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Blood gene expression of Toll-like receptors in SLE patients with lupus nephritis or neuropsychiatric systemic lupus erythematosus

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Abstract

Background To determine differences in the blood innate gene expression signatures of systemic lupus erythematosus (SLE) patients across various organ manifestations and disease activity, with a focus on lupus nephritis (LN) and central nervous system (CNS) involvement.

Methods Toll-like receptor family (TLR 1–10) mRNA expression was investigated in peripheral blood mononuclear cells from patients with SLE ($n=74$) and healthy controls ($n=34$). We compared patients with histologically confirmed active LN or neuropsychiatric systemic lupus erythematosus (NPSLE) with patients without these symptoms. The expression of TLR mRNA was determined by RT–qPCR using a high-throughput SmartChip Real-Time-qPCR system (WaferGen). Multivariate analysis and nonparametric statistics were used for data analysis to assess the associations between TLRs and disease activity and severity.

Results TLR4 (0.044 vs. 0.081, $p=0.012$) was upregulated and TLR10 (0.009 vs. 0.006, $p=0.0007$) was downregulated in the whole cohort of SLE patients compared to healthy controls. A comparison of the active LN group with participants without kidney involvement revealed increased expression of TLR2 (0.078 vs. 0.03, $p=0.009$), and TLR5 (0.035 vs. 0.017, $p=0.03$). Moreover, a significant difference was observed in TLR9 expression between inactive LN and the control group (0.014 vs. 0.009, $p=0.01$), together with borderline correlation in TLR2 expression (0.04 vs. 0.03, $p=0.06$). Receiver operating characteristic (ROC) curve analysis revealed that TLR1 and TLR2 expression were the best potential diagnostic markers for active LN. The NPSLE group showed upregulation of TLR1 (0.088 vs. 0.048, $p=0.01$), TLR4 (0.173 vs. 0.066, $p=0.0003$) and TLR6 (0.087 vs. 0.036, 0.007). Our correlation analysis supported the close relationships among the expression of individual TLRs in the whole lupus cohort and its subgroups.

Conclusion Our study revealed differences in TLR expression between a lupus cohort and healthy controls. Additionally, our analysis provides insight into specific TLR expression in cases with severe organ manifestations, such

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as LN and NPSLE. The multiple mutual relationships of TLRs demonstrate the activation of innate immunity in SLE and suggest promising targets for future therapies or diagnostics.

Keywords Systemic lupus erythematosus, Innate immunity, Disease activity, Lupus nephritis, Toll-like receptors

Background

The pathophysiology of SLE is complex and involves interactions between factors and cells involved in innate and adaptive immunity. Innate immune receptors such as Toll-like receptors (TLRs) have the unique ability to recognise pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and self-molecules in autoimmune diseases, thus inducing activation of the immune system by linking innate and adaptive immune responses [1]. TLRs are classified according to their location. Extracellular TLRs (TLR1,2,4,5,6) respond to bacterial or fungal PAMPs and DAMPs, while intracellular TLRs (TLR3,7,8,9) are located on the endosomal membrane and respond to pathogen nucleic acids as well as self-nucleic acids [2]. They are usually expressed by cells of the immune system but can also be expressed by a specific organ tissue [3].

A number of studies have demonstrated that different members of the TLR family are involved in the pathogenesis of SLE [4, 5]. The expression of TLRs varies in different clinical manifestations of the disease [6]. Some TLRs (TLR7 and TLR8) are potential therapeutic targets for monoclonal antibodies [7].

The role of innate immunity in SLE development is already known. However, there is limited knowledge regarding the differences and similarities in TLR family members based on specific types of organ damage. This study focused on the possible differences between the expression of TLR family members in SLE patients with severe disease manifestations, such as active lupus nephritis (LN), and patients with neuropsychiatric systemic lupus erythematosus (NPSLE). This study analysed the gene expression of TLR family members in a clinically defined group of SLE patients to evaluate the potential of TLRs for the diagnosis of organ involvement.

Methods

Study subjects

The study was designed as an observational cross-sectional single-centre analysis of a cohort of patients from the Rheumatology Department of the University Hospital in Olomouc, Czech Republic. The primary objective of the study was to compare the expression of TLRs in patients with SLE and healthy controls. The secondary objectives were to investigate differences in TLR expression between patients with kidney or central nervous system (CNS) involvement and to compare these results with disease activity scores, major antibody levels and disease-related organ damage.

The depersonalised and anonymous data and samples were collected and stored after signed written informed consent was obtained for data collection from all participants and healthy controls. The study was approved by the local ethical committee (Multicenter Research Ethics Committee of the University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic). The study was performed in accordance with the Helsinki Declaration of 1964 and its later amendments.

All SLE patients enrolled in the study met the 2019 EULAR/ACR classification criteria for SLE [8]. Disease activity was quantified using the Systemic Lupus Eryhematosus Disease Activity Index 2000 (SLEDAI-2 K) [9]. The degree of organ damage that has accumulated since disease onset was assessed using the Systemic Lupus International Collaborating Clinics American College of Rheumatology Damage Index (SLICC/ACR DI) [10].

