

A Flowcytometry Study of Complement Regulatory Proteins Expression on Peripheral Blood Cells in Rheumatoid Arthritis Patients

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Abstract: Background: Inappropriate activation or blockage of inhibition of the complement system could cause tissue damage in autoimmune diseases particularly rheumatoid arthritis (RA). CD55 and CD59 are proteins with complement regulatory (Creg) properties that ensure cell and tissue integrity when this system is activated. **Objective:** The aim of this study was to evaluate the expression of CD55 and CD59 complement regulatory proteins on peripheral blood cells of RA patients and its association with disease activity. **Subjects and Methods:** Seventy RA patients clinically diagnosed and classified as RA according to the American College Of Rheumatology/European Leagues against Rheumatism (ACR/EULAR) revised criteria for the classification of RA (2010), were included in our study with mean age of 40.32±2.27 years, including 58 (82.9 %) females and 12 males (17.1 %). They were attending the Department of Rheumatology and Rehabilitation, Faculty of Medicine, Sohag University during the period from June 2012 to August 2013. The clinical parameters of disease activity were determined, including the 28-joint disease activity score (DAS28), C-reactive protein (CRP), and rheumatoid factor (RF) levels. The patients were subdivided into active disease group (n=50) with DAS28 score higher than 5.1 (Group I); and remission group (n=20) with DAS28 score less than 2.6 (Group II). Twenty healthy individuals with mean age 33.44±7.09 years, including 14 (70%) females and 6 males (30%) were randomly selected as the control group (Group III). Flowcytometric analyses of expression of CD55 and CD59 complement regulatory proteins on erythrocytes, T lymphocytes, B lymphocytes, and neutrophils of all the study population were performed. The correlations between the expression of CD55 and CD59 complement regulatory proteins on peripheral blood cells and disease activity parameters of patients with RA were determined. **Results:** In RA patients, CD55 and CD59 were significantly decreased on red blood cells in comparison to control group. The mean fluorescence intensity (MFI) of CD55 and CD59 on RBCs were highly significantly lower in RA patients both in active and in remission stage of the disease than those of healthy controls ($p < 0.01$). As MFI for CD55 on RBCs was 9.07 ± 4.05 arbitrary units for patients and 21.33 ± 5.87 for healthy group. CD59 MFI was 28.87 ± 7.40 in patient group and 47.4 ± 7.41 in healthy group. The MFI of CD55 on neutrophils was significantly lower ($p < 0.05$) in RA patients in active stage of the disease and was highly significantly ($p < 0.01$) lower in remission stage of the disease than those of healthy controls. As MFI of CD55 on neutrophils was 85.63 ±13.02 arbitrary units for patients and 93.44 ± 6.65 for healthy group. CD59 MFI was 78.79 ± 12.29 in patient group and 78.94 ± 6.16 in healthy group. The MFI of CD55 and CD59 on B Lymphocytes were significantly lower in RA patients in remission stage of the disease than those of healthy controls. As MFI of CD55 on B Lymphocytes was 3.87 ± 2.99 arbitrary units for patients and 4.94 ± 2.76 for healthy group. CD59 MFI was 2.05 ± 1.62 in patient group and 2.78 ±1.45 in healthy group. Only the MFI of CD59 on T lymphocytes was significantly lower ($p < 0.05$) in RA patients in active stage of the disease. As MFI of CD55 on T Lymphocytes was 20.22 ± 6.36 arbitrary units for patients and 23.27 ± 4.66 for healthy group. CD59 MFI was 21.93 ± 5.42 in patient group and 26.16 ± 4.60 in healthy group. In addition, a significant positive correlation between CD55 and CD59 expression on the patients' peripheral blood cells and the disease activity was found; as besides confirming the decreased expression of CD55 and CD59, it was demonstrated that the higher the disease activity, the lower their expression on peripheral blood cells. **Conclusion:** The expression of CD55 and CD59 is down-regulated on peripheral blood cells of patients with RA; which may contribute to the pathogenesis of RA and it can be an indicator of disease activity and help in patients' follow-up.

