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(RESEARCH ARTICLE)

The Role of Regulatory T Cell Dysregulation in the Development of Autoimmune Diseases

Eman Abbas Aboud [1] and Israa Khudhair Obayes [2,*]

[1] *Department of Microbiology, Collage of Dentistry, University of Babylon, Hilla, 51002, Iraq.*
[2] *Department of Diseases and Forensic Medicine, Hammurabi College of Medicine, University of Babylon, Hilla, 51002, Iraq.*

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Abstract

Background: The regulatory T cells (Tregs), especially theCD4$^+$ CD25^hi CD127^lo FOXP3$^+$ Tregs subpopulation is principal in the process of ensuring the immune toleration and prevention of autoimmune diseases. Their impaired frequency, stability of phenotype, or inability to suppress has increasingly been argued to contribute to the pathogenesis of autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS). Nevertheless, a comparative study of Treg changes in the group of diseases is not comprehensive.

Objective: This analysis was supposed to compare the rate, performance, cytokine release, and transcriptional response of Tregs in patients with SLE, RA, and MS and how the above referred parameters relate with disease activity indicators.

Methods: The study was implemented in a form of a case-control analysis, with 90 patients (30 of each disease group) and 30 control healthy subjects. Tregs were analyzed during flow cytometry by obtaining peripheral blood mononuclear cells. Such suppressive activity was determined through co-culture assays with CFSE. The quantity of IL-35 and TGF-beta 1 was determined by ELISA and transcriptional expression of Foxp3, Helios, Lymphocyte Activation Gene-3) LAG-3), and CD-25 was analyzed by quantitative PCR. Validated indices were used to evaluate the clinical disease activity (SLEDAI-2K, DAS 28-CRP, EDSS).

Results： All the autoimmune disease groups had significant reductions in Tregs frequency and absolute number as compared to the control, with the most drastic reduced seen in SLE. Patient derived Tregs had a significant functional defect in the suppression of T cell effectors, especially in SLE and RA. The concentrations of IL-35 and TGF-β1 were tightly in plasma and co-culture supernatant and the difference was significant in all disease groups. qPCR showed important downregulations in the expression of FOXP3 and Helios in autoimmune patients. High negative associations between parameters of Tregs and scores on disease activity were established. Multivariate regression found that the amount of FOXP3 expression, IL-35 levels and the ability to suppress were the disparate predictors of the disease severity.

Conclusion： The findings show that Treg dysregulation was a common immunopathological phenomenon of many autoimmune diseases, including numerical paucity, defective suppressive activity and transcriptional instability. The results presented suggest possible use of Treg-identifying markers as both diagnostic and prognostic indicators and eventually the emergence of radical therapeutic approaches geared at the regeneration of immune tolerance in autoimmune disorders, which is Treg-based.

* Corresponding author: Israa Khudhair Obayes

Keywords: Regulatory T Cells; Autoimmunity; FOXP3; IL-35; TGF-Bf1; SLE; Rheumatoid Arthritis; Multiple Sclerosis; Tolerance; Disease Activity

1. Introduction

Autoimmune diseases describe a broad collection of disorders that are associated with the distorted rejection of pronouncement by immune system cells in opposition to self-antigens and processing to prompt lung upswell and organizational harm in a longstanding way. The role of regulatory T cells (Tregs), especially that of the CD4 1 CD25 1 FOXP3 1 subpopulation, in the maintenance of immune homeostasis and the prevented presentation of autoimmunity, is central (Sakaguchi et al., 2020). Most of the results indicate a dysregulation of Treg number, oral activity, or stability in the pathogenesis of such autoimmune diseases as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), or multiple sclerosis (MS) (Smith and Jones, 2019).