Subgroups were created based on the clinical manifestations of the disease. The diagnosis of LN was confirmed by kidney biopsy. Patients were divided into two groups according to the activity of the LN, as assessed by the Renal Domain Score of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-R). The groups were named the Active LN-SLE group, with SLEDAI-R scores ≥ 4, and the Inactive LN-SLE group. The SLEDAI-R was calculated by summing the SLEDAI-2 K item scores for proteinuria > 0.5 g/day, hematuria and pyuria (both > 5 cells/HPF, infection excluded) and cellular casts [11]. Patients without kidney impairment were classified into the No LN-SLE group. Another subgroup, called CNS-SLE, consisted of patients with NPSLE and CNS involvement according to the ACR criteria [12]. Patients with both active LN and NPSLE were excluded from the subgroup analysis. Furthermore, the expression of TLR was evaluated in relation to the administration of corticosteroids or antimalarials in the entire cohort of patients with SLE.

The control group consisted of age- and sex-matched healthy volunteers. In all healthy controls, recent vaccinations, infections, and the use of immunosuppressive drugs as well as the presence of inflammatory autoimmune diseases in first- or second-degree relatives were excluded by questionnaire.

Sample processing

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood collected in K3EDTA tubes by Ficoll density gradient centrifugation (Sigma–Aldrich,

Germany) and stored in Tri reagent (Sigma–Aldrich, Germany) at − 80 °C until analysis. Total RNA was extracted using a Direct-zol RNA kit (Zymo Research, USA) according to the manufacturer's recommendations. After reverse transcription with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland), qPCR was performed in a 100-nl reaction volume containing LightCycler 480 SYBR Green I Master mix (Roche, Switzerland) using a high-throughput SmartChip Real-Time-qPCR system (WaferGen, USA), and the relative mRNA expression was calculated using phosphoglycerate kinase 1 as a reference gene as reported previously [13].

Statistical analysis

IBM SPSS Statistics for Windows, Version 23.0 Armonk, NY: IBM Corp. statistical software was used for statistical processing. Multivariate analysis (Andrews curves, Associate rules, Spearman correlation coefficient and Kruskal–Wallis test) was used for data analysis. The Mann-Whitney U-test was used to compare TLR expression according to treatment. All tests were performed at the 0.05 level of significance. Differences for which the p value was less than 0.05 were considered to be statistically significant and are indicated by an asterisk. According to the receiver operating characteristic (ROC) curve analysis, areas under the ROC curve (AUCs) greater than 0.7 are considered to be of fair discriminating value for predicting diagnostic tests. Optimal cut-off values were established based on Youden's J statistic [14, 15].

Results

Participants

A total of 74 white patients fulfilled the inclusion criteria and were enrolled in the study. The group of healthy controls consisted of 34 volunteers. The demographic and clinical characteristics are presented in the Table 1., while the current and previous medications, along with the main comorbidities, is presented in Additional file Table 4. The group of patients with active LN, hereafter referred to as the Active LN-SLE group, included 11 patients (mean age 35 years, min–max 19–57 years, female/male 10/1) and 14 patients (mean age 35 years, min–max 22–54 years, female/male 11/3) with histologically confirmed but inactive LN at the time of sampling were considered the Inactive LN-SLE group. The most prevalent class of LN among patients with SLE and renal impairment, as determined by kidney biopsy, was class IV in 44% of cases and class II in 28%. The distribution

Table 1 Demographic and clinical characteristics of the enrolled patients

	SLE patients		**Control group**	
Number of patients	74		34	
Age mean (min–max)	40 (19–74)		40 (24–50)	
Female/Male	65/9		29/5	
	SLE (*n* = 74)			
Duration of disease (years) mean (min–max)	11.3 (1–38)			
Nonsmokers/Stop smoking/Smokers (percentage)	64.9/16.2/18.9			
SLEDAI-2 K mean (min–max)	7.0 (0–43)			
SLICC/ACR DI mean (min–max)	1.21 (0–8)			
C3 level (g/l) mean (min–max)	0.88 (0.2–1.72)			
C4 level (g/l) mean (min–max)	0.14 (0.06–0.3)			
	SLE cohort	**Active LN-SLE group**	**Inactive LN-SLE group**	**No LN-SLE group**
Number of patients	74	11	14	47
Anti ds DNA IgG positive	46 (62.2%)	6 (54.5%)	12 (85.7%)	28 (59.6%)
Anti ds DNA IgG (IU/ml) mean when positive (min–max)	80.47 (21– >200)	141.3 (24–200)	96.4 (26–200)	91.1 (21–200)
Anti-Smith antibody positive	16 (21.6%)	3 (27.3%)	3 (21.4%)	8 (17%)
Anti-nucleosome antibodies positive	61 (82.4%)	10 (100%)	12 (85.7%)	37 (78.7%)
Anti-nucleosome antibodies mean when positive (IU/ml) (min–max)	105.37 (1.7– >200)	145.3 (32– >200)	145.5 (46– >200)	122.5 (27– >200)
Active LN– Proteinuria (mean - g/day)		1.5 (0.36–6.03)		
Proteinuria (> 0.5 g/day)/Hematuria/Pyuria (number of patients)		11/4/7		

SLE Systemic lupus erythaematosus, LN lupus nephritis, TLR Toll–like receptor, SLEDAI 2 K Systemic Lupus Eryhematosus Disease Activity Index 2000, SLICC/ACR DI Systemic Lupus International Collaborating Clinics American College of Rheumatology Damage Index, C3 Complement Component 3, C4 Complement Component 4, Anti–dsDNA Anti–Double–stranded DNA Antibodies,, ACR American College of Rheumatology

of the histological classes of LN in subgroups is presented in Additional file Table 5. A total of 47 patients with SLE (No LN-SLE group) did not have kidney impairment (mean age 43 years, min–max 20–74 years, female/male 43/4). No statistically significant difference has been identified between antibody levels in the aforementioned study subgroups (Additional file Table 6). Six patients were diagnosed with NPSLE (mean age 47 years, min–max 20–60 years, female/male 6/0). A total of nine patients (12% of the SLE cohort) were not currently undergoing antimalarial treatment. All of these patients were included in the No LN-SLE group. Furthermore, 22 patients (30% of the SLE cohort) were not currently receiving corticosteroid treatment. Of these, three were from the Inactive LN-SLE group (21% of the Inactive LN-SLE group). The remaining 19 patients (40% of the No LN-SLE group) were part of the No LN-SLE group.