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1.Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by progressively destructive joint inflammation, destruction of

articular cartilage and bone and synovial hyperplasia (*Kim et al., 2007*). Rheumatoid arthritis affects approximately 1% of the world population, being two to three times more common in women (*Szabo-*

Taylor et al., 2012). Inappropriate activation or blockage of inhibition of the complement system could cause tissue damage in rheumatoid arthritis (RA) (*Pourazar et al., 2005*). The complement system represents the first defense line of innate immunity; it acts facilitating the phagocytosis of immune complexes, pathogens, and apoptotic cells and forming the membrane attack complex (MAC), resulting in cell lysis (*Zipfel, 2009*). Normal cell membranes express complement regulatory (Creg) proteins that regulate activation of the complement system and provide essential protection against self damage (*Ruiz-Arguelles and Llorente, 2007*). The major human cell surface Creg proteins are; CD59 (membrane inhibitor of reactive lysis—MIRL), which is a complement membrane inhibitor that blocks assembly of the MAC by binding to C8 and C9 (*Kimberley et al., 2007*), and CD55 (decay accelerating factor—DAF), which accelerates the disassembly of preformed C3 and C5 convertases (*Christmas et al., 2006*). These Creg proteins are present on the cell surface of whole blood cells. It has been reported that the production and the expression of some of these complement regulatory proteins are altered in autoimmune diseases (*Alegretti et al., 2012*). In this work, we tried to evaluate the expression of CD55 and CD59 on peripheral blood cells of RA patients in comparison to healthy controls using flow cytometry and its correlation with the disease activity.

2. Patients And Methods

Our study was carried out in the Departments of Rheumatology and Rehabilitation, Orthopedic Surgery and Medical Microbiology and Immunology, Faculty of Medicine, Sohag University during the period from June 2012 to August 2013. Patients clinically diagnosed and classified as RA according to the American College Of Rheumatology revised criteria for the classification of RA (*Aletaha et al., 2010*). All patients were evaluated clinically with 28 joints disease activity score (DAS28); depending on number of joint with tenderness and swelling (*Prevo et al., 1995*). A group of 50 approved RA patients (Group I) active disease group with DAS28 score > 5.1; including 10 (20%) males and 40 (80%) females with age range from 25 to 57 years (mean age 40.62±9.33 years). A group of 20 RA patients (Group II) in remission with DAS28 score < 2.6 were included in our study including 2 (10%) males and 18 (90%) females with age range from 33 to 58 years (mean age 45.63±7.69 years). In addition to 20 healthy volunteers (Group III) with no history of RA or other autoimmune diseases were involved in the present study; 14 (70%) females and 6 (30%) males

with age range from 25 to 45 years (mean age 33.44 years).

Exclusion criteria: Patients found to be suffering from osteoarthritis, tubercular arthritis, infective arthritis, rheumatic fever, pulmonary tuberculosis, and any other systemic disease as renal disease, diabetes mellitus, hepatic diseases, hypertension, or ischemic heart disease. All candidates were subjected to:

- Complete history taking; personal history, family history and history of autoimmune diseases.
- Full clinical evaluation and orthopedic examination for signs of deformity.
- The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF) levels were determined using standard laboratory methods.
- Blood samples were collected intravenously from all the study population for Flow Cytometric analysis of CD55 and CD59 on Peripheral Blood Cells; Blood was collected in glass tubes containing EDTA in order to prepare erythrocytes, lymphocytes and neutrophils for flow cytometry running. The subpopulations analyzed were
 - (1) CD3 (T lymphocytes): CD3/CD55, CD3/CD59.
 - (2) CD19 (B lymphocytes): CD19/CD55, CD19/CD59.
 - (3) neutrophils CD55, neutrophils CD59.
 - (4) RBCs CD55, RBCs CD59.
- CD3-PC5 {phycoerythrin-cyanin 5 (PE–CyTM5)}, CD19-ECD {energy coupled dye (ECD)}, CD55-PE {phycoerythrin (PE)}, and CD59-FITC {Fluorescein isothiocyanate (FITC)} conjugated antibodies were used purchased from *Beckman Coulter, France*.
- **For red blood cells (RBCs) staining**, 100 uL of diluted blood (with an optimal dilution with phosphate-buffered saline (PBS) to achieve 10000 RBC/uL) were placed into polystyrene tubes (*Beckman Coulter, France*) and subjected to two-colour staining with 10 uL/test of fluorochrome-conjugated monoclonal antibodies (MoAbs) antiCD55PE and anti CD59FITC antibodies (*Beckman Coulter, France*). After 20 min incubation at room temperature in the dark, samples were resuspended in 1 mL of PBS. After 20 min incubation at room temperature in the dark, cells were analysed by the flow cytometer.
- **For neutrophils, B and T lymphocytes staining**, 100 uL of whole blood were placed into polystyrene tubes and were subjected to four-colour staining with 10 uL of each of fluorochrome-conjugated MoAbs; anti CD55PE, antiCD59FITC, antiCD3PC5 and antiCD19ECD

