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ORIGINAL ARTICLE

Expression of T helper 17 cells Retinoid Acid Related Orphan Receptor Gammat (RORγt) mRNA in Systemic Lupus Erythematosus

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ABSTRACT

Background: *Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by various immunological abnormalities, including dis-regulating activation B lymphocytes with subsequent productionof a large quantity of autoreactive-antibodies. It is also hypothesized that T helper-17 lymphocytes (TH-17) may have a role in this disease. The aim of the present work was to determine the role of TH-17cells expressing the retinoid acid related orphan receptor gamma t (RORγt) mRNA in the pathogenesis of SLE disease.* ***Methodology:*** *The study was conducted on 30 female SLE patients fulfilling SLICCA /ACR criteria for SLE classification and 30 healthy subjects sex- and age-matched apparently as control group with no previous history of autoimmune diseases. SLE Disease Activity Index was calculated for SLE patients. Level of expression of (RORγt) mRNA of IL-17 were measured in all patients and control by quantitative Real Time Polymerase chain reaction (Q PCR).* ***Results****: The mean ± SD of RORγt mRNA expression levels in SLE patients (3.6±6.1) was significantly reduced compared to that of controls (11.7±13.7) (p= 0.008).Neither the clinical features of SLE nor the laboratory parameters have significant relationship with RORγt expression.* ***Conclusion:*** *The reduction of RORγt mRNA expression in TH-17 lymphocytes may point out to the regulatory protective role of TH-17 in the pathogenesis of SLE. Agents that block the functions of these cells should be tried.*

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the production of multiple auto-antibodies, complement activation and immune-complex deposition, causing tissue and organ damage[1].It is a multisystem disorder affecting virtually all systems such as joints, skin and blood in 80 -100 % of cases, kidneys, central nervous system and cardio-vascular in over 50% of cases, while thrombosis associated with presence of anti- cardiolipin antibody in 10% of cases[2].SLE disease is of a marked female predominance.

Naïve CD4 T lymphocytes (TH) cells are activated by the antigenic stimulation of T cell receptor (TCR) and are subsequently differentiated into three different subsets of effector helper cells (TH-1, TH-2, TH-17) in order to boost the immune responses[1].Their differentiation depends on cytokines that present early during immune response, involvement of transcription activation and genetic modification of cytokine genes[3].Cytokine signaling induces the activation of specific transcription factors to promote lineage-specific cytokine production [4,5].

TH-1 cells require STAT1and STAT4 transcription factors for their development and production of IFN-γ. They are mainly devoted to protection against intracellular microbes.TH2 cells require STAT6 and GATA-3transcription factors for their development and production of IL-4, -5, -9and -13 cytokines. They are involved in the protection against gastrointestinal nematodes, but are also responsible for allergic disorders. The balance between Th1/Th2 cells is important for inducing autoimmune and allergic immune responses[4].

TH-17 cells are the more recently described T Helper lymphocyte subsets. They are differentiated from naïve CD4 cells after stimulation with proinflammatory cytokines (IL-6, IL-1, IL-23) produced by Dendritic cells and also by the anti-inflammatory cytokine (TGF-β)[6]. These cytokines induce the expression of a master regulator transcription factor called retinoic acid-related orphan receptor γt (RORγt) which is important in the development of TH-17 cells as

well as drive the inflammatory response of these cells. In addition, it orchestrates the induction of transcription of the genes encoding IL-17 and the related cytokine IL-17F in naïve CD4 T helper cells.[7]

TH-17 cells secrete a distinctive set of immunoregulatory cytokines, including IL-17A, IL-17F, IL-22, and IL-21. These cytokines collectively play roles in inflammation and autoimmunity and in response to extracellular pathogens and fungi mainly by inducing neutrophilic inflammation. TH-17 cells also cooperate with TH-1 in phagocyte-mediated elimination of microbes.[8]

IL-17 has a potential to induce the production of additional inflammatory cytokines (TNF) and chemokines and to promote recruitment of inflammatory cells such as monocytes and neutrophils to the inflamed organ. Increased number of TH-17 cells as well as high serum levels of IL-17 have been demonstrated in patients with autoimmune diseases such as psoriasis and inflammatory bowel diseases. The contribution of TH-17 to the development and pathogenesis of several inflammatory disorders is uncertain.[9]

Pharmacological antagonism of RORγt that blocks the functions of TH-17 cells and improved methods of identifying these cells would have therapeutic potential that allow earlier treatment, and may prevent irreversible tissue damage of autoimmune disorders.[10]

The **aim** of the current work was to determine the role of TH-17 cells expressing the retinoid acid related orphan receptor gamma t (RORγt) mRNA in the pathogenesis of SLE disease.