TLR family mRNA expression in patients with SLE versus healthy controls

The relative mRNA expression of the TLR family members was compared between the SLE patients and healthy controls. The aim was to identify differences between these groups. Compared to healthy controls, the relative mRNA expression of TLR4 was upregulated in the SLE group (0.044 vs. 0.081, $p = 0.012$; Table 2A, Fig. 1). Conversely, TLR10 expression was significantly lower in the SLE group than in the healthy control group (0.009 vs. 0.006, $p = 0.0007$; Table 2A, Fig. 1). Furthermore, increased expression of TLR6 and TLR7 and decreased expression of TLR3 were observed, although the differences did not reach statistical significance.

TLR family expression in active LN-SLE, inactive LN-SLE and no LN-SLE patients

To investigate the clinical manifestation-specific innate immune gene expression pattern, we compared SLE patients without renal impairment with patients with active and inactive LN according to the SLEDAI-R. A comparison of the active LN group with participants without kidney involvement revealed increased expression of TLR2 (0.078 vs. 0.03, $p = 0.009$), and TLR5 (0.035 vs. 0.017, $p = 0.03$). Moreover, a significant difference was observed in TLR9 expression between inactive LN and the control group (0.014 vs. 0.009, $p = 0.01$), together with borderline correlation in TLR2 expression (0.04 vs. 0.03, $p = 0.06$; Table 2B, Fig. 2).

Prediction of active LN based on TLR mRNA expression

ROC analysis identified TLR1 and TLR2 as potential predictive markers for active LN among all of the studied TLRs in the entire cohort of 74 patients with active LN versus those without. TLR1 had an AUC of 0.707 (95% CI 0.556–0.857, $p = 0.020$), and TLR2 had an AUC of 0.715 (95% CI 0.564–0.866, $p = 0.015$) (Fig. 3, Additional file Fig. 5). The optimal cut-off value for TLR1 mRNA expression in active LN was 0.0636, with a specificity of 0.787 and sensitivity of 0.615. The optimal cut-off value for TLR2 mRNA expression was 0.0282, with a specificity of 0.607 and sensitivity of 0.769. In the case of a summation of TLR1 and TLR2 values for the purpose of improved LN prediction, the sum is 0.095, exhibiting high sensitivity (0.846) but less specificity (0.689).

In conclusion, if a patient tests positive for TLR1 and/or TLR2 (at least one test), there is a high probability of active LN presence (0.923; 95% CI (0.640–0.998)). If a patient tests negative for both TLR1 and TLR2, there is a high probability that kidney damage is not caused by active LN (0.885; 95% CI (0.778–0.953)).

Correlation between TLR mRNA expression and disease activity score

Multivariate analysis was used to assess the associations between TLR1-10 mRNA expression and the SLEDAI-2 K score, SLICC/ACR DI score, complement C3 and C4 levels and anti-ds DNA antibody level in all patients with SLE. This analysis confirmed a statistically significant association between TLR9 levels and C4. Higher TLR9 mRNA levels were associated with active LN (0.0103, $p = 0.035$) and low complement C4 levels (-0.0759, $p = 0.039$) (Additional file Fig. 6). A correlation between TLR4 mRNA expression and the SLEDAI-2 K score was also demonstrated (Additional file Fig. 7). However, when the Spearman correlation was applied, no correlation was found. No other significant correlations were detected between TLR1-10 mRNA expression and the activity score or complement or antibody levels.

Correlation between TLR mRNA expression and treatment

A significant difference was observed between patients who were off and on corticosteroid therapy in TLR7 and TLR8 expressions. Patients who received corticosteroid treatment exhibited statistically significantly higher levels of TLR7 (0.011 vs. 0.023, $p = 0.039$) and TLR8 (0.031 vs. 0.05, $p = 0.018$) (Additional file Table 7). Upon dividing the patients into subgroups according to renal impairment, no statistically significant difference in TLR expression was observed. It is notable that patients undergoing corticosteroid therapy exhibited heightened SLE activity, as evidenced by elevated SLEDAI scores (3.95 vs. 7.62, $p = 0.022$). Mann-Whitney U-test showed no significant difference between patients on and off antimalarial therapy in TLR values.

