$CD4^+$ $CD25^+$ $CD127^{low}$ Regulatory T Cells as Indicator of Rheumatoid Arthritis Disease Activity

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by disturbed immune regulation, inducing a progressive cartilage and bone destruction. Despite enrichment of T regulatory cell (T-regs) in synovial fluid, conflicting results are reported concerning T-regs in peripheral blood (PB) of RA patients. To determine possible correlation between the frequency of PB $CD4^+$ $CD25^+CD127^{low}$ (T-regs) with RA disease activity. Forty females with RA, classified according to the Disease Activity Score 28 (DAS-28), as highly active, mild-moderate or low disease activity; and 20 age and sex matched healthy controls, were enrolled to study $CD4^+$ $CD25^+$ $CD127^{low}$ T- regs in PB by flow cytometry. Active RA patients had lower frequency of the $CD4^+$ $CD25^+$ $CD127^{low}$ T- regs compared to those with mild-moderate or low disease activity (P <0.001). The frequencies of the T- regs showed negative correlation with the DAS-28 (*P*<0.01). In conclusion, $CD4^+$ $CD25^+$ $CD127^{low}$ T-regs is significantly lower in highly active RA patients compared to patients with lower activity or controls.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammatory cell infiltration, synovial cell proliferation, cartilage and bone destruction with extra-articular manifestations including cardiovascular, pulmonary, psychological and skeletal disorders (McInnes and Schett 2011). The breaking of self-tolerance is a hallmark of rheumatoid arthritis leading to the production of auto-antibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (CCP) (Alunno *et al.,* 2015)**.** During the development of the disease, a wide variety of cells, including B cells, macrophages, dendritic cells (DCs), neutrophils, fibroblasts, and granulocytes infiltrate into relatively a vascular synovium. These cells release pro-inflammatory cytokines, such as tumor necrosis factor α(TNF-α), Interleukin 1 (IL-1), IL-2, IL-6, IL-15 and IL-23, that contribute to the activation of macrophages, synoviocytes, chondrocytes and osteoclasts; and stimulation of cell proliferation (Sakaguchi *et al.,* 2008). In contrast to these inflammatory cells, naturally occurring regulatory T-cells (n T regs), are shown to play an essential role in establishing the balance between pro and anti-inflammatory mechanisms in the periphery and maintaining self-tolerance. Such n Tregs typically are described as CD4+ T cells with high levels of CD25. Also, n Tregs demonstrate low expression of CD127 in combination with CD25 expression which distinguishes them from activated CD4+CD25+CD127+ conventional effector T cells (Ciebiada *et al.,* 2013). The ability of Tregs to suppress T-cell responses, and thereby to regulate immune reactions, ascribes to them a key role in the pathophysiology of autoimmune diseases and makes them an interesting target for treatment (Moradi *et al.,* 2014). T regs have the capacity to actively block immune responses, inflammation and tissue destruction by suppressing the functions of various cell types and processes, including classical T helper cells (T H), B-cell antibody production, affinity maturation, CD8+ Cytotoxic T Lymphocyte (CTL) granule release ,Antigen

Presenting Cell (APC) function and maturation state (Mempel *et al.,* 2006). Tregs mediate these functions mainly by 4 mechanisms including; 1) production of suppressive cytokines, 2) direct cytolytic activity, 3) cytokine (IL-2) deprivation, and 4) cell contact-induced cell modulation (Tang & Bluestone, 2008). Data about the frequency of T regs in peripheral blood of RA vary throughout the literature, in which it is described as decreased (Kawashiri et al. 2011). Similar (Möttönen *et al.,* 2005), and increased (Han *et al.,* 2008), compared to healthy controls. This controversy is partly due to the lack of one specific T reg marker and the resulting differences in the identification of T regs. CD4+ CD25+ FoxP3+ is accepted as the most reliable phenotype of T reg cells. Moreover, FoxP3 is an intracellular molecule, detection of which requires fixation and permeabilization of cells and the fixed cells cannot be used in studies of T reg function. Intracellular FoxP3 staining is hardly usable on a daily clinical routine basis in large series of samples (several steps of incubation, washing, long incubation times and use of isotype control) (Sakaguchi et al 2010). CD4+ $CD25^+$ $CD127^{low}$ isolated Tregs showed the best reached T reg population regarding purity, function, stability and in vitro expansion capacity, promising isolation of pure T reg populations with high suppressive functionality (Ukena *et al.,* 2011). The proportion of CD4+CD25+$CD127^{low}$Treg in the peripheral blood of patients with RA may be normal, however functionally they are unable to regulate Interferons γ (IFN -γ) and TNF-α production and display an inability to suppress the highly arthritogenic cytokines (McGovern, 2012). Rebuilding the balance by boosting the number and/or function of Treg cells attracted considerable attention as potential treatment of autoimmune diseases (Zhang *et al.,* 2013).

