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Relationship between CD69/CD3^{+} Ratio of activated T-cells and some immunological parameters for SLE patients

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Abstract

Systemic lupus erythematosus **(SLE**) considered as one of the chronic auto-immune multi systemic diseases affects females rather than males especially during childbearing age. A fluctuating nature of SLE disease activity deprive this disease from any of monitoring test, at likewise both of SLE disease activity index (**SLEDAI**) criteria or even the laboratory analyses do not confirm such disease activity, especially when a patient have normal serological results although during the disease activity (flare). This study done as a case control study (retrospective study), where a total sample size included (86) freshly peripheral blood samples, which subdivided into: a first the disease group comprised from (66) blood samples voluntary obtained from SLE patients whose attended Al-Yarmouk teaching hospital and Al-karama teaching hospital during the period (October/2017-June/2018) after official approvals were obtained from health institutions. however, the disease group were be divided into active group (flare) and in active group (remission) according to SLE disease activity index (SLEDAI) criteria by a specialized consultant. while the second group included (18) blood samples obtained from healthy individuals (controls) whose matched with a disease group in gender and age.

This study based on an immunological evaluation of (CD69 upon CD3^{+} ratio) percentages at peripheral blood T-lymphocytes tested as SLE disease activity monitoring marker, owing to that purpose these markers (CD69/CD3^{+}) were be isolated from freshly blood T-lymphocytes. therefore were be analyzed by Immunophenotyping flow cytometer of multi-color immunofluorescent staining. Whereas such ratio (CD69 upon CD3+ marker) obtained from data analysis (CY flow is the software part of flow cytometer). such ratio set up (CD69/CD3) test as Comparing with other laboratory analyzes to investigate the activity of SLE disease mentioned as; Anti-nuclear antibodies test, Anti-double stranded-DNA antibodies test, as well as SLE activity index scores. Furthermore thatCD69 type II of C-lectin membrane bound receptor, described as early activation marker for T-lymphocytes, that tested for such aim while the results gave insurance about over expression of CD69 upon CD3^{+} T-cells for the active (flare) group with a highly significant (P value= 0.0001).While healthy controls have (P value=0.05) as having no significant level.

Also there was a hallmark among this study groups between (CD69 upon CD3+) ratio and age as intervals of (9) years, such ratio markedly elevated at (21and59 years) that have a highly significant differences (p value=0.0001), at another word systemic lupus erythematosus disease effect females at highly significant (P value= 0.0001).anti-nuclear anti bodies (ANA) test signified a positive correlation of disease group CD69 upon CD3+ blood T-cells highly significant differences (p value=0.0001) within low significant level, But (ds-DNA) test indicated low significant differences with(CD69 upon CD3+ blood T-cells ratio) of the disease group. Moreover the most

important indicative parameter for disease activity that systemic lupus erythematosus disease activity index whereas the (CD69 upon CD3+) ratio a positive correlation matching with SLEDAI score for SLE disease groups, furthermore that active group registered elevated levels (mean ±SD = 30.07±1.89, and the mean=30.00) respectively at (8) and (9) scores. In conclusion According to these results which have been showing that CD69 gave a vigorous indication as early activating marker for revealing the activity of SLE disease rather than SLEDAI which consider as a real time of the clinical manifestations appearance.

Key Words: Systemic lupus Erythematosus, CD69 percentage, CD3 percentage, Anti-nuclear Abs, SLEDAI.

Introduction

Systematic lupus erythematosus (SLE) can be defined as a chronic systemic inflammatory autoimmune disorder (Syh.Jae *et al*., 2017).Characterized by impairment within tolerance to the self-antigen and formation of many different auto-antibodies with the ability to attack multiple organs, and systems, including the blood vessels, skin, kidney, nervous system (Vitales-Noyola *et al*., 2017),Thus, In patients of systemic lupus erythematosus the pathogenicity consider as a complex condition and the loss of tolerance to self -antigen due to the consequence of multiple genetic risk factors, environmental effectiveness, deficiency in immune regulatory mechanisms (Noyola *et al*., 2017;Novelli *et al*., 2018), as well as the gender specially effects women at child-bearing age (Wigren*et al*., 2015). The complex nature of (SLE) disease described as unpredictable characteristic and fluctuating course. In spite of developing methods for disease activity measurement but still lack of consent around the optimum criteria about (SLE) remission as well as flare (Medina-Quiñones *et al*., 2016).