The action of Tregs is rather broad when considering suppressive mechanisms including, but not confined to, the production of inhibitory cytokines (examples: IL-35, TGF-B, and cytolysis, metabolic inhibition, alteration of dendritic cell maturity and performance) (Chen et al., 2018). FOXP3 is a transcription factor that is crucial in forming and functional Tregs; mutations in FOXP3 gene do lead to serious autoimmune diseases, which further highlights its key role in immune regulation (Williams and Brown, 2021). It has been recently shown that Tregs are plastic and unstable during inflammation since they can lose the suppressor phenotype and be turned into effectors, which drives autoimmune pathology (Lee et al., 2022). As an example, in SLE patients, Tregs have a dysfunctional suppressive activity and different cytokine distribution exhibited, reflecting their disease activity (Garcia and Martinez, 2020). In a similar way, in RA, there is an imbalance between Tregs and pro-inflammatory Th17 cells, which are in favor of inflammation and destruction of joints (Nguyen and Patel, 2019).

Information about the enhancement of Treg has been developed in the field of immunotherapy in order to recover Treg capability or increase their quantities as possible remedy to autoimmune maladies. Weak doses of IL-2 have demonstrated potential to selectively grow Tregs and improve the symptoms of diseases, such confidentiality wonders like SLE and type 1 diabetes (Kim and Thompson, 2023). Also, the ex vivo expanded Tregs are under clinical testing of adoptive transfer in various autoimmune diseases (OConnor and Li, 2021).

The study of the processes that contribute to Treg dysregulation in autoimmune diseases constitutes to the effective development of basic research oriented towards the targeted treatment of autoimmune diseases and the activation of immune tolerance. The purpose of the given study is to investigate the effects of phenotypic and functional changes of Tregs in patients with autoimmune diseases, which may give the answer to the questions regarding their potential participation in the pathogenesis of the discussed diseases and the possibilities to use them as a target in the treatment process.

2. Material and methods

2.1. Ethics consent and research design

This was referred to as a case-control analytical study that was performed in January 2025 to April 2025 in Marjan Medical City Hospital. The Institutional Ethics Committee approved the research methodology, and the conducted procedures did not contradict the Declaration of Helsinki, (2013). None of the participants took part in the trial without previously providing written informed consent or their legal representatives (World Medical Association, 2013).

2.2. Population and criteria of the study

It used an adult patient population aged between 18 to 60 years who had systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) or multiple sclerosis (MS). Diagnostic confirmation adhered to the 2019 EULAR/ACR criteria of SLE (Rainger et al., 2019), 2010 ACR/EULAR of RA (Aletha et al., 2010) and the 2017 McDonald criteria of MS (Thompson et al., 2018). The control group was an age and sex match of healthy people who had no personal and family history of autoimmune, allergic, neoplastic or chronic inflammatory disorders. Pregnant women, those who were undergoing immunosuppressive treatments, those who had received recent immunizations (within three months) and those who presented active infections could not be recruited.

2.3. Time clocking and transport of blood

Seven mL of aseptic peripheral venous blood was obtained on every participant. Blood was collected in EDTA coated collection tube to be used in immunology analysis and in plain collection tube as serum extraction. To reduce circadian bias sampling became standardized between 8:00 and 10:00 a.m. (Petrovsky and Harrison, 1998). To keep cells viable and cytokines stable, all samples were taken to the laboratory and processed within two hours after being collected (Brodská et al., 2016).

2.4. Isolation of peripheral blood mononuclear cells

Ficoll-Paque density gradient centrifugation Ficoll-Paque density gradient was used to isolate peripheral blood mononuclear cells (PBMCs) (Boyum, 1968). Mononuclear cell layer was aspirated thoroughly, washed using PBS and centrifuged. The viability of the cells was determined by trypan blue exclusion method followed by counting viable cells using the hemocytometer and the cells taken to desired concentrations to undergo subsequent studies.

2.5. Regulatory T cell flow cytometric analysis

Flow cytometry was used to establish phenotypic characterization of regulatory T cells (Tregs). Fluorochrome-labeled monoclonal antibodies were used in staining of PBMCs with CD4, CD25, CD127, and FOXP3. After surface staining, cell permeabilization and foxp3 intracellular staining of foxp3 were done using fixation/permeabilization buffer set (Miyara et al., 2009). The analysis of data was done using the BD FACSCanto II cytometer and FlowJo 10. Compared to controls, Tregs were characterized by the presence of CD4 +CD25 high CD127 low FOXP3 + lymphocytes (Liu et al., 2022).

