Original Article

Iranian Journal of Immunology
https://iji.sums.ac.ir

Quantitation of CD4^{+}/CD25$^{+/high}$/CD127$^{low/-}$ Regulatory T-cells in Rheumatoid Arthritis Patients

Samaneh Delavari[1,2,3], Mehri Ghafourian[2,4], Elham Rajaei[5], Karim Mowla[5], Ata Ghadiri[1,2*]

[1]Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, [2]Department of Immunology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, [3]Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, [4]Fertility, Infertility and Perinatology Research Center, [5]Department of Internal Medicine, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Background: Rheumatoid arthritis (RA) is the most common rheumatoid disease of unknown etiology, determined by the articular cartilage destruction and bone loss. The hallmark of RA is the defect in immune tolerance. Regulatory T cells (Treg) play a critical role in the protection of peripheral tolerance.

Objective: To assess the percentage of CD4^{+}/CD25$^{+/high}$/CD127$^{low/-}$ Treg cells in peripheral blood of RA patients as compared with the healthy individuals.

Methods: The number of CD4^{+}/CD25$^{+/high}$/CD127$^{low/-}$ Treg cells was assessed by multicolor flow cytometry. The clinical disease activity of RA patients was determined by disease activity score 28 (DAS-28). The correlations of DAS-28 and erythrocyte sedimentation rate (ESR) with Treg cells were evaluated.

Results: The percentage of CD4^{+}/CD25$^{+/high}$/CD127$^{low/-}$ Treg cells in peripheral blood of RA patients significantly decreased as compared with the healthy individuals (P= 0.0002). The percentage of CD4^{+}/CD25$^{+/high}$/CD127$^{low/-}$ Treg cells negatively correlated with DAS-28 and ESR.

Conclusion: This study concludes that the defect of Treg cells plays a vital role in the pathogenesis of this disease. Further studies are necessary to determine the role of Treg cells in the clinical course of rheumatoid arthritis.

Keywords: DAS-28, Flow Cytometry, Regulatory T Cells, Rheumatoid Arthritis

*Corresponding author:
Ata Ghadiri,
Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
Tel: +98 61 3373838
Fax: + 98 61 33362537
Email: ata.ghadiri@hotmail.fr

Cite this article as:
Delavari S, Ghafourian M, Rajaei E, Mowla K, Ghadiri A. Quantitation of CD4+/CD25$^{+/high}$/CD127$^{low/-}$ Regulatory T-cells in Rheumatoid Arthritis Patients. *Iran J Immunol.* 2021; 18(3):179-187, doi: 10.22034/iji.2021.68100.1365.

Received: 2018-08-22
Revised: 2020-12-18
Accepted: 2020-12-26

INTRODUCTION

Rheumatoid arthritis (RA), an autoimmune and progressive disease, is described by synovial inflammation and bone destruction (1, 2). RA affects approximately 0.18 -1.07% of adults worldwide (3, 4), and its estimated prevalence in Iran is 0.33% (5). The actual etiology of this illness has yet to be identified.; however, the immune system, as well as genetic, and environmental factors have been implicated in its incidence (6, 7). B-cells, T-cells, as well as the interplay of pro-inflammatory cytokines ، have a

major role in the pathogenesis of RA. The pathology of this disease is specified by defective peripheral immune tolerance as well as immune system dysregulation along with a dominance of inflammatory cells (8, 9). These pathological immune events mostly appear at the synovial membrane where the invasion or activation of mononuclear cells as well as the development of new blood vessels lead to synovitis (10). The immune system recruits several pathways to preserve self-tolerance and overpower the auto-reactive T-cells (11). Among these pathways, T regulatory (Treg) cells play a key role in maintaining immune tolerance via balancing the action mechanisms of pro-inflammatory and anti-inflammatory cytokines (12-16). As well as failure to suppress pro-inflammatory cytokines such as interleukine-6 (IL-6) and tumor necrosis factor (TNF-α) released by monocytes and activated T-cells, the suppression of effector T-cell proliferation is among the major functions of regulatory T-cells. Above all, Tregs prevent the development of autoimmunity through the suppression of autoreactive lymphocytes that are induced by transforming growth factor-beta (TGF-β) and IL-10 (17). Treg cells have been defined as $CD4^+$ T-cells showing high expression of $CD25^+$ (IL-2 receptor α-chain) (18, 19). These cells are distinguished through their expression of forkhead box protein p3 (Foxp3) transcription factor. The defect of Treg cells or their absence is associated with the aggravation of autoimmune diseases, indicating that Treg cells have a vital role to play in the protection through self-tolerance (18, 20, 21). In recent years, the number and function of regulatory T-cells have been investigated in RA patients, but the frequency of Treg cells in peripheral blood (PB) has been different across reports (22-29). Because of these controversies, further studies must be conducted to analyze the count and quality of regulatory T-cells among RA patients. This study has examined the ratio of $CD4^+/CD25^{+/high}/ CD127^{low/-}$ regulatory T-cell populations in RA patients relative to healthy controls. Moreover, we analyzed the correlation between Treg cells with disease activity score in 28 joints (DAS-28) and erythrocyte sedimentation rate (ESR) among patients.