METHODOLOGY

This study was conducted on thirty adult patients with SLE Patients were selected from rheumatology outpatient's clinic of AL-Zahraa hospital, Cairo, Egypt, during the period from June 2015 till October 2016. SLE patients fulfilling the American College of Rheumatology (ACR) revised criteria for the diagnosis of SLE were included.[11]Patients with clinical manifestations of SLE such as malar rash, photosensitivity, alopecia, oral ulcers arthritis, nephritis, neuropathy and hypertension were included in the study. Current medications were recorded. Patients with diabetes mellitus, malignancies and those with a diagnosis of mixed connective tissue disease were excluded. The study also included thirty age and sex matched apparently healthy control subjects with no family history of autoimmune diseases. All patients were subjected to the following: Full medical history, general and rheumatologic examination: including age, disease duration, and drug treatment in use. Assessment of disease activity for patients by the systemic lupus disease activity index (SLEDAI). SLEDAI was calculated for all patients to active SLE patients who SLEDAI >6 points and inactive SLE patients who SLEDAI <6 points.[12]Informed oral consent was obtained from all subjects. Laboratory investigations such as complete blood picture (CBC) and erythrocytes sedimentation rate (ESR), kidney and liver functions, 24-hour protein in urine, autoimmune profile for systemic lupus (including anti-double stranded DNA (anti-ds DNA), anti-nuclear antibodies (ANA), and complement components C3 and C4 levels) were assessed. Measurements of quantification of RORγt mRNA expression level by Real time PCR (Q-PCR) for all patients and control. The protocol of the study was approved by a research ethical committee of the faculty.

Sample

Five ml of venous blood were withdrawn from each patient and control subjects under complete aseptic conditions into vacutainers containing EDTA. Peripheral blood mononuclear cells were separated and prepared for the measurement of RORγt mRNA expression in TH-17 cells.

Methods

1- Separation of blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated within four hours of sample collection by centrifugation on a density gradient lymphocyte separation medium (Histopaque 1077, Sigma). The buffy coat layer of the PBMNs cells containing lymphocytes was collected and stored at -20˚C till the time of use, in Q PCR.[13]

2- Quantification of RORγt mRNA expression level by Real time PCR (Q-PCR):

To quantify the relative levels of expression of Retinoid Acid Related Orphan Receptor gamma t (RORγt) mRNA in peripheral blood mononuclear cells (PBMCs) of patients and controls,Quantitative Real Time Polymerase chain reaction (Q-PCR)was utilized.[10]using AB Applied Biosystems P/N 4322171instrument,Applied Biosystems, Inc., Foster City, CA (USA).The primers for Q-PCR are listed in Table 1 and a constructing a standard curve (Fig 1) was applied.

a. RNA extraction and purification

Isolation of RNA is a prerequisite for the analysis of gene expression

The total RNA was extracted from 30 cases and 30 control samples from the buffy coat layer of the PBMNs using the QIAamp RNA Blood mini kit, Cat. no. 52304 (Applied Biosystems), following the manufacturer 's recommendations.

b. Reverse transcription and PCR amplification

Purified RNA was then used for one-step reverse transcription and real-time PCR amplification using the TaqMan one-Step RT-PCR master mix reagents kit (Applied Biosystems P/N 4322171), AB Applied Biosystems 7300 instrument and PeQ Lab thermal cycler.

Primers and Probes:

The specific forward and reverse primers and probesfor the amplification of RORγt gene and internal control primers and probes of the reference gene glyceraldehyde adenosine diphosphate hyderogenase. (GAPDH)are listed later.