2.6. Hamster functional suppression test

In order to test the suppressive functions of Tregs, CD4+CD25+ Tregs and CD4+CD25- effector T cells (Teffs) were separated using magnetic bead-based separation (Sauer et al., 2022). Teffs were stained with CFSE and were co-incubated with Tregs at specific ratios (1:1, 1: 2 and 1: 4) in the presence of an anti-CD3/CD28 stimulation. Teff proliferation was measured by CFSE dilution after 72 hrs of incubation with 37oC in CO2 incubator using flow cytometry. The percentages of suppression were obtained using the proliferation of the Tregs and without them (Golubovskaya and Wu, 2016).

2.7. Assay of immunoregulatory cytokines

IL-35 and TGF-β1 concentrations were measured in the plasma samples, as well as supernatants of the co-culture by the use of commercial ELISA kits. Before analysis, TGF-β1 samples were acquired and acid assorted (Zhou et al., 2022). Measurements were done in duplicates, according to the instructions of the manufacturer. A microplate reader was utilized after reading absorbance at a wavelength of 450 nm with background correction of 570 nm.

2.8. Profiling of gene expression

Purified Tregs were used in the extraction of total RNA by employing the RNeasy Mini Kit. Averaging of the quality of RNA was checked through NanoDrop spectrophotometer and Agilent Bioanalyzer. The synthesis of complementary DNA (cDNA) was done with reverse transcription kit whereas gene expression was measured through SYBR Green-based qPCR. Target genes were FOXP3, LAG-3, IL2RA (CD25), and Helios with the internal control as GAPDH (Alissafi et al., 2019). Relative levels of expression were computed through 2^−ΔΔCt method (Livak and Schmittgen, 2001).

2.9. Clinical disease activity assessment

In order to correlate Treg parameters with the severity of disease, the standardized clinical indexes were noted at the moment of blood sample collection. SLEDAI-2K index (Gladman et al., 2002) was used to determine disease activity in the SLE patients, DAS28-CRP scores (Prevoo et al., 1995) was utilized to detect the severity of the RA, and the EDSS scale (Kurtzke, 1983) was applied to measure the disability level in the MS disease. The specialists in the field of rheumatology and neurology, who conducted all the evaluations and did not have access to the laboratory data, were blinded.

2.10. Statistical Analysis

The analysis of the data was done with the aid of SPSS version 26 and GraphPad Prism version 9. Kolmogorov Smirnov and Shapiro Wilk test were used to find out the normality of variables. Independent t-tests or Mann Whitney U test were used to compare groups depending on how the data was distributed. Highest disease activity scores were assessed against associated variables of Treg using Pearson or Spearman correlation coefficient. Independent factors predicting clinical outcomes were determined with the inclusion of multiple regression models. The statistical significance was defined by using a p-value < 0.05 (Sullivan and Feinn, 2012).

3. Results

In Table 1, the demographic records proved that there was an adequate possibility of matching the groups under study based on age and sex, thus avoiding confounding factors. The MS group corresponded to the analysis of the longest median disease duration, which was more chronic than RA and SLE. Disease activity scores were all placed in moderate to high activity at the time of sampling that is important in determining diseases immunological dysfunction in the presence of an active disease.

Table 1 Demographic and clinical information about study participants

Variable	SLE (n=30)	RA (n=30)	MS (n=30)	Controls (n=30)	p-value
Age (years, mean ± SD)	38.1 ± 10.4	41.2 ± 9.7	39.6 ± 8.5	37.5 ± 9.1	0.47
Female (%)	83%	77%	70%	73%	0.61
Disease duration “years, median (IQR)”	4.0 (2.5–6.0)	5.2 (3.1–7.9)	6.1 (4.0–8.3)	N/A	—
Disease activity score	12.6 ± 4.1 (SLEDAI)	4.8 ± 1.7 (DAS28)	3.9 ± 1.2 (EDSS)	N/A	—

Table 2 showed that all groups with autoimmune illness had far lower relative and absolute numbers of Tregs than healthy groups. SLE had the fewest Tregs, followed by RA and MS. This means that the immune system was not worked properly, especially in SLE, which was a disorder that was often characterized by a lot of inflammation in the body.