MATERIALS AND METHODS

Our case group was comprised of 25 patients with RA diagnosed based on 2010 criteria of American College of Rheumatology (ACR) and European League with Rheumatism Collaborative (EULAR) (30). All the patients were newly diagnosed with RA disease and they had received no immunosuppressive drugs. None of the RA patients showed signs of other rheumatologic, inflammatory, and autoimmune diseases like psoriatic arthritis, systemic lupus erythematosus, and diabetes mellitus. RA patients were tested for ESR and rheumatoid factor (RF). Swollen joint count (SJC) and tender joint count (TJC) were evaluated by a rheumatologist. Besides, disease activity score in 28 joints (DAS-28) was estimated based on ACR, and scales of DAS-28 (0-9.4) were as follows: Remission:<2.6; low: ≥2.6 to <3.2; moderate: ≥3.2 to ≤5.1 and severe: >5.1. (31, 32). The patient's age range was 18-72 years. Patients' blood samples and the healthy individual were matched in terms of age and sex. Healthy individuals, if females, were not, in their gestation period and had no history of autoimmune or metabolic systemic disorders.. All patients signed a written informed consent form. This study was approved by the Ahvaz Jundishapur University of Medicine's Ethics Committee. (IR.AJUMS.REC.1394.633).

Sample Collection and Flow Cytometry Analysis

After the diagnosis of RA in patients, PB samples were drawn from them and kept in heparinized tubes. We used Ficoll density-gradient centrifugation (Ficoll Pague, Baharafshan, Iran) to separate peripheral blood mononuclear cells (PBMCs). The

isolated mononuclear cells were washed twice with phosphate-buffered saline (PBS) and subsequently resuspended in $1x10^6$ cells/ml for further analysis. The purified cells were employed for the flow cytometry staining to detect T reg cells. All antibodies were made by the eBioscience Company (San Diego, CA, USA). The isolated cells were stained for 30 minutes in darkness applying fluorescein isothiocyanate (FITC)–labeled monoclonal antibody (mAb) against CD4 (Clone RPA-T4), phycoerythrin (PE)-labeled mAb against CD25 (clone BC96), and allophycocyanin (APC)-labeled mAb against CD127 (clone eBioRDR5) via standard protocols. The cutoff value for all cell surface markers was determined according to isotype controls staining (33). For each marker, the relevant mouse isotype controls were provided by the eBioscience Company. After staining the surface markers, the cells were again washed by PBS. Data taken from 1×10^5 cells per sample were acquired making use of the FACSCalibur flow cytometer (Becton Dickinson, USA), and the FlowJo program was employed for data analysis (Tree Star, Ashland, USA).)The cells subject to analysis were gated for lymphocytes via forward and side scatter, $CD4^+$ $CD25^+$ cells were then gated, the CD25 gate was drawn against CD127, and the cells negative for CD127 marker were selected.

Statistical Analysis

The Mann-Whitney U test was applied for comparison between patients and the healthy controls of Treg $CD4^+/CD25^{high/+}CD127^{low/-}$ and Treg $CD4^+/CD25^{high}$ cells. The correlations between DAS-28, ESR, and Treg cells in patients were analyzed by Spearman's coefficient test. All data were analyzed using SPSS version 20 (SPSS Inc., Chicago, IL, USA), and graphical presentation was conducted by GraphPad Prism version 6 software (GraphPad Software, Inc., USA). For all tests, $P<0.05$ was considered as the significance level.

RESULTS

The demographic features of the study groups are shown in Table 1. The mean age and ratio of male to female among patients were 45.2±7.1 years and 1:4, respectively, and the mean age of the healthy controls was 48.3±5.9 years. The mean ESR was 44.3 in the patients' population. Serum RF was positive in 72% of patients, and the mean TJC and SJC of patients were reported about 9.9 and 7.4, respectively. Based on DAS-28, 19 patients were in the mild state and six patients were in the moderate group. The age distribution of RA patients did not indicate any correlation with DAS-28 ($P=0.32$, $r=0.15$).