antibodies (**Beckman Coulter, France**). After 20 min incubation at room temperature in the dark, 1.0 mL of Versalyse™ (**Beckman Coulter, France**) was added and lysis was allowed for 10 min at room temperature in the dark.

Cells were analysed and 10000 events were acquired by **Beckman Coulter Epics-XL flow cytometer** using System II software version 3.0 (**Coulter, USA**). Membrane intensity of CD55, CD59, CD3, and CD19, which is proportional to the number of CD55, CD59, CD3 and CD19 epitopes on the membrane, was estimated in the gated subpopulations by two-parameter histograms, and the relative mean fluorescence intensity (MFI) of each sample indicated the amount of MoAb bound to CD59 or CD55 expressed on peripheral blood cells was recorded.

Statistical Analysis

Statistical differences were determined by using analysis of variance (ANOVA) and Student's t-test. Results were expressed as mean \pm standard deviation

of the mean (SD). Data was analyzed using STATA intercooled version 9.2. Qualitative data was compared using either Chi square test or fisher exact test. The probability (*P value*) was considered significant if *p value* was < 0.05 , highly significant if *p value* was < 0.01 and *P value* < 0.001 , and insignificant if *p value* > 0.05 . The relationship between the studied measures was assayed by Pearson's linear correlation coefficient.

3.Results

To study the CD55 and CD59 expression on the erythrocytes, neutrophils, B lymphocytes and T lymphocytes in RA patients and normal healthy people, the specific MoAbs and flowcytometry technology was applied. Cell Quest software was used and the histogram resultant analysis of 1×10^4 cells were obtained and the mean fluorescence intensity (MFI) for each independent analysis is shown in the representative graph (Fig. 1).

Table 1: Descriptive data of subjects included in the study.

	Active disease group (50 patients) (Group I)	patients in remission (20 patients) (Group II)	Healthy control group (20) (Group III)
Age			
Mean (\pm SD)	40.62 \pm 9.33	45.36 \pm 7.69	33.44 \pm 7.09
Median (range)	40 (25-57)	45 (33-58)	33 (25-45)
Sex			
Females	40 (80%)	18 (90%)	14 (70%)
Males	10 (20%)	2 (10%)	6 (30%)

Table 2: Clinical and laboratory features of RA patients.

	Active disease group	patients in remission
Duration of the disease		
Mean (\pm SD)	5.82 \pm 5.01	10.27 \pm 3.95
Median (range)	4 (0.3-20)	12 (4-15)
Rheumatoid Factor		
Positive	42 (84%)	20 (100%)
Negative	8 (16%)	0 (0.00)
ESR		
Mean (\pm SD)	48.90 (20.04)	23.27 (3.32)
Median (range)	45 (25-97)	25 (17-29)
DAS28*		
Active	18 (36%)	0 (0.00)
Medium	20 (40%)	0 (0.00)
Mild	12 (24%)	0(0.00)
Remission	0 (0.00)	20 (100%)

*DAS28; the 28-joint Disease Activity Score

Results of flowcytometry analysis of the expression of CD55 and CD59 complement regulatory proteins on peripheral blood cells of RA patients:

Table 3: The mean fluorescence intensity (MFI) of CD55 and CD59 on peripheral blood cells of RA patients with active disease and controls.