For every patient and control subject two PCR reaction mixtures were prepared for measurement of RORγt mRNA and GAPDH mRNA expression in PBMCs. The Reaction mix tubes were run by real-time reverse transcription (Q-PCR with cycle threshold for each of the RORγt and GAPDH genes were recorded, Fig (1)

Primers and probes sequences used in the study

Primers and probes	**5'-sequence-3'**
Sequence-specific primer for RORγt gene	F. primer: 5′ -TTTTCCGAGGATGAGATTGC- 3′
	R. primer: 5′ -CTTTCCACATGCTGGCTACA- 3′
TaqMan Probe sequence for RORγt gene	FAM 5′-AAGACTCATCGCCAAAGCAT-3′ TAMRA
Primers for GAPDH gene	F. primer: 5′ - GAAGGTGAAGGTCGGAGTC- 3′
	R. primer: 5′ - GAAGATGGTGATGGGATTTC- 3′
TaqMan Probe sequence for GAPDH gene	VIC 5′- CAAGCTTCCCGTTCTCAGCC-3′ MGB

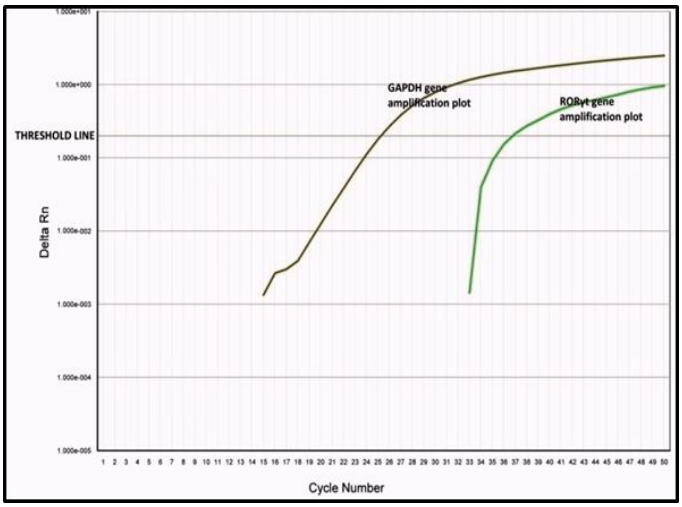

Fig. 1: Amplification plot showing ΔRn vs. Cycle view

Statistical analysis:

Data were analyzed using Statistical Program for Social Science (SPSS) version 20.0. Quantitative data were expressed as mean± standard deviation (SD). Qualitative data were expressed as frequency and percentage. Student's t-test was used for comparisons between the mean value of two groups. A p-value <0.05 is considered significant and p<0.001 is considered highly significant.

RESULTS

The demographic data of patients with SLE:

The current study enrolled thirty SLE patients diagnosed according to SLICCA /ACR criteria and thirty apparently healthy persons who served as a control group. SLE patients were 30 females their ages ranged from 19 to 40 years with mean age (36.5±8.19) years and duration of disease ranged from 1 to 11 years with mean ± SD (6.21±2.44) years. SLEDI of SLE patients were ranged from 1 to 7 with mean (4.03±2.01). The most common manifestations of the disease were nephritis (76.7%), malar rash (56.7%), arthritis (56.7%) oral ulcers (53.3%) and photosensitivity (46.7%) (Table 1).

Laboratory investigations of patients and controls:

Significant increases in TLC (/cmm), ALT (IU/L), AST (IU/L)and creatinine (mg/dl), while significant decrease in Hb (g/dl) were found in SLE patients than controls, table (2)

Quantification of RORγt mRNA expression levels in TH-17 cells by Q-PCR:

The amount of RORγtmRNA was expressed as Relative Quantitation (RQ). The mean RORγt mRNA

expression levels in SLE patients was significantly reduced (**3.63 ± 6.16)** compared to the expression levels in the control group (**11.75 ± 13.78)** (p= 0.008).Figure (2).

Relationship between RORγt mRNA expression levels and different clinical data:

There were no significant relationship between the mean ± SD of RORγt mRNA expression levels and any of the clinical data Table (3).

Correlation of RORγt mRNA expression levels with laboratory parameters:

There were no statistical significant correlation of RORγt mRNA expression level with disease activity, also there were no statistical significant correlations were found between RORγt mRNA expression levels and any of the laboratory parameters, Table (4).