We assessed Treg $CD4^+CD25^{high}$ and Treg $CD4^+$ $CD25^{+/high}$ $CD127^{low/-}$ cells in PB of RA patients and the healthy controls by the flow cytometry. RA patients had considerably lower number of Treg $CD4^+CD25^{high}$ and Treg $CD4^+$ $CD25^{+/high}$ $CD127^{low/-}$ cells ($P=0.003$ and $P=0.0002$, respectively) (Table 2 and

Table 1. Characteristics of the study population

Characteristics	RA Patients	Healthy Controls
Number (M/F)	25 (5/20)	25 (7/18)
Age (Years)	45.2±7.1	48.3±5.9
ESR (mm/h)	44.3±23.2	-
TJC	9.9±3.8	-
SJC	7.4±3.5	-
RF Positive, n (%)	18 (72%)	-
DAS-28	5.5±0.8	-

ESR: Erythrocyte sedimentation rate; TJC: Tender Joint count; SJC: Swollen Joint count; RF: Rheumatoid factor; DAS28: Disease activity score in 28 joins. Values are mean±SD.

Figure 1).

Figure 2 demonstrates dot plots for an RA patient. The red gates show the percentage of $CD4^+$ $CD25^{+/high}$ $CD127^{low/-}$ Treg cells.

We evaluated the correlation between the ratio of Treg $CD4^+CD25^{high}$ $CD127^{low/-}$ cells in peripheral blood of RA patients with markers of disease activity such as DAS-28 and ESR in RA patients. The $CD4^+$ $CD25^{+/high}$ $CD127^{low/-}$ Treg cells were inversely correlated with DAS-28 (P=0.0003) and ESR (P=0.0006) (Table 2).

Based on the scatter plot shown below, an inverse correlation is observed between Treg $CD4^+CD25^{high}$ $CD127^{low/-}$ cells with DAS-28 and ESR. This means that Treg cells decreased in patients by increasing DAS-28 and ESR values (Figure 3).

Table 2. Prevalence of the Treg cells subsets in RA patients and healthy controls.

Treg Subsets	Marker	HC (n=25)	RA (n=25)	P value
Treg Subset 1	$CD4^+$ $CD25^{high}$	2.6±0.14	1.5±0.09	0.003*
Treg Subset 2	$CD4^+$ $CD25^{high}CD127^{low}$	2.1±0.13	1.3±0.14	0.0002*

* Significant difference / Values are mean±SD.

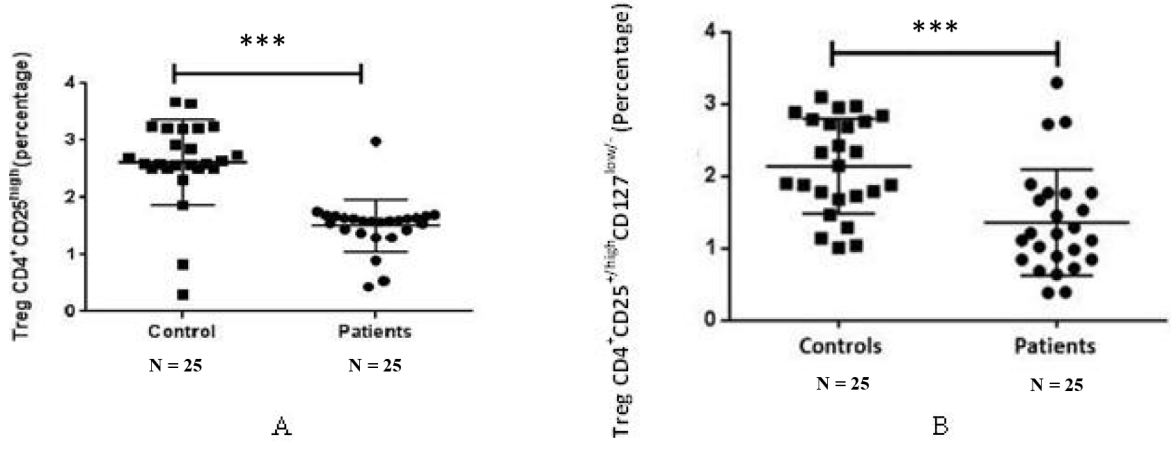

Figure 1. The percentages of $CD4^+CD25^{high}$ Treg cells (A) and $CD4^+$ $CD25^{+/high}$ $CD127^{low/-}$ Treg cells (B) in peripheral blood of RA patients and healthy controls. The mean frequencies of Treg $CD4^+CD25^{high}$ cells and $CD4^+$ $CD25^{+/high}$ $CD127^{low/-}$ Treg cells in healthy controls were significantly higher than patients (P=0.003, P=0.0002, respectively). Asterisks show the significance of the differences. Significant when less than 0.001. Values are mean±SD.