TLR family expression in CNS-SLE versus no CNS-SLE patients

The increased relative mRNA expression of TLR1 (0.088 vs. 0.048, $p = 0.01$), TLR4 (0.173 vs. 0.066, $p = 0.0003$) and

Table 2 Relative TLR mRNA expression according to SLE subgroup and healthy control

A) SLE patients vs. healthy controls

Gene	Mean (95% CI)		P value
	Controls	SLE	
TLR1	0.0482 (0.005–0.107)	0.053 (0.006–0.176)	0.599
TLR2	0.039 (4.75×10^{-5}–0.208)	0.040 (1.7×10^{-4}–0.213)	0.590
TLR3	0.002 (2.51×10^{-5}–0.017)	0.0009 (2.46×10^{-5}–0.013)	0.122
TLR4	**0.044 (0.008–0.118)**	**0.081 (0.011–0.503)**	***0.012****
TLR5	0.021 (3.94×10^{-5}–0.76)	0.020 (2.81×10^{-5}–0.103)	*0.976*
TLR6	0.03 (6.4×10^{-4}–0.117)	0.041 (0.004–0.230)	*0.323*
TLR7	0.014 (1.56×10^{-4} –0.039)	0.019 (6.63×10^{-5}–0.104)	*0.655*
TLR8	0.043 (0.004–0.190)	0.047 (2.42×10^{-4}–0.241)	*0.682*
TLR9	0.010 (2.5×10^{-5}–0.022)	0.012 (4.69×10^{-5}–0.073)	*0.408*
TLR10	**0.009 (5.0×10^{-4}–0.022)**	**0.006 (2.71×10^{-5}–0.430)**	***0.0007****

B) Active LN-SLE vs. Inactive LN-SLE vs. No LN-SLE Patients

Gene	Mean (95% CI)			P value		
	Active LN-SLE	Inactive LN-SLE	No LN-SLE	Active vs. Inactive LN-SLE	Active vs. No LN SLE	Inactive vs. No LN SLE
TLR1	0.061 (0.017–0.094)	0.044 (0.007–0.102)	0.051 (0.006–0.175)	0.087	0.194	0.377
TLR2	**0.078 (0.007–0.213)**	0.041(0.002–0.088)	**0.030 (1.6×10^{-4}–0.117)**	0.291	**0.009***	0.066
TLR3	5.7×10^{-4}(5.7×10^{-5}–0.003)	6.4×10^{-4}(2.9×10^{-5}–0.002)	0.001 (2.5×10^{-5}–0.014)	0.545	0.567	0.603
TLR4	0.076 (0.033–0.147)	0.06 (0.011–0.261)	0.079 (0.015–0.325)	0.077	0.269	0.544
TLR5	**0.035 (1.2×10^{-4}–0.103)**	0.02(2.8×10^{-5}–0.070)	**0.017 (4.7×10^{-5}–0.102)**	0.202	**0.037***	0.883
TLR6	0.039 (0.015–0.097)	0.055 (0.014–0.23)	0.036 (0.004–0.168)	0.936	0.134	0.171
TLR7	0.017 (0.006–0.039)	0.022(0.005–0.06)	0.0188 (6.6×10^{-5}–0.241)	0.599	0.344	0.383
TLR8	0.050 (0.008–0.096)	0.051 (0.012–0.714)	0.041 (2.4×10^{-4}–0.039)	0.687	0.101	0.136
TLR9	0.017 (6.5×10^{-5}–0.061)	**0.017 (0.003–0.073)**	**0.009 (4.7×10^{-5}–0.39)**	0.676	0.142	**0.019***
TLR10	0.007 (6.5×10^{-5}–0.017)	0.005 (2.5×10^{-4}–0.02)	0.006 (2.7×10^{-5}–0.061)	0.467	0.273	0.777

C) CNS-SLE vs. No CNS-SLE patients

Gene	Mean (95% CI)		P value
	CNS-SLE	No CNS-SLE	
TLR1	**0.088 (0.038–0.150)**	**0.048 (0.006–0.176)**	**0.0199***
TLR2	0.020 (4.0×10^{-4}–0.083)	0.041 (1.6×10^{-4}–0.213)	0.0986
TLR3	3.36×10^{-4} (7.9×10^{-5}–5.9×10^{-4})	0.001 (2.46×10^{-5}–0.014)	0.8946
TLR4	**0.173 (0.084–0.295)**	**0.066 (0.011–0.325)**	**0.0003***
TLR5	0.008 (0.002–0.014)	0.022 (2.81×10^{-5}–0.104)	0.3839
TLR6	**0.087 (0.025–0.168)**	**0.036 (0.004–0.23)**	**0.0070***
TLR7	0.048 (1.96×10^{-4}–0.104)	0.017 (6.63×10^{-5}–0.094)	0.1534
TLR8	0.096 (0.02–0.242)	0.04 (2.4×10^{-4}–0.172)	0.1437
TLR9	0.009 (3.91×10^{-4} –0.039)	0.012 (4.69×10^{-5}–0.073)	0.2179
TLR10	0.017 (5.93×10^{-4}–0.062)	0.005 (2.71×10^{-5}–0.02)	0.3102

P values less than 0.05 were considered to indicate statistical significance and are indicated by an asterisk. SLE Systemic lupus erythaematosus, LN lupus nephritis, TLR Toll–like receptor, CNS– central nervous system

TLR6 (0.087 vs. 0.036, $p=0.007$) was observed in comparison to No CNS-SLE patients. (Table 2C, Additional file Fig. 4).

Correlations between individual TLR mRNA expression

Multiple significant correlations were identified between the expression of individual TLRs in the entire lupus cohort. Specifically, TLR1 correlated with TLR2, TLR4, TLR5, TLR7, TLR8, and TLR10. TLR2 also correlated with TLR5 and TLR8, TLR3 with TLR9, TLR4 with TLR6-9, and TLR5 with TLR8 and TLR10 (Table 3, Additional file Fig. 8). The multiple mutual relationships of TLRs and their correlation with disease activity demonstrate the activation of innate immunity in SLE.