	Active disease group (50 patients) (Group I)	Healthy control group (20) (Group III)	P value
RBCs CD55			
Mean (\pm SD)	9.07 (4.05)	21.33 (5.87)	<0.0001
Median (range)	9.27 (3.36-17.9)	22.4 (15.1-30.8)	*HS
RBCs CD59			
Mean (\pm SD)	28.87 (7.40)	47.4 (7.41)	<0.0001
Median (range)	25.5 (18.8-42.6)	43.3 (39.3-57.1)	HS
Neutrophils CD55			
Mean (\pm SD)	85.63 (13.02)	93.44 (6.65)	0.02
Median (range)	90.4 (54.1-96.3)	95.2 (76.8-99.5)	*S
Neutrophils CD59			
Mean (\pm SD)	78.79 (12.29)	78.94 (6.16)	0.48
Median (range)	82.7 (51.7-95.2)	79.3 (67.4-88.3)	*NS
CD19 CD55			
Mean (\pm SD)	3.87 (2.99)	4.94 (2.76)	0.27
Median (range)	3.01 (0.21-10.6)	4.92 (0.34-8.99)	NS
CD19 CD59			
Mean (\pm SD)	2.05 (1.62)	2.78 (1.45)	0.14
Median (range)	1.61 (0.35-6.42)	2.81 (0.24-4.88)	NS
CD3 CD55			
Mean (\pm SD)	20.22 (6.36)	23.27 (4.66)	0.27
Median (range)	20.5 (7.01-30.4)	23.7 (17.6-31.6)	NS
CD3 CD59			
Mean (\pm SD)	21.93 (5.42)	26.16 (4.60)	0.04
Median (range)	22.5 (10.5-33.6)	26.5 (19.9-32.6)	S

Values were expressed as mean and median (range). CD; Cluster of differentiation, CD3; T-lymphocytes, CD19; B-lymphocytes; RBCs; Red blood cells; *NS= not significant.*S: significant, *HS: Highly significant.

Table 4: The mean fluorescence intensity (MFI) of CD55 and CD59 on peripheral blood cells of RA patients in remission and controls.

	Patients in remission (20 patients) (Group II)	Healthy control group (20) (Group III)	P value
RBCs CD55			
Mean (\pm SD)	9.13 (3.11)	21.33 (5.87)	0.0002
Median (range)	10.2 (4.14-13)	22.4 (15.1-30.8)	HS
RBCs CD59			
Mean (\pm SD)	36.17 (3.87)	47.4 (7.41)	0.001
Median (range)	36.2 (30-42.1)	43.3 (39.3-57.1)	HS
Neutrophils CD55			
Mean (\pm SD)	78.1 (16.40)	93.44 (6.65)	0.002
Median (range)	83.7 (37.3-93.8)	95.2 (76.8-99.5)	HS
Neutrophils CD59			
Mean (\pm SD)	75.89 (8.26)	78.94 (6.16)	0.73
Median (range)	80.4 (61.7-84.4)	79.3 (67.4-88.3)	NS
CD19 CD55			
Mean (\pm SD)	2.13 (1.23)	4.94 (2.76)	0.02
Median (range)	1.97 (0.65-4.18)	4.92 (0.34-8.99)	S
CD19 CD59			
Mean (\pm SD)	1.43 (0.74)	2.78 (1.45)	0.03
Median (range)	1.39 (0.46-2.64)	2.81 (0.24-4.88)	S
CD3 CD55			
Mean (SD)	16.63 (8.23)	23.27 (4.66)	0.14
Median (range)	20.7 (6.25-25.4)	23.7 (17.6-31.6)	NS
CD3 CD59			
Mean (\pm SD)	21.86 (7.29)	26.16 (4.60)	0.18
Median (range)	23.7 (11.8-33)	26.5 (19.9-32.6)	NS

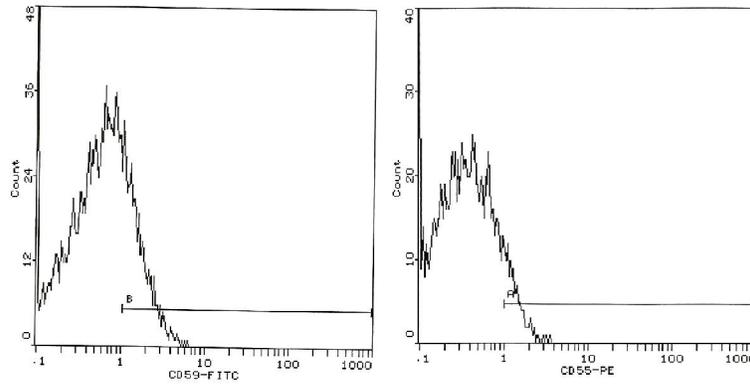


Figure 1 (a)