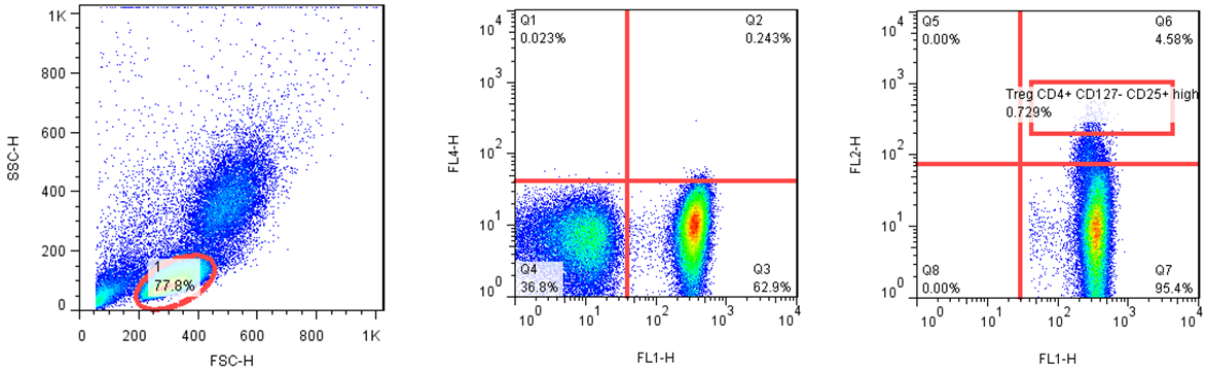

Figure 2. Gating strategies and repertoire of CD^{4+} $CD25^{+/high}$ $CD127^{low/-}$ regulatory T cells in peripheral blood of RA patient. We first gated lymphocytes via forward and side scatte, then $CD4^+$ $CD25^+$ cells gated and the CD25 gate was drawn against CD127, and cells that were negative for the CD127 marker were selected Peripheral blood mononuclear cells were stained with anti-CD4 (FITC)(FL1), anti-CD25 (PE)(FL2), anti-CD127 (APC)(FL4) antibodies and analyzed by flow cytometry.

Table 3. Spearman's correlation test

		DAS-28	ESR
Treg $CD4^+CD25^{+/high}$ $CD127^{low/-}$	Correlation Coefficient	-0.76**	-0.88**
	P value	0.0003	0.0006
	N	25	25

** Correlation is significant when less than 0.05.

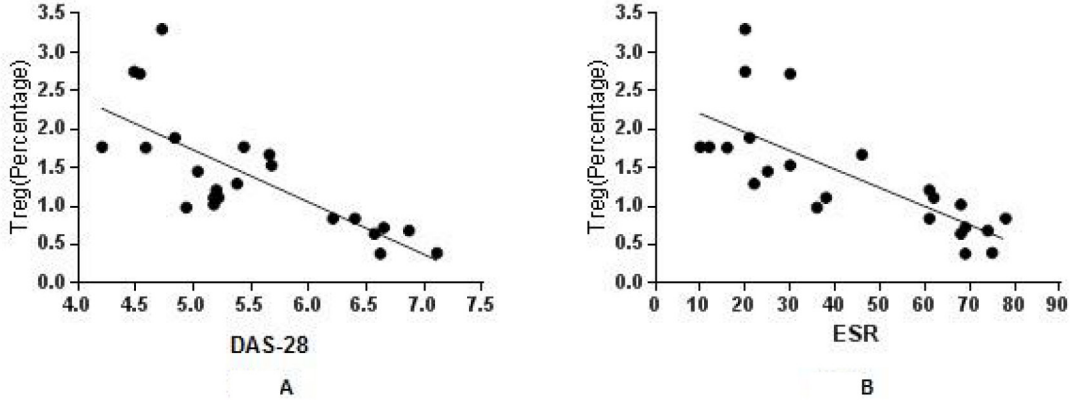

Figure 3. Scatter plot between Treg and DAS-28, ESR in RA patients. Plot (A) shows correlation between Treg and DAS-28 (P=0.0003, r=-0.76). Plot (B) shows correlation between Treg and ESR (P=0.0006, r=-0.88). Treg: Regulatory T cell, DAS-28=disease activity score in 28 joints, ESR=erythrocyte sedimentation rate.