Table 1: The demographic data of patients with systemic lupus erythmatosis

Variables	Range	Mean±SD
Age (years)	19-40	36.5±8.19
Disease duration (years)	1-11	6.21±2.44
SLEDI	1-8	4.03±2.01
Clinical data of 30 SLE patients	**NO**	**%**
Malar Rash	17	56.7
Photosensitivity	14	46.7
Alopecia	9	30
Oral ulcers	16	53.3
Arthritis	17	56.7
Nephritis	23	76.7
Neuropathy	8	26.7
Serositis	6	20
Hypertension	9	30
*DVT	6	20
Raynaud's phenomenon	6	20

**Deep venous thrombosis DVT*

Table 2: Laboratory investigations of patients and controls

Variables	Patients	Controls	P. value
	Mean ± SD	Mean ± SD	
Hb (g/dl)	9.7 ± 3.11	13.9± 1.4	< 0.001
TLC (/cmm)	8,058±1.0037	5,9±1.25	< 0.001
ALT (IU/L)	32 ± 34.8	19.0 ±5.3	0.04*
AST (IU/L)	39.9 ± 38.7	23.7±7.8	0.02*
Creatinine (mg/dl)	1.70±1.60	0.78±0.30	< 0.05

**Significant P- value < 0.05*

Table 3: Relationship between the mean ± SD of RORγt mRNA expression and different clinical data

Clinical Data	No (%)	Mean of RORγt mRNA	P value
Malar rash Positive	17 (56.6%)	4.215 ± 5.321	0.850
Negative	13 (43.3%)	2.532 ± 3.166	
Arthritis Positive	17(56.6%)	4.46 ± 5.73	0.51
Negative	13(43.4 %)	2.24 ± 3.20	
Nephritis Positive	23 (76.7%)	3.66 ± 4.63	0.79
Negative	7(23.3 %)	2.51 ± 1.34	
Neuropathy Positive	8 (26.7%)	1.98 ± 1.40	0.31
Negative	22(73.3%)	2.38 ± 5.11	
Serositis Positive	6 (20%)	2.56 ± 1.89	0.72
Negative	24(80%)	3.85 ± 6.71	
Hypertension Positive	9(30%)	3.32 ± 7.98	0.85
Negative	21(70%)	2.60 ± 4.56	
DVT Positive	6 (20%)	5.32 ± 9.35	0.36
Negative	24(80%)	2.99 ± 1.68	
Alopecia Positive	9 (30%)	4.87 ± 7.66	0.70
Negative	21(70%)	1.93 ± 2.92	

P >0.05 is not statistically significant, p≤0.05 is statistically significant, P≤0.01 is highly significant.

Table 4: Correlation of RORγt mRNA expression levels with laboratory parameters and SLEDI

RORYT mRNA expression levels		
Laboratory parameters	**RORYT mRNA expression**	**p-value**
age /years	-0.308	0.098
Hb (g/dl)	-0.108	0.570
TLC/10^3/cm	0.150	0.428
ALT (IU/ml)	-0.205	0.277
AST (IU/ml)	-0.221	0.240
Creatinine (mg/dl)	-0.105	0.581
Proteinuria (mg/dl)	0.147	0.632
Platelet count(cmm)	0.059	0.755
ESRmm/hr	0.043	0.823
SLEDI	0.169	0.369

No significant correlation between RORγt mRNA expression levels with laboratory parameters

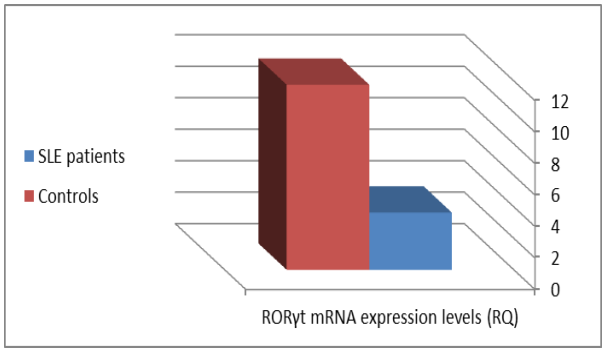

Fig. 2: The mean expression of RORγt mRNA (RQ) of the studied groups; significant reduction of RQ in patients compared to control subjects (p = 0.008).

