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TOLL-LIKE RECEPTORS 4 AND 9 EXPRESSION IN SYSTEMIC LUPUS ERYTHEMATOSUS AND DERMATOMYOSITIS: RELATION TO CLINICAL STATUS AND DISEASE ACTIVITY

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ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder affecting almost all organs and tissues. Dermatomyositis (DM) is a chronic muscle disorder that leads to muscle destruction. Although DM mechanisms remain unclear, there is an evidence of autoimmune origin. Toll-like receptors (TLRs) are the key initiators of innate and adaptive immune response due to high production of proinflammatory mediators and activation of antigen presentation. We used qPCR to investigate the expression of *TLR4* and *TLR9* in peripheral blood mononuclear cells (PBMCs) from SLE and DM patients, as well as muscle tissue biopsies from the DM patients, to explore their role and study their correlations with clinical manifestations and disease activity. Our findings showed a significant increase in *TLR4 and TLR9* expression levels in PBMCs from SLE patients and muscle biopsies from DM patients. Such results emphasize the role of TLR signaling and innate immune system in the pathogenesis of both diseases.

Keywords:
Gene Expression, qPCR, Innate Immunity and Autoimmune Diseases.

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INTRODUCTION

SLE is an autoimmune disorder of multifactorial origin, in genetically predisposed subjects, environmental factors, such as viral infections and smoking, induce the breakdown of self-tolerance eventually triggering autoimmune response ***(Tsokos, 2011).*** It is characterized by spontaneous lympho-proliferation, aberrant activation of T and B cells and production of auto-antibodies against nuclear DNA and / or other nuclear proteins ***(Mu et al., 2012).***

DM is a chronic muscle disorder, clinically characterized by features of symmetric proximal muscle weakness. DM is a microangiopathy affecting the skin and muscle, in which the early activation and deposition of complement causes the lysis of endomysial capillaries and muscle ischemia. Perimysial and perivascular inflammation with CD4+T cell, B cells, and macrophage infiltrations are the histological features of DM ***(Kim et al., 2010).***

TLRs is one of a group known as pattern recognition receptors that play a critical role in protective immunity against invading microorganisms. Pathogens, especially bacteria, have molecular structures that are not shared with their host and are relatively invariant named as Pathogen Associated Molecular Pattern (PAMPs) that are recognized by those TLRs such as bacterial DNA, flagellin, viral double stranded RNA ***(Pradhan et al., 2012).***

At least 12 mammalian TLR family members have been identified, TLR1, -2, -4, -5 and -6 are on the cell surface and they interact with components found on the surface of pathogens whereas TLR3 and -7, -8 and -9 - are expressed within intracellular vesicles and their ligands must be taken up into the endosome to result in activation ***(Blasius and Beutler, 2010).***

Intracellular TLRs, apart from being able to recognize PAMPs they are also capable of recognizing endogenous ligands which are named Danger Associated Molecular Patterns (DAMPs) ***(Denk et al., 2012).*** In SLE patients, impaired apoptosis and invalid cell debris clearance lead to increased concentration of serum nucleic acids (ssRNA, dsRNA, and DNA), which are well-known ligands for TLR so Immune response is induced by not only microbial infection, but also by sterile tissue damage and degeneration product ***(Ma et al., 2015).***

TLR activation leads to expression of genes such as inflammatory cytokines and co-stimulatory molecules. Eventually, antigen-specific

acquired immunity is developed as a result of TLR-dependent gene expression pattern together with phagocytosis-mediated antigen presentation ***(Husebye et al., 2006).*** Yet, the exact roles of TLRs in the adaptive immune response are less well understood, offering an important area of future study ***(Iwasaki and Medzhitov, 2010).***

This study was carried out to investigate the expression *TLRs 4* and *9* in the peripheral blood mononuclear cells (PBMCs) of patients with SLE and DM as well as muscle tissue specimens from patients with DM and study their correlations with clinical manifestations and disease activity of both diseases.

