average of 4.5% [4]. Individuals with ENL are seen in pain, having erythematous nodules of skin, fever and systemic malaise. Immune responses causing ENL are triggered by high loads of fragmented bacilli in skin tissue. The pathophysiology of ENL has not been fully understood, but immune complexes and cellular immune mechanisms thought to be involved [5, 6].

In addition to the conventional CD4 subsets of Th1 and Th2 cells, additional subsets of Th17 and Regulatory T (Treg) cells were discovered initially in models of autoimmunity followed subsequently in various human diseases, including infectious diseases caused by mycobacteria. CD4 subsets of Th17 and Treg cells have been shown to play a major role in disease-associated immunopathology in leprosy [7].

Treg is a subset of T cells that are involved in suppressing the immune response, typically to inhibit excessive immune reactions. Treg cells have a suppressor/inhibitory role and regulate inflammation and maintain tolerance. Treg cells express Forkhead Box P3 (FoxP3), which plays a role in suppressing the immune response. FoxP3 may work alongside Transforming Growth Factor Beta (TGF-β) to down-regulate T cells responses, leading to the antigen-specific anergy associated with LL, whereas ENL or type 2 reaction occurs mostly in LL patients [8]. Based on that, the aim of our study was to analyze Treg FoxP3 and TGF-β mRNA expressions in type 2 reactions ENL with *Mycobacterium leprae* infection.

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2. MATERIALS AND METHODS

2.1. Patients

Forty-nine diagnosed multibacillary (MB) leprosy patients attending the Dermatovenereology Clinic of Leprosy Subdivision, Sanglah General Hospital, Denpasar, Bali, Indonesia were included in the study. Leprosy type was determined on the basis of WHO classification and bacterial index. The study group included 25 leprosy patients with ENL (erythema nodusum leprosum) as type 2 leprosy reaction and 24 non-ENL leprosy patients. Twenty-five patients were included in the study as healthy controls. All ENL and non-ENL patients received WHO standard regimen. Exclusion criteria included clinical evidence of other infections such as tuberculosis. In this study, levels of Treg FoxP3 and TGF-β mRNA were identified with the Real-time PCR method.

2.2. Nucleic Acid Isolation

Nucleic acid was extracted from blood according to the method described by Hatta *et al*. using GuSCN (guanidiniumisothiocyanate) [9]. Whole blood was mixed with 900 µl of lysis buffer L6 (5.25M GuSCN, 50 mM Tris-HCl, 20 mM EDTA, 0.1% Triton X100), vortexed, and centrifuged at 1,000 rpm for 5 min. Samples were lysed by incubation at 18°C for 15 minutes and 20µl of diatom suspension was added. The suspension was centrifuged at 12,000 x g for 15 seconds and then washed with washing buffer L2 (5.25M GuSCN in 0.1M Tris-HCl, pH 6.4). The suspension was rinsed with 70% ethanol and acetone, and dried by incubation at 56°C for 10 minutes. The suspension was mixed with 60 µl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer and incubated at 56°C for 10 minutes. After sedimentation by centrifugation, the RNA was collected. RNA was reverse transcribed into cDNA using reverse transcriptase according to the manufacturer's instructions and stored at -20°C until Real-time PCR was performed.

2.3. Real-time PCR Assay

Real-time assay targeting the FoxP3 and TGF-β gene was used. In brief, 25µl of reaction mixtures containing 5µl extracted total nucleic acids and 0.5µM of each primer was mixed with 25µl Brilliant II SYBR®. Relative quantitative real-time PCR was performed using Brilliant II SYBR® following the manufacturer's instructions. The primers for FoxP3, TGF-β, and GAPDH were synthesized by Macrogen, Seoul, South Korea. The primers are Treg: F: 5′-GGC ACT CCT CCA GGA CAG-3′ and R: 5′-GCT GAT CAT GGC TGG GCT CT-3′ [10]; TGF-β: F: 5′-CCA ACT ATT GCT TCA GCT CCA-3′ and R: 5′-TTA TGC TGG TTG TAC AGG-3′ [11]; and GAPDH: F: 5′-GAA GGT GAA GGT CGG AGT-3′ and R: 5′-GAA GAT GGT GAT GGG ATT TC-3′ as housekeeping genes [12].