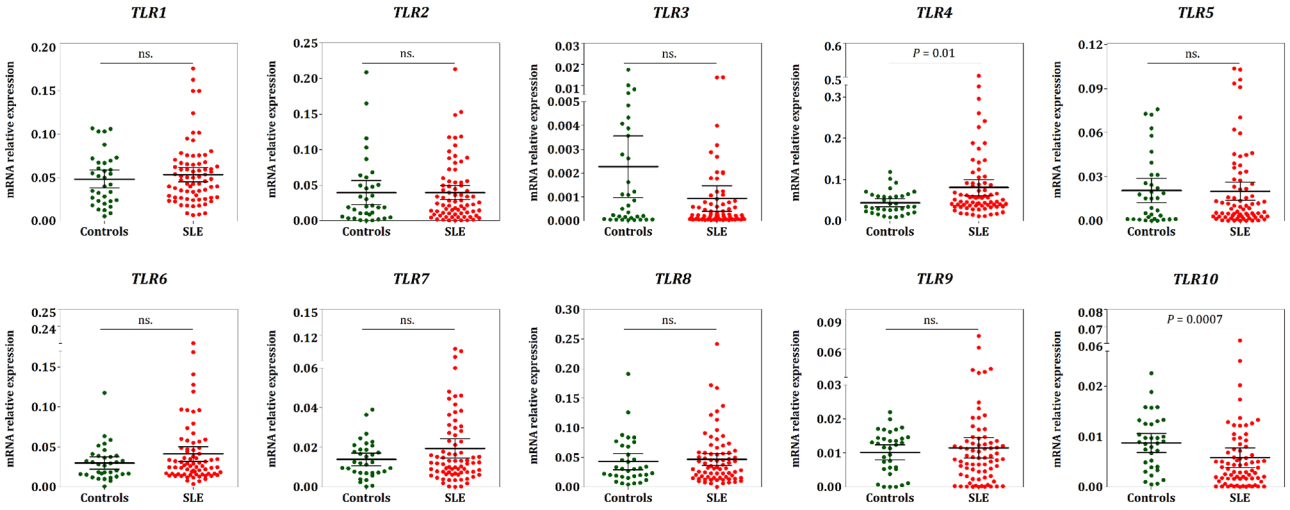

Fig. 1 Toll-like receptor mRNA relative expression– SLE patients vs. healthy controls. The relative mRNA expression of the TLR family members was compared between patients with SLE and healthy controls. Compared to that in healthy controls, the relative mRNA expression of TLR4 was upregulated in the SLE group (Table 2A). Conversely, TLR10 expression was significantly lower in the SLE group compared to healthy control group (Table 2A). Furthermore, increased expression of TLR6 and TLR7 and decreased expression of TLR3 were observed, although the differences did not reach statistical significance

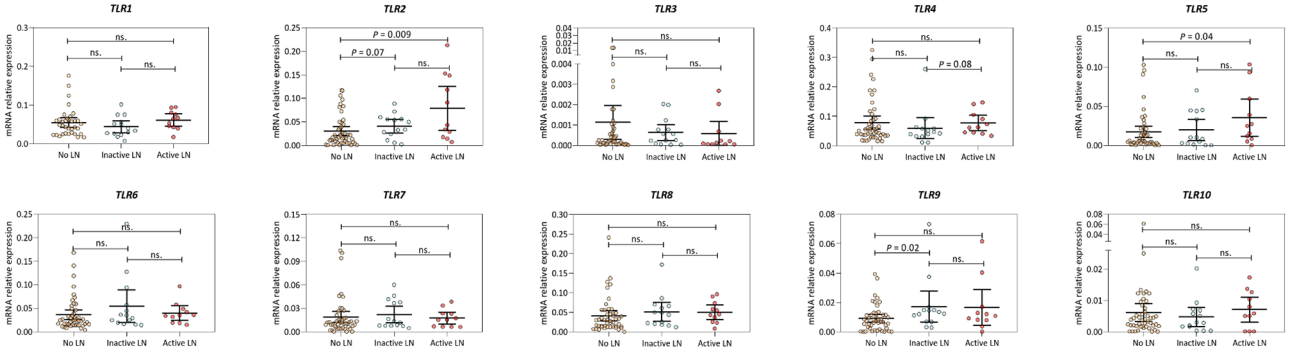

Fig. 2 TLR family expression in Active LN-SLE, Inactive LN-SLE and No LN-SLE patients. The objective of this study was to investigate the relationship between innate immune gene expression and clinical manifestations in patients with SLE without renal impairment and in patients with active and inactive LN according to the renal SLEDAI. Our findings revealed a significant upregulation of the TLR2 and TLR5 (Table 2B). Furthermore, TLR2 and TLR9 expression was increased when the No LN-SLE and Inactive LN-SLE groups were compared. Additionally, a significant distinction was observed in TLR4 expression when the Active and Inactive LN-SLE group were compared

Discussion

This study further supports the important role of TLR in SLE and its severe organ manifestations and offers insight into TLR activation, which is characteristic of kidney and central nervous system involvement. The specific blood profile of TLRs in the LN makes it suitable for minimally invasive diagnostics of active LN but also highlights a novel attractive treatment strategy for SLE by targeting TLR signalling cascades using antagonists or monoclonal antibodies that selectively prevent extracellular or endosomal TLR ligation [7].

