Biomedical & Pharmacology Journal, March 2026. Vol. **19**(1), p. 545-550

Correlation Between Regulator T Cell Levels and Acetylcholine Receptor Antibody Levels and Clinical in Patients with Myasthenia Gravis

Elta-Diah Pasmanasari*, William Tanyawan, Dodik-Tugasworo Pramukarso, Retnaningsih Retnaningsih, Jimmy-Eko-Budi Hartono and Suryadi Suryadi

Department of Neurology, Dr. Kariadi Hospital/Medical Faculty of Universitas Diponegoro, Semarang, Indonesia.
*Corresponding Author E-mail: eltadiah@gmail.com

https://dx.doi.org/10.13005/bpj/3373

(Received: 10 November 2025; accepted: 16 December 2025)

Myasthenia Gravis (MG) is an autoimmune disease characterized by muscle weakness due to disorders of the neuromuscular junction. Most patients with Myasthenia gravis have autoantibodies against acetylcholine receptors (AChR). Regulatory T cells (Tregs) are thought to be involved in the pathophysiology of MG, where they influence the maturation and proliferation of B cells (both in the thymus and peripherally). This showed that Tregs play a significant role in the production of autoantibodies in MG disease. The study aims to analyze the correlation among Treg levels, AChR levels, and clinical severity in patients with myasthenia gravis. This is a descriptive analytical study with a cross-sectional design. Outpatients with Myasthenia Gravis in Dr. Kariadi General Hospital in Semarang who met the inclusion and exclusion criteria had their blood drawn and demographic data collected through the electronic medical record. The Treg level was measured by flow cytometry, and the antibody level was measured by ELISA. The correlation between Treg and AChR levels was analysed using the Spearman test, and the association between Treg levels and clinical severity was analysed using the Mann-Whitney test. The research results show a significant, weakly correlated inverse relationship between Treg cell levels and AChR antibodies. (p = 0.049, r = -0.406) and has no significant association with MGFA-PIS score (p = 0,427)

Keywords: Antibody AChR; Autoimmune Disease; Myasthenia Gravis; Neuromuscular Junction; T-regulatory Cell.

Myasthenia Gravis (MG) is an autoimmune disease characterized by muscle weakness due to disorders of the neuromuscular junction (NMJ).[1] Most patients with MG have autoantibodies against acetylcholine receptors (AChR) and a small proportion are seropositive for antibodies to Muscle-specific kinase (MuSK), low-density lipoprotein receptor-related protein 4 (LRp4), or agrin. Regulatory T cells (Treg) are thought to be involved in the pathophysiology of MG, where Treg affect the maturation and proliferation of B cells (both in the thymus and peripherally).[2-3]

Treg cells express FOXP3, release inhibitory cytokines, such as transforming growth factor (TGF-â) and IL-10, and suppress the immune response by inhibiting the function of effector T cells and other antigen-presenting cells. In MG, Treg cell numbers have also been reported to be lower than in normal persons, as the controls, especially in patients with thymoma-

associated MG or those without immunotherapy. Furthermore, the number of CD4+CD25+FOXP3+ Treg cells decreases during the active phase of anti-AChR antibody-positive MG and increases after immunotherapy, and the degree of Treg cell change correlates with the QMG score.[4-6] The proportion of Treg cells was significantly lower in the generalized MG group with anti-AChR antibody-positive MG without thymoma compared to ocular MG and the control group. Treg cells are thought to be involved in the clinical course of MG (including MG patients with thymoma).[7-8]

The thymus plays a crucial role in the pathogenesis of AChR-positive MG. Negative selection of T cells occurs in the thymus. In patients with thymic hyperplasia/thymoma, this selection process is disrupted, contributing to the release of autoreactive CD4+ and CD8+ cells and the activation of peripheral autoreactive B cells, which then produce autoantibodies to the acetylcholine receptor in myasthenia gravis. Therefore, Treg cells play a significant role in the production of autoantibodies in MG.[6,8]

This study aims to analyze the correlation between Treg levels and AChR antibody levels in the serum of myasthenia gravis patients to identify a potential Treg-based therapy for MG.