DISCUSSION

There is a hypothesis about immune disorder and intolerance in various collagen vascular diseases such as Behçet, systemic lupus erythematosus, and rheumatoid arthritis, but the exact mechanisms are not clear. Treg cells are thought to play a crucial role in the inhibition of autoimmunity (11, 14, 34-36). There is a recognized role for Treg cells as suppressors for auto-reactivation of the immune system, and several studies have supported this idea (34, 37, 38). However, in RA patients, the findings of studies are controversial (34). Enumerating the Treg cells of RA patients could be a valuable index to indicate the pathogenesis of RA and help settle likely target biomarkers for successful treatment of the disease (11, 38). To infer the defacement of immunosuppressive drugs that can potentially interfere with the Treg population, we sampled only newly diagnosed RA patients who had not taken any immunosuppressive drugs. In the current study, we found that Treg $CD4^+$/ $CD25^{+/high}$/ $CD127^{low/-}$ cells ratio in the patients was significantly lower than the healthy group (P=0.0001), which had an inverse correlation with DAS-28 (r=-0.76 and P=0.0003) and ESR (r=-0.88 and P=0.0006). These findings are comparable to some previous investigations (22, 39-44). Kawashiri et al. have suggested that the frequency of $CD4^+CD25^{high}CD127^{low/-}$ Treg cells in PB of RA patients considerably decreased relative to the healthy group, and this frequency had a negative relationship with CRP, DAS28, and ESR in patients with RA (P<0.0001) (40). Li et al. evaluated the apoptosis status of Treg $CD4^+CD25^{high}Foxp3^+$ cells and their correlation with RA patients' clinical activity. Furthermore, they reported a decline of Treg cells in RA patients with active disease, and considerably higher expression of Fas, Caspase-3, and Caspase-8 was observed in Tregs of RA patients compared with the control group. The expression of Bcl-2 anti-apoptotic protein on Tregs did not indicate any noteworthy differences among active RA patients and the healthy group. Besides, there was a positive relationship between the rates of apoptosis with Caspase-3 expression in Tregs and DAS-28 of active RA patients. (45). Ren

et al. evaluated the level of Tregs with $CD4^+$/ $CD25^-$/$Foxp3^+$ markers by the flow cytometry and determined the expression of soluble programmed death-1 (SPD-1) in Treg cells applying western blot. It was observed that the frequency of Treg cells decreased in the peripheral blood of RA patients ($P<0.001$) and that the PD-1 level was higher than the healthy controls ($P<0.001$). It has been suggested that PD-1 may suppress the rate of $CD4^+$/ $CD25^-$/ $Foxp3^+$ in patients with RA (43). Moreover, Cao and et al. in a murine model of RA demonstrated that the number of $CD4^+/CD25^+$ Treg cells decreased in juvenile idiopathic arthritis (JIA), spondyloarthropathies (SpA), and psoriatic arthritis (PsA), and they showed that Tregs with $CD4^+/CD25^+$ markers reduced in PB of patients as well (46). This finding was similar to ours in the RA patients. Despite the similarity between the results of Cao and the present study, there were two major differences in between. Firstly, Cao's analysis focused on patients with active rheumatoid arthritis having positive RF and erosions in X-Ray. Treg cells may migrate from blood to joint surface to suppress the inflammatory process; whereas our patients were newly diagnosed and most of them had no bony erosion because of the lack of long-standing disease, most Treg cells were only produced in response to inflammation and did not migrate to peripheral blood. Secondly, the mouse models in the study of Cao had various collagen vascular diseases with multiple treatments and were under immunosuppressive drugs while we assessed only RA patients without any anti-inflammatory drug history that may show a false reduction of Treg cells. Zare et al. stated that the number of Treg cells in patients with RA was lower than that of healthy subjects. Moreover, it is assumed that this population serves as a reservoir for actual Tregs (10). Contrary to studies that reported decreasing quantity of Tregs in RA patients as well as our findings, some previous investigations have revealed that Treg cell number increased in RA patients (23, 47, 48). Van Amelsfort et al. observed that the percentage of Treg was higher in peripheral blood and synovial fluid of RA patients than in the controls (23). Similar results were reported by Wang et al. who suggested that increasing frequencies of Treg cells could shift toward Th17 cells, but Treg cells have a suppressive function in peripheral blood of RA patients (47). If this finding is true, it may demonstrate how the increasing number of Treg cells could not prevent autoreactivity in RA patients. The results regarding the frequency and functional activity of Tregs may be related to several factors. First, patient selection could influence the proportion of the Treg population. In our study, we included newly diagnosed RA patients who had moderate disease activity and did not undergo suppressive treatments; however, in Van Amelsfort's study, patients were chosen randomly and received various suppressive drugs such as methotrexate, corticosteroids, and TNF blockers. Moreover, the age of participants in their study was higher than in our patients with an average of 10 years with a mean disease duration of about 16 years. Active inflammation in Van Amelsfort's study population suggests a negative feedback loop where $CD4^+CD25^+$ regulatory T-cells are generated by ongoing inflammation. Furthermore, the frequency and modulatory effect of Treg cells may differ in various stages of RA and as the disease progresses. The increasing presence of Tregs in the chronic inflammatory phase as observed in Van Amelsfort's study may even be harmful to homeostatic control and the natural course of an effective immune response.