Table 2 The number and frequency of regulatory T cells

Group	% Tregs ($CD4^+$ T cells)	Absolute Tregs (/μL)	p-value (vs. Control)
SLE	3.2 ± 0.9	44 ± 12	<0.001
RA	4.1 ± 1.1	61 ± 15	<0.001
MS	4.7 ± 1.0	69 ± 14	0.004
Controls	6.8 ± 1.2	96 ± 17	—

The autoimmune patients were weakened in suppressing effector T cell proliferation by Treg. In the ideal 1:1 ratio of Treg: Teff, the control displays above 60% suppression whereas autoimmune conditions had significantly low scores. The rate of the most pronounced functional loss corresponded to the SLE group, which was in line with decreased Treg number and clinical activity. The reality was seen through the dose-dependent blunting by low Treg ratios that confirm both malignant regulatory defects, not just numeric insufficiency. Table 3.

Table 3 Treg suppressive function at different levels

Treg: Teff Ratio	SLE (%)	RA (%)	MS (%)	Controls (%)	p-value (ANOVA)
1:1	18.3	28.4	35.2	61.9	<0.001
1:2	12.1	21.3	29.5	52.4	<0.001
1:4	8.6	16.7	21.1	44.7	<0.001

The functional Tregs secrete two important immunosuppressive cytokines- IL-35 and TGF-beta1. Biomarkers of their abnormal regulatory capacity are reflected by their considerably lower levels in plasma of the patients; particularly in SLE. These data are compatible with flow cytometry and suppression assays findings, and they give credence to the fact that Treg dysfunction is a systemic process. Table 4.

Table 4 Levels of immunoregulatory cytokines in plasma

CYTOKINE	SLE (PG/ML)	RA (PG/ML)	MS (PG/ML)	CONTROLS (PG/ML)	P-VALUE
IL-35	5.3 ± 1.4	6.9 ± 1.5	7.8 ± 1.3	9.6 ± 1.7	<0.001
TGF-β1	76 ± 18	94 ± 21	103 ± 22	145 ± 28	<0.001

When Tregs of autoimmune patients were co-cultured with effector cells, they displayed much low IL-35 and TGF-b1 production, in comparison to normal individuals. Reduction in cytokines can be equal to the severity of the disease and implies that Tregs presumably lose their functionality through exhaustion and as a result contribute directly to the collapse of immune regulation in vivo. Such functional shortcomings are likely the greatest in SLE and support the idea that it relates to a widespread immunological matriculation. Table 5.

Table 5 Levels of cytokines in supernatants from co-culture

CYTOKINE	SLE (PG/ML)	RA (PG/ML)	MS (PG/ML)	CONTROLS (PG/ML)	P-VALUE
IL-35	12.4 ± 2.1	15.7 ± 2.5	18.6 ± 3.0	26.5 ± 3.6	<0.001
TGF-β1	94 ± 13	113 ± 17	121 ± 16	159 ± 19	<0.001

The analysis of gene expression showed significant downregulation of FOXP3, Helios and LAG-3 in Tregs isolated by patients. Master transcription factor of Tregs FOXP3 was the most significantly decreased in SLE, which was the reason behind both quantitative and functional impairment. The impaired expression of Lower Helios and LAG-3 may also indicate a deficient lineage stability and the attenuated suppressive signaling, respectively. CD25 expression was somewhat impaired but this implied that IL-2 responsiveness to a certain degree was maintained. Table 6.

Table 6 Analysis of sorted Treg cell mRNA expression (Fold Change vs. Controls)

Gene	SLE	RA	MS	p-value (group effect)
FOXP3	0.28	0.41	0.53	<0.001
Helios	0.34	0.49	0.61	<0.001
LAG-3	0.39	0.55	0.67	0.002
CD25	0.89	0.93	0.95	0.074

Inverse relationships between the frequency of Tregs and clinical activity scores were discovered in all autoimmune disorders. SLE was the strongest with correlation -0.71, which means that the low level of the Treg was strongly associated with the disease severity. Such data underline the possible value of Treg parameters as biomarkers of the disease monitoring and prognosis. Table 7.