DISCUSSION

Systemic lupus erythematosus (SLE) is an autoimmune disorder of unknown cause that can affect multiorgans.[14] T cell-medicated autoimmunity and glomerular injury are critical for persistent renal damage closely related to impaired quality of life.[15] Th-17 differentiation is inhibited by IFNγand IL-4, therefore strong Th1 and Th2 responses tend to suppress Th-17 development. Th17 cells are of central interest in the field of autoimmunity. There is accumulating evidence that Th17 cells regulate inflammatory and autoimmune diseases, also contribute to the pathogenesis of many inflammatory diseases such as psoriasis, inflammatory bowel disease, rheumatoid arthritis and multiple scelerosis via the production of IL-17A and IL-17F and IL-22[16]. In addition, recent studies in human SLE indicate a central role for interleukin-17 (IL-17) in the pathogenesis of lupus nephritis (LN)

In the present study the reduced RORγt mRNA expression levels of Th 17 cells are in agreement with the results of [17] who found that in patients of SLE, IL-22level , a cytokine involved in inflammatory processes and secreted by Th17 cells was decreased. .Their data indicated a possible role of Th17 as a protective factor in SLE. Plasma IL-22 levels were also significantly decreased in SLE in the study of[18,19] found that Treg cells were increased in SLE patients compared to normal controls In addition of [20]and [21].explained the reduction of RORγt mRNA expression and subsequently Th17 by the increased expression of Foxp3transcription factor of the regulatory T cells that inhibits the transcription of RORγt mRNA expression level of Th 17 cells. Moreover, [22] demonstrated increased urinary Foxp3 mRNA in patients with lupus nephritis and found to be correlated with disease activity and predicting a poor therapeutic response**.**

A wide spectrum of cytokines of Th1 and Treg namely, IFNα, IFNγ, IL-10, TGF-β, IL-12, IL-25 and IL-27negatively regulate Th17 cells and this finding may explained our result are increased in SLE, indicating that Th17 cells and RORγt relative expression are reduced. Furthermore Treg cells cytokines were reported to be increased in SLE. The study of[23,24] showed that IFNα by dendtritic cellsare elevated in many lupus patients that blocks IL-17 secretion by human PBMCs[25,26] reported that IFNγ down regulates Th17 differentiation in lupus models). This may indicate that RORγt, whose expression is necessary for IL-17 production, is limited in lupus patients. In addition, IL-12, a cytokine that down regulates RORγt expression[27], was found to be increased in SLE patients especially those manifesting with lupus nephritis[28]. Interestingly, 76.7% of our studied SLE patients manifested with different stages of nephritis. Thus, these studies could provide evidence of limited Th17 responses in SLE that are consistent with our findings of significant reduction of RORγt expression, the key transcription factor of Th 17 cells, in lupus patients.

None of the clinical features of SLE as lupus nephritis or arthritis and the autoimmune laboratory parameters have shown any significant relationships regarding RORγt relative expression, as well as no correlation between RORγt mRNA expression levels with laboratory parameters. Similarly,[17] found also a decrease in IL-22 in SLE patients with no significant difference regarding serum IL-22 level between SLE patients with nephritis and without nephritis. In our study we found no significant correlation between RORγt mRNA expression levels and disease activity. This was in agreement with[19] who found that Treg cells were increased in SLE patients compared to normal controls, but not related to any clinical syndromes or disease activity. In contrast Kwan et al.[29] also reported that urinary mRNA expression of Th17-related genes including RORγt was up-regulated in SLE patients, but the degree of up-regulation was inversely proportional to the disease activity. Kwan et al.[29] also reported no increase in the levels of plasma IL-17 or IL-17 RNA expression in peripheral blood lymphocytes of lupus nephritis patients and suggested a regulatory role of Th17-related cytokines in pathogenesis of lupus nephritis. Also on the other hand another study was done on 134 SLE patients by Cheuk Chun et al 2012. [30] who found that urinary level of IL17 correlated significantly with disease activity. These finding suggested that Th17 have a role in pathogenesis rather than regulatory finding. These studies suggest that RORγt is a key regulator of immune homeostasis and highlight its potential as a therapeutic target in inflammatory diseases.

CONCLUSION

The reduction of RORγt mRNA expression level in the peripheral blood mononuclear cells may point out to the regulatory protective role ofTh-17 in the pathogenesis of SLE..

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