Patients and Methods

Forty RA female patients recruited from inpatient and outpatient clinics of the Internal Medicine Department-Rheumatology division AL-Azher University Hospital fulfilling the new 2010 EULAR/ACR criteria (Aletaha *et al.,* 2010).Twenty subjects of the same age and sex without rheumatic disease or other autoimmune diseases were selected as apparently healthy control subjects. An oral consent was taken from each patient and healthy volunteers after explaining the purpose of the study which was approved by the local faculty ethics committee. Patients with other autoimmune disorders affecting T reg as SLE, Behcet disease, myasthenia gravis, bronchial asthma, chronic liver diseases and type 1 diabetes or those receiving any biologic therapy were excluded. All patients were free of infection, malignant diseases, cardiovascular complaints or other inflammatory diseases. Pain was assessed by a 0–100 mm horizontal visual analogue scale (VAS) (McCormack *et al.,* 1988). Measurement of the 28-joint count of tender and swollen joint with calculation of the disease activity score (DAS-28) for each RA patient was done. Patients with score $\leqslant$3.2 were classified as having low disease activity, >3.2 to $\leqslant$5.1 were classified as having moderately active disease while >5.1 denoted highly active disease (Prevoo *et al.,* 1995).

Patients were divided into 3 groups according to disease severity estimated by the DAS-28 score.

Group 1: Eleven patients with severe activity (DAS score >5.1). Group 2*:* Eleven patients having mild to moderate severity (DAS score from 3.2 to 5.1) Group 3*:* Eighteen patients having low disease activity (DAS score <3.2). All of them were compared to twenty healthy control volunteers.

All Patients groups were subjected to the following:

1- Proper history including age, marital status, smoking habit, disease duration and drug used.

2- Laboratory investigations including: Complete blood picture (Hb, TLC and platelets count were determined on Sysmex KX-21N (Sysmex, Japan),), Alanine transaminase (ALT), Aspartate transaminase (AST), serum urea and creatinine were determined on Cobas C 311 from Roche. ESR was done by Westergreen method, assessment of CRP and RF by latex agglutination test, Anti CCP was assessed by enzyme quantitative immunoassay using INOVA Diagnostic, Inc (QUANTA Lite CCP3 IgG ELISA 704535) on ELISA system (Reader A_3 1851 & Washer 909) from das (Italy).

T reg assessment was done by flow cytometry.

Sample collection

Eight ml of blood were collected aseptically from each subject, each sample was divided in to 3 parts: 5ml were dispended in 2 sterile tubes containing EDTA one for Complete Blood Count (CBC) and erythrocyte sedimentation rate (ESR) and the other was sent immediately for the lab for flow cytometric analysis of T regs, and the remaining part was centrifuged at 200xg for 10 minutes and serum was obtained for assessment of liver and kidney functions as well as C-reactive protein (CRP), RF and anti CCP.

Flowcytometric analysis of Tregs

It was done on EPICSXL flow cytometry using system 11version 3software with a standard 3- color filter configuration using R&D system kits (Minneapolis-Minnesota-USA), (carboxyluorescien (CFS) conjugated monoclonal anti human CD4 (lot number LKNO4) phycoerythrin (PE) conjugated monoclonal antihuman CD25 (lot number LLBo4), peridininchiorophyll protein complex (percp) conjugated antihuman CD127 (lot number ABUWO2).Lymphocytes were gated via their forward and side scatter properties then CD4+ T cells were identified based on their expression of CD4. The gated CD4+ T cells were assessed for both CD25 expression where CD4+, CD25 high T cells were discriminated from CD4+ CD25 dim T cells. Finally CD4+, CD25 high T cells were assessed for CD127 low cells. Treg cells were expressed as a percent of CD4+ T cells.