There are also several reports indicating no significant difference in Treg cells of peripheral blood among RA patients (33, 49-51). Fessler et al. stated that the frequency of Treg $CD4^+/CD25^+/CD28^+/Foxp^{3+}$ cells was equal in PB of both groups ($p=0.988$) (49). In the present study, a negative correlation was found between Treg cells with DAS-28 and ESR. Also, de Paz et al. showed a negative relationship between Treg $CD4^+$/

$CD25^{-}/Foxp3^{+}$ and DAS-28 (52). Contrary to our findings and those of de Paz, Zare et al. found no associations between frequencies of Treg cells and DAS-28 (11), which may be because merely ntreg cells have been investigated in their study. Our study's strength is that we chose patients who had recently been diagnosed and had not yet received any treatment. Patients in previous studies received a variety of treatments that could have influenced the frequencies of Treg cell population regarding the potency of treatment. One of the study's shortcomings that cannot be overlooked is that we only looked at the frequency of one subset of Treg cells in RA patients but did not assess the function of Treg cells. Further multicentric studies with a larger number of patients seem to be necessary to determine the function and frequency of different subsets of T regulatory cells populations for understanding and treating RA in the clinical course of the disease and performing subsequent follow-up of patients.

CONCLUSION

Our current findings suggest that decreasing proportion of the Treg population in peripheral blood of RA patients is associated with the pathogenesis of the disease and also the disease activity. Further functional analysis in these patients may clarify the status of regulatory T-cells as well as their specific immunomodulatory role in RA individuals.

ACKNOWLEDGMENT

We appreciate the patients' and healthy people's participation in this study. . This research was supported by Cellular and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences (Grant No: CMRC.3491). This essay is based on Samaneh Delavari's thesis for her master's degree in immunology from AJUMS.

Conflicts of Interest: None declared.

REFERENCES

1. Boissier M-C, Semerano L, Challal S, Saidenberg-Kermanac'h N, Falgarone G. Rheumatoid arthritis: from autoimmunity to synovitis and joint destruction. Journal of autoimmunity. 2012;39(3):222-8.
2. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. New England Journal of Medicine. 2011;365(23):2205-19.
3. Litwic A, Edwards MH, Dennison EM, Cooper C. Epidemiology and burden of osteoarthritis. British medical bulletin. 2013;105(1):185-99.
4. Alamanos Y, Voulgari PV, Drosos AA, editors. Incidence and prevalence of rheumatoid arthritis, based on the 1987 American College of Rheumatology criteria: a systematic review. Seminars in arthritis and rheumatism; 2006: Elsevier.
5. Kordasiabi MC, Akhlaghi M, Baghianimoghadam MH, Morowatisharifabad MA, Askarishahi M, Enjezab B, et al. Self Management Behaviors in Rheumatoid Arthritis Patients and Associated Factors in Tehran 2013. Global journal of health science. 2016;8(3):156.
6. Smolen JS, Aletaha D, Koeller M, Weisman MH, Emery P. New therapies for the treatment of rheumatoid arthritis. The Lancet. 2007;370(9602):1861-74.
7. Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. Nature Reviews Drug Discovery. 2003;2(6):473-88.
8. Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. Rheumatology. 2012;51(suppl 5):v3-v11.
9. Hashemi V, Farrokhi AS, Tanomand A, Babaloo Z, Hojjat-Farsangi M, Anvari E, et al. Polymorphism of Foxp3 gene affects the frequency of regulatory T cells and disease activity in patients with rheumatoid arthritis in Iranian population. Immunology Letters. 2018;204:16-22.
10. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature. 2003;423(6937):356-61.
11. Zare H-R, Habibagahi M, Vahdati A, Habibagahi Z. Patients with active rheumatoid arthritis have a lower frequency of ntregs in peripheral blood. Iranian Journal of Immunology. 2015;12(3):166.
12. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. The Journal of