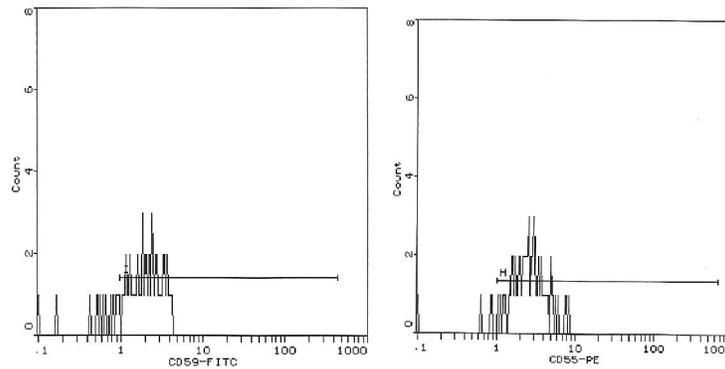


Figure 1(b)

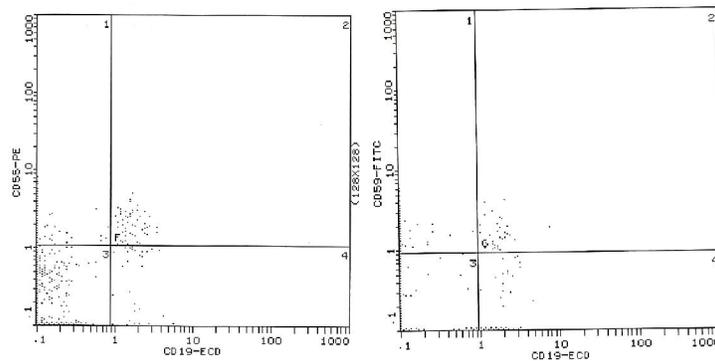


Figure 1(c)

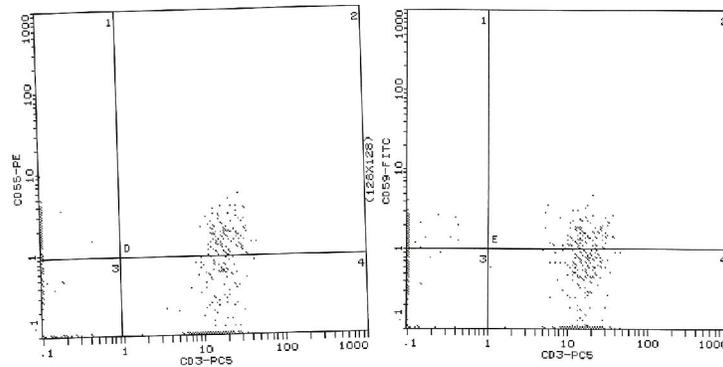


Figure 1(d)

Figure 1: Univariate histograms of CD55 and CD59 expression on (a) RBCs (b) neutrophils (c) B lymphocytes (d) T lymphocytes from the same sample.

- **Red Blood Cell analyses:** The mean fluorescence intensity (MFI) of CD55 and CD59 on RBCs were highly significantly lower in RA patients both in active and in remission stage of the disease than those of healthy controls ($p < 0.01$) (Tables 3 & 4).
- **Neutrophils analyses:** Only the MFI of CD55 on neutrophils was significantly lower ($P < 0.05$) in RA patients in active stage of the disease (Table 3); and was highly significantly ($p < 0.01$) lower in remission stage of the disease than those of healthy controls (Table 4). The MFI of CD59 on neutrophils were lower in RA patients (both in active and in remission stage of the disease) than those of healthy controls; but this difference was statistically insignificant ($P > 0.05$) (Tables 3 & 4).
- **B Lymphocytes analyses:** The MFI of CD55 and CD59 on B Lymphocytes were lower in RA patients than those of healthy controls; but this difference was statistically insignificant ($P > 0.05$) (Table 3). The MFI of CD55 and CD59 on B Lymphocytes were significantly lower in RA patients in remission stage of the disease than those of healthy controls (Table 4).
- **T Lymphocytes analyses:** Only the MFI of CD59 on T Lymphocytes was significantly lower ($P < 0.05$) in RA patients in active stage of the disease (Table 3); The MFI of CD55 and CD59 on T Lymphocytes were lower in RA patients in remission stage of the disease than those of healthy controls; but this difference was statistically insignificant ($P > 0.05$) (Table 4).