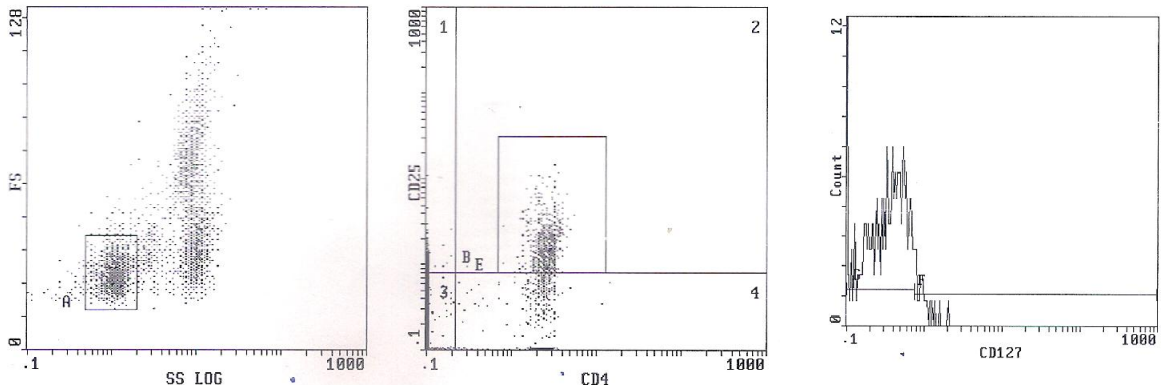

Figure 1$_{a.}$ A flow histogram of a patient with active RA.

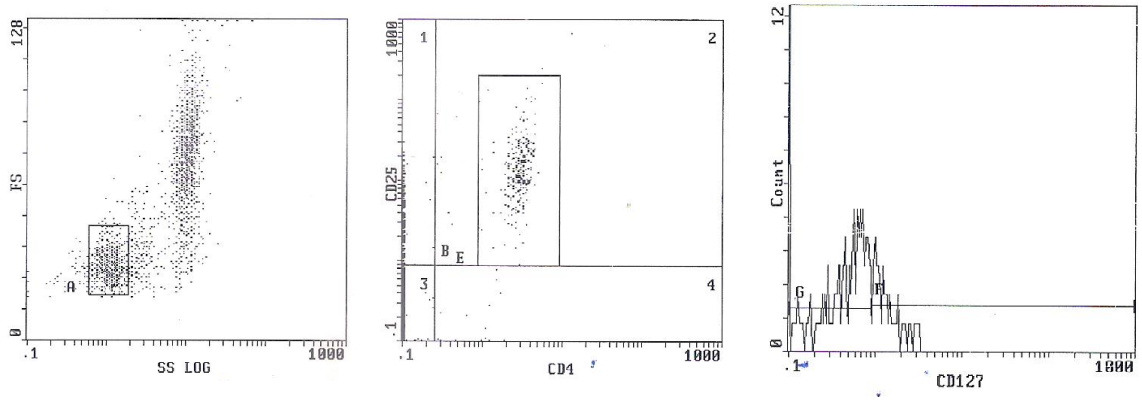

Figure 1$_{b.}$ A flow histogram of RA patient with moderate activity.