Immunology. 1995;155(3):1151-64.
13. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunological reviews. 2001;182(1):18-32.
14. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. The FASEB Journal. 2012;26(6):2253-76.
15. Wang L, Wang C, Jia X, Yu J. Circulating Exosomal miR-17 Inhibits the Induction of Regulatory T Cells via Suppressing TGFBR II Expression in Rheumatoid Arthritis. Cellular Physiology and Biochemistry. 2018;50(5):1754-63.
16. Scheinecker C, Göschl L, Bonelli M. Treg cells in health and autoimmune diseases: New insights from single cell analysis. Journal of Autoimmunity. 2020;110:102376.
17. Yap H-Y, Tee S, Wong M, Chow S-K, Peh S-C, Teow S-Y. The pathogenic role of immune cells in rheumatoid arthritis: Implications in clinical treatment and biomarker development. Cells. 2018;7(10):161.
18. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004;22:531-62.
19. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+ CD25high regulatory cells in human peripheral blood. The Journal of Immunology. 2001;167(3):1245-53.
20. Sakaguchi S. Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. Nature immunology. 2005;6(4):345.
21. Zhang X, Zhang X, Zhuang L, Xu C, Li T, Zhang G, et al. Decreased regulatory T-cell frequency and interleukin-35 levels in patients with rheumatoid arthritis. Exp Ther Med. 2018;16(6):5366-72.
22. Lawson C, Brown A, Bejarano V, Douglas S, Burgoyne C, Greenstein A, et al. Early rheumatoid arthritis is associated with a deficit in the CD4+ CD25high regulatory T cell population in peripheral blood. Rheumatology. 2006;45(10):1210-7.
23. van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4+ CD25+ regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis & Rheumatology. 2004;50(9):2775-85.
24. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. Journal of Experimental Medicine. 2004;200(3):277-85.
25. Raghavan S, Cao D, Widhe M, Roth K, Herrath J, Engström M, et al. FOXP3 expression in blood, synovial fluid , and synovial tissue during inflammatory arthritis and intra-articular corticosteroid treatment. Annals of rheumatic diseases. 2009;68(12):1908-15.
26. Han GM, O'Neil-Andersen NJ, Zurier RB, Lawrence DA. CD4+ CD25 high T cell numbers are enriched in the peripheral blood of patients with rheumatoid arthritis. Cellular immunology. 2008;253(1):92-101.
27. Möttönen M, Heikkinen J, Mustonen L, Isomäki P, Luukkainen R, Lassila O. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. Clinical & Experimental Immunology. 2005;140(2):360-7.
28. Behrens F, Himsel A, Rehart S, Stanczyk J, Beutel B, Zimmermann SY, et al. Imbalance in the distribution of functional autologous regulatory T cells in rheumatoid arthritis. Annals of rheumatic diseases. 2007;66(9):1151-6.
29. Kailashiya V, Singh U, Rana R, Singh NK, Dash D, Kailashiya J. Regulatory T Cells and Their Association with Serum Markers and Symptoms in Systemic Lupus Erythematosus and Rheumatoid Arthritis. Immunological Investigations. 2019;48(1):64-78.
30. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis & Rheumatism. 2010;62(9):2569-81.
31. Anderson JK, Zimmerman L, Caplan L, Michaud K. Measures of rheumatoid arthritis disease activity: Patient (PtGA) and Provider (PrGA) Global Assessment of Disease Activity, Disease Activity Score (DAS) and Disease Activity Score With 28-Joint Counts (DAS28), Simplified Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI), Patient Activity Score (PAS) and Patient Activity Score-II (PASII), Routine Assessment of Patient Index Data (RAPID), Rheumatoid Arthritis Disease Activity Index (RADAI) and Rheumatoid Arthritis Disease Activity Index-5 (RADAI-5), Chronic Arthritis Systemic Index (CASI), Patient-Based Disease Activity Score With ESR (PDAS1) and Patient-Based Disease Activity Score Without ESR (PDAS2), and Mean Overall Index for Rheumatoid Arthritis (MOI-RA). Arthritis care & research. 2011;63(S11):S14-S36.
32. Prevoo M, Van Hof M, Kuper H, Van Leeuwen M, Van De Putte L, Van Riel P. Modified disease activity scores that include twenty-eight-joint