There is a problem in (SLE) patients monitoring mainly exist at the determine disease activity, due to the contradiction in serological parameters which sometimes give normal values while The signs and symptoms have shown clearly appearance in (SLE) patients, (Saber *et al*.,2007).T-cells consider as a key player in (SLE) disease, because of their helpful effects that inducing auto-reactive B cells, also their ability to escape into objective organs and tissues causing their harmful effects; thus, T-lymphocyte at most importance not only in evaluating disease activity, pathophysiology; but also for characterize predictive biomarkers as well as most medication targets (Moulton *et al*., 2011).CD69 expression is the earliest induced marker on the T-lymphocyte surface after TCR/CD3 complex activation, this expression and appearance of CD69 on plasma membrane of the activated lymphocytes is go faster than expression of CD25 (Cibriàn et al., 2017). The expression of CD69 on T-cells surfaces at peripheral blood may give a novel insight indicator about monitoring of early activated T-cells population leading to level of T-cell mediated immune responses can also be expected in the body (Su *et al*., 1997).

Materials and Methods

Study population:

Whole samples population of such study were (86) individual samples subdivided into the first part "Disease group" that included (Active group and Inactive group) their samples obtained from both of (In-patients and out-patients) whom attended Al-SHAFAA DIALYSIS CENTER, The Consultancy Clinics of AL-Yarmouk Teaching Hospital. As well as patients who attended Al-Hayat Dialysis Center, Al-Karama teaching hospital.Whereas a second part of the study were "Healthy group" obtained

from selected (18) healthy voluntaries individuals. during the period (October/2017-June/2018). As the following details:

(a) Disease group: sub-divided according to SLEDAI into:

(a1) Active group (disease flare): The samples were being obtained from (32)patients; (1)male and (31) female with aging ranged(13-60years) systemic lupus erythematosus (SLE) patients diagnosed according to disease activity assessed with [(SLEDAI) SLE Disease Activity Index] by specialized physicians.

(a2)Inactive group (SLE remission): these Samples were obtained from (36) patients; (3) males /(33) females with aging ranged (13-60years) diagnosed by a specialized physicians as SLE patients within remission status according to (SLEDAI).

(b) Healthy control group: The samples were being obtained from the (18) healthy Individuals (2) males, (16) females their age ranged (13-60years) volunteer after take their conceptions. All samples were being tested which gave absolutely healthy condition insurance**.**

Samples collection:

All samples have been collected at the same manner from each individual, which described as about (5ml) of whole venous blood have been collected under sterile conditions; then they were subdivided into the followings:

- About (2.5ml) of whole venous blood poured into heparin tube for CD3 & CD69 flow cytometric analyses.
- About (2.5ml) of whole blood poured into plane tube, waiting until blood clotting ;then must be centrifuged about (3000rpm) for (3minutes) in order to separate a serum from clotted blood, the serum were be isolated by micropipette and separated for ANA, and ds-DNA testsabout (1ml) poured into a plane tube then stored at (-20˚C).

  That CD3 and CD69 are both used as vitro identification for both CD markers cell expression antigens diagnosed by BD FACs™ brand flow cytometer.

Flow cytometer: one improved table flow cytometer (Cy Flow) instrument used for detecting and analyzing of T-lymphocytes CD3 and CD69 markers, that must be equipped for appropriate fluorescence laser excitation at (405nm, 488nm, and 635nm) with appropriate software (Cy View) software for data acquisition as well as analysis.