MATERIALS AND METHODS

This study is a descriptive-analytical, cross-sectional design . Sample of MG patients treated at the outpatient clinic of Dr. Kariadi General Hospital, Semarang. Ethical approval for this study was obtained from the Institutional Research Ethics Committee, and all procedures followed the principles of research ethics. This study was conducted between January 2024 and March 2024 at the Neurology Outpatient Clinic at Dr. Kariadi General Hospital, Semarang. Data for patients who met the inclusion and exclusion criteria were collected from the electronic medical

Table 1. Correlation of Tregs and CD4CD25 to AChR antibodies

Variable	Mean (SD)	AChR-Ab	
		P	*r*
Treg	12,92 ± 14,04	0.049*	-0.406
$CD4^+$	2971,46 ± 2585,53	0.016*	0.488
$CD25^+$	193,58 ± 188,55	0.041*	0.419

*Spearman test

Table 2. Characteristics of the data on Treg cell and AChR antibody levels

Variable			*p*	
			Treg	AChR-Ab
Gender, n (%)	Frequency	Mean (SD)	0.696*	0.570*
Man	5 (20.8)			
Woman	19 (79.2)			
Age, year, mean ± SD		49.79 (± 12.62)	0.571#	0.265#
Onset, year, mean ± SD		5.09 (± 4.60)	0.550#	0.196#
MGFA-PIS/MG Composite Score, n (%)				
MM-2	12 (50)		0.427**	0.938**
MM-3	11 (45.8)			
CSR	1 (4,2)			
Intervention history, n (%)				
Thymectomy	6 (25)		0.257*	0.505*
Plasmapheresis	10 (41.7)		0.619*	0.412*
Immunoglobulin	2 (8.3)		0.531*	0.531*
Steroid	12 (50)		0.686*	0.273*

*independent t-test #Spearman **Man-Whitney MM-2: The patient has received only low-dose cholinesterase inhibitors (<120 mg pyridostigmine/day) for at least 1 year.MM-3: The patient has received cholinesterase inhibitors or other symptomatic therapy and some form of immunosuppression during the past yearCSR: The patient has had no symptoms or signs of MG for at least 1 year and has received no therapy for MG during that time. There is no weakness of any muscle on careful examination by someone skilled in the evaluation of neuromuscular disease. Isolated weakness of eyelid closure is accepted.

record. The inclusion criteria consist of MG patients who have been diagnosed by meeting 2 of the three criteria (clinical, laboratory and/or electrophysiological), Adult patients aged>18 years, and patients/families agree to be research participants by signing the Informed consent and the exclusion criteria consist of patients with a history of infection in the last 2 weeks, patients with comorbidities (infection for the previous 2 weeks, other neurologic disorder and other autoimmune diseases). We tried to control for other comorbid factors that may influence AChR levels in serum using those exclusion criteria. Blood samples were taken to measure Treg levels and AChR antibody levels. Treg cell levels were measured by flow cytometry, and AchR antibody levels were measured by ELISA. Twenty-four patients met the criteria and had their blood drawn.

Data were analyzed using SPSS version 26, with a significance level set at $p < 0.05$. The Spearman test was used to analyse correlations among Treg levels and AChR levels, age, and disease onset. The Mann-Whitney test was used to assess the association between Treg levels and disease severity (MGFA-PIS). The independent t-test was used to analyse the association between Treg level and gender and intervention history.

RESULTS

There were 24 patients, including 19 females and nine males. The study subjects were assessed for their characteristics and their effects on Treg cell levels and AChR antibodies. The results for $CD4^+$, $CD25^+$ cells, Treg cells, and their impact on AChR antibodies showed a negative correlation between Treg cell levels and AChR antibody levels, meaning that higher Treg cell levels were associated with lower AChR antibody levels (Table 1). The study did not show any effect of confounding variables (sample characteristics) on Treg cell levels or AChR antibodies (Table 2). In assessing the impact of Treg cell levels and AChR antibodies on the patient's clinical condition (MGFA-PIS/MG Composite Score), no effect was observed (Table 3).