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. All PCR assays were performed in triplicate, and data were analyzed with the PCR results using Bio-Rad CFX Manager 3.1 software (Biorad, USA). Instrument detection system using the comparative threshold cycle method was used as previously described by Yajima *et al*. [12]. Standard curves were generated and indicated excellent amplification efficiency (90–100%) [13].

2.4. Statistical Analysis

Analysis of variant (ANOVA), Chi-square test and odds ratio (OR) calculation were used; $p<0.05$ was considered statistically significant. SPSS ver. 16.0 was used for statistical analysis.

3. RESULTS

Twenty-five multibacillary leprosy patients with an ENL type 2 reaction were included as a case group. Another 25 multibacillary leprosy patients without ENL were included as the control group, but one patient was dropped off because of the elevated liver function test. Twenty-five healthy persons were included as healthy control. The characteristics of leprosy and healthy control patients are given in Table **1**.

Table 1. Characteristics of ENL, non-ENL, and healthy control patients.

Characteristics	Cases ENL Patients (n = 25)	Controls Non-ENL Patients (n = 24)	Healthy Controls (n =25)
Age (y.o) (mean ± SD)	35±12	35±12	34±12
Sex (n) Male Female	19 6	19 5	19 6
Leprosy type BL LL	21 4	24 0	NA NA
Bacterial Index + 1 + 2 + 3 + 4 + 5 Morphological Index	0 2 8 13 2 6.4±19.6	4 9 5 6 0 17.3±29.6	NA NA

The mean of Treg FoxP3 mRNA expression in ENL patients was 13.3 ± 2.9, whereas in non-ENL patients was 11.6 ± 4.1 and in healthy controls was 9.3 ± 1.2. The mean of TGF-β mRNA expression in ENL patients was 11.7 ± 2.7, whereas in non-ENL patients was 9.5 ± 3.6 and in healthy controls was 9.3 ± 1.2. ANOVA analysis for Treg FoxP3 and TGF-βmRNA level between ENL, non-ENL patients and healthy control group showed significance at $p<0.05$.

Treg FoxP3 mRNA expression between ENL and non-ENL patients group, by using cut off point at 25th percentile, showed OR = 3.00 (95% CI 0.24 – 157.49; p = 0.310); between ENL patients and healthy controls group, by using cut off point at 25th percentile, showed OR = 6.50 (95% CI 1.47 – 59.32; p = 0.004). TGF-β mRNA expression between ENL and non-ENL patients group, by using cut off point at median, showed OR = 2.67 (95% CI 0.64 – 15.60; p = 0.110); between ENL patients and healthy controls group, by using cut off point at median, showed and OR = 5.5 (95% CI 1.20 – 51.06; p = 0.011), as shown in Table **2**.

Table 2. mRNA expression (Treg FoxP3 and TGF-β) of ENL, non-ENL, and healthy controls patients.

mRNA Expression (fold change)	Cases ENL Patients	Controls Non-ENL Patients	Healthy Controls	Odds Ratio
Treg FoxP3	13.3±2.9[a]	11.6±4.1[a]	9.3±1.3[a]	OR 3.00 (CI 0.24-157.49); p 0.310[b]
				OR 6.50 (CI 1.47-59.32); p 0.004[c]
TGF-β	11.7±2.7[d]	9.5±3.6[d]	9.3±1.2[d]	OR 2.67 (CI 0.64-15.60); p 0.110[e]
				OR 5.5 (CI 1.20-51.06); p 0.011[f]

(a) $p<0.05$; (b) cut off point at 25th percentile, case *vs* control; (c) cut off point at 25th percentile, case *vs* healthy control; (d) $p<0.05$; (e) cut off point at median, case *vs* control; (f) cut off point at median, case *vs* healthy control.

4. DISCUSSION

The result of this study showed the mean age of the ENL patients group (35 ± 12 years old) has no differences from the non-ENL patients group (35 ± 12 years old) or healthy control group (34 ± 12 years old). Age is apparently not a risk factor for ENL, although a cohort study suggested an increased prevalence of ENL incidence was seen in patients diagnosed with leprosy in their adolescence, and a lower risk for those older than 40 (adjusted OR = 0.69, CI 0.5 – 0.94), but further researches are needed to support these findings [4].

The result of this study showed more male patients (76%) were found than female patients. Commonly, gender is not a risk factor for ENL. Some studies appear to challenge this, as a large hospital study in India found a male predominance, and a large Indian cohort reported a higher risk for women. These differences, however, may be due to differences in health-seeking behavior [14].