MATERIALS & METHODS

After approval of the study scheme by the ethical committee of Faculty of Medicine, Benha University, and obtaining an informed consent from participants, 32 SLE patients and 17 DM patients were enrolled into the current study. These patients were selected during regular follow-up at the outpatients' clinic and the inpatients' unit of the Rheumatology and Rehabilitation Department, Benha University Hospitals between November 2013 and March 2014 according to the SLICC and Bohan and Peter classification criteria for SLE and DM, respectively. Twenty age and sex matched apparently healthy volunteers were recruited as a control group.

All patients were subjected to full history taking, complete clinical examination. Disease activity was evaluated according to the Systemic Lupus Erythematosus Disease A ctivity Index (SLEDAI) score (***Bombardier et al., 1992)***, assessment of SLE-related disease damage was done according to the Systemic Lupus International Collaborating Clinic (SLICC) damage index ***(Gladman et al., 1992)***. In patients with DM, assessment of disease activity was done according to DM disease activity score (DAS) ***(Bode et al., 2003)***. A muscle biopsy was taken from vastus lateralis muscle for the detection of *TLR4* and *TLR9* from all DM patients as well as 10 of the control subjects who accepted the procedure with a "semi-open" muscle biopsy technique under local anesthesia.

Peripheral blood samples were withdrawn from all subjects and processed properly for routine investigations and separation of PBMCs using FICOLL-PAQUE™ PLUS density-gradient medium (GE Healthcare, USA) according to the manufacturer's instructions ***(Bøyum, 1968).***

RNA isolation and quantitative RT PCR: All PBMCs samples and muscle specimens were immediately used for total RNA extraction using RNeasy mini kit (QIAgen, Germany) as previously described ***(Salway et al., 2008).*** RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used, according to the manufacturer's instructions, for total RNA extraction ***(Wiame et al., 2000)*** and cDNA aliquots were stored at -80ºC till further processing. *TLR4* and *TLR9* gene expression assay of each sample was performed in duplicates using Maxima SYBR Green/ROX qPCR Master Mix (Thermoscientific / Fermentas, USA) by StepOne™ Real-Time PCR System (Life Technologies, USA). Detection and quantification of each gene was expressed as relative mRNA level compared with a standard housekeeping gene (GAPDH) according to the $2^{-\Delta\Delta Ct}$ method ***(Livak & Schmittgen, 2001).*** The primers sequences are as follows: ***TLR4*** Forward: 5′-CTTATAAGT GTCTGAACTCCC-3′, Reverse: 5′ -TACCAGCACGACTGCTCAG-3′, ***GAPDH*** Forward: 5′-CACCACCATGGAGAAGGCTG-3′, Reverse: 5′-GTGATGGCATGG ACTGTG-3′ ***(Szebeni etal., 2008)*** and ***TLR9*:** Forward 5′-TGGTGTTGAAGGACAGTTCTCTC-3′ and Reverse: 5′-CACTCG GAG GTT TCCCAGC-3′ ***(Mortaz et al., 2010).***

Statistical analysis: Analysis of data was performed using SPSS (version 16, Statistical Program for Social Science). Comparisons were performed by the Chi-square test for qualitative variables. Mann Whitney and Student's t-test were used for quantitative variable and Spearman's correlation coefficients (*r*) were calculated for detection of nonparametric correlations between variables in one group.

RESULTS

This study included 32 SLE patients, 28 females **(87.5%)** and 4 males **(12.5%) (F/M =7/1).** Their ages ranged between17-45 years with a mean of **(30.1 ±9.4)** years and disease duration from 9 months to 19 years with a mean of 3.44 ± 4.01 years. Clinical and laboratory characters are shown in tables (1&2).

The current study included 17 DM patients as well (15 females, 88% and 2 males 12%) **(F/M =7.5/ 1).** Their ages ranged between 9 and 52 years with a mean of **(22.6 ±15.2)** years and disease duration ranging from 18 months to 15 years with a mean of 3.74 ± 3.01 years.