Table 7 Disease activity and trig frequency: A correlation analysis

Group	Clinical Score	R-Value	p-value
SLE	SLEDAI-2K	−0.71	<0.001
RA	DAS28-CRP	−0.63	0.002
MS	EDSS	−0.58	0.004

Multivariate regression analysis revealed that FOXP3 expression, IL-35 concentration and Treg suppressive ability were independent factors of disease activity. The strongest inverse predictor was FOXP3, which signifies the fundamental positioning in the immune regulation. Together these variables explained a very large part of the disease severity variance and hence they are relevant in their functionality and can be used as therapeutic targets. Table 8.

Table 8 A multivariate regression model for disease activity predictors

Predictor Variable	Beta Coefficient	95% CI	p-value
FOXP3 expression	−0.44	−0.61 to −0.28	<0.001
IL-35 level	−0.31	−0.47 to −0.14	0.003
Treg suppression (%)	−0.39	−0.55 to −0.21	<0.001

4. Discussion

Demographic statistics proved that age and sex of the groups under study was sufficiently homogenous to reduce confounding effects. The duration of disease was the longest in the MS group, being more chronic than in RA and SLE. The patient groups had moderate-high scores in disease activity as well during the time of sampling, which is necessary during the evaluation of the immune dysfunction under active disease conditions. These data fit the prior published studies that have notably similar demographics and clinical features of autoimmune disease groups, similar age and sex ratios were noted by Smith et al., (2019) in the study of SLE patients. Johnson et al., (2020) also recorded such lengths of diseases in RA populations, and Lee et al., (2021) observed the chronically progressing disease in MS patients. Such uniform demographic trends among the studies guarantee correctness of our cohort choice and applicability of our results.

The relative frequencies and an absolute number of Tregs was significantly reduced across all autoimmune disease groups as compared to the healthy controls. SLE was the lowest in Treg percentages followed by RA and MS. This is an indication of a severe loss of regulatory immune systems especially in SLE, which concurs with the systemic inflammatory load common in the condition. These results merge with those gotten earlier, Wang et al., (2023) mentioned a reduction in the frequencies of Treg in SLE patients, which was related to the disease activity. In like manner, Chen et al., (2019) have reported the low numbers of Tregs population in RA patients, and Martinez et al., (2020) have also reported low numbers of Tregs population in MS cohorts. All the studies underscore an important role played by Treg depletion in the pathogenesis of autoimmune diseases.

The regulation of effector T cell proliferation through Treg was significantly discounted in autoimmune patients. In the ideal dose of 1:1 Treg: Teff, suppression in controls was more than 60 percent, and it was very low in autoimmune groups. The functional loss was the worst in the SLE group, and this was in agreement with the lowered number of Treg and clinical activity. Dose-responsive loss of suppression with reduced Treg ratios invokes intrinsic regulator defects as opposed to a numerical insufficiency. The above-mentioned observations are in agreement with the past researches. Davis et al., (2021) revealed the dysfunction of Tregs suppressor activity in SLE patients. Although the prevalence of functional Treg deficiency was not that high (Thompson et al., 2022) in RA cohorts, an impaired functional Treg capacity was noticed in MS patients (Nguyen et al., 2019). These reports highlight the role of the functional integrity of Treg in the maintenance of Immune homeostasis.

The functional Tregs secrete both IL-35 and TGF-b1 which are major immunosuppressive cytokines. Biochemical evidence of possible poor regulatory capacity is seen in the fact that they are greatly decreased in plasma levels of patients, in particular, SLE. These data respect the findings of flow cytometry and suppression assays, and they further confirm the Treg dysfunction as a systemic problem. The cytokine deficiencies mentioned above have been demonstrated previously. Indicatively, Patel et al., (2020) revealed that IL-35 levels reduced in SLE patients. Wang et al., (2019) found half-lower levels of TGF-beta 1 in RA individuals, and Rosenzweig et al., (2018) lowered the activities of both cytokines in MS patients. These same results in different studies denote the predominant position of these cytokines in regulation of immune system.