The mosaic of TLR expression in SLE patients published thus far in the literature includes a number of interesting aspects. SLE is commonly associated with the overexpression of TLR7, which leads to IFNα production [4, 5]. Genetic or environmental signals, including female sex due to the presence of the TLR7 gene on the X chromosome [16], a high-fat diet and a cytokine environment, including the level of type I interferons, lead to the overexpression of TLR7 and increase the susceptibility of individuals to SLE [4]. Two pathways of B-cell activation lead to the development of autoreactive antibody-secreting cells in SLE: the extrafollicular response and the germinal centre reaction. These pathways can enhance the production of autoantibodies [17]. TLR7 plays a key role in both responses [16]. Targeted inhibition of TLR7-stimulated inflammatory cytokine production has already been tested in lupus mouse and monkey models and is a promising therapeutic option for the treatment of SLE [7].

TLR2 and TLR4 are membrane-bound receptors that recognise bacterial wall components and endogenous components of dying cells [6, 18]. Upregulated TLR2 mRNA expression has been found in SLE patients and

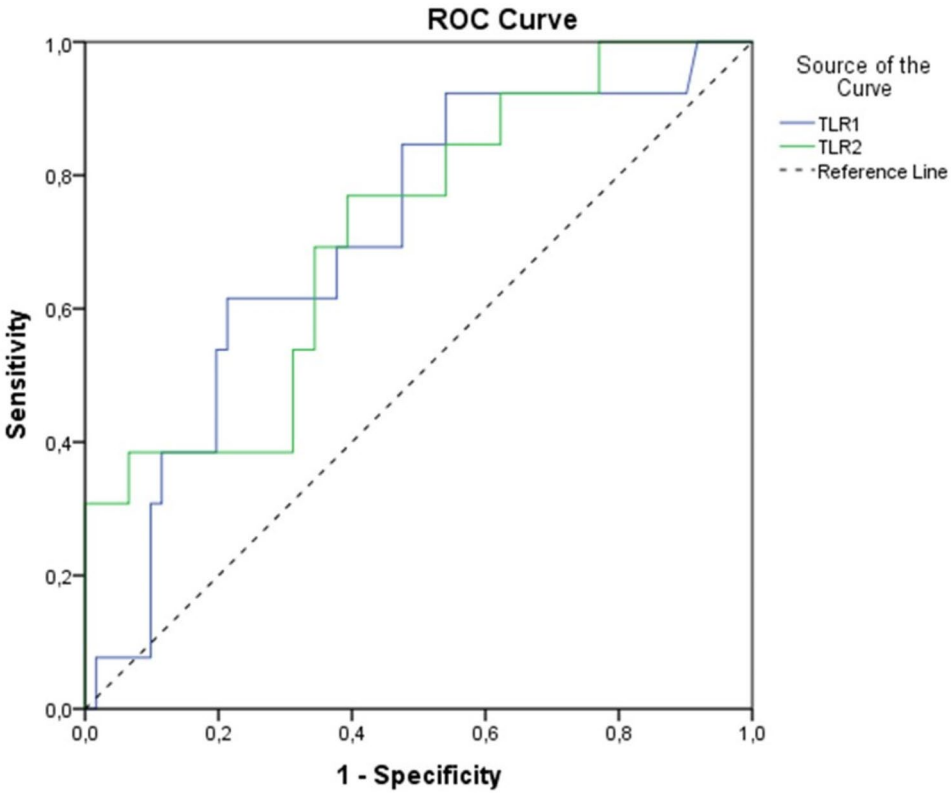

Fig. 3 ROC analysis of TLR1 and TLR2 mRNA expression. ROC analysis indicated that TLR1 and TLR2 were potential predictive markers for active LN among the studied TLRs. The area under the curve (AUC) of TLR1 was 0.707, with a 95% confidence interval (CI) of 0.556–0.857 and a p value of 0.020. The AUC of TLR2 was 0.715, with a 95% CI of 0.564–0.866 and a p value of 0.015. AUC values above 0.7 were regarded as a reasonable discriminating value for predicting diagnostic tests

Table 3 Correlations of Toll-like receptor (TLR) mRNA expression levels

	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
TLR1	1.000	*0.397**	0.106	*0.679**	*0.276**	0.201	*0.539**	*0.703**	-0.044	*0.270**
TLR2	*0.397**	1.000	0.120	0.150	*0.646**	-0.132	0.156	*0.472**	0.053	0.151
TLR3	0.106	0.120	1.000	0.066	0.150	-0.067	-0.017	-0.067	*0.264**	-0.047
TLR4	*0.679**	0.150	0.066	1.000	-0.007	*0.383**	*0.554**	*0.635**	*-0.250**	-0.046
TLR5	*0.276**	*0.646**	0.150	-0.007	1.000	-0.048	-0.024	*0.393**	0.165	*0.331**
TLR6	0.201	-0.132	-0.067	*0.383**	-0.048	1.000	0.171	*0.463**	0.052	-0.013
TLR7	*0.539**	0.156	-0.017	*0.554**	-0.024	0.171	1.000	*0.570**	-0.094	0.225
TLR8	*0.703**	*0.472**	-0.067	*0.635**	*0.393**	*0.463**	*0.570**	1.000	-0.066	0.207
TLR9	-0.044	0.053	*0.264**	*-0.250**	0.165	0.052	-0.094	-0.066	1.000	*0.381**
TLR10	*0.270**	0.151	-0.047	-0.046	*0.331**	-0.013	0.225	0.207	*0.381**	1.000