DM patients presented with various clinical features, all patients (100%) had heliotrope rash and /or gottron sign and papules , 14

patients (82%) had Constitutional symptoms in the form of fever, malaise, weight loss , 12 patients (71%) had calcinosis, 10 (59%) had arthritis, 4 patients (23.5%) had muscle power grade 3 and 14 (82%) had muscle power grade 4 of the shoulder and /or pelvic girdle muscles. The control group included twenty subjects (14 females, 70%) and 6 males, 30 %) **(F/M = 7/3).** Their ages ranged between 10-54 years with a mean of **(31.3±13.3)** years.

The data retrieved from our study revealed that the expression levels of *TLR4* and *TLR9* were significantly higher in the PBMCs from SLE patients compared to that of the controls (P<0.05). Table (3)

In addition, *TLR4* expression levels in the PBMCs of SLE were significantly higher in those patients who had malar rash (P<0.05), alopecia (P<0.05), arthritis (P<0.05) and pericarditis (P<0.05). On the other hand, *TLR4* mRNA expression showed a significant negative correlation with corticosteroid doses (r=-0.54, P<0.05). Regarding *TLR9* expression levels, they were higher in SLE patients with nephritis (P<0.05) and positively correlated with the protein /creatinine ratios (***r***=0.47, P<0.05), the SLICC damage indices (***r***=0.59, P<0.05) and negatively correlated with C3 levels (***r***=-0.53, P<0.05).

In DM, *TLR4* and *TLR9* expression levels in the PBMCs showed insignificant difference between the patients and the control subjects (P>0.05) for both genes. However, their expression levels in the muscle biopsies from DM patient group were significantly higher (P<0.05) than in the muscle tissue from the control Table (4). Comparing the muscle biopsies to the PBMCs in DM patients showed significant higher *TLR4* and *TLR9* expression levels (both P<0.05).

In the muscle biopsies from DM patients, both *TLR4* and *TLR9* expression levels, positively correlated with disease activity scores (DAS) (P<0.05) as well as with creatine kinase (IU/L) levels (P<0.05, respectively). Furthermore, our results indicated that *TLR9* expression levels were significantly higher in patients who had calcinosis (P<0.05). However, only *TLR4* expression levels exhibited a significant negative correlation with corticosteroid doses (P<0.05).

Table (1): Clinical characteristics of SLE patients

	No.= 32	Percentage (%)
Fever	18	56
Malar rash	26	81
Alopecia	15	47
Oral ulcer	31	97
Arthritis	29	91
Nephritis	16	50
Respiratory	13	41
Cardiac	8	25
Neuropsychiatric	3	9
SLEDAI score	Active disease 21	66
	Inactive disease 11	34
SLICC damage index	0 21	66
	1 11	34

***No.:* total number, SLEDAI =the Systemic Lupus Erythematosus Disease Activity Index, SLICC= Systemic Lupus International Collaborating Clinic.**

Table (2): Laboratory characteristics of SLE patients

	No.= 32	Percentage (%)
C3 (mg/dl)	Low 13	41
	Normal 19	59
C4 (mg/dl)	Low 13	41
	Normal 19	59
ANA Titre	Positive 32	100
	Negative 0	0
Anti ds- DNA (U/ml)	Positive 16	50
	Negative 16	50
	Mean	**SD**
ESR (mm/1st hour	71.25	31.49
Hb (gm/dl)	10.30	1.54
WBCS (thousands/ml)	5.08	2.44
Platelets (thousands/ml)	282.50	104.71

C3= complement 3**, C4 =** complement 4**, ANA=** anti- nuclear antibodies**, Anti ds -DNA=** anti double stranded deoxy nucleic acid**.**

Table (3): *TLR4* and *TLR9* gene expression in PBMCs from SLE patients as compared to control subjects

	SLE	Control
TLR4 (RU) Mean ± SD	*93.8±74.6	46 ±11.3
TLR9 (RU) Mean ± SD	*87.9 ±74.9	8.5 ± 5.7

RU: Relative Units, * $P < 0.05$ significant

Table (4): *TLR4* and *TLR9* gene expression in PBMCs and muscle biopsies from DM patients as compared to control subjects