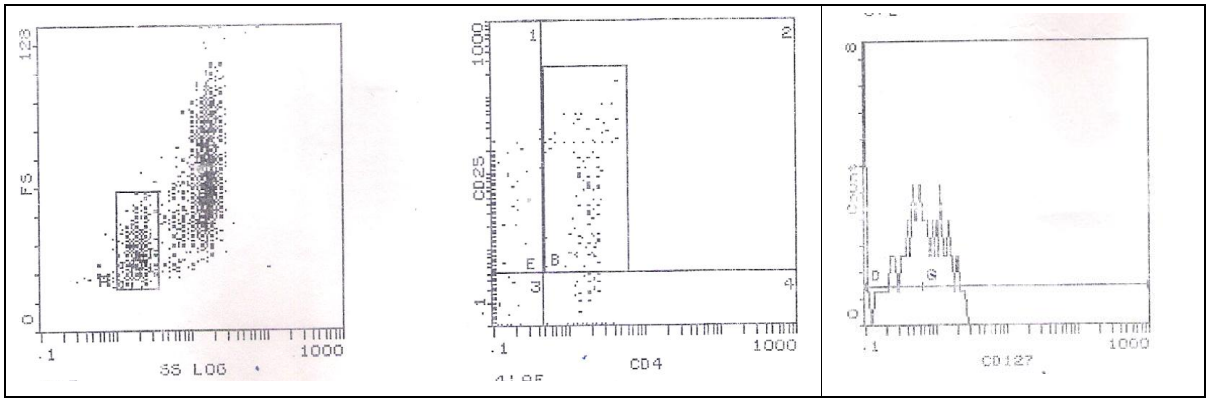

Figure 1c. A flow histogram of RA patient with low disease activity

Figure (1 a, b c). The percentage of lymphocytes is represented as A region. The percentage of CD4+ represented as the sum of (B2 + B4). Region CD4+CD25 high represented as E region. The percentage of CD4+ CD25 high CD127 low represented as G region.

Statistical Analysis

Results were collected, tabulated, and statistically analyzed by personal computer and statistical package SPSS version 10 Chicago, USA).Two types of statistics were performed: Descriptive statistics - for example, mean *(X)* and SD - and analytic statistics. Quantitative variables were compared using unpaired t-test and one way ANOVA test. Qualitative variables were compared using Chi square and test of proportion. Spearman correlation coefficient (r) is a test used to measure the association between two quantitative variables. The P value of less than 0.05 was considered as statistically significant.

Results

A total of 40 female patients with established RA were included in the study. The mean ± (SD) of age is 51±8.3 years and disease duration of 6±3.02 years. Their DAS-28 score was 6.46±0.6. VAS pain score was 30.22 ±22.Twenty age and sex matched healthy subjects were also included in the study and served as the control group. The descriptive data of the patients and controls are shown in (Table 1).

To study peripheral blood regulatory T cells (T reg) in RA patients and to test their link to the degree of activity; peripheral blood cells were examined from RA subjects with different disease activity score-28 (DAS-28) and compared to healthy controls (Table 2).

There was significant increase in DAS score in patients having active disease (6.464±0.645) and who have mild to moderate activity (3.836±0.798) when compared to patients with low disease activity (1.867±0.423), P <0.001 (Table 2). There was no significant difference in the Treg % between patients and controls (5.418± 3.9 and 6.115 ±3.4, respectively) P >0.05. But among the populations with RA; Treg cell frequency was lowest in patients with highly active RA (2.303±1.331). In contrast, the T reg cells frequency of patients with low disease activity (6.031±3.91) was similar to that of healthy controls (6.115±3.43) (Table 2). There was a significant increase in C-reactive protein (CRP) of RA patients compared to control. Also, there was a significant increase in CRP in patients having highly active disease and who have mild to moderate activity when compared to patients with low disease activity, P<0.001 (Table 2). There was a significant increase in erythrocyte sedimentation rate (ESR) of patients compared to control. Also, there was a significant increase in ESR in patients having

marked disease activity and who have mild to moderate activity when compared to patients with low disease activity, P <0.001 (Table 2). Comparison between patients and controls as regard anti-cyclic citrullinated peptide (Anti CCP) anti bodies revealed significant increase in Anti CCP antibodies in patients having active disease and who have mild to moderate activity when compared to patients with low disease activity, P <0.001 (Table 2). There was no significant difference between patients and controls regarding total leucocytic count (TLC), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), urea and creatinine, P >0.05. There was a statistically significant decrease in haemoglobin level in RA patients compared to control, P <0.01 (Table 1).