- **flow- cytometer kits:**I- CD3(SK7) monoclonal mouse anti-human, Π -PE Mouse Anti-Human CD69Becton, Dickinson and company BD Bioscience, BD Bioscience Pharmingen™/USA.
- **Procedure:** About 20ml from both of CD3 fluorochrome- conjugated monoclonal- antibody and CD69 fluorochrome-conjugated monoclonal antibody to 100ml of freshly whole blood in (12x75-mm) capped polystyrene test tube; at the same time.Thoroughly vortexes and incubated 15-30 minutes in the dark place at room temperature (20°C-25°C)

  -About 2ml added of 1x BD FACS lysine solution, then thoroughly vortex and incubated for 10 minutes in dark place at room temperature.Tube centrifugation at 300g (gravity=1500rpm) for 5minutes .Washing (3times) by adding buffer solution, discharging supernatant .Add (2ml) from (FITC) solution into the tube.

  Appropriated place for single test tube in the Partec Cy Flow® instrument; each tube place on appropriated place and tested the resultsappeared on the specific data show within special program named as (Cy View software).

Whereas Anti-double stranded DNA, Anti-nuclear antibody tested by using Immunofluorescence technique:

Indirect- Immunofluorescence the samples were incubated with antigen substrate while unreacted antibodies washed off; such substrate incubated within conjugate labeled with specific fluorescein; slides viewed under fluorescence microscope while auto antibodies give an apple green fluorescence appearance according to(CDC).

- **Immunofluorescence kits :I- NOVA lite ANA plus mouse kidney &stomach, Π -NOVA lite ds-DNA Crithidialuciliae, A werfern company /USA.**

Procedure:

- First step all samples and reagents must be placed until reached at room temperature (20-26˚C).
- all samples must be mixed by Vortex.
- reagents must be mixed gently.
- A warning; the slides must be never dried.
- Adding samples after dilution by PBS into wells of the slides, then The slides incubated in appropriated suitable moist chamber for 30±5 minutes.then washing by diluted washing buffer, After excess PBS shaken off ; the slides retained onto the humid chamber, immediately cover each well with one drop of fluorescent conjugate, then incubated 30±5, At the same manner of step5 repeated for 3 times, A cover slip placed on a paper towel. Mounting medium placed in a continuous line to the bottom edge of the cover slip. Excess PBS must be removed then cover slip placed by touching the lower edge.
- Those done for all positive samples by making serial (2 fold) dilutions from initial screening dilution (i.e. 1:40, 1:80 …1:5120).
- The fluorescence grade determined by using the below criteria:
  - ✓ (+1)Lowest specific fluorescence that provide clear differentiation of nuclear or/and cytoplasmic staining from the background fluorescence.
  - ✓ (+2)Positive clear differentiated fluorescence.
  - ✓ (+3) Bright apple green fluorescence.
  - ✓ (+4)Brilliant apple green fluorescence. (Loveridge *et al*., 1980)

Statistical analysis:

Analyses of data were carried out using the available statistical package of SPSS-24 (Statistical Packages for Social Sciences- version 24). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values).The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means or Paired-t-test for difference of paired observations (or two dependent means), or ANOVA test for difference among more than two independent means. The significance of difference of different percentages (qualitative data) was tested using Pearson Chi-square test (χ^2-test) with application of Yate's correction or Fisher Exact test whenever applicable. Statistical significance was considered whenever the P value was equal or less than (0.05), (Daniel*et al*., 2012).

Results:

Patients with SLE disease (66) freshly blood samples, whereas active group were (32) samples, while in-active group were (36) samples, finally health controls were (18) samples. There were a significant differences represented on the following tables:

Distribution of studied groups according to age & gender:the study groups were be divided into four age group (interval of 9 years) the main age group of SLE patients were (40-49 years) represented on (table 1).