Table 3. Correlation between Treg and AChR antibody levels and patient clinical outcomes (MGFA-PIS/MG Composite Score)

Variable	MGFA-PIS/MG Composite Score *p*
Treg	0.427*
AChR Antibody	0.938*

*Spearman test

DISCUSSION

This study found a significant inverse correlation between Treg and AChR antibody levels, indicating that Treg cells affect antibody levels. Treg were measured with flow cytometry, which showed $CD4^+CD25^+CD127^-$. Interestingly, the statistical analysis showed that the correlation between Treg levels and $CD4^+$ or $CD25^+$ alone was positive. (Table 1) Both $CD4^+$ and $CD25^+$ play essential roles in the self-tolerant immune system, and the removal of either leads to an autoimmune response. Removing $CD4^+$ cells may reduce $CD25^+$ expression, while $CD127^+$ expression was down-regulated in the $CD4^+$ T cell subset.[9,10]

Treg cells act as regulators in the immune system, controlling the production of many cytokines. The regulatory role involves regulating B-cell activity. The fact that antibodies are produced by B cells suggests that Treg cell levels can indirectly affect antibody levels. Interleukin changes in MG may be directly influenced by regulatory T cell defects, as T cells can influence many immune cells. The difference observed compared with previous studies may be due to our research not separating patients with a history of thymectomy and steroid use, which may also affect Treg cell levels and AChR antibody levels in the study subjects. In this study, we also found a significant correlation between $CD4^+$ and $CD25^+$ levels and AChR antibody levels, with a moderate strength of relationship, indicating that antibody levels were influenced by $CD4^+$ and $CD25^+$ cell levels, with a positive relationship: higher $CD4^+$ and $CD25^+$ cells were associated with higher antibody levels. As we know, in the calculation of Treg, which is a comparison of $CD25^+$ levels with $CD4^+$ so, Treg levels are greatly influenced by $CD4^+$ and $CD25^+$ levels, where in the previous theory, $CD4^+$ T cells in MG patients with positive AChR antibodies play an essential role in its

pathogenesis, helping B lymphocytes to produce AChR antibodies. The increased percentage of $CD25^+$ and $CD4^+$ cells in these patients is also consistent with a study by Balandina et al[11] showing an increase in $CD25^+$ cells of 20% vs. 10% in controls and in five thymus glands showing that $CD25^+$ expression and $CD4^+$ cells were increased in patients with positive titers of AChR antibodies, from 12% to 35% of $CD4^+$ cells, 10 to 16% of $CD25^+$ cells compared to controls.[11]

The study found no significant association between age, onset, gender, and history of therapy (Thymectomy, Plasmapheresis, Immunoglobulin, and Steroids) and Treg cell levels and AChR antibody levels in patients. This finding is in accordance with previous studies, which show that, although physiologically age-related changes occur — namely, decreased thymopoiesis and peripheral differentiation of Tregs during aging — some data indicate an accumulation of Tregs in secondary lymphoid organs in both mice and humans. This is associated with increased survival of old Tregs compared to young Tregs, due to the loss of selective expression of proapoptotic proteins. In addition to these quantitative changes, numerous studies have evaluated the immunosuppressive function of Tregs during aging. However, the results are often inconsistent and contradictory. Current data on quantitative and qualitative changes in Tregs related to age do not fully explain the increased risk of autoimmunity, cancer, and infection simultaneously in the elderly. The number of Tregs plays a determining role in regulating the entire immune system, as too many can trigger excessive immune suppression, while too few can trigger an autoimmune response.[12]

The current study found no significant relationship between thymectomy and AChR Antibody levels. This finding is also consistent with that reported in the study by Wang et al,[13] which found no significant difference in AChR Antibody levels either before thymectomy or 1 week after the procedure.[13] In previous studies, thymectomy was associated with improved clinical symptoms in MG patients after 3 years compared with those who did not undergo thymectomy.[14] In contrast Nabe et al[15] found that there is a decreasing of AchR antibody level after thymectomy in patients with thymoma without myasthenia gravis.[15] A previous study showed that 21% of patients who underwent thymectomy achieved complete remission, while 76% experienced significant improvement, as evidenced by a decrease in MGFA grade.[14] Another study showed that MG patients who underwent thymectomy experienced complete remission in 21% of patients and thymectomy was one of the strongest predictors for the prognosis of MG patients.[16] Studies from previous studies showed that there was a significant difference in Treg levels between MG patients who did not undergo thymectomy and patients who were not given immunosuppressants compared to Treg levels in patients who had a history of thymectomy and received immunosuppressants. From these studies, it can be seen that thymectomy may reduce and inhibit the inflammatory process and lymphocyte proliferation, thereby reducing damage and improving clinical symptoms, even though the overall antibody levels remain high.[13,14,15] The finding of a positive association between the scoring MGFA-PIS and Treg levels in these patients is also in line with research by Xu WH et al[6] and Masuda M et al[8], which showed higher levels of Treg cells in patients with better quality of life.[6,8]