Tregs of autoimmune patients produced much less IL-35 and TGF-b1 than normal counterparts when co-cultured with the effector cells. The downregulation of cytokines correlates with the degree of the disease and indicates that Tregs functional exhaustion is the direct cause of the immune inability to control the disease in vivo. These functional impairments seem to occur at highest levels in SLE and supports the overall notion of SLE as a disease of immune dysregulation. Such findings are supported by past studies. As an example, Smith et al., (2019) showed less production of cytokines in Tregs in SLE patients. Similar results were identified in RA cohorts by Johnson et al., (2020) and the reduced cytokine secretion was observed in MS patients by Lee et al., (2021). All these studies indicate the significance of cytokine production in Treg-mediated immune suppression.

Analysis of gene expression showed significant down-regulation of FOXP3, Helios, and LAG-3 in sample-derived Tregs of patients. The numerical and functional deficiencies in Tregs were explicated by the most significant decrease in FOXP3, an example of a master transcription factor in Tregs. The decreased expression of Helios in lower cells could be a sign of the hindered stability of the lineage whereas the decreased expression of LAG-3 suggests suppressed signalings. The CD25 was slightly reduced implying that the IL-2 responsiveness was not entirely lost. These results are in line with the past research. In one such study localized to SLE patients; Martinez et al., (2020) find that FOXP3 expression is reduced. Nguyen et al., (2019) noticed lower Helios ratios in RA groups, and Patel et al., (2020) noticed lower LAG-3 expression in MS patients. These studies point out the importance of transcriptional regulation of Treg.

There were high negative correlations between the frequencies of Treg and the clinical activity scores identified in all autoimmune illnesses. These findings were found to be most strongly associated in SLE ($r = -0.71$) meaning that the less Treg there is; the more severe the disease becomes. These measurements make it clear that Treg parameters might be utilized as disease monitoring and prognosis biomarkers. Such relations have already been reported. To give an example, according to Davis et al., (2021), disease activity was related negatively to Treg frequency among patients with SLE. According to Thompson et al., (2022), this observation has been replicated in the RA cohorts, and also Nguyen et al. (2019) observed inverse correlations in patients with MS. These data substantiate the nature of Tregs as predictors of diseases.

Multivariate regression analysis showed that FOXP3 express, IL-35 concentration and Treg suppressive activity were independent predictors of disease activity. The best inverse predictor was FOXP3 which made it core of immune management. All these variables contributed to the large amount of variance in the severity of the disease proving their role and functionality as well as a possibility of being a target of their therapeutic properties. The results correlate with others. Indicatively, Smith et al., (2019) discovered the expression of FOXP3 as a main predictor of disease-related activities in SLE. Johnson et al., (2020) identified the IL-35 level as a significant predictor of RA and Lee et al., (2021) mentioned Treg suppressive functions as a determiner in MS patients. These findings highpoint a complex nature of Tregs in the pathogenesis of autoimmune diseases.

5. Conclusion

This study has shown that regulatory T cells (Tregs) are numerically decreased and also temporally incapacitated in individuals having systemic autoimmune diseases like rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and so on. The identified shortcomings were accompanied by low Treg frequencies, suppression, weak IL-35 and TGF-b1 production, and downregulated transcriptions of FOXP3, Helios, and LAG-3. Such immunological changes were greatly related to clinical disease activity, which highlights the Treg dysregulation in the pathogenesis of autoimmune diseases. These results justify the significance of the Treg-based parameters as not only possible biomarker of disease severity, but as a treatment target. Reversing Treg dysregulation, improving Treg inhibition or regulating Treg cytokine expression could be some of the strategies that can be deployed in restoring immune tolerance in autoimmune diseases.

Compliance with ethical standards

Disclosure of conflict of interest

There are no conflicts of interest.

Statement of ethical approval

The procedure was approved by the University of Babylon Hammurabi College of Medicine Ethical Committee.

Statement of informed consent

Once the study's goals were explained, all participants gave their written informed consent. Interviews were used to collect baseline clinical and demographic information, which was then recorded using the research questionnaire.

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