Table 1: general distribution of studied groups according age and gender

parameters		Disease group (68)				Healthy group (18)		P-value
		Active group (32)		**In-active group (36)**				
		No	**%**	**No**	**%**	**No**	**%**	
Age (years)	**<30**	3	9.3	3	8.3	3	16.7	0.894
	30---39	8	25.0	10	27.8	3	16.7	
	40---49	15	46.9	14	38.9	7	38.9	
	=>50	6	18.8	9	25.0	5	27.7	
	total	32	100	36	100	18	100	
Gender	**Male**	1	3.1	3	8.3	2	11.1	0.520
	Female	31	96.9	33	91.7	16	88.9	
***Significant difference between proportion using Pearson Chi-square test at 0.05 level**								

Frequency of CD69/CD3^{+}T-cells % Expression among the study groups: table(2) shows a general statistical parameters about CD69/CD3^{+}%T-cells as differentiated marker among tested groups there were a highly significant differences according to (p value=0.0001) between active and inactive groups, as well as among groups at (p value=0.0001, when p 0.05).

Table 2: case control differences depending on CD69/CD3$^+$ T-cells% Expression

CD69/CD3$^+$%		Active (Flares)	Non-Active (Remission)	Healthy (controls)
	Number	32	36	18
	Mean ±SD	23.08±8.37	13.73±5.21	1.52±0.97
	Standard Error of Mean	1.480	0.868	0.230
	Percentile 05th	7.45	8.36	0.25
	50th (Median)	27.00	12.20	1.48
	75th	29.76	18.00	2.38
	99th	32.00	26.00	3.34
	P value compared to healthy control	0.0001*	0.0001*	-
	P value compared to Remission	0.0001*	-	-
	P value compare All groups	0.0001#		
***Significant difference between two independent means using Students-t-test at 0.05 level.**				
#Significant difference among more than two independent means using ANOVA test at 0.05 level.				

CD69/CD3+T-cells ratio frequency among studied groups according to ANA test & ds-DNA test grads: the frequency of CD69/CD3+% T-cells for the studied groups according to ANA, and ds-DNA tests grades, those records a positive correlation of CD69/CD3+% within grads of both ANA at highest (mean ±SD, active 29.26±1.78, in active 17.02±7.20) at (3+) grade, as well as ds-DNA tests at highest (mean ±SD, active28.41±3.23, inactive23.37±2.10) at (3+) grade. While negative score gave a significant value among studied groups for both ANA and ds-DNA tests on(table 3).

Table 3:comparisons table ofCD69/CD3+T-cells ratio frequency expressing on study groups according to ANA test and ds-DNA test

parameters		CD69/CD3$^+$%						
		Active (Flares)		Non-Active (Remission)		Healthy control		P value
		No	Mean± SD (Range)	No	Mean± SD (Range)	No	Mean± SD (Range)	
ANA	Negative	1	6.83±	1	11.33±	18	1.52±0.97 (0.25-3.34)	0.0001#
	[+]	17	21.91±8.89 (7.45-32.0)	19	11.90±3.83 (6.95-20.97)			0.0001*
	[++]	8	22.97±7.32 (7.86-31.14)	10	15.50±5.39 (9.50-26.0)			0.024*
	[+++]	6	29.26±1.78 (26.0-31.0)	6	17.02±7.20 (8.90-25.0)			0.002*
	P value		0.052		0.101			
Ds-DNA	Negative	8	9.82±2.92 (6.83-15.34)	28	11.44±2.86 (6.95-19.0)	18	1.52±0.97 (0.25-3.34)	0.0001#
	[+]	10	28.36±2.41 (24.0-32.0)	3	19.00±2.00 (17.0-21.0)			0.0001*
	[++]	9	26.05±3.73 (19.0-30.10)	2	23.49±3.56 (20.97-26.0)			0.401
	[+++]	5	28.41±3.23 (23.0-31.14)	3	23.37±2.10 (21.0-25.0)			0.055
	P value		0.0001#		0.0001#			
*Significant difference between two independent means using Students-t-test at 0.05 level.								
#Significant difference among more than two independent means using ANOVA test at 0.05 level.								