Moreover, this study did not find a significant association between plasmapheresis and AChR antibody levels. The findings by Wang et al[17] found a decrease in AChR antibody levels after plasmapheresis but no significant relationship was found.[17] The findings in our study, where there was no relationship between steroid therapy and AChR antibody levels, are in accordance with the findings obtained in previous studies where MG patients with positive AChR antibodies who were given immunosuppressant therapy in the form of steroids, non-steroids, or combinations did not show any difference between AChR antibody seropositive and seronegative. The administration of immunosuppressants is not intended to reduce the quantitative levels of AChR antibodies in the body/blood, but rather to target a specific target. Steroids work by reducing cytokine production, thereby reducing the inflammatory process triggered by AChR antibody binding and minimizing damage to the postsynaptic membrane.[17] Azathioprine works by blocking nucleotide synthesis and T lymphocyte proliferation, while mycophenolate mofetil selectively inhibits T and B lymphocyte proliferation. Looking at the mechanism of action, immunosuppressants do not reduce the

number of antibodies in the body, but reduce and inhibit the inflammatory process and lymphocyte proliferation, so that the damage that occurs can be reduced, and this will affect improving clinical symptoms, even though the quantitative number of antibodies in the body is still high. Another study showed that repeated assessment of AChR antibody levels can help predict clinical status in patients undergoing immunosuppressive therapy.[18,19]

In the evaluation of Treg cell levels after immunotherapy, previous studies showed that Treg cells expressing FOXP3 play a role in the release of inhibitory cytokines (such as transforming growth factor [TGF-â] and IL-10), and are responsible for suppressing the immune response by inhibiting the function of effector T cells and other antigen-presenting cells.[4,6] In MG patients themselves, the number of Treg cells has also been reported to be lower than in standard controls, especially in patients with thymoma-related MG or those who did not undergo immunotherapy, in addition, the number of $CD4^+CD25^+FOXP3^+$ Treg cells decreased during the active phase of anti-AChR antibody-positive MG and increased after immunotherapy, and the degree of Treg cell change correlated with the QMG-13 score. Another study showed that the MG model, in which mice were injected with plasma from MG patients with low Treg levels, resulted in significant muscle weakness, which correlated with Treg levels in clinical MG. The different findings in our study may be due to Treg cell and AChR antibody levels being measured only once, after interventions with varying time intervals, and to the lack of data on these levels before therapy.[20,21,22]

A limitation of this study is that we cannot control for confounding variables, such as different medications and relapse frequency, that may influence the level of AchR antibody in patients' serum.

CONCLUSION

There is a significant correlation between Treg cell levels and AChR (acetylcholine receptor) antibody levels in patients with myasthenia gravis (MG). Age, disease onset, gender, clinical condition, and prior therapy (thymectomy, plasmapheresis, immunoglobulin, and steroids) do not show a significant correlation with Treg cell levels or AChR antibodies in MG patients in this study, which may differ from previous studies.[12-15]

ACKNOWLEDGEMENT

The authors would like to thank the Faculty of Medicine, Diponegoro University, the laboratory team, and Yayasan Myasthenia Gravis Indonesia (YMGI) for their support and contribution to this research.

Funding source

The author(s) received no financial support for the research, authorship, and/or publication of this article

Conflict of interest

The author(s) do not have any conflict of interest.

Data availability statement

This statement does not apply to this article.

Ethics statement

Ethical approval was granted by the Health Research Ethics Committee No. 194/EC/KEPK/FK-UNDIP/V/2024, Faculty of Medicine, Diponegoro University.

Informed consent statement

Participants in this study have been informed that their blood will be drawn and that their data will be collected through the electronic medical record, and have completed the hospital's informed consent form.

Clinical trial registration

This research does not involve any clinical trials

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Not Applicable.

Author contributions

ED Pasmanasari: Idea of the study, data collection, manuscript writing, and data analysis; W Tanyawan: Data collection and manuscript writing; DT Pramukarso: Editor and reviewer in data analysis; R Retnaningsing: Idea of the study and reviewer of the manuscript draft; JEB Hartono: Reviewer of manuscript draft; S Suryadi: Reviewer of manuscript draft

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