Table 1. Laboratory data of rheumatoid arthritis (RA) patients and controls

Variables	(RA) patients Mean ±(SD)	Controls Mean ±(SD)	*P* value
TLC 103/Cmm)	8.108±3.46	7.675± 2.158	NS
Hemoglobin (gm/dl)	10.898±0.99	11.520±0.648	< 0.01
platelets(103/cmm)	287.100±90.3	285.000 ± 87.233	NS
ALT(U/L)	20.5±9.95	17.950± 7.904	NS
AST(U/L)	21.5±9.36	17.350 ±5.556	NS
Urea (mg/dl)	23.350±8.7	27.650±8.928	NS
Creatinine (mg/dl)	0.708±0.22	0.845 ± 0.332	NS
ESR 1st hour (mm)	43.0±33.5	10.5 ±4.774	<0.001
CRP (mg/l)	21.65±15.3	4.2±2.7	<0.001
Anti CCP (unit)	285.797±278.52	15.250	< 0.001
Tregulatory cells (Treg %)	5.418±3.918	6.115± 3.434	NS

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, DAS28: disease activity score in 28 joints,
Anti-CCP: cyclic citrullinatedce peptide antibody; TLC: total leucocytic count, ALT: alanine aminotransferase, AST: aspartate aminotransferase. *P*>0.05 is not significant (NS)

Table 2. Comparison between patient groups and control regarding clinical and laboratory parameters (mean ±SD).

Variables mean± SD	High activity	Mild-moderate activity	Low activity	Controls	*P* value
DAS(28)	6.464±0.645	3.836±0.798	1.867±0.423		<0.001
TLC(103/Cmm)	8.982± 4.382	7.609 ±1.861	7.878 ±3.670	7.675± 2.158	NS
Hemoglobin (gm/dl)	10.527 ±0.837	10.927 ±1.09	11.106 ±1.01	11.520 ±0.648	<0. 05
platelets.(103/cmm)	304.000 ±74.158	278.273 ± 101.678	282.167±95.609	285.000±87.233	NS
CRP (mg/l)	32.1±15.0	21.5±3.1	11.0 ± 5.1	4.2 ±2.7	<0.001
ESR (mm/hr)	87.818 ±17.792	48.273± 7.458	12.444±7.040	10.500 ±4.774	<0.001
Anticcp (unit)	35.545(mean rank)	34.0	24.222	15.250	<0.001
Treg %.	2.303 ±1.331	7.528 ±3.982	6.031 ±3.910	6.115±3.434	<0. 05

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, Anti-CCP: cyclic-citrullinated peptide antibody DAS28: disease activity score in 28 joints, TLC: total leucocytic count. *P*>0.05 is not significant (NS)

We investigated the correlation between the frequency of peripheral blood Treg cells and the markers of disease activity such as CRP, ESR and DAS28 in the RA patients (Table 3). The frequencies of CD4+ CD25+ CD127low/- (Treg) cells were not correlated with CRP or ESR. However there was a significant negative correlation between T-reg percentage and DAS in all stages (P<0.01) (Figure 2). T-reg cell percentages did not correlate with any laboratory tests in RA patients at any stage of activity (Table 3). There was a positive significant correlation between ESR and CRP and DAS (P<0.001) in RA patients, but there was no significant correlation between ESR and Anti-CCP (Table 4).

Table 3. Correlations between Tregs and different clinical and laboratory parameters in RA patients

Correlations		
	T regulatory cells	
	r	*P*-value
CRP	-0.241	NS
ESR	-0.295	NS
Anti- CCP	-0.060	NS
ALT	-0.270	NS
AST	-0.097	NS
Urea	-0.044	NS
Creatinine	-0.211	NS
WBC	-0.095	NS
Hb	0.135	NS
Platelets	-0.034	NS
DAS	-0.348	0.028

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, Anti-CCP: cyclic Citrullinated Peptide Antibody.

DAS28: disease activity score in 28 joints, TLC: total leucocytic count. ALT: alanine aminotransferase , AST: aspartate aminotransferase.

P>0.05 is not significant (NS)

Table 4. Correlations between ESR, CRP, Anti-CCP and DAS in RA patients

	ESR	
	r	*P*-value
CRP	0.817	<0.001
Anti-CCP	-0.112	NS
DAS	0.949	<0.001

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, Anti-CCP: Cyclic Citrullinated Peptide Antibody DAS-28: disease activity score in 28 joints.