CD69/CD3+T-cells ratio frequency among studied groups according to SLE disease activity index (SLEDAI) scores: The most important evaluating parameter is SLE disease activity index (SLEDAI), which displayed on table (4). whereas the studied groups distributed according to SLEDAI scores ranged (0-9) scores for each of studied groups (Active, In active, healthy controls). Likewise, although most active group patients stilled at score (4),but the (Mean ± SD) significantly positively corresponds with SLEDAI scores whereas reached at peak value of this study at (9) score

Table 4: comparisons table of CD69/CD3+T-cells ratio frequency expressing on study groups according to SLE disease activity index (SLEDAI) scores.

CD69/CD3^{+}%							
SLEDAI Scores	**Active (Flares)**		**Non-Active (Remission)**		**Healthy control**		**P value**
	No	**Mean ±SD (Range)**	**No**	**Mean ±SD (Range)**	**No**	**Mean ±SD (Range)**	
0	-	-	**3**	**13.66±0.15(13.5-13.8)**	**18**	**1.52±0.97(0.25-3.34)**	**0.0001 ***
1	-	-	**10**	**9.99±1.79 (6.95-12.73)**			
2	-	-	**11**	**14.35±5.00 (8.90-21.0)**			
3	-	-	**12**	**16.31±6.37 (8.36-26.0)**			
4	**11**	**16.01±7.48 (7.45-26.4)**	-	-			
5	**6**	**21.90±9.94 (6.83-31.0)**	-	-			
6	**4**	**26.13±2.78 (22.0-28.0)**	-	-			
7	**5**	**29.26±0.78 (28.0-29.8)**	-	-			
8	**5**	**30.07±1.89 (27.0-32.0)**	-	-			
9	**1**	**30.00±**	-	-			
0.002#		0.033#					

the correlation between CD69/CD3$^+$%T-cells with SLEDAI scores showed no shared points among the studied groups which displayed on Figure (1) as following:

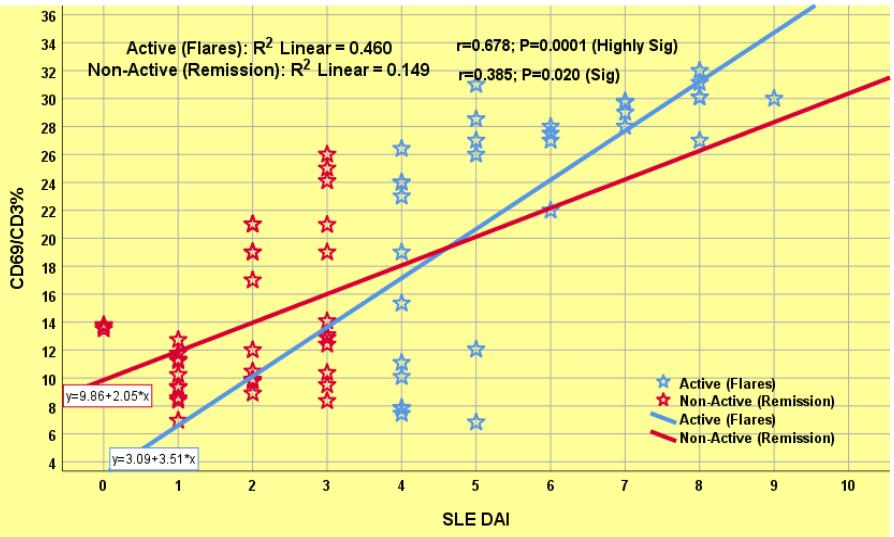

Fig. 1: Linear Regression of CD69/CD3$^+$ depending on SLEDAI scores for each of SLE active group that indicated highly significant positive correlation while SLE in active g group low significant positive correlation.

Discussion:

The present study detects SLE disease age ranged (21-59 years) among samples of SLE disease group patients. These results corresponds with Al-Haidary (2004) peak range of SLE Iraqi patients(20-69 years), while (14-61 years) by Al-Saady (2010), also This study results corresponds with Arabs 'researches disease like Al Dhanhani *et al*.(2017) reported about Arab United Emirates mean age (mean ±SD=28.6± 12.4 years), also Al youssuf *et al*.(2016) in Saudi Arabia SLE disease (mean age onset=29.3 years). Moreover Ku *et al*.(2016), referred to range of Chine's' women age was (9-40).