	DM	Control
PBMCs TLR4 (RU) Mean ±S.D	42.1 ±11.9	46 ±11.3
PBMCs TLR9 (RU) Mean ±S.D	6.9 ±2.2	8.5 ±5.7
Muscle TLR4	*52.4 ±11.7	6.9 ±2.3
Muscle TLR9	*8.3 ±2.3	0.6 ±0.5

RU: Relative Units, * $P \leq 0.05$ significant

DISCUSSION

Autoreactive B cells, present in the lymphoid tissue of healthy individuals remain silent due to presence of self-tolerance mechanisms. Upon failure of self-antigen tolerance, the antigen receptors co-engage with self-reactive antibodies, leading to activation of these autoreactive B cells and development of autoimmune diseases. TLRs that have been triggered by self-antigens, are suggested to respond to signals received by innate immune system, by induction of the secretion of inflammatory cytokines, thereby engaging lymphocytes to support an adaptive, antigen-specific immune response ***(Pradhan etal., 2012) (Lim & Staudt, 2013).***

Our findings revealed significantly higher mRNA expression regarding *TLR4* and *TLR9* in PBMCs from SLE patients as compared to that of control subjects.

As far as we know, this is the first study to assess *TLR4* expression in PBMCs from SLE patients using qRT-PCR. The present results denote that it was upregulated in the patient group as compared to the control group. In spite of the lack of previous studies using the same technique, this finding supports the previous results showing that the protein expression of many TLRs including TLR4 and 9 in PBMCs of SLE patients compared to control subjects ***(Wong et al., 2010)***. However, this was inconsistent with flow cytometry results of the study by ***Migita et al. (2007)***. This controversy might be attributed not only to the different techniques, but also to different cell subpopulation as well as different patients' characteristics.

Furthermore, the significantly increased *TLR9* expression in SLE group is in agreement with former studies using qRT–PCR **(*Komatsuda et al., 2008*) (*Mu et al. , 2012*),** and more recently using flow cytometry ***(Klonowska-Szymczyk et al., 2014).***

In our SLE patients, *TLR4* expression was significantly higher in those with malar rash, alopecia, arthritis and cardiac manifestations (all $P<0.05$). While, *TLR9* expression was significantly higher in patients with nephritis, positively correlated with protein/creatinine ratios, and mean SLICC damage indices ($P<0.05$).

Previous experimental studies highlighted the role of *TLR4* in the pathogenesis of SLE. It has been shown that overexpressing *TLR4* alone mice is sufficient to induce lupus-like autoimmune disease. ***(Liu et al., 2006)*** Moreover, *TLR4* deficient mice showed significant decrease of autoantibodies rates**.** Interestingly, *TLR4* has been reported to synergize with *TLR7&9* ligands. Upon activation, *TLR4, 9* transmit the signals through different adaptor molecules to activate dendritic cells (DCs), increase circulating cytokines levels specially IFN-I and finally enhance the disease ***(Crampton et al., 2014).***

The present results regarding higher mRNA expression of *TLR4* in SLE patients with malar rash, alopecia, arthritis and cardiac manifestations, could be attributed to the increased level of interferon type I (IFN-I) due to the continuous activation of TLR pathways by nucleic acids containing immunologic complexes (***Elkon and Stone, 2011***). IFN-alpha in turn, induces many interferon- inducible genes

and its level correlates with SLE activity and more detrimental clinical disease forms (***Rönnblom and Eloranta, 2013***).

The role of *TLR4* and *TLR9* activation in pathogenesis of arthritis might be explained by their importance in differentiation of DCs into T-helper1 (Th1) cells ***(Abdollahi –Roodsaz et al., 2008) (Davila & Kolls, 2010)***. In a mouse model of arthritis, aberrant *TLR4* expression has been shown to disrupt the balance of Th1 and Th2 lymphocytes ***(Wong et al., 2010);*** an imbalance that is thought to be of pathogenic significance in rheumatoid arthritis ***(Dolhain et al., 1996).*** Moreover, *TLR4* deficiency in mice models confers protection against the inflammatory process of rheumatoid arthritis due to the lowered capacity to produce interleukin-17 ***(Wong et al., 2010).***

Interestingly, animal studies of SLE have implicated TLR9 as a receptor for hypomethylated DNA. SLE patients have higher level of nuclear debris with hypomethylated CpG-DNA that may serve as autoantigens to trigger TLR9 expression and exacerbate the inflammatory process through increased inflammatory cytokines levels including IFN-I ***(Yasuda et al., 2009***) ***(Christensen et al., 2006***).