TLR Toll like receptor, Spearman correlation coefficients marked with asterisks are significant at the 0.05 level

is associated with increased production of IL-17 [19]. Overexpression of TLR4 can also lead to lupus progression and the presence of lupus nephritis [20]. TLR5 recognises bacterial flagellin, which activates both innate inflammatory responses and the development of adaptive immunity [21]. The role of TLR5 in SLE remains questionable. There are several single nucleotide polymorphisms (SNPs), such as the TLR5-rs5744168 SNP, that are significantly more frequent in SLE patients with LN [22], but another study revealed no association between this polymorphism and SLE [23]. Increased activation of TLR3 in peripheral mononuclear cells (PBMCs), B cells and T cells has also been observed in SLE patients [24] and may be associated with various SLE phenotypes [25]. TLR8 expression is upregulated in SLE patients, especially in females, due to the presence of 17β-estradiol, which is a potential factor in SLE development [26, 27]. However, some studies have indicated that TLR8 may also inhibit TLR7-mediated autoimmunity and renal inflammation [28]. In contrast, activation of TLR9 has an

antagonistic effect and may be protective against lupus-like diseases due to its ability to limit the stimulatory activity of TLR7 [29].

Our study revealed a decrease in TLR10 mRNA expression in patients with SLE in comparison to healthy controls. TLR10 is a plasma membrane receptor that is highly expressed in monocytes, dendritic cells and B cells. Several reports have shown that this receptor has an anti-inflammatory function by inhibiting the production of inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor α (TNF-α) [30–32]. All of these cytokines are elevated in patients with SLE and are associated with increased disease activity [33]. Our results confirmed a significant downregulation of TLR10 in SLE patients, as previously described [34]. This finding suggests a possible link between SLE development and its activity during TLR10 downregulation in our study group. TLR10 was also downregulated in active rheumatoid arthritis (RA) patients [13] and in patients with microscopic polyangiitis [35]. This finding supports its possible role in autoimmune disease development. Despite these findings, SNPs in the TLR10 gene have been reported to be associated with increased susceptibility to RA, hip osteoarthritis, and other diseases [36, 37]. In contrast, TLR7 and TLR9 mRNA expression, which are most often associated with the development of SLE, was only slightly upregulated in our SLE cohort, and the difference from healthy subjects did not reach statistical significance. Nevertheless, the elevation of TLR9 has been demonstrated in patients with Inactive LN.

Up to 40% of SLE patients develop severe organ manifestations, such as lupus nephritis. This is a risk factor for increased morbidity and all-cause mortality and can lead to progression to end-stage renal disease [38]. Numerous studies have investigated the involvement of TLRs in the pathogenesis of LN. The specific role of TLRs in kidney injury is complex. The available experimental and clinical data indicates that TLRs are involved in the pathogenesis of immune complex glomerulonephritis and intrarenal inflammation [6]. Inflammation of kidney tissue in LN can result from several pathways. Mesangial cells and other renal parenchymal cells express TLR1–4 and TLR6. Renal cells respond to TLR activation by secreting interleukins and chemokines [39, 40]. Another mechanism is that immune complex deposits containing TLR agonists activate glomerular mesangial cells and contribute to immune complex glomerulonephritis [6]. TLR2-mediated activation of podocytes and endothelial cells at the glomerular filtration barrier results in the aggravation of albuminuria [6]. Higher expression of TLR9 was detectable in the tubulointerstitial compartment and glomeruli of LN patients. A significant increase in TLR5 gene expression in LN biopsies has been also observed, confirmed by intense and diffuse staining for TLR5 only in LN tubules [41]. Overexpression of various polymorphisms of TLRs (TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9) has been found in LN patients [6]. In our study, we confirmed the significant overexpression of two members of the TLR family (TLR2 and TLR5) in active LN patients and TLR2 and TLR9 when compared Inactive LN with patients without kidney involvement.

In our study, TLR1 and TLR2 were suggested as possible diagnostic markers for active LN. We proposed cut-off values for the mRNA expression of these receptors. The positivity of TLR1 or TLR2 predicts lupus nephritis with high sensitivity and high predictive value of a negative test. Furthermore, if both are positive, there is a high specificity for active LN and also a high predictive value for a negative test. These findings highlight the potential role of TLR2 as a biomarker of active LN in our study. However, further studies are needed to confirm the high expression of TLR1 and TLR2 as possible diagnostic or therapeutic targets in LN. The overexpression of TLR1 may be related to proinflammatory cytokine release, activation of type I IFN signalling leading to podocyte injury and proteinuria in LN patients [42]. TLR1 is a cell surface receptor that is localised on monocytes, macrophages, dendritic cells and B cells. TLR1 recognises bacterial lipoproteins and glycolipids in complex with TLR2, and its overexpression has been also detected in Lyme disease, sepsis, candidaemia and leprosy [43].

Little is known about the role of TLR6 in the literature. TLR6 is a cell surface TLR that mainly recognises microbial membrane components to induce an inflammatory response [44]. TLR6 forms a heterodimer with TLR2 to mediate the cellular response to gram-positive bacteria, mycoplasma, fungi, some viruses and even protozoa [45]. According to one study, two specific coding and regulatory regions of TLR6 were enriched in LN, although this did not reach statistical significance [41]. Apart from this difference, our study confirmed the results of previous research.