P>0.05 is not significant (NS)

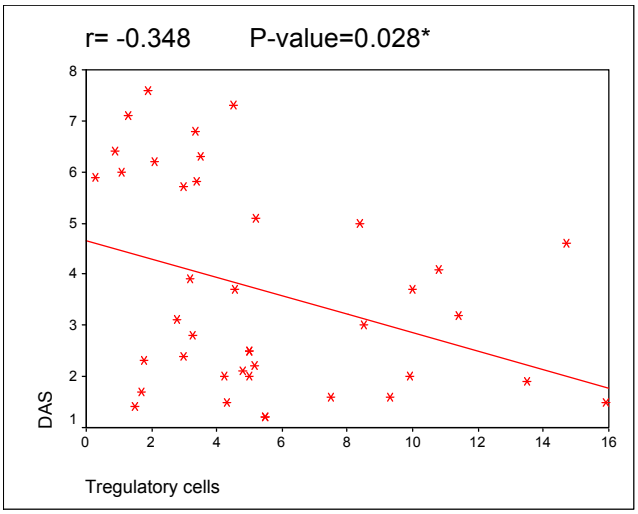

Figure 1. Correlation between Tregs and DAS.

Discussion

In this study the frequency of nTreg cells in peripheral blood of highly active RA group were significantly decreased when compared with healthy controls and patients with low disease activity P<0.05. Data reported by Al-Zifzaf *et al.,* (2015) showed that the distribution of FoxP3+CD4+CD25+high cells (Tregs) revealed a highly significant decrease in the frequency of T reg cells in RA patients compared to healthy controls. They suggested

that regulatory cells are recruited to sites of inflammation in an attempt to suppress disease, resulting in a relative reduction in the peripheral blood population. T regs may contribute to the pathogenesis of RA and may be an indicator of disease activity. Regulatory T cells could be an ideal target for therapies to induce durable remission of autoimmune and inflammatory disease such as rheumatoid arthritis. Enhancing the activity and increasing T reg cells numbers would be beneficial for inhibition of pathologic inflammation without blocking protective immune responses (Haque *et al.,* 2014).

Li *et al.,* (2014) found that the frequency of Tregs in active RA patients was decreased. They also found that the level of serum IL-10 was significantly lower in active RA group than in healthy controls and the addition of exogenous IL-10 inhibited T regs apoptosis in active RA patients. Their data described a novel role for IL-10 in RA via down regulation of T reg apoptosis, providing a potential approach to treat RA patients via modulate the serum IL-10. In contrary, Möttönen *et al* (2005) studied Treg in RA patients and found no deference between RA patients and control subjects.

The American College of Rheumatology 2008 and 2012 recommendations regarding treatment decisions using of biologic and non-biologic disease-modifying anti-rheumatic drugs (DMARDs) have included the DAS28 as one of the preferred outcome measures for its good psychometric properties (reliability, validity, responsiveness) and feasibility of using in clinical assessment .In this study there was a negative correlation between Tregs and DAS in all groups, but there was a lack of correlation between Treg percentages in the blood and all laboratory tests. However; *Kawashiri et al* (2011) found that the frequency of CD4+CD25high-CD127low/- T cells was negatively correlated with CRP, ESR, and DAS28, respectively. Anti cylic-citrullinated peptide antibodies (Anti-CCP) are the most specific known marker for RA diagnosis in adults. Anti-CCP develops several years before the first clinical manifestation of arthritis and is highly predictive of progression from undifferentiated arthritis to definite RA (van Venrooij et al 2011).

In the current study; there was a significant increase in Anti-CCP antibodies in RA patients compared to controls (P<.001), but there was no significant difference between the three groups of RA patients. Thus, the presence of Anti-CCP, but not its titer, is a predictor of disease activity. This was previously reported by Humphreys *et al.,* (2014) who found that presence of Anti-CCP, but not its titer, at baseline is a predictor of disease activity. In this study there was a positive significant correlation between ESR, CRP and DAS28 in RA patients. Wells *et al.,* (2009) compared DAS28-CRP with DAS28-ESR. They concluded that while the DAS28-CRP yielded a better EULAR response more often than the DAS28-ESR, the validation profile was similar to the DAS28-ESR, indicating that both measures are useful when assessing disease activity in patients with RA.

In conclusion, Lower abundance of T regulatory cells in peripheral blood of active rheumatoid arthritis patients highlight the significance of these cells in the pathogenesis of rheumatoid arthritis and can be used an indicator of disease activity.

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