Our results showed that *TLR9* relative expression was significantly higher in SLE patients with positive anti-dsDNA antibodies ***(P<0.05)***. This is consistent with reports detecting a positive correlation between increased expression of *TLR9* mRNA and the presence of anti-ds DNA antibody (***Mu et al., 2012)***.

One possible mechanism is that autoantigens can bind B cell receptors and trigger *TLR9* expression, which results in B cell activation, proliferation, production and class-switching of autoantibody (***Leadbetter et al., 2002***) ***(Lin et al., 2004)*** **.** This suggestion was ascertained by another study which showed that the production of anti-dsDNA antibodies was inhibited in *TLR9*-deficient lupus-prone mice (***Christensen et al., 2006***).

On the contrary, a negative correlation was observed between anti-dsDNA autoantibodies and the *TLR9* mRNA content in cells (***Komatsuda et al., 2008).*** Nevertheless, the difference may be explained by the heterogeneity of studied populations ***(Lartigue et al., 2009***).

On the other hand, *TLR4* mRNA expression in SLE patients inversely correlated with corticosteroid doses. The occurrence of profound suppression of *TLR 1, -2, -3, -4, -6* and *9* in liver sinusoidal

endothelial cells in response to dexamethasone treatment, has been reported previously ***(Broering etal., 2011).*** Moreover, *TLR9* expression negatively correlated with C3 levels, which is consistent with a recent study ***(Ghaly et al., 2013).***

It has been stated that muscle biopsies of myositis patients show a significantly increased expression of *TLR2*, *TLR3*, *TLR4*, and *TLR9* in the skeletal muscle and infiltrating cells as well as the enhanced expression of many cytokines including type 1 IFNs, suggesting that TLRs are engaged in the milieu of affected muscle and subsequently downstream genes are activated ***(Kim et al., 2010).***

In our study, *TLR4* and *TLR9* were upregulated in the muscle tissue of DM group versus that of the control group. Though, their expression showed insignificant difference in PBMCs from subjects of both groups.

These results are in agreement with the results of a former study reporting that the mRNA expression levels of *TLR4* were significantly higher in the muscle tissue of the patients group compared to those of the controls ***(Brunn et al., 2012).***

The possible mechanisms of TLR overexpression stems from the contribution of either various microbial pathogens considered as etiologic agents, or the endogenous molecules and cytokines that are generated during muscle damage or immune response. Our study showed that within DM group, *TLR4* and *TLR9* expressions in muscle tissue were statistically significantly higher ($P<0.05$) than that in the PBMCs of the same group and are statistically significantly correlated with the DM Disease Activity Score (DAS) and CK levels. However, only *TLR4* expression negatively correlated with corticosteroid doses.

Excessive physical activity and strenuous exercise in normal individuals leads to modest elevations in serum muscle enzymes such as creatine kinase (CK), whereas myositis patients generally show a significant increase in CK, suggesting that skeletal muscle leakiness and damage occur in this disease. It is likely that some DAMPs leak from the injured skeletal muscle and engage their receptors on both skeletal muscle and immune cells, thereby perpetuating the inflammatory process ***(Brunn et al., 2012).***

CONCLUSION

Our results may suggest a detrimental role of TLR4, TLR9 signaling and innate immune system in the pathogenesis of SLE and DM through activation of TLR pathway. Moreover, the results propose that targeting TLRs and their signaling pathways may lead to development of a new class of efficacious drugs for down-streaming the auto-inflammatory response and control disease activity in both SLE and DM disorders.

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