It is already established that antimalarial treatments have an immunomodulatory effect. However, our study did not identify any significant difference in the expression of specific TLRs in patients undergoing antimalarial therapy and those who were not. The impact of corticosteroid treatment on TLRs expression in patients with autoimmune diseases remains poorly understood. The results of our study indicated an elevated expression of TLR7 and TLR8 in the subgroup that had been treated with corticosteroids. One potential explanation for this observation is that the patients in this subgroup exhibited a more active disease state, as indicated by their SLEDAI scores. This is consistent with the literature, which predominantly associates TLR7 and TLR8 with SLE [4, 5, 16, 26, 27]. However, our data on the impact of therapy

on TLR expression are limited by the fact that the blood samples were not collected before and during treatment.

Neuropsychiatric systemic lupus erythematosus (NPSLE) is one of the least understood and most complex subtypes of SLE. NPSLE can affect both the central and peripheral nervous systems with a wide range of symptoms. The most common symptoms observed in our patients were organic brain syndrome, seizures, and lupus headache, with a predominance of diffuse CNS manifestations. According to previous studies, two pathological mechanisms contribute to NPSLE development. The ischemic or thrombotic pathway is associated with immune complexes, the complement system and antiphospholipid antibody positivity, while the autoimmune or inflammatory pathway is characterised by autoantibody, proinflammatory cytokine, chemokine, microglial and C1q production [46, 47]. The specific role of TLRs in the development of NPSLE is poorly understood due to the complexity of the problem. The concentration of high-mobility group box protein 1 (HMGB1) in serum and cerebrospinal fluid was elevated in patients with seizure disorders, accompanied by the overexpression of TLR4 mRNA in PBMCs from these patients [48]. Our study confirmed that the overexpression of TLR4 in these patients was accompanied by increased expression of TLR1 and TLR6.

The aim of our study was to investigate the relationship between disease activity and TLRs. We did not find any significant correlation between TLRs and scoring systems such as the SLEDAI-2 K, SLICC/ACR DI, or major antibody and complement levels. The only significant association observed was between TLR9 mRNA relative expression and active lupus nephritis. Additionally, an inverse correlation was found between TLR9 mRNA expression and C4 complement levels. Low C4 complement levels have been shown to be a useful marker for diagnosing and measuring activity in patients with SLE and may also predict renal flares in some patients [49].

Several significant correlations were found between the expression of individual TLRs across the lupus cohort, demonstrating the interconnectedness and complexity of innate immune activation leading to the development of SLE.

The strengths of this study are the detailed analysis of the expression of TLRs in a real-world clinical setting in a group of lupus patients and the statistical evaluation of the mutual relationships of TLRs. The authors of the study are aware of some constraints of their results. First, the study was performed as a cross-sectional analysis in a real-world setting of patients at different stages of the disease. Secondly, the study was performed in an ethnically homogeneous population of the Central Moravia region. It would be of interest to compare the results with those of an analysis of patients of different ethnic backgrounds or of multicentric cohorts. Finally, our study group focused mainly on patients with active LN and NPSLE. Patients with other SLE phenotypes and other different subsets of disease were not included. The authors of this study are aware that the utilisation of transcriptomics in smaller clinical settings may be constrained by elevated costs and the necessity for specific equipment.

Conclusion

The TLR family plays a key role in the innate immune response as well as in the pathogenesis of autoimmune rheumatic diseases. We found heterogeneity in TLR expression according to the main clinical manifestations of SLE. In our study, overexpression of TLR2 was found in both the active and inactive LN groups. ROC curves indicate that the expression of TLR1 and TLR2 is indicative of the presence of active LN and the discriminative values between active LN and other patients with inactive or no LN were established. These findings highlight the potential role of TLR2 as a biomarker of active LN in our study. In addition, the expression and role of TLRs in NPSLE are not well described in the literature. Although our study cannot provide a detailed analysis of the possible pathogenetic link between the expression of TLRs and the development of CNS involvement, it does provide some insight into this issue. Given the small number of patients with this severe manifestation in SLE, further investigation is needed.

In this context, an analysis of innate gene expression patterns can provide useful information for understanding the molecular mechanisms of SLE, which may impact patient prognosis and treatment outcomes. However, the practical use of determining TLR expression requires additional data.

Abbreviations

Anti-dsDNA	Anti-double-stranded DNA antibodies
ACR	American College of Rheumatology
CNS	Central nervous system
C3	Complement component 3
C4	Complement component 4
DAMPs	Damage-associated molecular patterns
HMGB1	High-mobility group box protein 1
IFNα	Interferonα
IL	Interleukin
HPF	High-power field
LN	Lupus nephritis
mRNA	Messenger ribonucleic acid
NPSLE	Neuropsychiatric systemic lupus erythematosus
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral mononuclear cells
SLE	systemic lupus erythematosus
SLEDAI-2 K	Systemic Lupus Eryhematosus Disease Activity Index 2000
SLICC/ACR DI	The Systemic Lupus International Collaborating Clinics American College of Rheumatology Damage index
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α

Supplementary Information

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Supplementary Material 1

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Author contributions
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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The depersonalised and anonymous data and samples were collected and stored after signed written informed consent for data collection was obtained from all participants and healthy controls. The study was approved by the local ethical committee (Multicenter Research Ethics Committee of the University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic). The study was performed in accordance with the Helsinki Declaration of 1964 and its later amendments. No animal data were used in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Footnote
The findings in this manuscript were presented in virtual poster format at the Annual European Congress of Rheumatology, EULAR 2024, Vienna, Austria, is available in the EULAR Abstract Archive.

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