More farther this study confirmed within a peripheral blood CD69/CD3+%upon T-cells as SLE disease activity indicator,CD69/CD3+% T-cells population described by Saber *et al*. (2007) as a much informative about CD69 expression receptor, whereas also explained by Vitales-Noyola *et al*. (2017) as a stimulator molecule involved activation of various immune cells.Therefore; a current study presents an actual considered differences among groups under study depending on CD69/CD3+T-cells ratio as immunological marker which pointed a highly significant correlation (p value 0.0001) with SLE disease activity, that played as an early inducible marker for T-lymphocytes activation as well as migration, Radulovic *et al*. (2013). our results approved with Martin *et al*.(2010) whose reported about SLE patients whereas ensured that CD69 considered as early activation receptor within an intrinsic differentiation program where determined immune inflammatory process. Whereas the previous researchers results corresponds with a current study, like Su *et al*.(1997) and Syh.Jae *et al*.(2017).Anti-nuclear anti-bodies test remains essential for SLE patients that may be assess on predicting the clinical patterns of SLE disease and its prognosis Ahmed *et al*.(2017) while Perumalla *et al*.(2016) mentioned the test as diagnostic SLE criteria, but also pointed about lupus ANA negative patients.According to the present study that signaled as a highly significant among the groups with (p value 0.0001), where may be represented as a diagnostic tool through

giving early indication, also signify between active vs. inactive as highly (p value; 0.0001, 0.024, 0.002, respectively), which may giving an indication about the highly correlation between elevated CD69/CD3+%T-cells of peripheral blood with increasing anti-nuclear anti-bodies levels as a result of T-cells activity which described by Moulton et al.(2011) as provides supporting for B-cells auto reactivity whereas Pisetky *et al*.(2018) disagreed this study about ANA test whose considered such test as independent indication about disease activity but corresponds with this results about ds-DNA test, which gave a sufficientdifferentiation between active vs. inactive SLE patients, whereas the measuring of ds-DNA Abs may be giving a potential helpful biomarker for SLE disease activity assessment by Xu *et al*.(2018), but our results shows no any significant correlation within disease activity, that may be explained by Su *et al*.(1997) as the correlation of anti-double stranded DNA Abs with disease activity reflects impairment, when SLE clinical activity not absolutely products of B-cells alone, the activation of T helper2 capable to direct the reaction toward humeral immunity additionally to their effects on B-cells activation and Abs production, which observed in central nervous system lupus patients have been reported within elevated SLEDAI scores but low anti-double stranded DNA titers. Additionally, Anand *et al*.(2002), whose corresponds these results also referred about subpopulation T-cells; (CD3+ CD4- CD8-, and double negative dendritic T-cells) which highly expressed CD60 that enhancing anti-DNA auto-antibodies production. Which gave an explanation about lost correlation between ds-DNA and (CD69/CD3+T-cells%). Finally, also the results corresponds with Compagno (2015) about ds-DNA.Actually, SLE disease activity index was a basic requisite for CD69/CD3+% T-cells evaluation test whereas successfully signify a highly correlation with its scores reached to (mean ±SD >30± %) at (9) score. also besides that disease activity assessed by measuring SLEDAI scores, while "weighted average of SLEDI scores>10" by Kakati *et al*.(2015), whose observed the correlation between numbers of flares and SLEDAI scores, where the sum of the clinical appearance described as "flare". Therefore, this study patients selected within the scores lower than (average >10 score) in order to investigate the early activation process prior disease flare. Anyway SLEDAI described by Yee *et al*.(2011) as the index condensed on recurrent or the new manifestations but failed to capture earliest activity (on-going activity). However that SLE patients (flare) within disease activity initially at score (4), while the peak at score at (9) according to this study. There were many researcher investigated the SLEDAI with CD69/CD3+T –cells such as, Saber *et al*.(1997), Su *et al*.(2007), Vitales-Noyola *et al*.(2017), and Hervier *et al*.(2011).

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