Leprosy displays a clinical spectrum that is determined by the host immunological response against *M. leprae*. Two types of spontaneous acute inflammatory phenomena are known to occur in the complex evolution of leprosy. Reactions are classified into two main types: type 1 reaction, also commonly known as reversal reaction (RR), and type 2 reaction, commonly known as Erythema Nodosum Leprosum (ENL) [15]. Type 2 reaction occurs more often in the patients around the LL pole of the leprosy spectrum with a heavy load of bacilli. High bacterial load and diffuse infiltration in skin lesions are regarded as important risk factors. Type 2 reaction is characterized by painful and tender red papules or nodules on the skin, the typical signs of erythema nodosum, accompanied by systemic symptoms including fever, joint

pain, edema, proteinuria and malaise [16].

Leprosy is characterized histologically by a spectrum of different granulomatous skin lesions, reflecting patients' immune responses to *M. leprae*. Treg represents up to 10% of CD4(+) T cells found in the peripheral blood of normal human beings. Although no single marker uniquely identifies this suppressor subpopulation, Treg is defined as CD25 (interleukin-2 [IL-2] receptor α-chain)-expressing CD4(+) T cells, which express the transcription factor forkhead box protein P3 (FoxP3), a master regulator for the development and function of Treg [17].

The direct role of FoxP3 is a transcription factor known as CD4(+)CD25(+)FoxP3(+) Treg to suppress the immune system. Although they are pivotal in the regulation of Treg in leprosy, its reactional states have been investigated [18]. These findings may explain the Treg, as measured by CD4(+)CD25(+)FoxP3(+) functions, which occur throughout the leprosy spectrum [19].

Treg is critically involved in balancing the reactivity of the immune system and preventing autoimmunity. Parallel to its role in preventing autoimmune reactions, Treg has been shown to control excessive inflammatory responses against pathogens. However, strict control of T effector cell responses by Treg may favor infection and promote pathogen persistence [17]. Treg appears to control *M. leprae* major infections by modulating the effector immune response via TGF-β and immunosuppression. The development and maintenance of CD4(+)CD25(+)FoxP3(+) Treg cells depend on TGF-β. CD4(+)CD25(+)FoxP3(+) Treg transcription factor induced by TGF-β in the nucleus has been shown to negatively regulate immune responses [20].

The result of this study showed higher Treg FoxP3 and TGF-β mRNA expressions found in type 2 reaction ENL patients, compared to non-ENL leprosy and healthy control group. This result was similar to the study that showed a significantly lower frequency of Treg but the higher FoxP3 expression in ENL. Using flow cytometry in ENL, the absolute numbers and proportion of Treg were shown to be significantly lower during ENL, although FoxP3 expression, a marker used to define Treg, was higher [21]. ENL is characterized by deficient Treg and increased Treg FoxP3 expression. It is known that there is an association between leprosy and overproduction of TGF-β, which were identified significantly higher in patients compared to healthy controls ($p<0.001$) [18].

Interestingly, an increase of FoxP3 mRNA expression by peripheral blood mononuclear cells in ENL patients compared to LL controls has also been reported. CD4(+)FoxP3(+) Treg cells producing TGF-β were increased in stable lepromatous patients, which may explain the anergy associated with this leprosy type [7]. Another study showed adherent to ENL secretes higher TGF-β1. FoxP3 expression in Treg has also been linked to the *M. leprae* proliferation seen in patients with LL, who are found to have higher frequencies of Treg, TGF-β, and FoxP3 expression linked. Skin lesions and antigen-stimulated peripheral blood mononuclear cells showed increased gene expression of TGF-β in LL [8].

The different result came from the study that showed lower CD4(+)CD25(+)FoxP3(+) Treg levels in ENL. Lower Treg was also shown in ENL patients [22]. Another study showed a markedly lower number of Treg in ENL patients than non-ENL and controls. This decrease was paralleled by decreased TGF-β expression. Biopsies from ENL and non-ENL patients before the reaction episodes showed a similar number of FoxP3 cells. However, in biopsies taken during the reaction, ENL patients showed a decrease in Treg, whereas non-ENL patients showed the opposite: Treg increased. Decreased expansion of Treg was found upon in vitro stimulation with *M. leprae*. Lower expression of FoxP3 Treg was also found in ENL patients [23].