- **Correlation between CD55 and CD59 expression on peripheral blood cells in RA patients:**

It was found that there was positive correlation between CD55 and CD59 expression on erythrocytes, neutrophils, B lymphocytes and T lymphocytes in RA patients $P < 0.05$ (Table 5).

Table 5 (a,b,c,d): Correlations between CD55 and CD59 expression on peripheral blood cells in RA patients.

a)

Group		Neu.CD59	
Active	Neu.CD55	Pearson Correlation	0.660
		Sig. (2-tailed)	0.001
Remission	Neu.CD55	Pearson Correlation	0.604
		Sig. (2-tailed)	0.049

b)

Group		CD3CD59	
Active	CD3CD55	Pearson Correlation	.352
		Sig. (2-tailed)	.017
Remission	CD3CD55	Pearson Correlation	.797
		Sig. (2-tailed)	.003

c)

Group		CD19CD59	
Active	CD19CD55	Pearson Correlation	.930
		Sig. (2-tailed)	.000
Remission	CD19CD55	Pearson Correlation	.852
		Sig. (2-tailed)	.001

d)

Group		RBC CD59	
Active	RBC CD55	Pearson Correlation	.757
		Sig. (2-tailed)	.000
Remission	RBC CD55	Pearson Correlation	.425
		Sig. (2-tailed)	.012

- **The correlation between the disease activity (measured by DAS-28) and the expression of CD55 and CD59 on peripheral blood cells in RA patients:**

We found that; the MFI of CD55 and CD59 was inversely related with DAS-28 values in all cell types; the more the disease activity the lower the expression of CD55 and CD59 on peripheral blood cells. However, this correlation was only significant in RBCs (highly significant in RBCs CD59; P value < 0.001) (Table 6).

Table 6: The correlation between disease activity and the expression of CD55 and CD59 on peripheral blood cells in RA patients.

		DAS-28
CD3CD55	Pearson Correlation	-0.052
	Sig. (2-tailed)	0.747
CD3CD59	Pearson Correlation	-0.210
	Sig. (2-tailed)	0.188
CD19CD55	Pearson Correlation	-0.178
	Sig. (2-tailed)	0.266
CD19CD59	Pearson Correlation	-0.251
	Sig. (2-tailed)	0.114
Neu.CD55	Pearson Correlation	-0.098
	Sig. (2-tailed)	0.541
Neu.CD59	Pearson Correlation	-0.066
	Sig. (2-tailed)	0.684
RBCs CD55	Pearson Correlation	-0.426
	Sig. (2-tailed)	0.006
RBCs CD59	Pearson Correlation	-0.535
	Sig. (2-tailed)	0.000

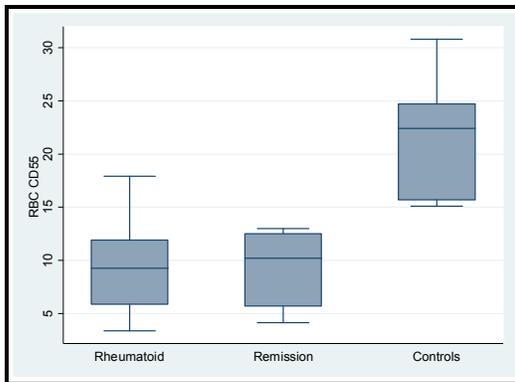


Figure 2(a)

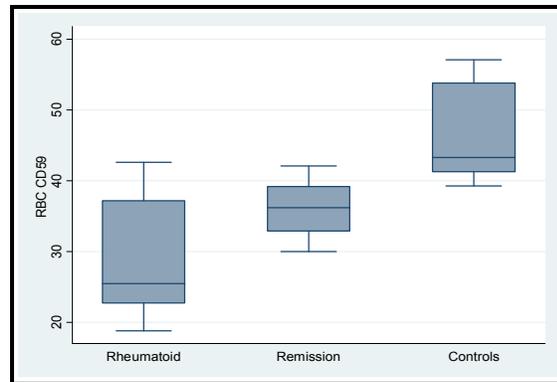


Figure 2(b)

Figure 2 (a & b): CD55 and CD59 expression of RBCs. The figure displays mean fluorescence intensity (MFI) of CD55 and CD59 on gated RBCs from RA patients both in active and remission stages of the disease and controls. *Significant statistical difference ($P < 0.05$).