counts development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis & Rheumatology. 1995;38(1):44-8.
33. Moradi B, Schnitzer P, Hagmann S, Rosshirt N, Gotterbarm T, Kretzer JP, et al. CD4+ CD25+/high CD127 low/-regulatory T cells are enriched in rheumatoid arthritis and osteoarthritis joints—analysis of frequency and phenotype in the synovial membrane, the synovial fluid , and peripheral blood. Arthritis research & therapy. 2014;16(2):R97.
34. Morita T, Shima Y, Wing JB, Sakaguchi S, Ogata A, Kumanogoh A. The Proportion of Regulatory T Cells in Patients with Rheumatoid Arthritis: A Meta-Analysis. PloS one. 2016;11(9):e0162306.
35. Nocentini G, Alunno A, Petrillo MG, Bistoni O, Bartoloni E, Caterbi S, et al. Expansion of regulatory GITR+ CD25 low/-CD4+ T cells in systemic lupus erythematosus patients. Arthritis research & therapy. 2014;16(5):444.
36. Göschl L, Scheinecker C, Bonelli M, editors. Treg cells in autoimmunity: from identification to Treg-based therapies. Seminars in Immunopathology; 2019: Springer.
37. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133(5):775-87.
38. Rosenzwajg M, Lorenzon R, Klatzmann D. Response to: 'Regulatory T cell frequencies in patients with rheumatoid arthritis are increased by conventional and biological DMARDs but not by JAK inhibitors' by Meyer <em>et al</em&gt. Annals of the Rheumatic Diseases. 2020:annrheumdis-2019-216598.
39. Kim J-R, Chae J-N, Kim S-H, Ha J-S. Subpopulations of regulatory T cells in rheumatoid arthritis, systemic lupus erythematosus, and Behcet's disease. Journal of Korean medical science. 2012;27(9):1009-13.
40. Kawashiri S-Y, Kawakami A, Okada A, Koga T, Tamai M, Yamasaki S, et al. CD4+ CD25highCD127low/-Treg cell frequency from peripheral blood correlates with disease activity in patients with rheumatoid arthritis. The Journal of rheumatology. 2011;38(12):2517-21.
41. Huang Z, Zheng B, Li T, Lu Y, Gu J, Han X. Study on expressions of CD25 and CD127 in CD4+ peripheral T lymphocytes of patients with rheumatoid arthritis. Zhonghua Yi Xui Zi Zhi . 2009;89(35):2476-80.
42. Daïen CI, Gailhac S, Audo R, Mura T, Hahne M, Combe B, et al. High levels of natural killer cells are associated with response to tocilizumab in patients with severe rheumatoid arthritis. Rheumatology. 2014;54(4):601-8.
43. Ren CF, Zhao YX, Hou CF, Luan L, Duan GQ, Li SJ, et al. expression of the soluble programmed death-1 protein in peripheral blood regulatory T cells and its effects on rheumatoid arthritis progression. Molecular medicine reports. 2017;15(1):460-6.
44. Al-Zifzaf DS, El Bakry SA, Mamdouh R, Shawarby LA, Ghaffar AYA, Amer HA, et al. FoxP3+ T regulatory cells in Rheumatoid arthritis and the imbalance of the Treg/TH17 cytokine axis. The Egyptian Rheumatologist. 2015;37(1):7-15.
45. Li N, Ma T, Han J, Zhou J, Wang J, Zhang J, et al. Increased apoptosis induction in CD4+ CD25+ Foxp3+ T cells contributes to enhanced disease activity in patients with rheumatoid arthritis through IL-10 regulation. Eur Rev Med Pharmacol Sci. 2014;18(1):78-85.
46. Cao D, van Vollenhoven R, Klareskog L, Trollmo C, Malmström V. CD25 bright CD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res Ther. 2004;6(4):R335.
47. Wang T, Sun X, Zhao J, Zhang J, Zhu H, Li C, et al. Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood. Annals of rheumatic diseases. 2014:annrheumdis-2013-204228.
48. Kravchenko P, Zhulai G, Churov A, Oleinik E, Oleinik V, Barysheva O, et al. Subpopulations of regulatory T-lymphocytes in the peripheral blood of patients with rheumatoid arthritis. Vestnik Rossiiskoi akademii meditsinskikh nauk. 2016(2):148-53.
49. Fessler J, Raicht A, Husic R, Ficjan A, Schwarz C, Duftner C, et al. novel senescent regulatory T-cell subset with impaired suppressive function in rheumatoid arthritis. Frontiers in Immunology. 2017;8.
50. Jiao Z, Wang W, Jia R, Li J, You H, Chen L, et al. Accumulation of FoxP3-expressing CD4+ CD25+ T cells with distinct chemokine receptors in synovial fluid of patients with active rheumatoid arthritis. Scandinavian journal of rheumatology. 2007;36(6):428-33.
51. Cribbs AP, Kennedy A, Penn H, Read JE, Amjadi P, Green P, et al. Treg cell function in rheumatoid arthritis is compromised by CTLA-4 promoter methylation failing to activate the indoleamine 2, 3-dioxygenase pathway. Arthritis & rheumatology. 2014;66(9):2344-54.
52. de Paz B, Prado C, Alperi-López M, Ballina-García FJ, Rodriguez-Carrio J, López P, et al. Effects of glucocorticoid treatment on CD25− FOXP3+ population and cytokine-producing cells in rheumatoid arthritis. Rheumatology. 2012;51(7):1198-207.