The different result also came from the study that shows no statistical difference in FoxP3 expression between TT, BT, BL, and LL [24]. No difference of TGF-β in ENL and non-ENL patients is also shown in another study [25]. Another study showed no statistically significant serum TGF-β difference between ENL, non-ENL and healthy control groups [18]. TGF-β mRNA expression was not increased in ENL patients in this study [7]. The conflicting results for FoxP3 could be due to the fact that FoxP3 mRNA may not be translated to functional FoxP3. The variability in FoxP3 and TGF-β expression may be due to whole peripheral blood mononuclear cells included in these studies.

Although ENL and non-ENL are perceived as being triggered by different immunopathogenic mechanisms, it is clear that both result from exacerbation of an ongoing immune response. The development of ENL is associated with a sharp reduction in both in situ and circulating Treg [21]. ENL develops in leprosy patients concomitantly with a reduction in the Treg frequency in peripheral blood and in situ. The high TGF-β expression would explain the downmodulation of Treg. TGF-β is known to have an important role in the maintenance of Treg [18].

In addition, we did not know the exact source of elevated serum TGF-β reported in leprosy (from Th3 or other cells), and whether it is a cause or a result within the disease process [18]. While FoxP3 expressing Treg producing TGF-β is increased in stable lepromatous leprosy patients, patients with reactions exhibit an imbalance in Treg populations. These data suggest that Treg and TGF-β may exert control on the inflammatory response during leprosy reactions [15].

CONCLUSION

From this study, it can be concluded that higher Treg FoxP3 and TGF-β mRNA expressions were found in type 2 reaction ENL patients compared to non-ENL and healthy control groups. The role played by Treg FoxP3 and TGF-β in type 2 reaction episodes can possibly provide a new target for the treatment of this still-challenging complication of leprosy. Furthermore, in vivo and in vitro studies on larger populations are recommended for a better understanding of this expression in leprosy.

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of Variance
BB	=	Borderline Borderline Leprosy
BL	=	Borderline Lepromatous Leprosy

BT	=	Borderline Tuberculoid Leprosy
CD4	=	Cluster of Differentiation 4
CD8	=	Cluster of Differentiation 8
CD25	=	Cluster of Differentiation 25
EDTA	=	Ethylenediaminetetraacetic Acid
ENL	=	Erythema Nodosum Leprosum
FoxP3	=	Forkhead Box P3
GAPDH	=	Glyceraldehyde 3-Phosphate Dehydrogenase
GuSCN	=	Guanidiumisothiocyanate
IL-2	=	Interleukin-2
LL	=	Lepromatous Leprosy
MB	=	Multibacillary
M. leprae	=	Mycobacterium Leprae
mRNA	=	Messenger Ribonucleic Acid
PCR	=	Polymerase Chain Reaction
RR	=	Reversal Reaction
SPSS	=	Statistical Package for Social Sciences
TGF-β	=	Transfoming Growth Factor Beta
Th1	=	T Helper 1
Th2	=	T Helper 2
Th17	=	T Helper 17
Treg	=	Regulatory T Cell
TT	=	Tuberculoid Leprosy

AUTHORS' CONTRIBUTIONS

MH, LMR and SP initiated and designed the study. MH, LMR, and YY drafted the manuscript. RD, MH, RAN and ARJ supervised the molecular biology experimental work. MS, RD, and YY carried out the real-time PCR and serological experiment. MH, YY, IGW, MDA, MW, and SP, MRP performed the statistical analysis. All the authors have read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics approval was issued by the ethics committee of Unit Penelitian dan Pengembangan (LITBANG) Faculty of Medicine, Universitas Udayana Denpasar, Indonesia No: 249/UN.14.2/Libang/2013.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All human research procedures were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was obtained from all participants.

AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study are available from the corresponding author, [M.H.], on reasonable request.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

We express our gratitude to the heads of the offices of the Ministry of Health, Republic of Indonesia, in Bali and to the leprosy officers in the Ministry of Health and in the provinces of Bali for their cooperation. We are obliged to the Head and staff of Sanglah General Hospital, Denpasar, Bali, Indonesia, for their valuable advice. We thank Mr. Romi and Mr. Marwani for excellent technical assistance. The voluntary cooperation of the leaders of the Bali villages enabled this study. We are especially indebted to all the patients and their families who cooperated voluntarily in this study.

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