4. Discussion

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by articular inflammation eventually; leading to joint destruction. Although the mechanism of RA pathogenesis is not fully understood, humoral and cellular immunity are known to be involved (Kim *et al.*, 2013). The identification and diagnosis of RA early in the disease course is very important, because early, intensive

treatment has been demonstrated to prevent joint damage, to preserve joint function, and to improve the work participation of patients (Grigor *et al.*, 2004). Several studies showing an association between complement activation and inflammatory responses in the diseased joints of RA patients or in individual cell types (Walport, 2001). The complement system is considered to be an important system in the control of infectious agents, with the ability to act as an effector

molecule to lyse many microorganisms and cells. In autoimmune diseases like RA, the complement system may become activated under inappropriate circumstances with different pathways and cause self-tissue damages (Davies, 1991). To prevent complement-mediated injury, normal cells have regulatory mechanisms constituted by proteins categorized into two major classes: soluble in biological fluids, such as properdin and factor H; and cell-membrane-anchored proteins, such as CD55 (or decay-accelerating factor – DAF), CD59 (or membrane inhibitor of reactive lysis – MIRL), CD46 (or membrane cofactor protein – MCP), and CD35 (or complement receptor type 1 – CR1) (Piccoli et al., 2011). The pattern of CRP expression in RA patients has not been well established. Defining that pattern of expression is important to assess its potential meaning in the development of the inflammatory process in these patients. It has been reported that the production and the expression of some of these complement regulatory proteins are altered in autoimmune disorders (Alahlafi et al., 2005). In this study; we performed a flowcytometry study of complement regulatory proteins CD55 and CD59 expression on peripheral blood cells in RA patients. Our study revealed significantly lower Creg proteins CD55 and CD59 expression on blood cells; erythrocytes, neutrophils, B lymphocytes and T lymphocytes from RA patients when compared with healthy controls, more marked in cases associated with higher disease activity. Although there were a few publications evaluating some of the Creg proteins in specific blood cells in RA patients, our study is one of few publications evaluating some of the membrane-bound Creg proteins in most peripheral blood cells in RA patients. This allows a clear view of the expression profile of these proteins and their relations with disease activity. We found that; the MFI of CD55 and CD59 was inversely related with DAS-28 values in all cell types investigated; erythrocytes, neutrophils, B lymphocytes and T lymphocytes. However, this correlation was only significant in RBCs (highly significant in RBCs CD59). These results agreed with those of Pourazar et al. (2005) who reported that; the expression of CD55 and CD59 is down-regulated on erythrocytes of patients with RA. Also, our results agreed with those of Arora et al. (1998) who reported that there was an inverse relationship between the relatively deficient CD59 expression on erythrocytes and in vitro complement activation. Previous analyses of CRP expression in the rheumatoid synovium have revealed an increase in CD55 and a decrease in CD59 compared to non-inflamed synovium. These findings suggest that CD59 can be the key to synovial membrane protection, and its lost could be associated with a higher susceptibility to damage by MAC

(Piccoli et al., 2011). The present study not only show the alteration in the molecule expression, but concluding correlation with the disease activity which can be used as a useful marker to detect active RA and disease progression in patients with RA. These results may be useful in the management of new modalities of therapy for the treatment of RA. Genetic engineering of complement regulators to increase their inhibitory activity seems feasible, and gene therapy might also be an option in the future (Huang et al., 2005) as an important key in treating such inflammatory autoimmune diseases.

Summary and Conclusion

It was evident that there were differences in the pattern of expression of Creg proteins on the peripheral blood cells in RA patients; there were diminished expression of CD55 and CD59 Creg proteins on erythrocytes, neutrophils, B lymphocytes and T lymphocytes. Moreover, these differences correlate with the disease activity and so, contribute to the pathogenesis of RA. And these levels may be used as a diagnostic tool in follow-up of the disease progression. Deeper understanding of these processes, and the role of Cregs, could be important for the development of novel therapies for the complement involvement in RA and other autoimmune-mediated diseases.

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